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Habilitační práce

Bakterie mléčného kvašení a jejich funkční vlastnosti

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Obsah

1	Úvod	3
2	Bakterie mléčného kvašení.....	4
2.1	Taxonomické zařazení bakterií mléčného kvašení	4
2.2	Metabolismus bakterií mléčného kvašení.....	7
2.3	Antimikrobiální metabolity bakterií mléčného kvašení	9
2.3.1	Organické kyseliny	10
2.3.2	Acetaldehyd.....	12
2.3.3	Diacetyl	12
2.3.4	Peroxid vodíku	13
2.3.5	Reuterin	14
2.3.6	Cyklické dipeptidy.....	15
2.3.7	Bakteriociny	15
2.4	Antimikrobiální aktivita, protektivní kultury	17
3	Bakterie mléčného kvašení jako probiotika	20
3.1	Stručná historie a definice probiotik.....	20
3.2	Nejčastěji používané mikroorganismy jako probiotika	22
3.3	Kritéria pro výběr probiotických mikroorganismů.....	23
3.3.1	Původ a charakteristika probiotických mikroorganismů	23
3.3.1.1	Odolnost vůči podmínkám trávicího traktu	23
3.3.1.2	Povrchové vlastnosti, adheze.....	27
3.3.1.3	Toxicita, faktory virulence	28
3.3.1.4	Antibiotická rezistence	29
3.3.2	Zdravotně-funkční vlastnosti probiotik, klinické studie.....	30
3.3.3	Technologické vlastnosti – aplikace probiotických mikroorganismů do potravin	32
3.3.3.1	Enkapsulace probiotických mikroorganismů	32
3.3.3.2	Metody enkapsulace probiotických mikroorganismů.....	33
3.3.3.3	Použití prebiotik	35
3.3.3.4	Aplikace probiotických mikroorganismů do nemléčných potravin.....	37
4	Selektivní stanovení bakterií mléčného kvašení a probiotických mikroorganismů	39
5	Závěr.....	44
6	Použitá literatura.....	45
7	Seznam zkratk.....	63
8	Seznam příloh.....	65
9	Přílohy	67

1 Úvod

Jedním z nejdůležitějších mezníků ve vývoji a utváření lidské civilizace byla bezpochyby schopnost vyrábět a následně skladovat větší množství potravin. Účinnou formou prodloužení trvanlivosti potravin se staly již před mnoha tisíci lety spontánní fermentace, které jsou závislé na biologické aktivitě mikroorganismů s produkcí řady metabolitů potlačující růst nežádoucí mikroflóry a které jsou jednou z nejstarších známých metod konzervování potravin. Přesný počátek používání spontánní fermentace mléka je obtížné určit, ale může to být doba 10 000 - 5 000 let př. n. l., kdy se měnil způsob života lidí, a z pouhých sběračů a lovců potravy se stávali zemědělci a výrobci potravin. Tato změna s sebou přinesla také domestikaci zvířat (skotu, ovcí, koz, buvolů a velbloudů) a je pravděpodobné, že nastala v různých částech světa v rozdílných dobách. Archeologické nálezy ukazují, že některé civilizace měly velmi vyspělé zemědělství a znaly metody výroby fermentovaných výrobků.

V současné době tvoří potraviny získané fermentací pomocí definovaných i nedefinovaných mikrobiálních kultur asi jednu třetinu světové potravinové spotřeby. Až do začátku 20. stol. zůstávala fermentace potravin neregulovaným procesem. Teprve objev jednotlivých druhů mikroorganismů a porozumění podstatě biochemických jevů umožnil rozvoj přesně řízených průmyslových procesů. Mechanismus účinku, prevalence a vývoj mikrobiální populace ve fermentačním procesu je v současnosti předmětem intenzivního výzkumu, včetně výzkumu vlastností jednotlivých mikroorganismů, jejich bezpečnosti a dopadu jejich konzumace na zdraví člověka.

Zcela nezastupitelnou roli v potravinářských, zemědělských i klinických aplikacích má skupina bakterií mléčného kvašení (BMK). Schopnost adaptace BMK na odlišné environmentální podmínky a jejich adhezní vlastnosti jim umožnily rozšíření v různých prostředích od potravinových matric, jako je mléko a mléčné výrobky, maso, zelenina, obilné produkty, až po výskyt v komenzální mikroflóře lidí a zvířat. Jejich metabolická aktivita je spojena nejen se samotnou fermentací sacharidových substrátů, ale také s produkcí mnoha prospěšných sloučenin s antimikrobiální a antioxidační aktivitou.

2 Bakterie mléčného kvašení

Pro výrobu potravin, a obecně v lidské výživě, se používá řada mikroorganismů, které řadíme mezi bakterie mléčného kvašení (BMK). Uplatňují se buď ve formě přesně definovaných mikrobiálních kultur, nebo jako „divoké“ kmeny, které se přirozeně vyskytují na použitých surovinách. V obou případech je jejich technologická funkce spojena s přeměnou přítomných substrátů (sacharidů, bílkovin, lipidů) na metabolity, které ovlivní výslednou chuť, vůni a konzistenci výrobků. Některé speciálně selektované doplňkové kultury podporují tvorbu aromatických sloučenin a ovlivňují texturní vlastnosti hlavně u fermentovaných mléčných výrobků a sýrů. Velká pozornost je v současné době věnována aplikaci tzv. protektivních kultur, které se vyznačují zvýšenou produkcí antimikrobiálně aktivních metabolitů, které potlačují růst technologicky nežádoucích mikroorganismů a mikroorganismů způsobujících onemocnění z potravin. V neposlední řadě se ze skupiny BMK rekrutuje celá řada probiotických kmenů s prokázanými pozitivními zdravotními účinky a pozitivním působením na fyziologické funkce konzumenta.

2.1 Taxonomické zařazení bakterií mléčného kvašení

Skupinu BMK je do určité míry velmi obtížné jednoznačně definovat. Na přelomu 20. století se termín „bakterie mléčného kvašení“ začal používat k označení „organismů okyselujících mléko“, nicméně základ současné klasifikace BMK položil svojí prací dánský vědec Orla-Jensen (1919). Kritéria používaná Orla-Jensenem (buněčná morfologie, způsob fermentace glukosy, teplotní rozsahy růstu a utilizace sacharidů) jsou pro klasifikaci BMK stále velmi důležitá, ale zavedení modernějších taxonomických nástrojů, zejména molekulárně biologických metod, podstatně zvýšily počet rodů BMK ze čtyř původně určených Orla-Jensenem (*Lactobacillus*, *Leuconostoc*, *Pediococcus* a *Streptococcus*) (Wessels *et al.*, 2004).

BMK jsou Gram-pozitivní chemoorganotrofní organismy, tyčinky nebo koky, které mají nízký obsah G+C (méně než 50%), jsou nepohyblivé, nesporulující, katalasa-negativní, acidotolerantní a postrádající cytochromy. Vyskytují se v prostředí bohatém na živiny, jako je rozkládající se rostlinný materiál, ovoce, zelenina, traviny, mléko či maso; jsou součástí komenzální mikrobioty úst a trávicího a vaginálního traktu obratlovců. Většina druhů je obecně dobře adaptována na růst v aerobním i anaerobním prostředí. Ke svému růstu nevyžadují přítomnost kyslíku, ale některé druhy jsou mikroaerofilní. BMK nejsou schopny syntetizovat

enzymy s prostetickou skupinou hemem (katalasa, peroxidasa), což má u některých za následek sníženou odolnost vůči toxicitě reaktivního kyslíku. Ke zhášení radikálů pak některé druhy využívají např. kovy (zinek, mangan nebo selen) (Salvatore *et al.* 2007), což potvrzuje i schopnost akumulace jejich vysokého množství v cytoplasmě.

Zástupci BMK se řadí do kmene *Firmicutes*, třída *Bacilli*, řád *Lactobacillales*. Rody, které jsou nejčastěji spojeny s výrobou potravin, jsou následující: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus* a také *Enterococcus*, *Oenococcus*, *Carnobacterium*, *Weissella* či již méně významnější *Aerococcus*, *Sporolactobacillus* *Tetragenococcus* a *Vagococcus* (Crowley *et al.*, 2013). Moderní genetické metody však objasňují stále více odlišností mezi těmito mikroorganismy, což může být dokladováno reklasifikací jednotlivých rodů. V roce 1985 byl např. rod *Streptococcus* rozdělen na *Lactococcus*, *Enterococcus*, *Vagococcus* a *Streptococcus*. První genom BMK byl publikován pro kmen *Lactococcus lactis* spp. *lactis* IL1403 v roce 2001 (Bolotin *et al.*, 2001; GenBank No. AE005176). Od té doby byla sekvenována řada průmyslově důležitých druhů. Genomy BMK mají nízký obsah G+C (34 – 46 %) a jsou typické tím, že kódují vysoce variabilní počet genů. Velikost genomu se pohybuje od 1,8 Mb u *L. gasseri* až po 3,3 Mb u *L. plantarum* (Siezen *et al.*, 2004; Li *et al.*, 2016; Wu *et al.*, 2017). Dostupnost sekvenovaných genomů umožňuje pochopit divergenci BMK. Redukce genů, jejich duplikace a horizontální přenos během evoluce hrály důležitou roli v současné struktuře genomu. Redukce genů je spojována s přizpůsobením se růstu na nutričně bohatých médiích. Dva rozdílné geno-fenotypy byly např. prokázány u *L. rhamnosus*, kdy se adaptace na prostředí bohaté na živiny ukázala ve změně nebo ztrátě biologických funkcí spojených s antimikrobiální aktivitou a odolností vůči stresu (Douillard *et al.*, 2013). Zjevný příklad redukce genomu nastal u *Streptococcus thermophilus*, který se liší od patogenních druhů *Streptococcus* ztrátou genů asociovaných s virulentními faktory (Pfeiler & Klaenhammer, 2007). Dalším příkladem může být zřejmě ztráta 600 – 1200 genů u *Lactobacillales* v porovnání s jejich předchůdcem třídou *Bacilli*, včetně genů kódujících některé biosyntetické enzymy a sporulaci (Douillard & de Vos, 2014).

K významným změnám identifikace dochází aktuálně u rodu *Lactobacillus*. Doposud u něj bylo popsáno 261 druhů. Již dříve byli z důvodu velké odlišnosti přearženi někteří zástupci do jiných rodů (*Carnobacterium* spp., *Oenococcus* spp. nebo *Weissella* spp.). V současné době však probíhá dramatická změna v reklasifikaci čeledi *Lactobacillaceae*, rodů *Lactobacillus*, *Paralactobacillus* a *Pediococcus*. V publikaci, která byla zveřejněna v dubnu tohoto roku (Zheng *et al.*, 2020), bylo na základě sekvenování celého genomu těchto bakterií vytvořeno 25 rodů – 2 pozmeněné rody *Lactobacillus* a *Paralactobacillus* a 23 nových

rodů (*Acetilactobacillus*, *Agrilactobacillus*, *Amylolactobacillus*, *Apilactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Dellaglioia*, *Fructilactobacillus*, *Furfurilactobacillus*, *Holzapfelia*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lapidilactobacillus*, *Latilactobacillus*, *Lentilactobacillus*, *Levilactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Liquorilactobacillus*, *Loigolactobacillus*, *Paucilactobacillus*, *Schleiferilactobacillus* a *Secundilactobacillus*). V původním rodu *Lactobacillus* bylo ponecháno pouze 38 druhů ze skupiny *Lactobacillus delbrueckii* (např. *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Lactobacillus helveticus*). Snahou autorů reklasifikace bylo lépe zařadit a zdokumentovat jednotlivé druhy tak, aby se heterogenita genomu projevila v taxonomii a bylo možno odlišit jednotlivé zástupce z hlediska ekologických a funkčních vlastností a adaptace na hostitele. Příklady nově vytvořených rodů pro nejčastěji používané laktobacily v potravinářských technologiích jsou uvedeny v Tab. 1.

Tab. 1. Příklad nového zařazení vybraných druhů laktobacilů významných z hlediska potravinářských technologií (dle Zheng *et al.*, 2020)

Basonyma (původní názvy)	Nové zařazení
<i>Lactobacillus casei</i>	<i>Lacticaseibacillus casei</i>
<i>Lactobacillus rhamnosus</i>	<i>Lacticaseibacillus rhamnosus</i>
<i>Lactobacillus plantarum</i>	<i>Lactiplantibacillus plantarum</i>
<i>Lactobacillus fermentum</i>	<i>Limosilactobacillus fermentum</i>
<i>Lactobacillus reuteri</i>	<i>Limosilactobacillus reuteri</i>
<i>Lactobacillus brevis</i>	<i>Levilactobacillus brevis</i>
<i>Lactobacillus kefir</i>	<i>Lentilactobacillus kefir</i>
<i>Lactobacillus sanfranciscensis</i>	<i>Fructilactobacillus sanfranciscensis</i>

V předkládané práci budou však dále použita původní jména druhů laktobacilů, tak jak byla publikována v citované literatuře.

Přesné zařazení druhů používaných pro potravinářské účely má význam i z hlediska zdravotní bezpečnosti. K tomuto účelu byl vytvořen panelem EFSA (Biohazard Panel) návod k posuzování bezpečnosti jednotlivých druhů a kmenů v potravinovém řetězci (Qualified Presumption of Safety). Seznam bezpečných mikroorganismů je každoročně aktualizován, ale jsou v něm obsaženy pouze druhy, o jejichž hodnocení požádal výrobce. Úplný seznam bezpečných druhů BMK, ale i dalších mikroorganismů pro výrobu potravin a krmiv, byl vytvořen v rámci projektu organizací International Dairy Federation a European Food and Feed

Cultures Association (Bulletin IDF 455, 2012), který byl naposledy aktualizován v roce 2018 (Bulletin IDF 495, 2018).

2.2 Metabolismus bakterií mléčného kvašení

Jako hlavní zdroj energie využívají BMK ke svému růstu jednoduché sacharidy a disacharidy. Většina druhů se přizpůsobila růstu v mléce díky získané schopnosti utilizace laktosy (Kelly *et al.*, 2010). Laktosa i jiné sacharidy mohou být do buňky přenášeny buď fosfoenolpyrutát-fosfotransferasovým systémem (PEP-PTS) nebo za účasti bílkovinných přenašečů. PEP-PTS, který je typický pro laktokoky, translokuje sacharidy zároveň s jejich fosforylací na úkor jedné molekuly fosfoenolpyruvátu (ekvivalent jedné molekuly ATP). Vícekomponentní systém PTS se skládá ze dvou obecných proteinů, enzymu I a proteinu HPr, a několika enzymů specifických pro daný sacharid (EIIA, EIIBC), které mohou obsahovat až čtyři domény (Neves *et al.*, 2005).

BMK jsou fermentující mikroorganismy (homofermentativní nebo heterofermentativní), jako hlavní produkt rozkladu sacharidů vytvářejí kyselinu mléčnou. V závislosti na rodu a druhu mohou dále tvořit kyselinu octovou, ethanol, kyselinu mravenčí, oxid uhličitý a další metabolity. Obligátně homofermentativní druhy, u kterých je podmínkou přítomnost fruktosa-1,6-difosfát aldolasy (EC 4.1.2.13), vytvářejí z hexos téměř výhradně kyselinu mléčnou (Embden-Mayeroffova dráha). Za určitých podmínek, jako je limitované množství sacharidů nebo růst na galaktose jako jediném zdroji energie a za anaerobních podmínek (formát C-acetyltransferasa (EC 2.3.1.54) je citlivá ke kyslíku), byla i u homofermentativních druhů zjištěna schopnost produkovat malé množství kyseliny mravenčí, octové nebo ethanolu. Mezi homofermentativní druhy jsou řazeny laktokoky, streptokoky, enterokoky, pediokoky a některé laktobacily. Obligátně heterofermentativní druhy využívají pentosa-fosfátovou dráhu s klíčovým enzymem fosfoketolasou (EC 4.1.2.9) za vzniku laktátu, ethanolu nebo acetátu a CO₂. Všechny heterofermentativní BMK jsou schopny využít pentosy. Jsou reprezentovány druhy rodu *Leuconostoc* a některými druhy laktobacilů. Některé druhy jako *L. plantarum*, *L. casei* nebo *L. rhamnosus*, které využívají jak homofermentativní tak heterofermentativní dráhu v závislosti na podmínkách prostředí, jsou zařazeny mezi tzv. fakultativně heterofermentativní skupinu mikroorganismů.

Kromě substrátové fosforylace sacharidů mohou BMK k získání energie využívat i další paralelní dráhy, jako jsou deaminace argininu a dekarboxylace aminokyselin. Při metabolismu

argininu vzniká L-citrulin, následně L-ornitin a karbamoylfosfát za uvolnění amoniaku a ATP (Arena *et al.*, 2002). Dekarboxylace aminokyselin je spojena s elektrogenním antiportovým systémem, který může působit proti intracelulární acidifikaci a zároveň vytvářet proton-motivní sílu, tj. zdroj energie pro organismy, které nejsou schopny respirace (Barbieri *et al.*, 2019).

Z potravinářského hlediska je důležitá rovněž schopnost některých druhů metabolizovat citráty, která je závislá na přítomnosti citrát permeasy a citrát lyasy (EC 4.1.3.6). U všech BMK využívajících citráty se citrát metabolizuje nejprve na acetát a oxalacetát, jehož dekarboxylací vzniká pyruvát (Pretorius *et al.*, 2019). Další produkce senzoryicky významných C4 sloučenin probíhá podobným způsobem jako u jiných organismů. V této dráze se syntetizuje α -acetolaktát (α -acetolaktát syntasa, EC 2.2.1.6), který je následně dekarboxylován na diacetyl, jehož redukcí vzniká acetoin a 2,3-butan-diol (Oberman *et al.*, 1982).

Pro růst BMK v mléce, kde jsou nízké koncentrace volných aminokyselin, i pro nejrozumnější technologické aplikace je důležitá jejich proteolytická aktivita. Ta je nejvíce prozkoumána pro rod *Lactococcus* a *Lactobacillus*. BMK jsou auxotrofní pro mnoho aminokyselin, jsou pro ně esenciální, a proto si vyvinuly dostatečný proteolytický systém. První částí tohoto systému je proteasa asociovaná s buněčnou stěnou (serinová proteasa podobná subtilisinu, cell envelope proteinase (CEP), také nazývána laktocepin (EC 3.4.21.96) nebo Prt) (Børsting *et al.*, 2015; Courtin *et al.*, 2002). Je umístěna mimo buňku a iniciuje rozklad kaseinu na středně velké peptidy. Jeden z prvních klasifikačních systémů rozdělil CEP do 3 skupin na základě jejich schopnosti degradovat různé části kaseinu: CEP typu PI přednostně hydrolyzuje β -kasein, zatímco CEP typu PIII také hydrolyzuje α_{S1} -kasein a κ -kasein; byla definována také mezilehlá skupina PI / PIII (Visser *et al.*, 1986). V současné době jsou známy některé specifické proteasy u jednotlivých druhů BMK – PrtP, PrtS, PrtR, PrtH, (Sadat-Mekmene *et al.*, 2011). Dále jsou vzniklé peptidy transportovány přes buněčnou membránu nejméně třemi různými typy specializovaných transportních systémů. Jsou popsány oligopeptidová permeasa (Opp), iontově vázaný transportér (DtpT) pro di- a tripeptidy a transportér ABC (Dpp) pro peptidy obsahující 2 až 9 aminokyselinových zbytků (Sung *et al.*, 2020). Uvnitř buňky jsou peptidy degradovány na aminokyseliny kombinovaným působením mnoha interních peptidas, jako jsou endopeptidasy (PepO, PepF, PepE a PepG), aminopeptidasy (PepN, PepC, PepS, PepA a PepL), tripeptidasy (PepT), dipeptidasy (PepD a PepV) a prolinově specifické peptidasy (PepQ, PepI, PepR, PepX a PepP) (Griffiths & Tellez, 2013). Proteolytickým štěpením kaseinu i syrovátkových bílkovin vzniká celá škála tzv. bioaktivních peptidů, u kterých byla prokázána řada fyziologických funkcí (Nielsen *et al.*, 2017;

Raveschot *et al.*, 2018; Worsztynowicza *et al.*, 2020). Největší pozornost je v tomto směru asi věnována tzv. antihypertenzním peptidům (Ile-Pro-Pro, Val-Pro-Pro) (Rubak *et al.*, 2020).

Lipolytická aktivita (triacylglycerol acylhydrolasy EC 3.1.1.3) je u BMK nízká a byla zjištěna většinou u některých druhů laktobacilů (Silva *et al.*, 2019). Ve starší literatuře byla popsána i u rodů *Lactococcus*, *Leuconostoc*, *Pediococcus* a *Streptococcus* (Meyers *et al.*, 1996). Její význam je akcentován u sýrů s dlouhou dobou zrání, u fermentované zeleniny nebo masných výrobků (Tanasupawat *et al.*, 2015), kde přispívá k rozvoji chuti a zároveň vytváří substráty pro další metabolické reakce za vzniku konečných produktů katabolismu.

Důležitými produkty anabolismu jsou exopolysacharidy (EPS). BMK mohou syntetizovat homopolysacharidy hlavně z jednotek glukosy (*L. acidophilus*, *L. crispatus*, *L. fermentum*) nebo fruktosy (*L. reuteri*), jako substrát používají sacharosu. Struktura heteropolysacharidů je velmi rozdílná, jsou většinou složeny z 3 – 8 molekul monosacharidů jako je glukosa, galaktosa, rhamnosa, fruktosa, polyoly a minoritně z aminocukrů N-acetyl-D-glukosaminu a N-acetyl-D-galaktosaminu. Produkce heteropolysacharidů hlavně u laktobacilů, laktokoků a streptokoků je podrobně prostudována; např. u *S. thermophilus* byly identifikovány geny pro několik desítek glykosyltransferas (Evivie *et al.*, 2017; Miao *et al.*, 2015). Některé EPS mohou být klasifikovány jako prebiotika či mít samy zdravotní benefity prokazatelné již při nízkých koncentracích.

I když jsou BMK autotrofní pro některé vitaminy, jsou zároveň v závislosti na druhu významnými producenty vitaminů skupiny B (foláty, vitamin B₁₂, riboflavin a thiamin) a vitaminu K₂ (LeBlanc *et al.*, 2020).

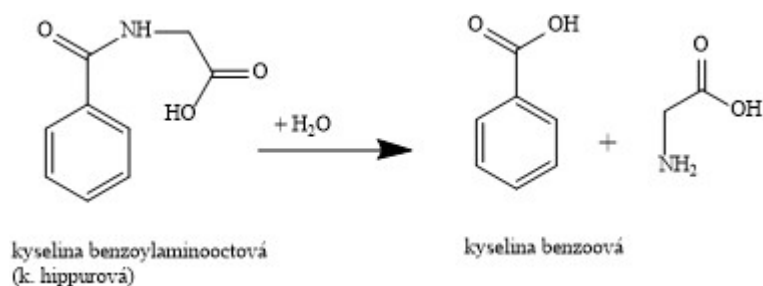
2.3 Antimikrobiální metabolity bakterií mléčného kvašení

Bakterie mléčného kvašení produkují celou řadu nízkomolekulárních antimikrobiálních látek, které mohou úspěšně potlačovat rozvoj kazící mikroflóry ve výrobcích i růst patogenních mikroorganismů, a to jak bakterií, kvasinek, plísní i některých virů. Jsou to hlavně organické kyseliny, ethanol, peroxid vodíku, C₄ sloučeniny, acetaldehyd, bakteriociny, reuterin a jeho deriváty, cyklické peptidy a některé další (Suskovic *et al.*, 2010).

2.3.1 Organické kyseliny

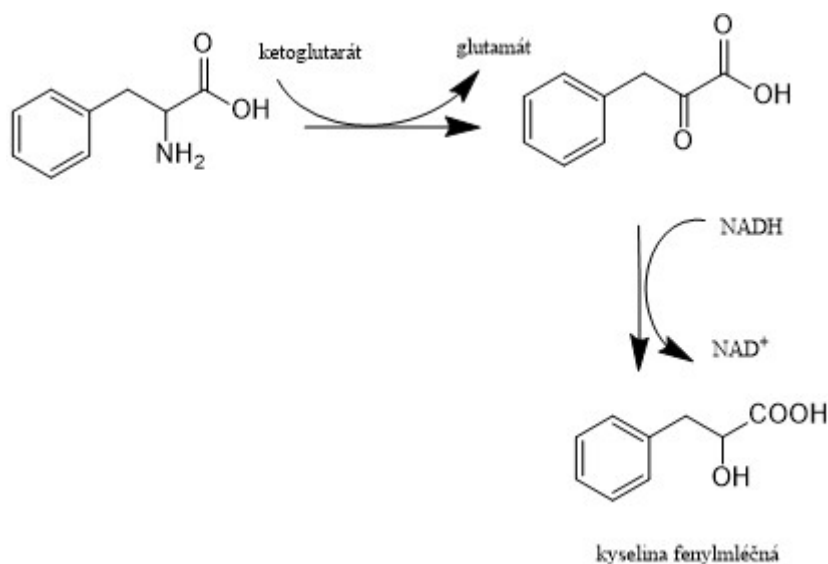
Kyselina mléčná (kyselina 2-hydroxypropanová) je hlavním produktem fermentace. Je to chirální molekula se dvěma optickými enantiomery. Vzniká podle druhu mikroorganismu buď jako L⁺ nebo D⁻ isomer. Kyselina L⁺ mléčná je preferována z hlediska výživy a farmaceutické aplikace a jako výchozí materiál při výrobě biopolymerů, což vyžaduje vysokou enantiomerní čistotu (Castro-Aguirre *et al.*, 2016). Kyselina D⁻ mléčná může být ve vyšších koncentracích pro člověka mírně toxická (Vitetta *et al.*, 2017). Produkce kyseliny mléčné a dalších organických kyselin u BMK (kyselina octová, propionová, mravenčí, jantarová, máselná) se liší v závislosti na kmenu, použitém růstovém médiu a růstových podmínkách (Özcelik *et al.*, 2017). Růst a produkci izomerů kyseliny mléčné a produkci kyseliny octové může ovlivnit přidavek mléčných i nemléčných složek do růstového média. Tato skutečnost byla potvrzena při přidavku syrovátky, syrovátkových bílkovin nebo sladu do mléka při kultivaci vybraných druhů laktobacilů. Výsledky ukázaly, že přidání sladu pozitivně ovlivnilo růst kmenů laktobacilů ve srovnání s růstem v mléce obohaceném o syrovátkové složky. Přidání sladu významně zvýšilo produkci D⁻ izomeru kyseliny mléčné u *L. acidophilus* CCDM 151 a *L. casei* CCDM 198 a produkci kyseliny octové u *L. casei* CCDM 198. (Horáčková *et al.*, 2014 – **Příloha I**). Naopak přidání lněného oleje v množství 0,6% hm. do mléka nemělo vliv na růst a produkci kyselin u *L. acidophilus* CCDM 151, zatímco acidifikační aktivita jogurtové kultury byla o něco nižší ve srovnání s čistým mlékem a spojená s nižším růstem *Streptococcus thermophilus*. Při přidavku lněné vlákniny v množství 7,6% hm. do mléka se stimuloval růst a produkce kyseliny mléčné u *L. acidophilus* CCDM 151 (Bialasová *et al.*, 2017 – **Příloha II**).

Mimo glykolýzu vzniká při kultivaci v mléce nebo na některých rostlinných substrátech i významné množství kyseliny benzoové při degradaci kyseliny benzoylaminoctové (kyselina hippurová) nebo při degradaci fenylalaninu (Park *et al.*, 2017) (Obr. 1). Její produkce byla prokázána u jogurtové kultury (Mroueh *et al.*, 2008).



Obr. 1. Vznik kyseliny benzoové z kyseliny hippurové v mléčných výrobcích

Známou antifungální látkou, která inhibuje růst plísní již ve velmi malých koncentracích, je kyselina fenylmléčná a její p-hydroxy derivát. Vznikají při degradaci fenylalaninu a tyrosinu pomocí nespecifické aminotransferasy - transaminace fenylalaninu na fenylpyruvát, kdy příjemcem aminoskupiny je kyselina α -ketoglutarová. Redukcí fenylpyruvátu vzniká fenyllaktát (Valerio *et al.*, 2016; Cortés-Zavaleta *et al.*, 2014) (Obr. 2). Kyselina fenylmléčná má velice rozsáhlou škálu antifungálního účinku, ale její aktivita byla prokázána i vůči nežádoucím bakteriím (Liu *et al.*, 2018; Mu *et al.*, 2012; Rodriguez *et al.*, 2012). Kyselina fenylmléčná má amfifilní charakter (hydrofobní benzenový kruh a hydrofilní karboxylová skupina), snadněji interaguje s lipidovými složkami i proteiny v buněčné membráně, a poté narušuje její propustnost a integritu. Rovněž interaguje s bakteriální DNA, jak bylo prokázáno u *Escherichia coli* a *Listeria monocytogenes* (Ning *et al.*, 2017).



Obr. 2. Vznik kyseliny fenylmléčné z fenylalaninu u BMK (upraveno dle Chaudhari & Gokhale (2016))

Během metabolismu aminokyselin u BMK může vznikat 2-hydroxyisokapronová kyselina, která inhibuje růst plísní. Jedním z producentů této antifungální látky je *L. reuteri*, který vytváří také kyselinu 4-hydroxyfenylmléčnou se stejným účinkem (Axel *et al.*, 2016).

Organické kyseliny mají různorodý způsob účinku, inhibují Gram-pozitivní i Gram-negativní bakterie, kvasinky i plísně, i když se jejich citlivost značně liší. Mechanismus působení organických kyselin spočívá ve snižování intracelulárního pH po průniku do buňky v nedisociované formě. Nedisociované formy kyselin vzhledem ke svému hydrofobnímu charakteru difundují skrze buněčnou membránu a poté uvnitř buňky disociují. Uvolněním H^+ iontů nastane okyselení cytoplasmy, inhibice aktivního transportu a zastavení různých

metabolických pochodů. Následně dochází k selhání protonmotivní síly v důsledku rozdílné rozpustnosti disociované a nedisociované formy organických kyselin v cytoplasmatické membráně (Reis *et al.*, 2012). Rozsah disociace závisí na pH prostředí (Ammor *et al.*, 2006). Při nízkém pH je velké množství kyseliny mléčné nedisociováno, a tudíž je prostředí toxické pro mnoho mikroorganismů. Při pH 5,0 inhibuje kyselina mléčná sporulující bakterie, ale není účinná vůči kvasinkám a plísním (Yang & Chang, 2000). Je také známo, že organické kyseliny a další metabolity s antifungální či antibakteriální aktivitou vykazují synergický efekt (Dagnas *et al.*, 2015; Cortés-Zavaleta *et al.*, 2014). V modelovém uspořádání byl prokázán synergický efekt kyseliny mléčné a octové k potlačení růstu plísně *Fusarium culmorum*. Obě kyseliny vykazovaly v prvních dnech růstu inhibiční účinky. Po 10 dnech kultivace však byla účinnost značně snížena. Kyselina octová přidaná do agarů v koncentraci 0,05 g/100g způsobila pouze malý rozdíl v růstu plísně vzhledem ke kontrole. Přídavek samotné kyseliny mléčné (pH agarů upraveno na 4,6) byl po 7 dnech neúčinný. Naopak při současném použití obou kyselin byl i po 10 dnech růst plísně potlačen, při okyselení agarů kyselinou mléčnou na pH 4,6 a následném přidavku kyseliny octové (0,1 g/100 g agarů) dosáhl průměr kolonie plísně pouze 0,5 cm. Synergický efekt obou kyselin potvrdil vhodnost použití heterofermentativních kmenů laktobacilů, které tyto kyseliny produkují, jako protektivních kultur (Horáčková *et al.*, 2018a – **Příloha III**).

2.3.2 Acetaldehyd

Acetaldehyd je důležitou aromatickou sloučeninou v mléčných výrobcích. Metabolickým prekurzorem syntézy acetaldehydu je glukosa a meziprodukty glykolýzy – pyruvát a acetyl-CoA (pyruvát dekarboxyláza EC 4.1.1.1). Dalším prekurzorem může být DNA (2-deoxyribosa-5-fosfát), ale nejdůležitější cestou je přeměna threoninu na acetaldehyd a glycin, reakce katalyzovaná threonin aldolase (EC 4.1.2.48). U jogurtové kultury, která produkuje nejvíce acetaldehydu, byl enzym s aktivitou threonin aldolasy identifikován pouze u *Streptococcus thermophilus*. Je to serinová hydroxymethyl transferasa (EC 2.1.2.1) (Liu *et al.*, 2019; Gezginc *et al.*, 2015).

2.3.3 Diacetyl

Diacetyl (2,3-butanedion) je vytvářen některými druhy BMK jako jsou leukonostoky, citrát využívající laktokoky nebo některé laktobacily během fermentace citrátů (Aunsbjerg *et al.*, 2015). Jeho produkce se zvyšuje s prodlužující se dobou fermentace. Jelikož se jedná o těkavou sloučeninu, může během technologických operací docházet k jeho ztrátám. Je prokázáno, že

diacetyl vykazuje antifungální účinek, i když mechanismus působení na růst plísní nebyl dosud objasněn (Oliveira *et al.*, 2014).

2.3.4 Peroxid vodíku

K produkci peroxidu vodíku dochází hlavně u mikrobiálních druhů, které postrádají enzymy pro jeho eliminaci, jako je katalasa nebo peroxidasa. U BMK je jeho produkce sledována zvláště u rodu *Lactobacillus*, ze skupiny *L. acidophilus*. Tato skupina zahrnuje několik blízce příbuzných druhů – *L. johnsonii*, *L. gasseri*, *L. acidophilus*, *L. helveticus*, *L. crispatus*. Řada z nich tvoří důležitou část vaginálního konsorcia (Tachedjian *et al.*, 2018; Martín & Suárez, 2010). Až 80 % izolátů laktobacilů z vaginálního traktu tvoří peroxid vodíku (Mijač *et al.*, 2006), což bylo potvrzeno i ve studii Kumherová *et al.* (2020 – **Příloha IV**). Zástupci druhu *L. crispatus* produkovaly 0,3 – 4,2 mg H₂O₂/l. Tyto izoláty vykazovaly zároveň zvýšenou aktivitu vůči *Candida albicans*, což je jeden z hlavních původců infekcí vaginálního traktu (Zangl *et al.*, 2019). Peroxid vodíku samotný nebo v kombinaci s halogenidem a peroxidasou, které jsou přítomny ve vaginálních sekretech, má silné toxické vlastnosti. Byly zdokumentovány studie *in vitro* potvrzující, že peroxid produkující laktobacily mohou potlačovat infekce HIV-1 (Schwebke, 2001), *Gardnerella vaginalis*, *Neisseria gonorrhea* a *Candida albicans* (Breshears *et al.*, 2015; Kumherová *et al.*, 2020 – **Příloha IV**), neboť peroxid generuje ve vaginální tekutině cytotoxické reaktivní molekuly kyslíku. Produkce peroxidu byla prokázána i u izolátů z potravin u rodu *Lactococcus* (Ito *et al.*, 2003), *L. delbrueckii* subsp. *lactis* (Batdorj *et al.*, 2007) nebo u nozokomiálních izolátů rodu *Enterococcus* (Moy *et al.*, 2004).

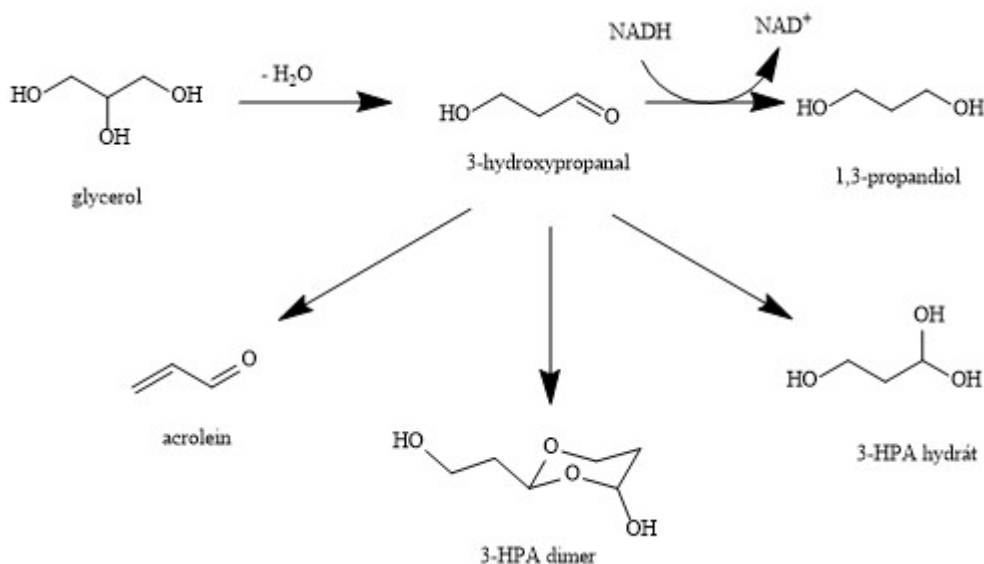
Peroxid vodíku je silné oxidační činidlo, ale reaguje pomalu s biologickým materiálem, pokud není přítomen katalyzátor, např. v podobě Cu²⁺ nebo Fe²⁺ v živých systémech (Thomas *et al.*, 1994). Antimikrobiální účinek H₂O₂ je s největší pravděpodobností výsledkem oxidace sulfhydrylových skupin způsobujících denaturaci řady enzymů a oxidaci membránových lipidů, čímž se zvyšuje propustnost membrány. Může vyvolávat také vznik volných radikálů, které poškozují DNA (Ammor *et al.*, 2006).

Peroxid vodíku je produkován během hlavních metabolických drah (získávání energie) za pomoci oxidas jako je pyruvát oxidasa (EC 1.2.3.3), laktát oxidasa (EC 1.13.12.4) nebo NADH peroxidasa (EC 1.11.1.1). Enzymatické pochody vedoucí ke vzniku peroxidu vodíku u BMK nejsou ještě zcela prozkoumány. Uvádí se také, že jeho vznik může být regulován NADH-dependent flavin mononukleotid reduktasou (EC 1.5.1.36) (Valladares *et al.*, 2015; Hertzberger *et al.*, 2014).

2.3.5 Reuterin

Reuterin byl původně popsán jako antimikrobiální látka produkovaná výlučně druhem *L. reuteri* (Talarico *et al.*, 1988). Do současné doby ale byla jeho přítomnost objevena i u dalších druhů laktobacilů, např. *Lactobacillus coryniformis* nebo *Lactobacillus collinoides* (Martín *et al.*, 2005; Sauvageot *et al.*, 2000).

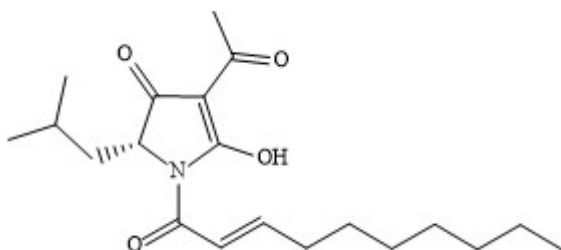
Bakterie produkují v logaritmické fázi růstu během metabolismu glycerolu (slouží jako vnější akceptor vodíku během fermentace glukosy) 3-hydroxypropanal (3-HPA) za účasti glycerol dehydratasy (EC 4.2.1.30) závislé na koenzymu vitaminu B₁₂. Následně je 3-HPA metabolizován na konečný produkt propan-1,3-diol oxidoreduktasou závislou na NAD⁺ (EC 1.1.1.20). Ve stacionární fázi se v prostředí hromadí převážně 3-HPA, který v roztoku vytváří reuterin, což je směs 3-HPA, jeho hydrátu (1,1,3-propanetriol), dimeru (2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxan) a akroleinu (Engels *et al.*, 2016). Základní schéma je uvedeno na Obr. 3. Produkce reuterinu byla potvrzena nejen v laboratorních kultivačních médiích, ale rovněž přímo ve fermentovaných výrobcích (Ortiz-Rivera *et al.*, 2017). Aktivita reuterinu je širokospektrální, je rozpustný ve vodě, stabilní v širokém rozmezí pH a rezistentní vůči působení enzymů (Asare *et al.*, 2020; Montiel *et al.*, 2014).



Obr. 3. Základní schéma vzniku reuterinu (3-HPA = 3-hydroxypropanal; hydrát a dimer), upraveno dle Asare *et al.* (2020)

Poměrně vzácným znakem u několika kmenů *L. reuteri* je produkce reutericyklinu (Gänzle *et al.*, 2000). Jedná se o N-acylovanou kyselinu tetramovou (Obr. 4)

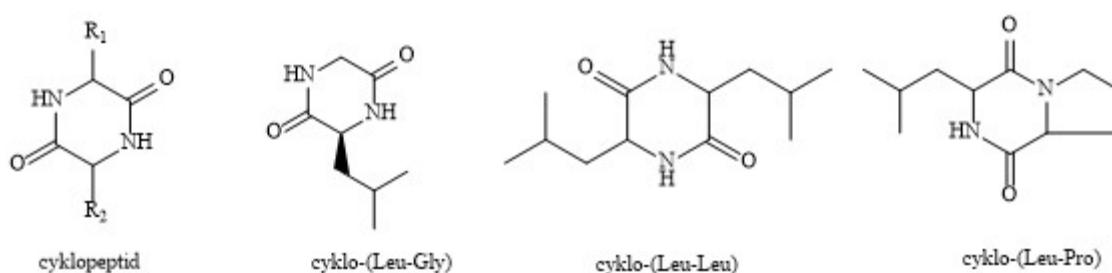
s bakteriostatickým a baktericidním účinkem proti Gram-pozitivním bakteriím, včetně *Staphylococcus aureus*, *Listeria innocua*, *Enterococcus faecium*, *Clostridium difficile* a rodu *Bacillus*, které způsobují zkažení chleba (Höltzel *et al.*, 2000; Lin *et al.*, 2015).



Obr. 4. Reutericyclin

2.3.6 Cyklické dipeptidy

Bakterie mléčného kvašení, zvláště z původního rodu *Lactobacillus*, produkují biologicky aktivní cyklické dipeptidy, u kterých byla ve většině případů potvrzena antifungální aktivita. Zaznamenan byl antifungální účinek u cyklických dipeptidů cyklo(Gly-L-Leu), cyklo(L-Phe-L-Pro), cyklo(L-Phe-trans-4-OH-L-Pro), cyklo(Phe-OH-Pro), cyklo(Leu-Leu) nebo cis-cyklo(L-Val-L-Pro), které byly produkovány druhy *L. plantarum*, *L. sakei*, *L. coryniformis* a *Pediococcus pentosaceus* (Kwak *et al.*, 2014; Sangmanee & Hongpattarakere, 2014; Dalié *et al.*, 2010; Yang & Chang, 2010). Příklady některých dosud identifikovaných peptidů pomocí X-ray krystalografie jsou uvedeny na Obr. 5. Citlivé k jejich účinku byly plísně *Ganoderma boninense*, *Aspergillus fumigatus*, *A. nidulans*, *Fusarium sporotrichioides*, *Penicillium commune* a *P. roqueforti*.



Obr. 5. Příklady cyklických peptidů s antifungální aktivitou produkovaných laktobacily

2.3.7 Bakteriociny

Bakteriociny jsou malé, ribosomálně syntetizované peptidy, které jsou antimikrobiálně účinné většinou proti úzce příbuzným rodům. Jejich účinek je založen na blokování syntézy buněčné stěny (váží se na lipid II) a vytváření pórů, kterými může unikat obsah cytosolu. Jejich

syntéza byla prokázána u řady Gram-negativních i Gram-pozitivních bakterií včetně BMK (Perez *et al.*, 2014). Na základě jejich struktury, vlastností i účinku jsou rozděleny do tří tříd (DeVuyst & Leroy, 2007) :

- třída I – lantibiotika: postranslačně modifikované peptidy obsahující lanthionin; rozdělují se dále na podtřídu I (lineární, nisin) a podtřídu B (globulární se záporným nebo nulovým nábojem);
- třída II – tepelně stabilní peptidy vyžadující specifické receptory, které zahrnují bakteriociny typu pediocin s vysokou antilisteriální aktivitou (pediocin, enterocin), dvoupeptidové s odlišnou primární strukturou (laktacin, plantaricin), cyklické, kde je kovalentně vázán N- a C-konec (laktocyklicin) a ne-pediocinové lineární peptidy (lakticin, weissellicin);
- třída III – velké tepelně labilní peptidy, ne zcela dobře charakterizované, jsou to lytické proteiny často klasifikované jako murein-hydrolasy (helveticin).

Navzdory rozmanitosti i potvrzení účinnosti antimikrobiálních peptidů produkovaných BMK jsou komerčně dostupné pouze nisin (komerční produkt NisaplinTM, 2,5 % nisin), pediocin (preparát ALTATM 2341) či lakticin 3147. Na základě Nařízení Evropského parlamentu a Rady (ES) č. 1333/2008 ze dne 16. prosince 2008 o potravinářských přídatných látkách je povoleno použití nisinu pouze v některých potravinách, a to jsou zakysaná smetana, maskarpone, zrající a tavené sýry, tepelně ošetřené masné výrobky, pasterovaná tekutá vejce a semolinové a tapiokové pudinky a jim podobné produkty. Bezpečné koncentrace jeho použití byly nedávno zrevidovány Panelem EFSA (EFSA, 2017). Vzhledem k nárůstu počtu kmenů rezistentních na antibiotika a rostoucí poptávce po minimálně zpracovaných potravinách je výzkum a izolace nových antimikrobiálních peptidů v současné době jedním z hlavních biotechnologických aplikací BMK, ať už pro potravinářský průmysl nebo pro aplikace v humánní a veterinární medicíně. Komerční aplikace je vázána na optimalizaci podmínek pro syntézu bakteriocinů a jejich následného čištění (Mora-Villalobos *et al.*, 2020). Možné zvýšení produkce nisinu bylo testováno po přidavku L-lanthionu získaného selektivní syntézou z cystin-dimethylesteru do kultivačního média (koncentrace 0,78; 3,9 a 7,8 mg/l). Syntéza nisinu se u všech testovaných kmenů *Lactococcus lactis* subsp. *lactis* v porovnání s kontrolním stanovením zvýšila (Feldekova *et al.*, 2020 – **Příloha V**).

Jako slibná technologie se ukazuje rovněž začlenění bakteriocinů do pružných fólií pro balení potravin. Antimikrobiální obal představuje oproti konvenčním obalům velkou

výhodu, protože se vyhýbá přidávání konzervačních látek přímo do potravin, což podporuje současný trend výroby „zdravějších“ potravin (Santiago-Silva *et al.*, 2009).

Potravinářský průmysl se v poslední době ale stále více zaměřuje na využití tzv. protektivních kultur, u kterých byla často prokázána i tvorba bakteriocinů zamezujících růstu nežádoucí mikroflóry (Da Costa *et al.*, 2019). Příkladem může být komerční kultura Bactoferm™ F-LC (Christian Hansen, Dánsko) obsahující *Pediococcus acidilactici* a *L. curvatus* s produkcí pediocinu a bavaricinu, které zamezují růstu *Listeria monocytogenes*.

2.4 Antimikrobiální aktivita, protektivní kultury

Bylo publikováno mnoho studií, které dokazují účinné potlačení růstu nežádoucích mikroorganismů pomocí BMK v modelových systémech i v potravinách. Jejich působení je chápáno jako multifaktoriální, neboť v prostředí se vždy uplatňuje kombinace různých antimikrobiálních metabolitů, které byly popsány v kap. 2.3. Jako příklad může být uvedeno několik rozsáhlých studií publikovaných v posledním období, ať už se týkají aktivity vůči bakteriím, kvasinkám, plísním nebo virům, i když každé z těchto oblastí by mohlo být věnováno samostatné pojednání.

Z hlediska antibakteriální aktivity vůči Gram-pozitivním i Gram-negativním bakteriím jsou prozkoumány stovky izolátů BMK. Nejčastěji se autoři zaměřují na potlačení růstu podmíněně patogenních bakterií, jako jsou *Bacillus* spp., *Clostridium* spp., *Staphylococcus aureus*, *Streptococcus* spp., *Listeria* spp., *Pseudomonas* spp., *Escherichia coli*, *Salmonella* spp., (Djadouni & Kihal, 2012).

Aktivita vůči kvasinkám již byla zmíněna v kap. 2.3.4. Z hlediska potravinářského má význam pro potlačení nežádoucí kontaminace druhy rodů *Kluyveromyces*, *Debaromyces* či *Candida* při výrobě mléčných výrobků typu jogurt, tvaroh nebo i při přípravě kvasů v pekárenském průmyslu (Cizeikiene *et al.*, 2013).

Antifungální aktivita je často spojována s činností druhu *L. plantarum*, který je schopen potlačovat růst řady plísní, jako jsou *Aspergillus niger*, *A. flavus*, *Fusarium culmorum*, *Penicillium roqueforti*, *P. expansum*, *P. chrysogenum* a *Cladosporium* spp. (Russo *et al.*, 2016). Účinné potlačení růstu plísně *Fusarium culmorum* bylo potvrzeno také ve studii Oliveira *et al.* (2015). Rozsáhlé review týkající se nejen antifungální aktivity BMK, ale i jejich schopnosti adsorbovat, degradovat nebo detoxikovat fungální mykotoxiny včetně ochratoxinů, aflatoxinů

a toxinů rodu *Fusarium*, bylo vypracováno autory Sadiq *et al.* (2019). Aktivita různých kmenů *L. plantarum*, *L. harbinensis* nebo *L. rhamnosus* vůči plísním byla testována také při výrobě jogurtu v pilotním měřítku (Leyva Salas *et al.*, 2018).

Aktivita vůči virům je testována především v souvislosti s probiotickými vlastnostmi některých kmenů BMK (viz kap. 3.3.2.). Její podstatou může být přímá interakce viru s bakteriální buňkou, jak bylo prokázáno u *Enterococcus faecium* a viru prasečí chřipky (Wang *et al.*, 2013). Dalším mechanismem je přímá stimulace imunitního systému (Kawashima *et al.*, 2011) nebo produkce aktivních metabolitů v případě antagonismu vůči HIV virům (Tyssen *et al.*, 2018) či virům chřipky (Kvak *et al.*, 2013). Zcela aktuálně je pozornost samozřejmě také zaměřena na použití určitých kmenů BMK proti viru způsobujícímu onemocnění COVID-19 (Baud *et al.*, 2020). I když jsou dostupná data zatím dosti omezená, byla zjištěna silná korelace mezi progresí onemocnění a složením střevní mikrobioty. Někteří pacienti s COVID-19 vykazovali intestinální mikrobiální dysbiosu s významnou redukcí počtu buněk rodů *Lactobacillus* a *Bifidobacterium* (Xu *et al.*, 2020). Jelikož některé BMK jsou schopny inhibovat ACE2 enzym či blokovat a interferovat s jeho receptorem, což je i hlavní hostitelský receptor viru SARS-CoV-2, předpokládá se jejich využití i při léčení této choroby (Olaimat *et al.*, 2020; Ayyash *et al.*, 2020). Například během fermentace mléka bylo prokázáno, že některé laktobacily uvolňují peptidy s vysokou afinitou k ACE (Chen *et al.* 2018). Zcela nedávno bylo publikováno, že kmen *Paenibacillus* sp. B38 přirozeně produkuje karboxypeptidasy homologní s ACE2 ve struktuře a funkci (Minato *et al.* 2020).

Pokud se vrátíme k aplikaci protektivních kultur v potravinářství, je nutné připomenout, že v současnosti existuje již celá řada komerčních mikrobiálních přípravků určených k potlačení nežádoucí mikroflóry, zvláště v mlékárenském a masném průmyslu. Mezi významné producenty se v tomto segmentu řadí firmy Christian Hansen (Dánsko) nebo Danisco (Dánsko). Ch. Hansen dodává protektivní kultury pro fermentované mléčné výrobky (FRESHQ[®]), pro polotvrdé a tvrdé sýry (BIOSAFE[™]), pro masné výrobky a zeleninové pokrmy (SAFEPRO[®]) či vína a fermentované nápoje (VINIFLORA[®]). Příklady protektivních kultur firmy Danisco jsou shrnuty v Tab. 2.

Tab. 2. Příklady komerčních protektivních kultur

Kultura	Účinek	Aplikace
HOLDBAC® YM-C Plus HOLDBAC® YM-B Plus	kontrola růstu kvasinek, plísni a některých heterofermentativních laktobacilů	čerstvé fermentované potraviny bílý sýr
HOLDBAC® LC	kontrola růstu leukonostoků, heterofermentativních laktobacilů a enterokoků	tvrdé a polotvrdé sýry
HOLDBAC® Listeria	kontrola růstu listerií	měkké a mazové sýry, masné výrobky, mleté maso
HOLDBAC® YM VEGE	zlepšení kvality	fermentované rostlinné produkty a nápoje
HOLDBAC® YM-XPM	kontrola růstu kvasinek a plísni	jogurty a fermentované mléčné výrobky
HOLDBAC® YM-XPB	kontrola růstu kvasinek a plísni	všechny typy sýrů

Důležitým aspektem aplikace protektivních kultur je rovněž jejich vzájemná kompatibilita s původními kyselinotvornými kulturami. Bylo zjištěno, že některé kmeny *L. plantarum* mohou potlačovat růst *L. delbrueckii* subsp. *bulgaricus* v jogurtové kultuře (Moreno-Montoro *et al.*, 2018; Horáčková – nepublikovaná data). Antimikrobiální účinek může být rovněž ovlivněn při vzájemné kokultivaci různých kmenů. V práci Horáčková *et al.* (2018b – **Příloha VI**) byla potvrzena snížená antifungální aktivita proti plísni *Fusarium culmorum* u kmene *L. plantarum* CCDM 583 v případě společné kultivace s dalšími kmeny *L. plantarum* CCDM 583 a MP2. Tento jev nebyl pozorován v testech proti *Penicillium expansum*. Vzájemná inhibice byla potvrzena agarovou difúzní metodou u všech tří kmenů laktobacilů testovaných proti sobě. Výsledky ukazují, že k zajištění požadovaných vlastností je nezbytný pečlivý výběr vzájemně kompatibilních kmenů. Je to důležité zejména v případech, kdy se k výrobě potravin používají další kultury. Různé kmeny laktobacilů se mohou ve smíšených kulturách navzájem inhibovat, což může vést k následnému snížení jejich antifungálního účinku.

Při aplikaci BMK do nemléčných výrobků se může pozitivně projevit také jejich metabolická aktivita při možnosti přeměny některých substrátů na antifungální metabolity. Jsou publikovány četné studie o metabolismu fenolických sloučenin pomocí BMK, které následně zvyšují antifungální a antioxidační aktivitu výsledných produktů. Souhrn výskytu fenolických látek, jejich význam a metabolismus některými druhy laktobacilů byl zpracován v práci Koval *et al.* (2020 – **Příloha VII**).

3 Bakterie mléčného kvašení jako probiotika

3.1 Stručná historie a definice probiotik

Nezastupitelnou roli hrají BMK spolu se zástupci rodu *Bifidobacterium* v konceptu probiotických aplikací. Ten vychází z historických empirických pozorování, která po staletí potvrzovala prospěšnost konzumace fermentovaných výrobků. První takovéto zmínky jsou uvedeny v ajurvédských spisech z doby asi 1000 let př. n. l. Bible zmiňuje, že Abraham vděčil za svoji dlouhověkost a plodnost konzumaci kyselého mléka. Autor nejvýznamnější přírodovědecké encyklopedie starého Říma Plinius Starší v 1. st. n. l. poprvé písemně popisuje, že některé nomádské kmeny „umí zahušťovat mléko na látku příjemné kyselosti“. V novověké Evropě byl fermentovaný výrobek (jogurt) představen králem Františkem I. Francouzským v roce 1542, kterému byl nabídnut tureckým lékařem pro léčbu těžkého průjmu. Ale teprve s rozvojem mikrobiologie a znalostí o jednotlivých druzích mikroorganismů byl na počátku 20. stol. položen základ konceptu tzv. probiotik v práci ruského vědce, ředitele Pasteurova institutu v Paříži a nositele Nobelovy ceny za imunologii, Ilji Mečnikova (*Prolongation of life: Optimistic Studies*, 1907). Přibližně ve stejnou dobu došlo ve stejném institutu k dalšímu důležitému objevu Henry Tissiera, který izoloval bifidobakterie (*Bacillus bifidum*) ze střevní flóry kojených dětí. Pozoroval, že tyto bakterie mohou u kojenců zmírnit průjem.

Vlastní termín „probiotikum“ byl však poprvé použit ve vědecké literatuře až v roce 1964 německým bakteriologem Wernerem Kollathem (Korhonen, 2009). Psal o probiotikách jako o protikladu ke škodlivým antibiotikům a definoval je jako účinné látky, které jsou nezbytné pro zdravý vývoj života. O rok později, v roce 1965, Lilley a Stillwell vydali publikaci *Probiotics: Growth-Promoting Factors Produced by Microorganisms*, kde jsou probiotika definována jako látky, které jsou vylučovány jedním mikroorganismem a které stimulují růst jiného organismu. V 70. letech 20. stol. byl termín používán pro doplňky stravy pro zvířata, které měly příznivý účinek na hostitelské zvíře ovlivněním jeho střevní flóry (Parker, 1974). Teprve v roce 1989 byla probiotika předefinována na živé mikroorganismy, které příznivě ovlivňují hostitele zlepšením jeho střevní mikrobiální rovnováhy (Fuller, 1989). Požadavek na viabilitu je zakotven rovněž v oficiální definici WHO/FAO z roku 2002 – „probiotika jsou živé mikroorganismy, které při podávání v dostatečném množství poskytují hostiteli zdravotní přínos“ (WHO/FAO, 2002). Tato definice byla později upřesněna s tím, že probiotika (probiotické preparáty a potraviny) musí mít definovány obsažené mikroorganismy

a požadovaný počet životaschopných buněk na konci doby expirace, musí být vhodně ověřeny jejich zdravotní benefity na konzumenta a musí být bezpečná pro zamýšlené použití (Hill *et al.*, 2014). Tyto body ve svém prohlášení pak zopakovala i Mezinárodní vědecká asociace pro probiotika a prebiotika (International Scientific Association for Probiotics and Prebiotics) (ISAPP, 2018), kde byly shrnuty následovně:

- charakteristika mikroorganismu dostatečná k identifikaci probiotika na úroveň rodu, druhu a kmene;
- zařazení a pojmenování probiotika podle vědecky platné nomenklatury;
- probiotický název obsahuje označení kmene;
- bezpečnost probiotika je prokázána pro zamýšlené použití;
- probiotický kmen je uložen v mezinárodní sbírce kultur;
- probiotikum má zdravotní přínos prokázaný alespoň v jedné studii na lidech;
- výrobky s probiotiky obsahují až do konce doby použitelnosti dostatečné množství mikroorganismů, které poskytují zdravotní přínos.

S postupujícími znalostmi o mikrobiotě lidského těla se v současné době objevují další termíny související s pozitivními zdravotními účinky mikroorganismů:

- postbiotika (probiocuticals) – metabolické produkty probiotik, bioaktivní sloučeniny se zdravotními benefity produkované během fermentace; mohou zahrnovat krátké mastné kyseliny, exopolysacharidy, enzymy, bílkoviny a peptidy, vitaminy, biosurfaktanty apod. (Kumar *et al.*, 2020; Nataraj *et al.*, 2020; Wegh *et al.*, 2019);
- paraprobiotika (parabiotika, ghost probiotics) – inaktivované mikrobiální buňky probiotik neporušené nebo lyzované obsahující buněčné složky, jako jsou peptidoglykany, kyselina teichoová, povrchové proteiny atd. (Nataraj *et al.*, 2020);
- psychobiotika - živý organismus, který při požití v dostatečném množství vytváří zdravotní přínos pro pacienty trpící psychiatrickým onemocněním (Dinan *et al.*, 2013); ovlivňují funkce a chování související s centrálním nervovým systémem zprostředkované osou střevo-mozek prostřednictvím imunitních, humorálních, nervových a metabolických cest (Cheng *et al.*, 2019).

3.2 Nejčastěji používané mikroorganismy jako probiotika

Původně byly probiotické mikroorganismy vybírány převážně z rodů *Lactobacillus* a *Bifidobacterium*, u kterých se jednalo ve většině případů o izoláty z trávicího traktu. Přehled nejběžnějších probiotik je uveden v Tab. 3. Pro úplnost jsou zmíněny i druhy, které nepatří mezi BMK. Výhodou aplikace BMK je, že mají doloženu dlouhou historii použití v lidské výživě, a tudíž jsou považovány za zcela bezpečné. Je nutno zdůraznit, že u každého druhu probiotika se vždy jedná o konkrétní kmeny, neboť probiotické vlastnosti jsou výhradně kmenově specifické. V současné době se ale již upouští od striktního požadavku na humánní původ probiotických kmenů, či v případě tzv. paraprobiotik od jejich životaschopnosti v gastrointestinálním traktu (GIT) (Zendeboodi *et al.*, 2020; Kesen & Aiyegoro, 2018).

Tab. 3. Nejběžnější druhy probiotických bakterií (vždy konkrétní kmeny)

původní rod <i>Lactobacillus</i>	ostatní BMK	rod <i>Bifidobacterium</i>	ostatní druhy
<i>L. acidophilus</i>	<i>Streptococcus thermophilus</i>	<i>B. animalis</i>	<i>Escherichia coli</i>
<i>L. gasseri</i>	<i>Enterococcus faecium</i>	<i>B. bifidum</i>	<i>Bacillus subtilis</i>
<i>L. crispatus</i>	<i>Enterococcus faecalis</i>	<i>B. breve</i>	<i>Bacillus coagulans</i>
<i>L. delbrueckii</i>	<i>Pediococcus acidilactici</i>	<i>B. lactis</i>	<i>Bacillus clausii</i>
<i>L. helveticus</i>	<i>Pediococcus pentosaceus</i>		<i>Clostridium butyricum</i>
<i>L. casei/paracasei</i>			<i>Saccharomyces cerevisiae</i> var. Boulardii
<i>L. plantarum</i>			<i>Propionibacterium freudenreichii</i>
<i>L. rhamnosus</i>			<i>Propionibacterium jensenii</i>
<i>L. reuteri</i>			

K doplnění přehledu mikroorganismů se zdravotním účinkem je nutné zmínit, že v důsledku stále se rozvíjejících znalostí o lidské střevní mikrobiotě a důsledcích střevní dysbiosy je pozornost upřena také na použití komenzálních bakterií GIT, které nespádají do skupiny BMK, ani bifidobakterií. Tyto mikroorganismy se označují jako „probiotika nové generace“. Byly většinou identifikovány na základě srovnávací analýzy složení mikrobioty mezi zdravými a nezdravými jedinci a patří do různých rodů. Nemají dlouhou historii bezpečného používání a jejich bezpečnost proto není považována za prokázanou (Martín & Langella, 2019). Z těchto komenzálních izolátů jsou nejčastěji zmiňovány druhy *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, *Bacteroides ovatus* a zástupci rodu *Clostridium*, klastyry IV, XIVa, XVIII (Zhang *et al.*, 2019; El Hage *et al.*, 2017; O'Toole *et al.*, 2017; Martín *et al.*, 2017; Heinken *et al.*, 2014).

3.3 Kritéria pro výběr probiotických mikroorganismů

Při výběru nových mikroorganismů jako probiotik nebo látek pro biologickou kontrolu je třeba vzít v úvahu řadu vlastností. Kritéria pro výběr probiotických mikroorganismů lze rozdělit do tří skupin:

- i. vlastnosti a charakteristika mikroorganismu;
- ii. funkční vlastnosti mikroorganismu, zdravotní účinky a studie;
- iii. technologické vlastnosti mikroorganismu.

Je nutné připomenout, že některá níže uvedená kritéria již nejsou v dnešní době akcentována. Jedná se např. o požadavek na izolát z trávicího traktu člověka, neboť se běžně používají probiotika, která byla izolována i z jiných zdrojů.

Všechna testovaná kritéria jsou pak striktně kmenově specifická a mohou se významně lišit v rámci jednoho druhu mikroorganismu.

3.3.1 Původ a charakteristika probiotických mikroorganismů

Dle původních předpokladů o lepší adaptabilitě mikroorganismů v těle bylo požadováno, aby probiotický mikroorganismus pocházel ze zdroje (ať už humánního nebo zvířecího), pro který je ve své aplikaci určen (WHO/FAO, 2002). Samozřejmě musí být přesné taxonomické určení a zařazení daného mikroorganismu.

3.3.1.1 *Odolnost vůči podmínkám trávicího traktu*

Vzhledem k tomu, že probiotika mají působit v trávicím traktu v živém stavu, musí být odolná vůči podmínkám trávicího traktu. V ústní dutině se setkávají s lysozymem a amylasami, daleko významnější stresové podmínky ale znamená prostředí žaludku (nízké pH, pepsin), žlučové soli a další trávicí enzymy v tenkém střevě. Hodnota pH v lidském žaludku se pohybuje od 1,5 během půstu do 4,5 po jídle. Obvykle se ve studiích odolnosti používá médium simulující prostředí žaludku o pH 2 – 3 a další s obsahem 0,3 – 2,0 % žlučových solí (Fontana *et al.*, 2013).

Tolerance ke žluči je jednou ze stěžejních vlastností probiotických mikroorganismů. Za normálních fyziologických podmínek se může v lidském tenkém střevě vyskytovat gradient koncentrace žlučových solí od 40 mmol/l k méně než 1 mmol/l (2 – 0,05 %) (Islam *et al.*, 2011). Někteří autoři uvádějí hodnoty pouze v rozmezí 2 – 1,5 % v první hodině trávení s následným poklesem na hodnotu 0,3 % (Noriega *et al.*, 2004). Kromě normální fyziologické funkce pro trávení tuků zajišťuje žluč svojí toxicitou silnou antimikrobiální aktivitu vůči mikroorganismům, které nejsou na přítomnost žlučových solí adaptovány. Hlavní složku žluči

(přibližně 50–60 % organických složek) tvoří žlučové kyseliny. Kyselina cholová a chenodeoxycholová jsou syntetizovány *de novo* v játrech z cholesterolu (prakticky pouze z LDL částic) multienzymovým procesem. Klíčovým enzymem této metabolické dráhy je cholesterol-7 α -hydroxylasa (EC 1.14.13.17), která katalyzuje přeměnu cholesterolu na 7 α -hydroxycholesterol. Na hydrofobní steroidní jádro je poté napojena aminokyselina glycin nebo taurin za vzniku hlavních molekul konjugovaných žlučových kyselin amfifilního charakteru (kyselina glykocholová, glykochenodeoxycholová, taurocholová a taurochenodeoxycholová). Konjugované i dekonjugované formy žlučových kyselin mají antibakteriální vlastnosti s prokázanou vyšší citlivostí Gram-pozitivních bakterií (Hofmann & Eckman, 2006).

Sekrece žluči je jedním z mechanismů, které udržují rovnováhu mezi lidským hostitelem a střevní mikrobiotou. Vliv žlučových solí na mikroorganismy byl intenzivně studován jak u patogenních bakterií, tak konkrétně i u probiotik (Merritt & Donaldson *et al.*, 2009; Bustos *et al.*, 2012). Tolerance k žluči umožňuje lepší kolonizaci v lidském GIT. Obecně se předpokládá, že kmeny izolované z GIT jsou odolnější vůči vlivu žlučových solí než kmeny z jiných zdrojů. V posledních letech však úsilí o nalezení nových probiotických kmenů vedlo k testům bakterií (zejména BMK), které pocházejí z různých fermentovaných mléčných a nemléčných produktů nebo zvířecího GIT. Příklady těchto studií jsou shrnuty v Tab. 4.

Tab. 4. Příklady testovaných mikroorganismů na odolnost vůči různým koncentracím žluči a jejich původ (dle Horácková *et al.*, 2018)

Mikroorganismus	Původ	Koncentrace žluči (% hm.)
BMK	fermentované olivy	0,5
<i>Lactobacillus</i> spp.	sýry	0,4 – 2,0
<i>Lactobacillus</i> spp., <i>Weissella</i> spp.	fermentovaná zelenina	0,3 – 0,5
<i>Lactobacillus</i> spp.	mléčné výrobky	0,15 – 0,45
<i>Lactobacillus</i> spp., <i>Pediococcus</i> spp., <i>Oenococcus oeni</i>	červené víno	0,06 – 1,0
<i>L. brevis</i> , <i>Pediococcus pentosaceus</i>	ječmen, olivy, maso	0,3 – 1,0
<i>Lactobacillus fermentum</i>	GIT drůbež, prase	0,3
<i>Lactobacillus plantarum</i> WCFS1		0,05 – 0,15
<i>Lactobacillus</i> spp., <i>Leuconostoc</i> spp.	ovocný nápoj	0,15 – 0,30
<i>Enterococcus durans</i>	měkký sýr	0,1 – 1,5
<i>Propionibacterium</i> spp.	DSM, ATCC	0,06 – 0,30
<i>Propionibacterium</i> spp.	sýry	0,06 – 0,30
<i>Bacillus</i> spp.	sójová omáčka	0,3
<i>Bacillus polyfermenticus</i>	kimči	0,3
kvasinky	zelené olivy	0,3

Bylo popsáno několik mechanismů, které vysvětlují schopnost mikroorganismů tolerovat a růst v prostředí žlučových solí. Jejich podrobnější popis je předmětem souhrnné práce Horáčková *et al.* (2018c – **Příloha VIII**). Stručně lze vyjmenovat následující:

- prvním možným mechanismem je změna ve fyzikálně-chemických vlastnostech buněčné stěny, zejména změny ve složení bakteriálních proteinů a membránových lipidů, které mohou přispět ke snížení difúze žlučových solí;
- druhou možností je využití efluxních pump - aktivní efluxní pumpy a transportéry mohou chránit buňky před toxickými sloučeninami, jako jsou žlučové soli, jiné detergenty, antibiotika, kovy nebo různé stresové faktory; efluxní pumpy jsou všudypřítomné systémy popsány jak pro Gram-pozitivní, tak Gram-negativní bakterie;
- intenzivně je studována aktivita hydrolasy žlučových solí (BSH), choloylglycinhydrolasy (EC 3.5.1.24), která může přispět nejen ke snížení toxicity žluči, ale je také spojena s terapeutickým účinkem snížení hladiny sérového cholesterolu.

Hydrolasa žlučových solí je konstitutivní intracelulární enzym, který katalyzuje hydrolýzu amidové vazby, kdy se aminokyselina (glycin nebo taurin) uvolňuje ze steroidního jádra žlučových kyselin. Enzym je klasifikován jako N-koncová nukleofilní hydrolasa s cysteinovým zbytkem na N-konci, jehož thiolová skupina je katalyticky aktivní místo (Begley *et al.*, 2005; Begley *et al.*, 2006). V aktivním centru jsou navíc zahrnuty cystein 2, arginin 18, kyselina asparagová 21, asparagin 175 a arginin 228. Enzym BSH vykazuje vysokou podobnost v aminokyselinové sekvenci s penicilin V amidasou izolovanou z *Bacillus sphaericus* (Ridlon *et al.*, 2006).

Přítomnost enzymu BSH byla zjištěna u bakterií pocházejících ze zažívacího traktu, jako např. u rodu *Lactobacillus* spp., *Bifidobacterium* spp. (Jarocki *et al.*, 2014; Kim *et al.*, 2008; Kumar *et al.*, 2013; Ren *et al.*, 2011; Tanaka *et al.*, 1999) nebo *Enterococcus* spp. (Chand *et al.*, 2016). Přesná funkce enzymu BSH nebyla dosud plně vysvětlena. Původní hypotézy uvádějí, že napomáhá při detoxikaci žluči a umožňuje tak lepší přežití v podmínkách GIT (De Smet *et al.*, 1995; Tanaka *et al.*, 2000). Bylo ale potvrzeno, že tento enzym je aktivní i u izolátů získaných z jiných zdrojů než z GIT, jako je syrové mléko, fermentované mléčné výrobky nebo fermentovaná zelenina, ale nebyl aktivní u některých komerčních probiotických kmenů (Horáčková *et al.*, 2020 – **Příloha IX**; Sedláčková *et al.*, 2015 – **Příloha X**; Reyes-Nava *et al.*, 2016). Pro rychlý screening BSH aktivity je většinou autorů používána plotnová metoda založená na precipitaci CaCl_2 s uvolněnou kyselinou cholovou, což vytváří halo-zóny kolem

pozitivních kolonií (Dashkevicz & Feighner, 1989; Ahn *et al.*, 2003). Při porovnání s výsledky metody za použití tenkovrstvé chromatografie (TLC) byla ale tato metoda vyhodnocena jako méně citlivá a v některých případech neprůkazná. TLC metoda umožňuje jednoduchým způsobem stanovit hydrolýzu glykocholátu i taurocholátu sodného po jejich přidavku do kultivačního média (Sedláčková *et al.*, 2015 – **Příloha X**).

U mikroorganismů se také liší schopnost dekonjugovat glykocholát a taurocholát. U druhu *L. acidophilus* byla zjištěna významnější aktivita enzymu ke glykocholátu sodnému. Čtyři ze sedmi kmenů BSH pozitivních u glykocholátu byly BSH negativní u taurocholátu (Horáčková *et al.*, 2020 – **Příloha IX**). To lze vysvětlit skutečností, že afinita ke glykocholátu může být důležitější při snižování žlučové toxicity, protože poměr glyko-konjugovaných a tauro-konjugovaných kyselin ve žluči je přibližně 3 : 1 (De Aguiar Vallim & Tarling, 2013).

Přítomnost a uspořádání genů kódujících enzym BSH je u BMK i bifidobakterií vysoce variabilní. V případě některých druhů laktobacilů – *L. fermentum* (Kumar *et al.*, 2013), *L. casei* (Zhang *et al.*, 2009), *L. gasseri* (Jiang *et al.*, 2010) – byl nalezen jen jeden gen. Naproti tomu byly dva geny potvrzeny u *L. acidophilus* NCFM (*bshA* a *bshB*) (McAuliffe *et al.*, 2005) a *L. johnsonii* (Elkins *et al.*, 2001). *L. salivarius* má dva geny (Bi *et al.*, 2013), ale Jiang *et al.* (2010) označili pouze jeden gen. U *L. plantarum* se autoři shodují na přítomnosti čtyř různých genů pro aktivitu BSH (Ren *et al.*, 2011; Lambert *et al.*, 2008). Také v práci Horáčková *et al.* (2020 – **Příloha IX**) byla u 12 testovaných kmenů *L. acidophilus* získaných z různých zdrojů potvrzena přítomnost dvou genů pro BSH, i když pouze 6 z nich vykazovalo aktivitu enzymu.

Skutečnost, že produkty dekonjugace při nízkém pH (způsobeného například aktivitou BMK) se mohou vysrážet a opustit střevní trakt, podporuje teorii, že BSH aktivita napomáhá detoxikaci žluči (Begley *et al.*, 2006; Liong & Shah, 2005). Tento závěr byl potvrzen pouze u kmenů, které byly kultivovány při nižší koncentraci žluči (0,3 %). Při vyšších koncentracích (0,5 a 1,0 %) docházelo k signifikantnímu snížení růstové rychlosti u BSH pozitivních i BSH negativních kmenů. Přídavek žluči navíc významně ovlivnil lag-fázi negativních kmenů (Horáčková *et al.*, 2020 – **Příloha IX**).

Dekonjugované žlučové soli však také vykazují silnou antimikrobiální aktivitu. Volné žlučové kyseliny (kyselina cholová a deoxycholová) mohou být toxičtější než jejich konjugované deriváty. Samotná kyselina cholová je hydrofobnější a může volně vstupovat do buněk (Oelschlaeger, 2010). Přídavek 0,01 % cholátu sodného zcela zastavoval růst BSH pozitivního kmene *L. acidophilus* CCDM 406. Při zvyšování pH média ale došlo ke snížení toxicity cholátu sodného. Při vyšším pH se mění poměr disociovaných a nedisociovaných molekul. Cholát je více disociovaný (kyselina cholová $pK_a = 6,4$), a proto méně toxický. Studie

růstu v prostředí žluči se obvykle provádí při neutrálním pH, ale kyselý obsah žaludku může na začátku tenkého střeva snížit pH přibližně na hodnotu 5 (Liong & Shah, 2005). Tento fakt může být proto v prostředí *in vivo* dost významný (Horáčková *et al.*, 2020). Někteří autoři proto mají tendenci konstatovat, že dekonjugace je spíše nepřátelskou reakcí na podporu autochtonních GIT mikroorganismů proti patogenům (Reyes-Nava *et al.*, 2014).

Pokud je mikroorganismus vystaven samostatně podmínkám žaludku a podmínkám tenkého střeva, nezohledňuje tento přístup postupný průchod žaludeční kyselostí následované žlučovými solemi *in vivo*. Důležité je také brát v úvahu, v jakém prostředí se mikroorganismus do trávicího traktu dostává, tzn., zda jsou testy prováděny s izolovanými buňkami nebo s buňkami v určité matrici. V práci Horáčková *et al.* (2011) byl porovnán růst několika druhů laktobacilů v prostředí žlučových solí. I když žlučové soli působí inhibičně, bylo zjištěno, že po určité době jsou laktobacily schopny jisté adaptace a obnovují růst v porovnání s kontrolním stanovením. V případě obsahu 0,3 % žluče to bylo téměř 100 %, v případě obsahu 1 % žluče až 80 %. Zásadní vliv na viabilitu mělo nízké pH (pH 2, simulující prostředí žaludku), které u izolovaných buněk způsobilo redukci buněk z koncentrace 10^9 KTJ/ml na koncentraci 10^6 – 10^0 KTJ/ml v závislosti na druhu. Pokud byl ale stejný test proveden v matrici fermentovaného mléka, nedocházelo u žádného z testovaných mikroorganismů k výraznému úbytku životaschopných buněk. Fermentované mléčné potraviny se zdají být velmi vhodným nosičem pro probiotické mikroorganismy.

3.3.1.2 Povrchové vlastnosti, adheze

Autoagregace a další metody měření povrchových vlastností, jako je hydrofobicita, zeta-potenciál a adheze k modelovým povrchům (mikrotitrační destičky, tkáňové kultury Caco-buňky, HT-buňky, kolagen aj.), jsou nejčastějšími metodami zjišťování adhezních schopností mikroorganismů *in vitro*, které mohou pomoci k posouzení případné adheze v GIT a následné kolonizace (Monteagudo-Mera *et al.*, 2019; Rokana *et al.*, 2018). Autoagregační schopnosti jsou u různých laktobacilů významně rozdílné (Horáčková *et al.*, 2011). Koagregační vlastnosti probiotických kmenů s patogeny a jejich schopnost vytěsnit patogeny z trávicího traktu představují důležitý obranný mechanismus hostitele proti infekci. Několik studií prokázalo koagregaci probiotik s patogeny, což inhibovalo tvorbu biofilmu, např. koagregace kmenů *L. reuteri* s patogenní kvasinkou *Candida albicans* (Matsubara *et al.* 2016; Jørgensen *et al.*, 2017). Kmeny, které jsou hydrofobní, vykazují obecně vyšší schopnost autoagregace a koagregace s patogeny (Collado *et al.*, 2008; Kos *et al.*, 2003), i když i tyto závěry nejsou v některých studiích plně potvrzeny (García-Cayuela *et al.*, 2014).

Uvedené vlastnosti jsou ovlivněny jednotlivými povrchovými strukturami buněk. V tomto směru je nejvíce prozkoumána charakteristika a složení S-vrstvy (Alp *et al.*, 2020; Do Carmo *et al.*, 2018), produkce exopolysacharidů (Ruas-Madiedo *et al.*, 2006), či specifický výskyt fimbrií u *L. rhamnosus* GG (Vargas García *et al.*, 2015). S-vrstva je krystalická vrstva proteinů na povrchu buňky, která se u laktobacilů vyznačuje vysokým obsahem hydroxylovaných a hydrofobních aminokyselin a nízkým obsahem sirných aminokyselin. Tyto proteiny mají molekulovou hmotnost mezi 25 -71 kDa a jsou obecně neglykosylované. I když funkce S-vrstvy není zcela známa – pravděpodobně slouží k ochraně buňky, je prokázáno, že slouží k adhezním vlastnostem buněk na buňky epitelu (Hynönen & Palva, 2013). Tvorba exopolysacharidů je obecně u BMK považována za důležitou z hlediska vlivu na senzorycké a texturní vlastnosti fermentovaných výrobků. Exopolysacharidy zvyšují viskozitu potravin, což přispívá k jejímu delšímu setrvání v GIT a k delší přechodné kolonizaci probiotickými bakteriemi (Evivie *et al.*, 2017; Miao *et al.*, 2015).

Rozdíly v experimentech pro stanovení tolerance vůči podmínkám žaludku (médium okyselené pomocí HCl nebo kyseliny mléčné, s nebo bez enzymů), rezistence vůči žluči (původ a koncentrace žluči) a hodnocení adheze (mukus, buněčné linie) velmi ztěžují porovnání výsledků jednotlivých studií. Kromě *in vitro* pokusů jsou tedy definitivním rozhodujícím kritériem studie klinické.

3.3.1.3 Toxicita, faktory virulence

Probiotické mikroorganismy nesmí produkovat žádné toxiny, ani vykazovat faktory virulence. Tento požadavek je s určitostí splněn u BMK i bifidobakterií, nicméně určitá obezřetnost je nutná u některých konkrétních druhů. Byly zaznamenány případy bakteriémie v přítomnosti *L. rhamnosus* (Gouriet *et al.*, 2012; Salminen *et al.*, 2004), *L. casei* (Tommasi *et al.*, 2008) i bifidobakterií (Esaiassen *et al.*, 2017), ale vždy pouze u těžce nemocných a imunosupresivních pacientů. U potencionálně probiotických druhů *Enterococcus faecium* a *E. faecalis* bylo popsáno několik virulentních faktorů (Elsner *et al.*, 2000).

Za další předpoklad výběru probiotického kmene je považována absence hemolytické aktivity (WHO/FAO, 2001). Hemolýza je známým faktorem virulence mezi patogenními mikroorganismy. BMK většinou vykazují α -hemolytickou aktivitu (Maragkoudakis *et al.*, 2006), ale u některých kmenů byla prokázána i α -hemolýza, která není považována za nebezpečnou (Argyri *et al.*, 2013).

3.3.1.4 Antibiotická rezistence

Jako u všech mikroorganismů, které se mají používat při výrobě potravin a krmiv, je i u probiotik pečlivě sledována jejich antibiotická rezistence z důvodu jejího možného horizontálního přenosu na potencionálně patogenní mikroorganismy. Kvůli potřebě rozhodnutí, zda je mikroorganismus rezistentní nebo citlivý k určitému antibiotiku, zpracoval úřad EFSA pro BMK a bifidobakterie metodiku stanovení rezistence a hraniční hodnoty minimálních inhibičních koncentrací pro nejběžněji používaná antibiotika (EFSA, 2012).

Znalost fenotypu je důležitá i při absenci přenositelné rezistence, neboť může mít velký význam při léčbě bakteriémie související s výskytem laktobacilů (Cannon *et al.*, 2005). Až na některé výjimky rezistence na antibiotika u probiotických bakterií (*Lactobacillus* spp., *Bifidobacterium* spp.) nepředstavuje bezpečnostní riziko, pokud jsou za fenotyp rezistence odpovědné mutace nebo mechanismy vrozené rezistence. Některé probiotické kmeny s vrozenou rezistencí mohou být užitečné pro obnovení střevní mikrobioty po léčbě antibiotiky (Gueimonde *et al.*, 2013). Ve většině případů jsou např. laktobacily rezistentní na vankomycin, tetracyklin, erythromycin a částečně chloramfenikol (Das *et al.*, 2020; Anisimova & Yarullina, 2019; Verraes *et al.*, 2013). V německé studii bylo pouze šest ze 473 zkoumaných potencionálně probiotických BMK izolovaných z lidských i zvířecích zdrojů multirezistentních vůči tetracyklinu a erythromycinu (Klare *et al.*, 2007).

Primární rezistence je charakteristická pro všechny kmeny daného druhu, přenos genů je v tomto případě vertikální. Naopak při horizontálním přenosu může docházet k předání genetické informace mezidruhově prostřednictvím mobilních elementů, jako jsou plazmidy, transposony a integrony. Doposud byly popsány tři hlavní způsoby výměny či přenosu genetického materiálu mezi bakteriemi:

- přímý kontakt donorové a recipientní buňky (konjugace);
- příjem genů z okolního prostředí buňky (transformace);
- prostřednictvím bakteriofágů (transdukce).

Za nejvýznamnější, a v potravinových matricích opakovaně prokázaný, je pokládán první ze jmenovaných způsobů (Rossi *et al.*, 2014). Zajímavá je i skutečnost, že horizontální přenos může urychlovat používání konzervačních látek (Cen *et al.*, 2020). Přenositelné geny antibiotické rezistence jsou u BMK vzácné, většinou jsou detekovány mobilní geny rezistence na tetracyklin (Gueimonde *et al.*, 2013). Největší riziko v tomto směru představují zástupci rodu *Enterococcus*, kteří jsou v některých zemích používány jako kyselé kultury a kteří jsou rovněž představiteli probiotických doplňků stravy (*E. faecium*, *E. faecalis*). *Enterococcus* spp. je schopen přenášet geny rezistence na vlastní druhy, nepatogenní bakterie i na některé

patogenní bakterie, jako je *Staphylococcus aureus* a *Listeria* spp. (Jahan *et al.*, 2015; Gazzola *et al.*, 2012; Hummel *et al.*, 2007). Vážný problém představují enterokoky v souvislosti s rostoucím výskytem multirezistentních kmenů a s výskytem nozokomiálních enterokokových onemocnění. Nejčastěji je u nich detekována rezistence na vankomycin a tetracyklin (Faron *et al.*, 2016). Je také zajímavé, že při studiu celkového rezistomu střevní mikrobioty bylo zjištěno, že ve střevech kojenců se vyskytuje snížená antibiotická rezistence u bifidobakterií v porovnání s tou, která je přítomna u bifidobakteriální střevní mikroflóry starších dětí. Tato zjištění podporují teorii, že mikrobiota kojenců je po antibiotické terapii náchylnější k disbiose a dlouhodobým poruchám ve srovnání s odolnější mikrobiotou dospělých.

3.3.2 Zdravotně-funkční vlastnosti probiotik, klinické studie

Vyhodnocení zdravotně-funkčních vlastností probiotik je velmi často diskutovanou a do jisté míry kontroverzní záležitostí. Na jedné straně je těmto mikroorganismům připisováno mnoho účinků, což je podporováno jejich ekonomickým významem. Globální trh s probiotiky dosáhl v roce 2017 výše 40 miliard USD a předpokládá se, že do roku 2023 vzroste na 64 miliard USD (Reid *et al.*, 2019). Na straně druhé někteří vědci poukazují na protichůdné důkazy o jejich zdravotních benefitech. Výsledkem této situace je, že doposud bylo oficiálně schváleno pouze jediné zdravotní tvrzení týkající se BMK – živé kultury v jogurtu nebo v kysaném mléce zlepšují trávení laktosy z výrobku u osob, které laktosu špatně tráví. Aby bylo možné tvrzení použít, musí jogurt nebo kysané mléko obsahovat nejméně 10^8 KTJ živých mikroorganismů kyselové kultury (*Lactobacillus delbrueckii* subsp. *bulgaricus* a *Streptococcus thermophilus*) na 1 gram (Nařízení Komise EU č. 432/2012). Častými důvody, které úřad EFSA uvádí pro neschválení žádostí o zdravotní tvrzení týkající se probiotik (dosud podáno více než 300 žádostí, více než 200 probiotik a jejich kombinací, více než 60 zdravotních benefitů), jsou nedostatečná charakterizace mikroorganismu, nepřesně definované účinky, nedostatek relevantních studií na lidech, nedostatečná kvalita studií či to, že ne všechny měřitelné údaje napovídají o zdravotních účincích.

K použití mikroorganismu jako probiotika je vždy nutné doložit minimálně dvě klinické studie, které byly designovány jako dvojitě zaslepené a placebem kontrolované (FAO/WHO, 2002). Většinou jim předcházejí studie *in vitro* a studie na gnotobiotických či běžných laboratorních zvířatech (Fontana *et al.*, 2013). Do dnešní doby byly publikovány stovky studií týkající se nejrůznějších zdravotních účinků při podávání probiotických mikroorganismů a

jejich detailní výčet by přesahoval rozsah této práce. Z nejčastěji uváděných benefitů lze jmenovat (Brüssow, 2019; Kerry *et al.*, 2018):

- stimulace imunitního systému (včetně působení na rozvoj různých druhů karcinomů); ovlivnění tvorby cytokinů a interleukinů;
- „dobrý zdravotní stav střev“;
- léčba průjemových onemocnění (akutní infekce způsobené nejrůznějšími patogenními mikroorganismy, rotavirové průjmy, cestovatelské průjmy, postantibiotické průjmy, průjemové stavy po chemoterapii či radioterapii apod.);
- léčba vaginálních infekcí;
- podpůrná léčba infekce *Helicobacter pylori*;
- podpůrná léčba, udržení remise u ulcerózní kolitidy a Crohnovy choroby,
- usnadnění trávení laktosy, produkce β -galaktosidasy; produkce dalších enzymů;
- metabolická funkce, produkce krátkých mastných kyselin;
- možnost snižování hladiny sérového cholesterolu;
- produkce vitaminů;
- antioxidační aktivita;
- produkce bioaktivních peptidů a jejich fyziologický účinek (nejčastěji snižování krevního tlaku);
- podpůrná léčba obezity;
- léčba ekzémů;
- prevence nekrotizující enterokolitidy u předčasně narozených dětí;
- použití u lidí s cystickou fibrózou a další.

Vhodným zdrojem pro posouzení účinků je, kromě jednotlivých studií, Cochrane Database of Systematic Reviews, která sumarizuje metaanalýzy získaných výsledků (www.cochranelibrary.com).

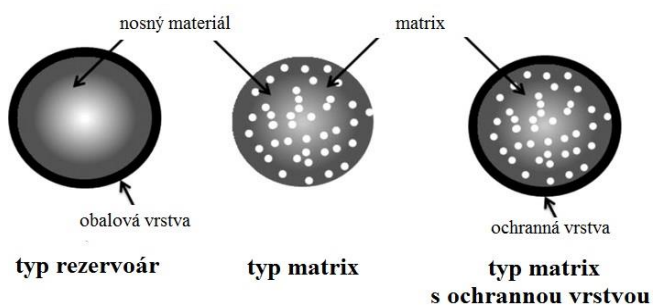
Aktuální data ukazují, že střevní mikrobiom je rovněž úzce spjat s fungováním nervové soustavy. Hledají se tudíž mikrobiální preparáty, které mohou pomoci v případě deprese, úzkosti a stresu (Cheng *et al.*, 2019). Nadějných výsledků je při použití probiotických BMK dosahováno rovněž při léčbě Alzheimerovy (Angelucci *et al.*, 2019) a Parkinsonovy choroby (Gazerani, 2019), zmírnění poruch autistického spektra (Abdellatif *et al.*, 2020), hyperaktivity (ADHD) (Partty *et al.*, 2015) a dalších.

3.3.3 Technologické vlastnosti – aplikace probiotických mikroorganismů do potravin

Výroba probiotických kultur pro aplikaci v potravinářství sleduje stejné požadavky jako výroba klasických („neprobiotických“) mikrobiálních kultur. Je kladen důraz na dobrou produkci biomasy, stabilitu mikroorganismů během mražení, lyofilizace a distribuce kultur, genetickou stabilitu a rezistenci vůči fágům (Markowiak & Śliżewska, 2017). Technologické požadavky při samotné výrobě probiotických produktů do jisté míry závisejí na druhu výrobku. V doplňcích stravy jsou používány lyofilizované buňky a jedinými sledovanými parametry jsou čistota preparátu a dostatečná viabilita buněk po celou dobu trvanlivosti. V případě aplikace do potravin musí být brán zřetel na další parametry, kterými jsou dostatečné pomnožení buněk v potravinové matrici (požadovaná koncentrace) a přežívání buněk během celého technologického procesu a během skladování potraviny. V neposlední řadě přídavné probiotické kultury nesmějí mít žádný negativní dopad na senzorické vlastnosti výrobku. Hlavními faktory prostředí, které ovlivňují životaschopnost probiotik během výroby, skladování a přepravy potravin, jsou změny v expozici ke kyslíku, změny relativní vlhkosti, teploty a pH (Rodrigues *et al.*, 2011).

3.3.3.1 Enkapsulace probiotických mikroorganismů

Pro potlačení méně příznivých podmínek, které mohou nastat v potravinách pro některé citlivější probiotické bakterie (bifidobakterie), ale také jako ochrana před nepříznivým prostředím při průchodu GIT, je možno přistoupit k jejich enkapsulaci. Enkapsulace je proces, při kterém dojde k zachycení jedné látky (vnitřní fáze, účinná látka) uvnitř jiné látky (obal). Během tohoto procesu vznikají částice většinou o velikosti 1 – 1000 μm ., které mohou být různého typu – rezervoárový, matrixový, kdy je účinná látka rozptýlena v obalovém materiálu ve formě kapiček, nebo matrixový typ s další obalovou vrstvou (Obr. 6). Enkapsulace probíhá obvykle ve třech krocích. Nejprve je rozptýlena aktivní složka do vhodné matrice, poté je matrice dispergována do proudu vzduchu či jiné kapaliny a nakonec dochází k chemické, fyzikálně-chemické či fyzikální stabilizaci utvořených kapslí (Burgain *et al.*, 2011).



Obr. 6. Hlavní typy enkapsulovaných částic (dle Burgain *et al.*, (2011))

V literatuře byla popsána metodika enkapsulace s využitím celé řady materiálů jako obalové/ochranné vrstvy, jako jsou např. polysacharidy z mořských řas (karagenan, alginát) (Chakraborty, 2017; Etchepare *et al.*, 2015), polysacharidy z rostlin (škrob a jeho deriváty) (Pankasemsuk *et al.*, 2016) či z bakterií (xanthan, gellanová guma) (Shu *et al.*, 2017) nebo živočišné proteiny (bílkoviny mléka, želatina) (Picot & Lacroix, 2004; Patarroyo *et al.*, 2020).

Princip tvorby gelu je u jednotlivých materiálů odlišný a ve svých vlastnostech se liší i vzniklé mikrokapsle, jak ukazuje Tab. 5.

Tab. 5. Nejčastěji používané materiály pro enkapsulaci buněk

Obalová vrstva	Princip tvorby enkapsulovaných částic	Odolnost vůči kyselému prostředí	Termostabilita
alginát	vyžaduje Ca^{2+}	ne	stabilní
xanthan	vyžaduje Ca^{2+}	ano	stabilní
κ -karagenan	vyžaduje Ca^{2+}	ano	citlivý
syrovátkové bílkoviny	tepelná denaturace	ano	stabilní
kasein	syřidlo	ano	stabilní
želatina	záhřev	ano	citlivý

3.3.3.2 Metody enkapsulace probiotických mikroorganismů

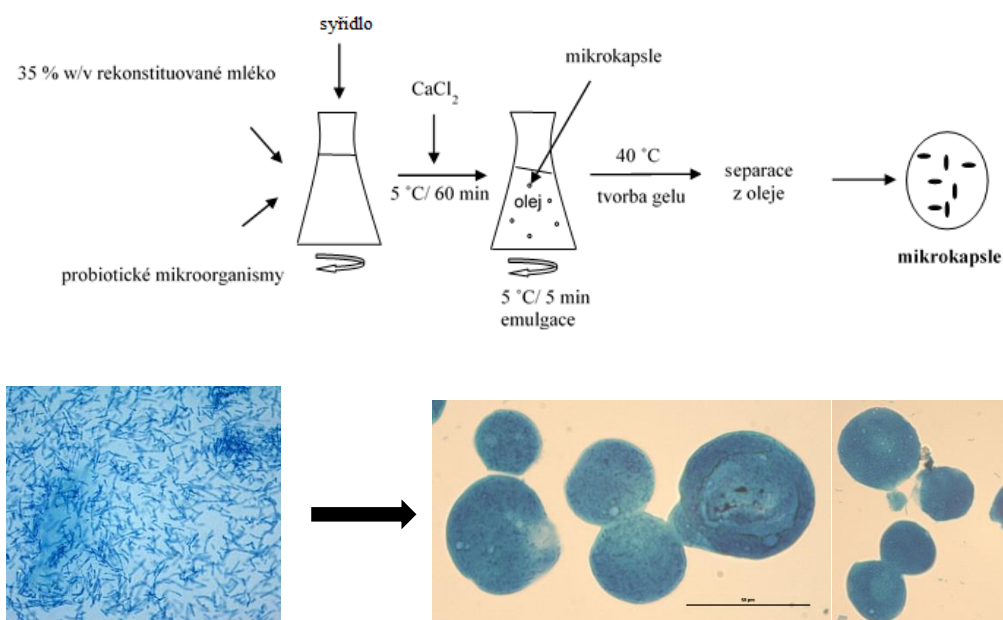
Mezi nejrozšířenější metody enkapsulace patří sprejové sušení. Jedná se o velmi účinnou, rychlou a především ekonomicky výhodnou metodu s širokým průmyslovým uplatněním, ale vzhledem k vyšší teplotě procesu nemusí být příliš vhodná pro mikroorganismy. Výsledné částice o velikosti 10 - 100 μm jsou získávány po pádu na dno sběrné nádoby (Tantratian *et al.*, 2018; Nedovic *et al.*, 2011).

Laboratorně široce používanou technikou je extruzní metoda, která je jednoduchá, má nízké náklady a zároveň díky mírným výrobním podmínkám zaručuje přežití vysokého počtu

buněk. Suspenze s obsahem mikroorganismů se protlačuje úzkou tryskou (např. i injekční stříkačkou), malé kapičky pak padají do roztoku, který je zpevní. Nejčastěji se využívá rozdílné rozpustnosti alginátu sodného a alginátu vápenatého. Velikost a tvar vytvořených kapslí závisí na průměru jehly a vzdálenosti mezi jehlou a hladinou roztoku chloridu vápenatého. Obvykle vzniknou kapsle o velikosti 1 - 5 mm (Mortazavian *et al.*, 2007; Krasaekoopt *et al.*, 2003).

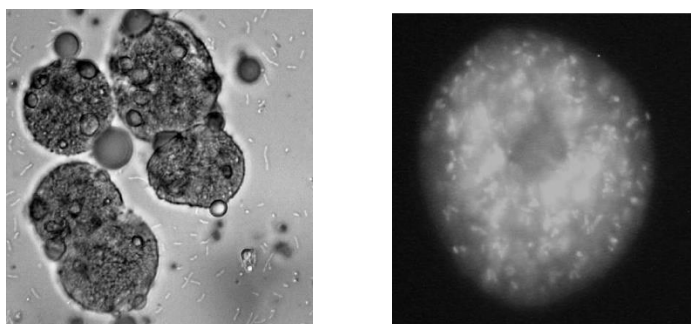
Další vhodnou metodou je emulzní způsob enkapsulace, při kterém je malý objem suspenze s mikroorganismy (disperzní fáze) přidán do velkého množství rostlinného oleje (spojitá fáze) a směs je homogenizována za vzniku emulze voda v oleji. Následně se pomocí různých solidifikačních procesů vytvářejí kapsle nerozpustné ve vodě o velikosti 20 μm – 2 mm. Od olejové fáze mohou být odděleny filtrací nebo odstředěním. Ve srovnání s technikou extruze má tato metoda výhodu vzniku menších částic a možného ovlivnění jejich velikosti. Určitou nevýhodou může být široký rozsah velikosti distribuce částic, ale výsledná velikost se dá ovlivnit změnou rychlosti míchání nebo poměrem vodné a olejové fáze (Burgain *et al.*, 2011; Kailasapathy, 2009). Velikost mikrokapslí je jedním z nejdůležitějších parametrů pro jejich další použití v potravinách, protože může významně ovlivnit senzorické vlastnosti produktu.

Ve studii provedené Heidebachem *et al.* (2009) se při emulzní enkapsulaci dle schématu uvedeném na Obr. 7 při použití rychlosti míchání emulze 500 rpm dosáhlo velikosti částic $d_{0,5} 68 \pm 5 \mu\text{m}$.



Obr. 7. Schéma emulzní enkapsulace buněk dle Heidebacha *et al.* (2009) a výsledné mikrokapsle získané tímto procesem (Lisová *et al.*, 2013 - Příloha XI)

Dle stejného postupu bylo v práci Lisová *et al.* (2013 – **Příloha XI**) dosaženo efektivní enkapsulace (viz obr. 8), ale při 500 rpm byly získány mikrokapsle o velikosti $d_{0,5}$ $196 \pm 37 \mu\text{m}$. Nepotvrdil se předpoklad, že při zvýšení rychlosti míchání bude dosaženo menší velikosti částic – při použití 1200 rpm bylo $d_{0,5}$ $193 \pm 24 \mu\text{m}$. Tato metoda byla dále inovována, a to tak, že k olejové fázi byl přidán sójový lecitin v množství 0,5 % hm. (Lisová, Plocková, Horáčková – užitný vzor, 2012). Při takovémto uspořádání pokusu byla snížena velikost vzniklých mikrokapslí na $79 \pm 3 \mu\text{m}$ při 500 rpm a $39 \pm 3 \mu\text{m}$ při 1200 rpm.



Obr. 8. Vizualizace mikrokapslí s *B. animalis* subsp. *lactis* Bb12. A – nativní preparát; B – FISH metoda (Lisová *et al.*, 2013 - **Příloha XI**)

3.3.3.3 Použití prebiotik

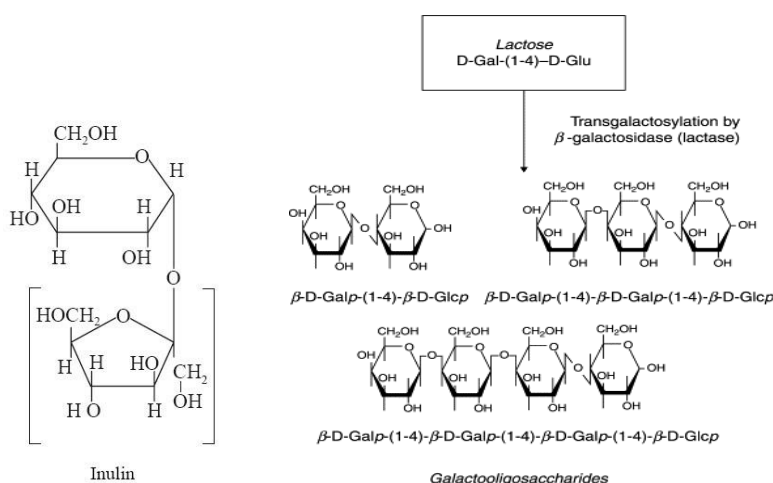
Další možností, jak zvýšit viabilitu a podpořit růst probiotických bakterií, je použití prebiotik. Definice prebiotických preparátů byla poprvé publikována v roce 1995, kdy byly popsány jako nestrávitelná složka potravy, která příznivě ovlivňuje hostitele selektivní stimulací růstu a aktivity omezených druhů (prospěšných) bakterií v tlustém střevě, a tím zlepšuje zdraví hostitele (Gibson & Reberfroid, 1995). Později byla definice upřesněna na „dietní prebiotika“ jako selektivně fermentovatelnou složku potravin, která má za následek specifické změny ve složení a/nebo aktivitě gastrointestinální mikrobioty, což přináší opět zdravotní benefity (Gibson *et al.*, 2010; Bindels *et al.*, 2015).

Prebiotika by měla splňovat následující kritéria:

- jsou odolná vůči kyselému pH žaludku;
- nejsou hydrolyzována trávicími enzymy, nejsou absorbována v GIT;

- mohou být fermentovatelná střevní mikrobiotou, její aktivita může být prebiotikem selektivně stimulována, což zlepšuje zdraví hostitele (Gibson *et al.*, 2010).

Mezi nejfrekventovaněji používaná prebiotika jak v laboratorních studiích, tak při aplikaci do potravin, patří fruktany (inulin, fruktooligosacharidy), galaktooligosacharidy, oligosacharidy sóji, v některých případech rezistentní škrob a některé další oligosacharidy (Vandeputte *et al.*, 2017; Macfarlane *et al.*, 2008; Zhou *et al.*, 2012) (Obr. 9).



Obr. 9. Nejčastěji používaná prebiotika v potravinářském průmyslu (převzato z Drakoularakou *et al.*, 2011)

Prebiotika mohou ovlivňovat samotný růst probiotik (De Souza Oliveira *et al.*, 2012, Akin *et al.*, 2007), ale mohou mít také vliv na jejich životaschopnost při průchodu GIT (Valero-Cases & Frutos, 2015). Při použití kombinace enkapsulace do mléčné bílkovinné matrice nebo do alginátových kapslí mikroorganismu *Bifidobacterium animalis* subsp. *lactis* Bb12 a přídavku inulinu byl sledován ochranný efekt při dlouhodobém skladování v mléce při chladírenské teplotě (Kumherová *et al.*, 2020 – **Příloha XII**). I když volné buňky Bb12 byly dosti stabilní, na konci doby skladování (6 týdnů) bylo jejich množství sníženo o 1,5 řádu, zatímco u enkapsulovaných buněk k žádnému poklesu nedošlo. Přídavek inulinu do kapslí v tomto případě neovlivnil počet buněk. Enkapsulované buňky významně odolnější vůči simulovaným podmínkám GIT (pokles počtu živých buněk o 0,9 – 2,8 řádu) oproti volným buňkám (pokles o 8 řádů). Přídavek inulinu ovlivňoval viabilitu buněk u enkapsulace do bílkovinné matrice, ale ne v alginátových kapslích.

3.3.3.4 Aplikace probiotických mikroorganismů do nemléčných potravin

Výrobky mlékárenského průmyslu, zvláště fermentované výrobky a kojenecká výživa, jsou dobrými nositeli probiotických mikroorganismů z hlediska jejich stability. Nicméně vzhledem k možnému výskytu laktosové intolerance nebo alergie na bílkoviny mléka u některých konzumentů či upřednostňování nemléčných potravin v případě vegetariánského způsobu stravování, narůstá na trhu poptávka i po probiotických nemléčných výrobcích. Aplikace a sledování stability v různých druzích potravin jsou předmětem celé řady vědeckých studií. Byly testovány ovocné a zeleninové džusy (Nguyen *et al.*, 2019; Martins *et al.*, 2013), cereální nápoje (Herrera-Ponce *et al.*, 2014) či čokoláda (Gadhiya *et al.*, 2018; Foong *et al.*, 2013). Upřednostňovanou surovinou pro přípravu fermentovaných výrobků je hlavně v asijských zemích sója nebo sójový nápoj (Sirilun *et al.*, 2017; Shimakawa *et al.*, 2003). Tyto výrobky pronikají v poslední době ve větší míře i na evropský trh. V práci Horáčková *et al.* (2015 – **Příloha XIII**) bylo prokázáno, že sójový nápoj je vhodný pro růst nejen klasické jogurtové kultury, ale i probiotických kmenů *B. animalis* subsp. *lactis* Bb12 a *B. bifidum* CCDM 94. I když byl u bifidobakterií potvrzen lepší růst v mléce v porovnání se sójovým nápojem, bylo i tohoto výrobku dosaženo koncentrace bifidobakterií více než 10^7 KTJ/ml po 16 h fermentaci při 37 °C, což splňuje požadavek Vyhlášky o požadavcích na mléko a mléčné výrobky, mražené krémy a jedlé tuky a oleje (Vyhláška č. 274/2019 Sb.) pro výrobky s bifidobakteriemi. Zajímavým výsledkem rovněž bylo, že kultura *B. bifidum* CCDM 94 byla schopna uvolňovat vyšší množství aglykonů genisteinu, daidzeinu a glyciteinu z glykosilovaných isoflavonů sóji. Glykosidy (genistin, daidzin, glycitin) jsou považovány za biologicky neaktivní, ale při fermentaci může docházet ke štěpení β -glykosidové vazby za uvolnění biologicky aktivních aglykonů (Hong *et al.*, 2012). I tento parametr by mohl být zohledněn pro výběr vhodné probiotické kultury do sójových výrobků.

Rozsáhlejší přehled o použití probiotik do nemléčných potravin byl nedávno zpracován v práci Aspri *et al.* (2020). Komerčně nejrozšířenějšími výrobky z této kategorie jsou ovocné džusy s vysoce stabilním *L. rhamnosus* GG (firma Valio). Výhodou tohoto typu „funkčních potravin“ je kromě obsahu mikroorganismů, vyšší obsah minerálů, vitaminů, vlákniny či antioxidantů.

Jak již bylo zmíněno, výběr potravinové matrice je důležitý pro životaschopnost probiotik během zpracování a skladování. Při výrobě ovocných, zeleninových a cereálních probiotických produktů bylo prokázáno, že kromě aktivity vody a pH závisí viabilita buněk také na výběru probiotických kmenů. Rostlinné matrice obsahují základní živiny pro mikroorganismy, ale jejich růst a viabilita může být omezena např. velmi nízkým pH ovocných šťáv, nedostatkem

volných aminokyselin, vysokým obsahem rozpuštěného kyslíku, přítomností antimikrobiálních látek, přítomností umělých barviv či tepelným ošetřením produktu. Na rozdíl od mléčných výrobků, které se skladují při nízkých teplotách, může být limitujícím faktorem také skladování při pokojové teplotě, což je běžné u mnoha nemléčných výrobků (Perricone *et al.*, 2015; Perricone *et al.*, 2014). Použití ochrany buněk enkapsulací, které není v souvislosti s aplikacemi do rostlinných nápojů příliš často v odborné literatuře zmiňováno, bylo zpracováno v publikaci Horáčková *et al.* (2018d – **Příloha XIV**). V práci byla potvrzena důležitost výběru vhodné rostlinné matrice pro daný mikroorganismus. Probiotický mikroorganismus *Bifidobacterium animalis* subsp. *lactis* Bb12 (jak volné buňky, tak buňky v mikrokapslích) nepřežíval v jahodovo-jablečném nápoji v dostatečném množství při skladování při 8 °C. Při skladování při 22 °C nebyly volné ani enkapsulované buňky v nápoji detekovatelné již po 14 dnech skladování. Naproti tomu v ananasovém džusu při skladování při pokojové teplotě i v chladu dosahovaly počty i po 4 týdnech hodnot $10^7 - 10^8$ KTJ/ml. Důležité je také zjištění, že na základě stanovení obsahu kyseliny mléčné a kyseliny octové nebyla zjištěna metabolická aktivita buněk ve výrobku (kultivace 24 h při 37 °C) a nebyly tak významně ovlivněny jeho sensorické vlastnosti. Na druhé straně to však znamenalo, že buňky nevykazovaly růst, což značí, že v případě výroby by musely být buňky do tohoto typu výrobku přidávány již v množství, které by bylo požadováno pro hotový produkt.

4 Selektivní stanovení bakterií mléčného kvašení a probiotických mikroorganismů

Použití laktobacilů a bifidobakterií jako přídatných, probiotických a protektivních kultur do nejrůznějších potravin je stále rozšířenější. S tímto rostoucím trendem roste také potřeba jejich přesné kvantifikace ve směsích s ostatními (základními) kulturami BMK, aby byla potvrzena jejich přítomnost v živém stavu a v množství, které je potřeba k zajištění zdravotního benefitu.

Dle obecně přijímaného konsenzu jsou za životaschopné buňky považovány ty, které se množí a vytvářejí kolonie na agarových půdách. Mikroorganismy však mohou existovat v různých růstových fázích a metabolických stavech v závislosti na podmínkách prostředí a jen pouze určitý stav zahrnuje aktivní replikaci s viditelnou tvorbou kolonií (Volkert *et al.*, 2008). Z hlediska probiotik však může i poškozená buňka nebo buňka, která se přizpůsobila stresovým podmínkám, jako je např. acidifikace (Lahtinen *et al.*, 2008), zajišťovat svoje funkční působení. Pro použití v praxi byly ale pro selektivní stanovení BMK doposud normovány pouze klasické kultivační metody na pevných agarrech.

Obecně se laktobacily nejčastěji kultivují na MRS médiu (de Man, Rogosa a Sharpe), což je nutričně bohaté médium, které je pro tyto mikroorganismy nezbytné, na kterém ale nelze odlišit jednotlivé druhy (Galat *et al.*, 2016). Postupy k selektivnímu odlišení různých druhů laktobacilů, které jsou doposud publikovány v odborné literatuře, jsou založeny většinou na použití různých kultivačních teplot, různého pH média, atmosféry, zdroje uhlíku nebo různých selektivních činidel jako jsou antibiotika, žlučové soli, barviva apod. (Talwalkar & Kailasapathy, 2004). Schválené mezinárodní normy však existují pouze pro selektivní stanovení druhů *Lactobacillus acidophilus* (ČSN ISO 20128), *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus* (ČSN ISO 7889; ČSN ISO 9232) a *Bifidobacterium* spp. (ČSN ISO 29981).

Probiotickým druhem, který se ve větší míře používá zvláště v kombinaci s jogurtovou kulturou, je *Lactocaseibacillus* (*Lactobacillus*) *casei*. Jeho selektivní stanovení však není žádným normativním dokumentem definováno. Kmeny označované jako skupina *L. casei* vykazují velkou míru fenotypické i genotypické heterogenity, která vyvolává diskuse ohledně zařazení jednotlivých druhů a kmenů do této skupiny. V minulosti byly na základě pouze fenotypických znaků (hlavně fermentace sacharidů) všechny druhy této skupiny označovány jako *L. casei* s pěti subspeciemi (*alactosus*, *casei*, *pseudoplantarum*, *rhamnosus* a *tolerans*). Později byla skupina rozdělena dle sekvence 16S rRNA (Bergey's Manual of Systematic

Bacteriology) na tři rozdílné druhy: *L. casei*, *L. rhamnosus* a *L. paracasei*, který se dále dělí do dvou podskupin *L. paracasei* subsp. *paracasei* a *L. paracasei* subsp. *tolerans* (Toh *et al.*, 2013; Sato *et al.*, 2012).

V literatuře bylo popsáno několik médií navržených pro selektivní stanovení *L. casei*. Již v roce 1998 porovnávali Shah a Ravula (1998) růst různých BMK a bifidobakterií na M17 agaru, na MRS agarech, kde byla glukosa nahrazena salicinem, sorbitolem, ribosou nebo glukonátem, s růstem na jimi navrženém tzv. LC agaru s bromkresolovou zelení a D⁺ ribosou s upraveným pH na 5,1. Potvrdili jeho selektivitu pro použitý kmen *L. casei*, neboť byl na tomto médiu inhibován jak růst *S. thermophilus* úpravou pH na 5,1, tak i růst *L. delbrueckii* subsp. *bulgaricus* díky jeho neschopnosti fermentovat ribosu. I když autoři potvrzují inhibici růstu také pro *L. acidophilus* a bifidobakterie, Van de Castele *et al.* (2006) ve své práci ukazuje, že ne všechny kmeny *L. acidophilus* na tomto médiu nerostou. Dalším navrženým způsobem pro stanovení *L. casei* v kombinaci s termofilními druhy BMK je snížení kultivační teploty na 15 °C s prodlouženou dobou kultivace na 15 dní (Champagne *et al.*, 1997). Takto dlouhá doba kultivace je však z provozního hlediska velmi nepraktická.

Při stanovení *L. casei*, *L. acidophilus*, *Bifidobacterium* spp. v přítomnosti *L. delbrueckii* subsp. *bulgaricus* a *Streptococcus thermophilus* bylo potvrzeno, že MRS-Bile (MRS agar s přídavkem 0,15 % žluči) a MRS-LP médium (MRS agar s přídavkem 0,2 % chloridu lithného a 0,3 % propionátu sodného) potlačuje růst obou mikroorganismů jogurtové kultury, ale ani jedno z těchto dvou médií není vhodné pro selektivní stanovení *L. casei* ve směsi s *L. acidophilus* a *Bifidobacterium* spp. Nicméně u výše zmiňované směsi mikroorganismů lze stanovit celkový počet kolonií *L. casei* a *L. acidophilus* pomocí MRS-Bile média, neboť inhibuje jogurtové kultury a také bifidobakterie, anebo celkový počet *L. casei* a *Bifidobacterium* spp. na MRS-LP médiu, které inhibuje růst jogurtové kultury a *L. acidophilus* (Vinderola & Reinheimer, 2000).

V současné době je asi nejrozšířenějším médiem pro selektivní stanovení *L. casei*, které doporučují i komerční dodavatelé probiotických kultur, MRS agar s přídavkem vankomycinu (1 mg/l), případně upravený přídavkem bromfenolové modři (Sutula *et al.*, 2012). Půda s vankomycinem je popsána ve studii Tharmaraj a Shah (2003) spolu s dalšími 18 médii v kombinaci s různými kultivačními podmínkami – aerobní, anaerobní, teploty kultivace 27, 30, 37, 43 a 45 °C po dobu 24 a 72 h a 7 – 9 dnů. MRS-vankomycin agar byl vyhodnocen jako selektivní při anaerobní inkubaci při 45 °C po dobu 72 h. Pouze při velmi malém rozdílu kultivační teploty (43 °C) doporučují autoři na stejném agaru selektivní stanovení *L. rhamnosus*. Pro selektivní odlišení *L. casei* a *L. paracasei* od *L. rhamnosus* byl navržen

modifikovaný rhamnosa-2,3,5-trifenyltetrazolium chlorid – LBS – vankomycin agar (M-RTL V agar) (Sakai *et al.*, 2010). Na tomto médiu tvoří *L. casei* a *L. paracasei* červené kolonie, zatímco *L. rhamnosus* kolonie růžové anebo bílé s červenou tečkou. Při srovnání MRS agaru s vankomycinem a M-RTL V agaru pro selektivní stanovení *L. casei* ve fermentovaných mléčných výrobcích, které obsahují směs *L. casei*, *L. acidophilus* LA-5, *Streptococcus thermophilus* a *L. delbrueckii* subsp. *bulgaricus*, je vhodnější MRS-vankomycin agar kultivovaný za anaerobních podmínek po dobu 48 h při 37 °C (Colombo *et al.*, 2014).

Selektivní médium pro stanovení mikroorganismů skupiny *L. casei* v kombinaci s jogurtovou kulturou bylo navrženo také v práci Di Lena *et al.* (2015): MRS agar s maltosou (místo glukosy), vankomycinem (10 g/l), L-cysteinem a s bromkresolovou červení.

V nejnověji publikované práci na toto téma bylo poprvé navrženo médium s chromogenními substráty umožňující selektivní stanovení *L. rhamnosus*, dvou kmenů *L. paracasei* subsp. *paracasei* a *Streptococcus salivarius* subsp. *thermophilus* na základě rozdílných aktivit β -galaktosidasy a β -glukosidasy. Tento agar byl suplementován rhamnosou nebo vankomycinem a inkubace byly prováděny při 37 °C nebo 44 °C pro zvýšení selektivity. Další chromogenní substrát byl použit k selektivnímu stanovení *L. delbrueckii* subsp. *bulgaricus* při 47 °C (Galat *et al.*, 2016).

Kultivační metody, jejichž výhodou je, že stanovují pouze živé buňky, mohou být samozřejmě nahrazeny méně časově náročnými qPCR metodami. Zde ale přesnost stanovení velmi závisí na přesnosti odběru a přípravě vzorku, správném výběru primerů a použití technik pro rozlišení živých a mrtvých buněk pomocí propidium monoazidu (PMA) či ethidium monoazidu (EMA) (García-Cayuela *et al.*, 2009; Nocker *et al.*, 2006). V práci Mühlhansová *et al.* (2016 – **Příloha XV**) byly porovnány kultivační plotnové metody pro stanovení *L. casei* a *L. acidophilus* s optimalizovanou metodou qPCR. Pro stanovení *L. casei* byl použit MRS agar s vankomycinem a LC agar s ribosou a bromkresolovou zelení; stanovení *L. acidophilus* probíhalo dle ČSN ISO 20 128). Výsledky kultivačních metod byly porovnávány s qPCR metodou a s qPCR metodou za použití ošetření buněk sterilním dimethylsulfoxidem s různou koncentrací interkalačních barviv PMA nebo EMA a optimalizace doby fotoindukce azidové skupiny LED lampou. Počet buněk stanovených plotnovou metodou byl u *L. casei* i *L. acidophilus* o 2 řády nižší než u qPCR a o 1,5 řádu nižší než u PMA-qPCR. Použití EMA-qPCR nebylo shledáno jako vhodné z důvodu málo reprodukovatelných výsledků. Doba fotoindukce neměla významný vliv na stanovení. Jako optimální metoda byla navržena PMA-qPCR s finální koncentrací PMA 50 mmol/l a dobou fotoindukce 15 min. Při ošetření buněk

cholátem sodným (0,5 % hm.) před barvením buněk PMA bylo dosaženo nejvyšší shody s hodnotami počtu buněk z kultivačním metod.

Z výše uvedeného přehledu je patrné, že selektivní stanovení druhu *L. casei* je věc velmi složitá. Dokladem důležitosti této problematiky je i nedávná iniciativa International Dairy Federation, která vyzvala jednotlivé členské státy k zapojení se do projektu Enumeration of *Lactobacillus casei/paracasei* in fermented milks and starter cultures.

Metoda qPCR byla otestována také pro stanovení druhu *L. helveticus* v matici sýra (Mühlhansová *et al.*, 2016 – **Příloha XVI**) s primery navrženými pro geny kódující peptidoglykan hydrolasy (Jebavá *et al.*, 2014).

Stejný pohled na danou problematiku je možné uplatňovat i u druhu *Lactiplantibacillus* (*Lactobacillus*) *plantarum*, jehož zástupci v podobě probiotických kmenů 299v nebo LP01 se začínají rovněž ve větší míře uplatňovat v kombinaci s dalšími zástupci BMK do fermentovaných potravin, i jako přídatná kultura do sýrů. *L. plantarum* je izolován především z rostlinných substrátů. Je to fakultativně heterofermentativní, mezofilní mikroorganismus, který roste v teplotním rozmezí 15 °C až 45 °C. Produkuje oba izomery kyseliny mléčné a některé kmeny mohou být značně rezistentní k NaCl (růst při 4 – 6 % hm. NaCl) (Todorov & De Melo Franco, 2010). V posledních letech se objevují stále častěji studie týkající se aplikace *L. plantarum* do jogurtů či fermentovaných rostlinných nápojů (Li *et al.*, 2020; Dan *et al.*, 2019; Moreno-Montoro *et al.*, 2018; Sidira *et al.*, 2017). Neexistuje však žádná uznaná metoda pro jeho selektivní stanovení, někteří autoři využívají přídavek vankomycinu (10 mg/l) do MRS média (Li *et al.*, 2017). V práci Veselá *et al.* (2019 – **Příloha XVII**) byly pro odlišení *L. plantarum* (ve studii testovány 4 kmeny) od *S. thermophilus* (4 kmeny) a *L. delbrueckii* subsp. *bulgaricus* (4 kmeny) testovány různé selekční faktory, a to teplota (30, 37, 42 a 45 °C), pH kultivační půdy (pH 5,2 a 5,6 a 6,2), aerobní a anaerobní podmínky, přídavek vankomycinu a použití maltosy, galaktosy, sacharosy, manitolu a sorbitolu namísto glukosy v MRS agaru.

Na základě vyhodnocení kombinace výše uvedených růstových podmínek a sacharidů v médiu bylo navrženo dostatečně selektivní médium pro odlišení *L. plantarum* od druhů jogurtové kultury. Nahrazení glukosy galaktosou bylo vysoce selektivní, ale protože kmeny *L. delbrueckii* subsp. *bulgaricus* v některých případech vykazovaly mírný nahodilý nárůst, byl zvolen klasický MRS agar s přídavkem vankomycinu (20 mg/l). Oproti normované metodě pro stanovení jogurtové kultury bylo nutno zvýšit kultivační teplotu na 45 °C. Pouze kmeny *L. delbrueckii* subsp. *bulgaricus* byly schopny růstu na MRS agaru, pH 5,2 anaerobně při této

teplotě. Kmeny *L. plantarum* tvořily na M17 agaru (dle ČSN ISO 7889 agar určený pro *S. thermophilus*) malé kolonie, ale při zvýšení kultivační teploty na 45 °C aerobně byl jejich růst potlačen a selektivně se mohl stanovit pouze počet *S. thermophilus*. Pro selektivní stanovení samotného druhu *L. plantarum* pak byla navržena kultivace na MRS agaru s vankomycinem (20 mg/l), pH 5,6 a aerobní kultivace při 30 °C po dobu 48 h (Veselá *et al.*, 2019 – **Příloha XVII**).

5 Závěr

Bakterie mléčného kvašení se podílejí na výrobě fermentovaných potravin ze surovin, jako je mléko, maso, zelenina a obiloviny. Tyto potraviny jsou významnou součástí potravinářského průmyslu a vyrábějí se za použití vybraných kmenů, které mají schopnost vytvářet požadované texturní a senzorické vlastnosti. Dlouhotrvajícím trendem studia a aplikace BMK je charakteristika nových kmenů, které lze izolovat z přírodních zdrojů nebo z tradičních fermentovaných potravin. Většina těchto kmenů se sice nestává součástí komerčních produktů, ale výzkum jejich vlastností napomáhá k pochopení a optimalizaci procesů při výrobě potravin. Hrají rovněž nezastupitelnou roli v zabezpečení mikrobiální nezávadnosti potravin produkcí antimikrobiálních látek. Do budoucna se jeví perspektivní možnosti využití některých metabolitů, zvláště bakteriocinů, i jako antibakteriálních přípravků ve veterinární i humánní medicíně při kauzální léčbě infekcí způsobených bakteriemi rezistentními k antibiotikům.

S postupujícími znalostmi o mikrobiotě lidského těla vyvstává také stále řada otázek spojených s významem probiotických kmenů BMK. Jejich nespornou výhodou při použití jako doplňků stravy, případně léčiv, při různých stavech disbiosy, při podpoře imunitního systému a dalších aplikacích je jejich naprostá bezpečnost. V budoucnu lze očekávat, že mohou být využity i geneticky modifikované kmeny pro specifické použití. Stále častěji se rovněž diskutuje o nutnosti personifikované selekce probiotického kmene nebo individuální mikrobiální terapie autoprobiotiky.

6 Použitá literatura

- Abdellatif B., McVeigh C., Bendriss G., Chaari A. (2020): The promising role of probiotics in managing the altered gut in autism spectrum disorders. *Int. J. Mol. Sci.* 21 (11): 4159. doi:10.3390/ijms21114159
- Ahn Y. T., Kim G. B., Lim K. S., Baek Y. J., Kim H. U. (2003): Deconjugation of bile salts by *Lactobacillus acidophilus* isolates. *Int. Dairy J.* 13: 303-311. doi:10.12691/ajmr-6-1-1
- Akin M. B., Akin M. S., Kirmaci Z. (2007): Effects of inulin and sugar levels on the viability of yogurt and probiotic bacteria and the physical and sensory characteristics in probiotic ice-cream. *Food Chem.* 104 (1): 93-99. doi:10.1016/j.foodchem.2006.11.030.
- Alp D., Kuleşan H., Korkut Altıntaş A. (2020): The importance of the S-layer on the adhesion and aggregation ability of lactic acid bacteria. *Mol. Biol. Rep.* 47: 3449-3457. doi:10.1007/s11033-020-05430-6
- Ammor S., Tauveron G., Dufour E., Chevallier I. (2006): Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 1- Screening and characterization of the antimicrobial compounds. *Food Control* 17: 454-461. doi:10.1016/j.foodcont.2005.02.006
- Angelucci F., Cechova K., Amlerova J., *et al.* (2019): Antibiotics, gut microbiota, and Alzheimer's disease. *J. Neuroinflammation* 16, 108. doi:10.1186/s12974-019-1494-4
- Anisimova E. A., Yarullina D. R. (2019): Antibiotic resistance of *Lactobacillus* strains. *Curr. Microbiol.* 76: 1407-1416. doi:10.1007/s00284-019-01769-7
- Arena M. E., Manca de Nadra M. C., Munoz R. (2002): The arginine deiminase pathway in the wine lactic acid bacterium *Lactobacillus hilgardii* X1B: structural and functional study of the *arcABC* genes. *Gene* 301: 61–66. doi:10.1016/s0378-1119(02)01083-1
- Argyri A. A., Zoumpopoulou G., Karatzas K. A., Tsakalidou E., Nychas G. J., Panagou E. Z., Tassou C. C. (2013): Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol.* 32: 282-91. doi:10.1016/j.fm.2012.10.005
- Asare P. T., Zurfluh K., Greppi A., Lynch D., Schwab C., Stephan R., Lacroix C. (2020): Reuterin demonstrates potent antimicrobial activity against a broad panel of human and poultry meat *Campylobacter* spp. isolates. *Microorganisms* 8: 78. doi:10.3390/microorganisms8010078
- Aspri M., Papademas P., Tsaltas D. (2020): Review on non-dairy probiotics and their use in non-dairy based products. *Fermentation* 6: 30. doi:10.3390/fermentation6010030
- Aunbjerg S. D., Honoré A. H., Marcussen J., Ebrahimi P., Vogensen F. K., Benfeldt C., Skov T., Knøchel S. (2015): Contribution of volatiles to the antifungal effect of *Lactobacillus paracasei* in defined medium and yogurt. *Int. J. Food Microbiol.* 194: 46-53. doi:10.1016/j.ijfoodmicro.2014.11.007
- Axel C., Brosnan B., Zannini E., Furey A., Coffey A., Arendt E. K. (2016): Antifungal sourdough lactic acid bacteria as biopreservation tool in quinoa and rice bread. *Int. J. Food Microbiol.* 239: 86-94. doi:10.1016/j.ijfoodmicro.2016.05.006
- Ayyash M., Olaimat A., Al-Nabulsi A., Liu S. Q. (2020): Bioactive properties of novel probiotic *Lactococcus lactis* fermented camel sausages: Cytotoxicity, angiotensin converting enzyme inhibition, antioxidant capacity, and antidiabetic activity. *Food Sci. Anim. Resour.* 40 (2): 155-171. doi:10.5851/kosfa.2020.e1
- Barbieri F., Montanari C., Gardini F., Tabanelli G. (2019): Biogenic amine production by lactic acid bacteria: A Review. *Foods* 8: 17. doi:10.3390/foods8010017
- Batdorj B., Trinetta V., Dalgalarondo M., Prevost H., Doussset X., Ivanova I., Haertlé T., Chobert J.-M. (2007): Isolation, taxonomic identification and hydrogen peroxide production by *Lactobacillus*

- delbrueckii* subsp. *lactis* T31, isolated from Mongolian yoghurt: inhibitory activity on food-borne pathogens. J. App. Microbiol. 103: 584-593. doi:10.1111/j.1365-2672.2007.03279.x
- Baud D., Dimopoulou Agri V., Gibson G. R., Reid G., Giannoni E. (2020): Using probiotics to flatten the curve of coronavirus disease COVID-2019 Pandemic. Front. Public Health 8:186. doi:10.3389/fpubh.2020.00186
- Begley M., Gahan C. G. M., Hill C. (2005): Interaction between bacteria and bile. FEMS Microbiol. Rev. 29: 625-651. doi:10.1016/j.femsre.2004.09.003
- Begley M., Hill C., Gahan C. G. M. (2006): Bile salt hydrolase activity in probiotics. App. Environ. Microbiol. 72: 1729-1738. doi:10.1128/AEM.72.3.1729-1738.2006
- Bi J., Fang F., Lu S., Du G., Chen J. (2013): New insight into the catalytic properties of bile salt hydrolase. J. Mol. Catal. B Enzym. 96: 46-51. doi:10.1016/j.molcatb.2013.06.010
- Bialasová K., Němečková I., Kyselka J., Štětina J., Solichová K., Horáčková Š. (2018): Influence of flaxseed components on fermented dairy product properties. Czech J. Food Sci. 36, 51-56. doi:10.17221/411/2017-CJFS
- Bindels L. B., Delzenne N. M., Cani P. D., Walter J. (2015): Towards a more comprehensive concept for prebiotics. Nat. Rev. Gastroenterol. Hepatol. 12(5): 303-310. doi:10.1038/nrgastro.2015.47
- Bolotin A., Wincker P., Mauger S., Jaillon O., Malarme K., Weissenbach J., Ehrlich S. D., Sorokin A. (2001): The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. 11(5): 731-753. doi:10.1101/gr.169701
- Børsting M. W., Qvist K. B., Brockmann E., Vindeløv J., Pedersen T. L., Vogensen F. K., Ardö Y. (2015): Classification of *Lactococcus lactis* cell envelope proteinase based on gene sequencing, peptides formed after hydrolysis of milk, and computer modeling. J. Dairy Sci. 98: 68-77. doi:10.3168/jds.2014-8517
- Breshears M. L., Edwards V. L., Ravel J., Peterson M. L. (2015): *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. BMC Microbiol. 15: 276. doi:10.1186/s12866-015-0608-0
- Brüssow H. (2019): Probiotics and prebiotics in clinical tests: an update. F1000Research, 8, F1000 Faculty Rev-1157. doi:10.12688/f1000research.19043.1
- Burgain J., Gaiani C., Linder M., Scher J. (2011): Encapsulation of probiotic living cells: From laboratory scale to industrial applications. J. Food Eng. 104 (4): 467-483. doi:10.1016/j.jfoodeng.2010.12.031
- Bustos A. Y., Saavedra L., Font de Valdez G., Raya R. R., Taranto M. P. (2012): Relationship between bile salt hydrolase activity, changes in the internal pH and tolerance to bile acids in lactic acid bacteria. Biotechnol. Lett. 34: 1511-1518. doi:10.1007/s10529-012-0932-5
- Cannon J. P., Lee T. A., Bolanos J. T., Danziger L. H. (2005): Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. Eur. J. Clin. Microbiol. Infect. Dis. 24: 31-40. doi:10.1007/s10096-004-1253-y
- Castro-Aguirre E., Iñiguez-Franco F., Samsudin H., Fang X., Auras R. (2016): Poly(lactic acid) - Mass production, processing, industrial applications, and end of life. Adv. Drug Deliv. Rev. 107: 333-366. doi:10.1016/j.addr.2016.03.010
- Cen T., Zhang X., Xie S., Li D. (2020): Preservatives accelerate the horizontal transfer of plasmid-mediated antimicrobial resistance genes via differential mechanisms. Environ. Int. 138: 105544. doi:10.1016/j.envint.2020.105544.
- Cizeikiene D., Juodeikiene G., Paskevicius A., Bartkienec E. (2013): Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. Food Control 31: 539-545. doi:10.1016/j.foodcont.2012.12.004

- Collado M. C., Meriluoto J., Salminen S. (2008): Adhesion and aggregation properties of probiotic and pathogen strains. *Eur. Food Res. Technol.* 226 (5):1065-1073.
doi:10.1007/s00217-007-0632-x
- Colombo M., Olivera A. E. Z., Carvalho A. R., Nero L. A. (2014): Development of an alternative culture medium for selective enumeration of *Lactobacillus casei* in fermented milk. *Food Microbiology* 39: 89-95. doi:10.1016/j.fm.2013.11.008
- Cortés-Zavaleta O., López-Malo A., Hernández-Mendoza A., García H. S. (2014): Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *Int. J. Food Microbiol.* 173: 30-35. doi:10.1016/j.ijfoodmicro.2013.12.016
- Courtin P., Monnet V., Rul F. (2002): Cell-wall proteinases PrtS and PrtB have a different role in *Streptococcus thermophilus*/*Lactobacillus bulgaricus* mixed cultures in milk. *Microbiology* 148: 3413-3421. doi:10.1099/00221287-148-11-3413
- Crowley S., Mahony J., van Sinderen D. (2013): Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends Food Sci. Technol.* 33: 93-109. doi:10.1016/j.tifs.2013.07.004
- ČSN ISO 20128 (560642): Mléčné výrobky - Stanovení počtu presumptivního *Lactobacillus acidophilus* na selektivní živné půdě - Technika počítání kolonií vykultivovaných při 37 °C
- ČSN ISO 29981 (560649): Mléčné výrobky - Stanovení počtu presumptivních bifidobakterií - Technika počítání kolonií vykultivovaných při 37 °C
- ČSN ISO 7889 (571420): Jogurt - Stanovení počtu charakteristických mikroorganismů - Technika stanovení počtu kolonií při 37 °C
- ČSN ISO 9232 (571421): Jogurt - Identifikace charakteristických mikroorganismů - (*Lactobacillus delbrueckii* subsp. *bulgaricus* a *Streptococcus thermophilus*)
- Da Costa R. J., Voloski F. L. S., Mondadori R. G., Duval E. H., Fiorentini A. M. (2019): Preservation of meat products with bacteriocins produced by lactic acid bacteria isolated from meat: Article ID 4726510. doi:10.1155/2019/4726510
- Dagnas S., Gauvry E., Onno B., Membré J.-M. (2015): Quantifying effect of lactic, acetic, and propionic acids on growth of molds isolated from spoiled bakery products. *J. Food Prot.* 78: 1689-1698. doi:10.4315/0362-028X.JFP-15-046
- Dalié D. K. D., Deschamps A. M., Richard-Forget F. (2010): Lactic acid bacteria – Potential for control of mould growth and mycotoxins. *Food Control* 21: 370-380.
doi:10.1016/j.foodcont.2009.07.011
- Dan T., Chen H., Li T., Tian J., Ren W., Zhang H., Sun T. (2019): Influence of *Lactobacillus plantarum* P-8 on fermented milk flavor and storage stability. *Front. Microbiol.* 9: 3133.
doi:10.3389/fmicb.2018.03133
- Das D. J., Shankar A., Johnson J. B., Thomas S. (2020): Critical insights into antibiotic resistance transferability in probiotic *Lactobacillus*. *Nutrition* 69: 110567. doi:10.1016/j.nut.2019.110567
- Dashkevich M. P., Feighner S. D. (1989): Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. *App. Environ. Microbiol.* 55: 11-16.
doi:10.1128/AEM.55.1.11-16.1989
- De Aguiar Vallim T. Q., Tarling E. J. (2013): Pleiotropic roles bile acids in metabolism. *Cell Metab.* 17: 657-669. doi:10.1016/j.cmet.2013.03.013
- De Smet I., Van Hoorde L., Woestyne V., Christiaens H., Verstraete W. (1995): Significance of bile salt hydrolytic activities of lactobacilli. *J. Appl. Bacteriol.* 79: 292-301.
- De Souza Oliveira R. P., Perego P., de Oliveira M. N., Converti A. (2012): Effect of inulin on the growth and metabolism of a probiotic strain of *Lactobacillus rhamnosus* in co-culture with *Streptococcus thermophilus*. *LWT - Food Sci. Technol.* 47 (2), 358-363.
doi:10.1016/j.lwt.2012.01.031

- De Vuyst L., Leroy F. (2007): Bacteriocins from lactic acid bacteria: production, purification, and food applications. *J. Mol. Microbiol. Biotechnol.* 13 (4): 194-199. doi:10.1159/000104752
- Di Lena M., Quero G. M., Santovito E., Verran J., De Angelis M., Fusco V. (2015): A selective medium for isolation and accurate enumeration of *Lactobacillus casei*-group members in probiotic milks and dairy products. *Int. Dairy J.* 47: 27-36. doi:10.1016/j.idairyj.2015.01.018
- Dinan T. G., Stanton C., Cryan J. F. (2013): Psychobiotics: A novel class of psychotropic. *Biol. Psychiatry* 74: 720-726. doi:10.1016/j.biopsych.2013.05.001
- Djadouni F., Kihal M. (2012): Antimicrobial activity of lactic acid bacteria and the spectrum of their biopeptides against spoiling germs in foods. *Braz. Arch. Biol. Technol.* 55 (3): 435-444. doi:10.1590/S1516-89132012000300015
- Do Carmo F., Rabah H., de Oliveira Carvalho R. D., Gaucher F., Cordeiro B. F., da Silva S. H., Le Loir Y., Azevedo V., Jan G. (2018): Extractable bacterial surface proteins in probiotic-host interaction. *Front. Microbiol.* 9: 645. doi:10.3389/fmicb.2018.00645
- Douillard F. P., de Vos W. M. (2014): Functional genomics of lactic acid bacteria: from food to health. *Microb. Cell Fact.* 13 (Suppl 1): S8. doi:10.1186/1475-2859-13-S1-S8
- Douillard F. P., Ribbera A., Kant R., Pietilä T. E., Järvinen H. M., *et al.* (2013): Comparative genomic and functional analysis of 100 *Lactobacillus rhamnosus* strains and their comparison with strain GG. *PLOS Genetics* 9(8): e1003683. doi:10.1371/journal.pgen.1003683
- Drakoularakou A., Rastall R., Gibson G.: 19 - Functional foods for the gut: probiotics, prebiotics and synbiotics, in *Functional Foods* (2nd Edition), Editor Maria Saarela, Woodhead Publishing, 2011, pp. 449-470.
- Durant S., Lugli, G. A., Mancabelli L., Turroni F., Milani C., Mangifesta M., Ferrario C., Anzalone R., Viappiani A., van Sinderen D., Ventura M. (2017): Prevalence of antibiotic resistance genes among human gut-derived bifidobacteria. *App. Environ. Microbiol.* 83 (3): e02894-16. doi:10.1128/AEM.02894-16
- EFSA (European Food Safety Authority) (2017): Safety of nisin (E 234) as a food additive in the light of new toxicological data and the proposed extension of use. *EFSA Journal* 15 (12): e05063.
- EFSA (European Food Safety Authority) (2007): Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA – Opinion of the Scientific Committee. *EFSA Journal* 5: 1-16.
- EFSA (European Food Safety Authority) 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J.* 10:2740.
- El Hage R., Hernandez-Sanabria E., Van de Wiele T. (2017): Emerging trends in “Smart Probiotics”: Functional consideration for the development of novel health and industrial applications. *Front. Microbiol.* 8: 1889. doi:10.3389/fmicb.2017.01889
- Elkins C. A., Moser S. A., Savage D. C. (2001): Genes encoding bile salt hydrolases and conjugated bile salt transporters in *Lactobacillus johnsonii* 100-100 and other *Lactobacillus* species. *Microbiology* 147: 3403-3412. doi:10.1099/00221287-147-12-3403
- Elsner H., Sobottka I., Mack D., Laufs R., Claussen M., Wirth R. (2000): Virulence factors of *Enterococcus faecalis* and *Enterococcus faecium* blood culture isolates. *Eur. J. Clin. Microbiol. Infect. Dis.* 19: 39-42. doi:10.1007/s100960050007
- Engels C., Schwab C., Zhang J. *et al.* (2016): Acrolein contributes strongly to antimicrobial and heterocyclic amine transformation activities of reuterin. *Sci. Rep.* 6: 36246. doi:10.1038/srep36246
- Esaiassen E., Hjerde E., Cavanagh J. P., Simonsen G. S., Klingenberg C. (2017): *Bifidobacterium* bacteremia: Clinical characteristics and a genomic approach to assess pathogenicity. *J. Clin. Microbiol.* 55 (7): 2234-2248. doi:10.1128/JCM.00150-17

- Etchepare M. A., Barin J. S., Cichoski A. J., Jacob-Lopes E., Wagner R., Fries L. L. M., de Menezes C. R. (2015): Microencapsulation of probiotics using sodium alginate. *Ciência Rural*. 45 (7): 1319-1326. doi:10.1590/0103-8478cr20140938
- Evivie S. E., Huo G. C., Igene J. O., Bian X. (2017): Some current applications, limitations and future perspectives of lactic acid bacteria as probiotics. *Food Nutr. Res.* 61(1): 1318034. doi:10.1080/16546628.2017.1318034
- Faron M. L., Ledebouer N. A., Buchan B.W. (2016): Resistance mechanisms, epidemiology, and approaches to screening for vancomycin-resistant *Enterococcus* in the health care setting. *J. Clin. Microbiol.* 54 (10): 2436-2447. doi:10.1128/JCM.00211-16
- Feldeková E., Solichová K., Horáčková Š., Kumherová M., Kyselka J. (2020): The impact of L-lanthionine supplementation on the production of nisin by lactococci. *Eur. Food Res. Technol.* 246: 845-851. doi:10.1007/s00217-020-03449-4
- Fontana L., Bermudez-Brito M., Plaza-Dia, J., Muñoz-Quezada S., Gil A. (2013): Sources, isolation, characterisation and evaluation of probiotics. *Br. J. Nutr.* 109 (S2): S35-S50. doi:10.1017/S0007114512004011
- Fontana L., Bermudez-Brito M., Plaza-Diaz J., Muñoz-Quezada S., Gil, A. (2013): Sources, isolation, characterisation and evaluation of probiotics. *Br. J. Nutr.* 109 (S2): S35-S50. doi:10.1017/S0007114512004011
- Foong Y. J., Lee S. T., Ramli N., Tan Y. N., Ayob M. K. (2013): Incorporation of probiotic into dark chocolate. *J. Food Qual.*, 36: 164-171. doi:10.1111/jfq.12028
- Fuller R. (1989): Probiotics in man and animals. *J. Appl. Bacteriol.* 66: 365-378. doi:10.1111/j.1365-2672.1989.tb05105.x
- Gadhiya D., Shah N. P., Pate, A. R., Prajapati J. B. (2018): Preparation and shelf life study of probiotic chocolate manufactured using *Lactobacillus helveticus* MTCC 5463. *Acta Aliment.* 47: 350-358. <https://akjournals.com/view/journals/066/47/3/article-p350.xml>
- Galat A., Dufresne J., Combrisson J., Thépaut J., Boumghar-Bourtchai L., Boyer M., Fourmestraux C. (2016): Novel method based on chromogenic media for discrimination and selective enumeration of lactic acid bacteria in fermented milk products. *Food Microbiol.* 55: 86-94. doi:10.1016/j.fm.2015.11.005
- Gänzle M. G., Hölzel A., Walter J., Jung G., Hammes W. P. (2000): Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. *Appl. Environ. Microbiol.* 66 (10): 4325-4333. doi:10.1128/aem.66.10.4325-4333.2000
- García-Cayuela T., Korany A. M., Bustos I., de Cadiñanos L. P. G., Requena T., Peláez C., Martínez-Cuestaa M. C. (2014): Adhesion abilities of dairy *Lactobacillus plantarum* strains showing an aggregation phenotype. *Food Res. Int.* 57: 44-50. doi:10.1016/j.foodres.2014.01.010
- García-Cayuela T., Tabasco R., Peláez C., Requena T. (2009): Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR. *Int. Dairy J.* 19 (6-7): 405-409. doi:10.1016/j.idairyj.2009.02.001
- Gazerani P. (2019): Probiotics for Parkinson's disease. *Int. J. Mol. Sci.* 20: 4121. doi:10.3390/ijms20174121
- Gazzola S., Fontana C., Bassi D., Cocconcelli P. S. (2012): Assessment of tetracycline and erythromycin resistance transfer during sausage fermentation by culture-dependent and -independent methods. *Food Microbiol.* 30: 348-354. doi:10.1016/j.fm.2011.12.005
- Gezginc Y., Topcal F., Comertpay S., Akyol I. (2015): Quantitative analysis of the lactic acid and acetaldehyde produced by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* strains isolated from traditional Turkish yogurts using HPLC. *J. Dairy Sci.* 98: 1426-1434. doi:10.3168/jds.2014-8447

- Gibson G. R., Roberfroid M. B. (1995): Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* 125 (6): 1401-1412. doi:10.1093/jn/125.6.1401
- Gibson G. R., Scott K. P., Rastall R. A., Tuohy K. M., Hotchkiss A., Dubert-Ferrandon A., Gareau M., Murphy E. F., Saulnier D., Loh G., *et al.* (2010): Dietary prebiotics: Current status and new definition. *Food Sci. Technol. Bull. Funct. Foods.* 7: 1-19. doi:10.1616/1476-2137.15880
- Gouriet F., Million M., Henri M., Fournier P.-E., Raoult D. (2012): *Lactobacillus rhamnosus* bacteremia: an emerging clinical entity. *Eur. J. Clin. Microbiol. Infect. Dis.* 31: 2469-2480. doi:10.1007/s10096-012-1599-5
- Griffiths M. W., Tellez A. M. (2013): *Lactobacillus helveticus*: the proteolytic system. *Front. Microbiol.* 4: Article 30. doi:10.3389/fmicb.2013.00030
- Gueimonde M., Sánchez B., de los Reyes-Gavilán C., Margolles A. (2013): Antibiotic resistance in probiotic bacteria. *Front. Microbiol.* 4: 202. doi:10.3389/fmicb.2013.00202
- Heidebach T., Först P., Kulozik U. (2009): Microencapsulation of probiotic cells by means of rennet-gelation of milk proteins. *Food Hydrocolloids* 23 (7): 1670-1677. doi:10.1016/j.foodhyd.2009.01.006
- Heinken, A., Khan, M. T., Paglia, G., Rodionov D. A., Harmsen H. J. M., Thiele I. (2014): Functional metabolic map of *Faecalibacterium prausnitzii*, a beneficial human gut microbe. *J. Bacteriol.* 196 (18): 3289-3302. doi:10.1128/jb.01780-14
- Herrera-Ponce A., Nevárez-Morillón G., Ortega-Ríva, E., Pérez-Vega S., Salmerón I. (2014): Fermentation adaptability of three probiotic *Lactobacillus* strains to oat, germinated oat and malted oat substrates. *Lett. Appl. Microbiol.* 59: 449-456. doi:10.1111/lam.12302
- Hertzberger R., Arents J., Dekker H. L., Pridmore R. D., Gysler C., Kleerebezem M., Teixeira de Mattos M. J. (2014): H₂O₂ production in species of the *Lactobacillus acidophilus* Group: a Central role for a novel NADH-dependent flavin reductase. *Appl. Environ. Microbiol.* 80 (7): 2229-2239. doi:10.1128/AEM.04272-13
- Hill C., Guarner F., Reid G., *et al.* (2014): The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11: 506-514. doi:10.1038/nrgastro.2014.66
- Hofmann A. F., Eckmann L. (2006): How bile acids confer gut mucosal protection against bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 103, 4333-4334. doi:10.1073/pnas.0600780103
- Höltzel A., Gänzle M., Nicholson G., Hammes W., Jung G. (2000): The first low molecular weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. *Angew Chem Int Ed Engl* 39: 2766-2768. doi:10.1002/1521-3773
- Hong G.-E., Mandal P. K., Lim K.-W., Lee, 2012 C.-H. (2012): Fermentation increases isoflavone aglycone contents in black soybean pulp. *Asian J. Anim. Vet. Adv.* 7: 502-511. doi:10.3923/ajava.2012.502.511
- Horácková Š., Mühlhansová A., Sluková M., Schulzová V., Plocková M. (2015): Fermentation of soymilk by yoghurt and bifidobacteria strains. *Czech J. Food Sci.* 33 (4): 313-319. doi:10.17221/115/2015-CJFS
- Horácková Š., Nováková T., Sluková M., Bialasová K., Plocková M. (2018a): Vliv laktobacilů na růst plísně *Fusarium culmorum* a *Penicillium expansum*. *Sborník Celostátní přehlídky sýrů 2018 - Výsledky přehlídek a sborník konference Mléko a sýry.* (ed. Štětina J., Čurda L.), 171-175, VŠCHT Praha 2018 (ISBN 978-80-7592-030-0).
- Horácková Š., Nováková T., Sluková M., Bialasová K., Kumherová M., Plocková M. (2018b): Antifungal activity of selected lactobacilli intended for sourdough production. *App. Food Biotechnol.* 5 (4): 213-220. doi:10.22037/afb.v%vi%i.22173

- Horáčková Š., Plocková M., Demnerová K. (2018c): Importance of microbial defence systems to bile salts and mechanisms of serum cholesterol reduction. *Biotechnol. Adv.* 36: 682-690. doi:10.1016/j.biotechadv.2017.12.005
- Horáčková Š., Rokytová K., Bialasová K., Klojďová I., Sluková M. (2018d): Fruit juices with probiotics – new type of functional foods. *Czech J. Food Sci.* 36: 284-288. doi:10.17221/39/2018-CJFS
- Horáčková Š., Sedláčková P., Sluková M., Plocková M. (2014): The influence of whey, whey component and malt on the growth and acids production of lactobacilli in milk. *Czech J. Food Sci.* 32 (6): 526-531. doi:10.17221/214/2014-CJFS
- Horáčková Š., Veselá K., Klojďová I., Berčíková M., Plocková M. (2020): Bile salt hydrolase activity, growth characteristics and surface properties in *Lactobacillus acidophilus*. *Eur. Food Res. Technol.* 246: 1627-1636. doi:10.1007/s00217-020-03518-8
- Horáčková Š., Žaludová K., Plocková M. (2011): Stability of selected lactobacilli in the conditions simulating those in the gastrointestinal tract. *Czech J. Food Sci.* 29: 30-35. doi:10.17221/283/2011-CJFS
- Hummel A., Holzapfel W. H., Franz C. M. A. P. (2007): Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food. *Syst. App. Microbiol.* 30 (1): 1-7. doi:10.1016/j.syapm.2006.02.004
- Hynönen U., Palva A. (2013): *Lactobacillus* surface layer proteins: structure, function and applications. *Appl. Microbiol. Biotechnol.* 97: 5225-5243. doi:10.1007/s00253-013-4962-2
- Chakraborty S. (2017): Carrageenan for encapsulation and immobilization of flavor, fragrance, probiotics, and enzymes: A review. *J. Carbohydr. Chem.* 36 (1): 1-19. doi:10.1080/07328303.2017.1347668
- Champagne C. P., Roy D., Lafond A. (1997): Selective enumeration of *Lactobacillus casei* in yoghurt-type fermented milks based on a 15 °C incubation temperature. *Biotechnol. Techniques* 11: 567-569. doi:10.1023/A:1018490822649
- Chand D., Ramasamy S., Suresh C. G. (2016): A highly active bile salt hydrolase from *Enterococcus faecalis* shows positive cooperative kinetics. *Process Biochem.* 51: 263-269. doi:10.1016/j.procbio.2015.12.006
- Chaudhari S. S., Gokhale D. V. (2016): Phenyllactic acid: a potential antimicrobial compound in lactic acid bacteria. *J. Bacteriol. Mycol.* 2 (5): 121-125. doi:10.15406/jbmoa.2016.02.00037
- Chen L., Zhang Q., Ji Z., Shu G., Chen H. (2018): Production and fermentation characteristics of angiotensin-I-converting enzyme inhibitory peptides of goat milk fermented by a novel wild *Lactobacillus plantarum* 69. *LWT – Food Sci. Technol.* 91: 532-540. doi:10.1016/j.lwt.2018.02.002
- Cheng L. H., Liu Y. W., Wu C. C., Wang S., Tsai Y. C. (2019): Psychobiotics in mental health, neurodegenerative and neurodevelopmental disorders. *J. Food Drug Anal.* 27 (3): 632-648. doi:10.1016/j.jfda.2019.01.002
- ISAPP 2018: Minimum criteria for probiotics. Zdroj: <https://isappscience.org/minimum-criteria-probiotics/>; staženo 21. 11. 2020
- Islam K. B., Fukiya S., Hagio M., Fujii N., Ishizuka S., Ooka T. (2011): Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 141 (5), 1773-1781. doi:10.1053/j.gastro.2011.07.046
- Ito A., Sato Y., Kudo S. *et al.* (2003): The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens. *Curr. Microbiol.* 47: 0231-0236. doi:10.1007/s00284-002-3993-1

- Jahan M., Zhanel G. G., Sparling R., Holley R. A. (2015): Horizontal transfer of antibiotic resistance from *Enterococcus faecium* of fermented meat origin to clinical isolates of *E. faecium* and *Enterococcus faecalis*. *Int. J. Food Microbiol.* 199: 78-85. doi:10.1016/j.ijfoodmicro.2015.01.013
- Jarocki P., Podleśny M., Glibowski P., Targoński Z. (2014): A new insight into the physiological role of bile salt hydrolase among intestinal bacteria from the genus *Bifidobacterium*. *PLoS ONE* 9 (12), e114379. doi:10.1371/journal.pone.0114379
- Jebavá I., Chuat V., Lortal S., Valence F. (2014): Peptidoglycan hydrolases as species-specific markers to differentiate *Lactobacillus helveticus* from *Lactobacillus gallinarum* and other closely related homofermentative lactobacilli. *Curr. Microbiol.* 68: 551-557. doi:10.1007/s00284-013-0512-5
- Jiang J., Hang X., Zhang M., Liu X., Li D., Yang H. (2010): Diversity of bile salt hydrolase activities in different lactobacilli toward human bile salts. *Ann. Microbiol.* 60: 81-88. doi:10.1007/s13213-009-0004-9
- Jørgensen M. R., Kragelund C., Jensen P. Ø., Keller M. K., Twetman S. (2017): Probiotic *Lactobacillus reuteri* has antifungal effects on oral *Candida* species *in vitro*. *J. Oral Microbiol.* 9 (1): 1274582. doi:10.1080/20002297.2016.1274582
- Kailasapathy, K. (2009). Encapsulation technologies for functional foods and nutraceutical product development. *Cab Reviews: Perspectives In Agriculture, Veterinary Science, Nutrition And Natural Resources*, 4 (33): 1-19. doi:10.1079/PAVSNNR20094033
- Kawashima T., Hayashib K., Kosakac A., Kawashimaa M., Igarashi T., Tsutsui H., Tsujic N. M., Nishimura I., Hayashib T., Obataa A. (2011): *Lactobacillus plantarum* strain YU from fermented foods activates Th1 and protective immune responses. *Int. Immunopharmacol.* 11 (2): 2017-2024. doi:10.1016/j.intimp.2011.08.013
- Kelly W. J., Ward L. J. W., Leahy S. C. (2010): Chromosomal diversity in *Lactococcus lactis* and the origin of dairy starter cultures. *Genome Biol Evol* 2: 729-744. doi:10.1093/gbe/evq056
- Kerry R. G., Patra J. K., Gouda S., Park Y., Shin H.-S., Das G. (2018): Benefaction of probiotics for human health: A review. *J. Food Drug Anal.* 26 (3): 927-939. doi:10.1016/j.jfda.2018.01.002
- Kesen M. A., Aiyegoro O. A. (2018): Beneficial characteristics and evaluation criteria of probiotics. *Int. J. Food Biosci.* 1 (1): 19-26.
- Kim G.-B., Lee B. H. (2008): Genetic analysis of a bile salt hydrolase in *Bifidobacterium animalis* subsp. *lactis* KL612. *J. Appl. Microbiol.* 105: 778-790. doi:10.1111/j.1365-2672.2008.03825.x
- Klare I., Konstabel C., Werne G., Huys G., Vankerckhoven V., Kahlmeter G., Hildebrandt B., Müller-Bertling S., Witte W., Goossens H. (2007): Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *J. Antimicrob. Chemother.* 59: 900-912. doi:10.1093/jac/dkm035
- Korhonen H. J.: Bioactive Components in Bovine Milk. In Park Y. W.: *Bioactive Components in Milk and Dairy Products*. John Wiley and Sons. London 2009, p. 29.
- Kos B., Šušković J., Vuković S., Šimpraga M., Frece J., Matošić S. (2003): Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J. Appl. Microbiol.* 94: 981-987. doi:10.1046/j.1365-2672.2003.01915.x
- Koval D., Plocková M., Kyselka J., Skřivan P., Sluková M., Horáčková Š. (2020): Buckwheat secondary metabolites: Potential antifungal agents. *J. Agric. Food Chem.* 68: 11631-11643. doi:10.1021/acs.jafc.0c04538
- Krasaekoopt W., Bhandari B., Deeth H. (2003): Evaluation of encapsulation techniques of probiotics for yoghurt. *Int. Dairy J.* 13 (1): 3-13. doi:10.1016/S0958-6946(02)00155-3

- Kumar R., Bansal P., Singh J., Dhanda S. (2020): Purification, partial structural characterization and health benefits of exopolysaccharides from potential probiotic *Pediococcus acidilactici* NCDC 252. *Process Biochem.* 99: 79-86. doi:10.1016/j.procbio.2020.08.028
- Kumar R., Rajkumar H., Kumar M., Varikuti S. R., Athimamula R., Shujauddin M., Ramagoni R., Kondapalli N. (2013): Molecular cloning, characterization and heterologous expression of bile salt hydrolase (Bsh) from *Lactobacillus fermentum* NCDO394. *Mol. Biol. Rep.* 40: 5057-5066. doi:10.1007/s11033-013-2607-2
- Kumar R., Rajkumar H., Kumar M., Varikuti S. R., Athimamula R., Shujauddin M., Ramagoni R., Kondapalli N. (2013): Molecular cloning, characterization and heterologous expression of bile salt hydrolase (Bsh) from *Lactobacillus fermentum* NCDO394. *Mol. Biol. Rep.* 40, 5057-5066. doi:10.1007/s11033-013-2607-2
- Kumherová M., Veselá K., Jokešová K., Kłodová I., Horácková Š. (2020): Influence of co-encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 with inulin and ascorbic acid on its viability. *Czech J. Food Sci.* 38 (1): 57-62. doi:10.17221/292/2019-CJFS
- Kumherová M., Veselá K., Kosová M., Mašata J., Horácková Š., Šmidrkal J. (2020): Novel potential probiotic lactobacilli for prevention and treatment of vulvovaginal infections. *Probiotics and Antimicrob. Proteins* (2020). doi:10.1007/s12602-020-09675-2
- Kwak M., Liu R., Kim M., *et al.* (2014): Cyclic dipeptides from lactic acid bacteria inhibit the proliferation of pathogenic fungi. *J. Microbiol.* 52: 64-70. doi:10.1007/s12275-014-3520-7
- Kwak M., Liu R., Kwon J., *et al.* (2013): Cyclic dipeptides from lactic acid bacteria inhibit proliferation of the influenza A virus. *J. Microbiol.* 51: 836-843. doi:10.1007/s12275-013-3521-y
- Lahtinen S., Ahokoski H., Reinikainen J., Gueimond, M., Nurmi J., Ouwehand A., Salminen S. (2008): Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. *Lett. App. Microbiol.* 46: 693-698. doi:10.1111/j.1472-765X.2008.02374.x
- Lambert J. M., Bongers R. S., de Vos W. M., Kleerebezem M. (2008): Functional analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus plantarum* WCFS1. *Applied and Environmental Microbiology* 74: 4719-4726. doi:10.1128/AEM.00137-08
- LeBlanc J. G., Levit R., Savoy de Giori G., LeBlanc A. M. (2020): Application of vitamin-producing lactic acid bacteria to treat intestinal inflammatory diseases. *Appl. Microbiol. Biotechnol.* 104: 3331-3337. doi:10.1007/s00253-020-10487-1
- Leyva Salas M., Thierry A., Lemaître M., Garric G., Harel-Oger M., Chatel M., Lê S., Mounier J., Valence F., Coton E. (2018): Antifungal activity of lactic acid bacteria combinations in dairy mimicking models and their potential as bioprotective cultures in pilot scale applications. *Front. Microbiol.* 9: 1787. doi:10.3389/fmicb.2018.01787
- Li Ch., Song J., Kwok L., Wang J., Dong Y., Yu H., Hou Q., Zhang H., Chen Y. (2017): Influence of *Lactobacillus plantarum* on yogurt fermentation properties and subsequent changes during postfermentation storage. *J. Dairy Sci.* 100 (4): 2512-2525. doi:10.3168/jds.2016-11864
- Li J., Zhao J., Wang X., Qayum A., Hussain M. A., Liang G., Hou J., Jiang Z., Li A. (2019): Novel Angiotensin-converting enzyme-inhibitory peptides from fermented bovine milk started by *Lactobacillus helveticus* KLDS.31 and *Lactobacillus casei* KLDS.105: Purification, identification, and interaction mechanisms. *Front. Microbiol.* 10: 2643. doi:10.3389/fmicb.2019.02643
- Li P., Gu Q., Zhou Q. (2016): Complete genome sequence of *Lactobacillus plantarum* LZ206, a potential probiotic strain with antimicrobial activity against food-borne pathogenic microorganisms. *J. Biotechnol.* 238: 52-55. doi:10.1016/j.jbiotec.2016.09.0120168-1656
- Li S., Tang S., He Q., Gong J., Hu J. (2020): Physicochemical, textural and volatile characteristics of fermented milk co-cultured with *Streptococcus thermophilus*, *Bifidobacterium animalis* or *Lactobacillus plantarum*. *Int. J. Food Sci. Technol.*, 55: 461-474. doi:10.1111/ijfs.14279

- Lilly D. M., Stillwell R. H. (1965): Probiotics: Growth-promoting factors produced by microorganisms. *Science* 12: 747-748.
- Lin X. B., Lohans C. T., Duar R., *et al.* (2015): Genetic determinants of reutericyclin biosynthesis in *Lactobacillus reuteri*. *App. Environ. Microbiol.* 81(6): 2032-2041. doi:10.1128/aem.03691-14
- Liong M. T., Shah N. P. (2005): Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. *Int. Dairy J.* 15: 391-398. doi:10.1016/j.idairyj.2004.08.007
- Lisová I., Horáčková Š., Kováčová R., Rada V., Plocková M. (2013): Emulsion encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 with the addition of lecithin. *Czech J. Food Sci.* 31: 270-274. doi:10.17221/188/2012-CJFS
- Lisová I., Plocková M., Horáčková Š. (2012): Mléčné bílkovinné mikrokapsle s obsahem probiotických mikroorganismů a lecitinu. Úžitný vzor 23219. Úřad průmyslového vlastnictví Praha 9. 1. 2012.
- Liu F., Wang F., Du L., Zhao T., Doyle M. P., Wang D., Zhang X., Sun Z., Xu W. (2018): Antibacterial and antibiofilm activity of phenyllactic acid against *Enterobacter cloacae*. *Food Control* 84: 442-448. doi:10.1016/j.foodcont.2017.09.004
- Liu W., Su X., Duo N., Yu J., Song Y., Sun T., Zha M., Menghe B., Zhang H., Sun Z., (2019): A survey of the relationship between functional genes and acetaldehyde production characteristics in *Streptococcus thermophilus* by multilocus sequence typing. *J. Dairy Sci.* 102 (11): 9651-9662. doi:10.3168/jds.2018-16203
- Macfarlane G. T., Steed H., Macfarlane S. (2008): Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J. Appl. Microbiol.* 104 (2): 305-344. doi:10.1111/j.1365-2672.2007.03520.x.
- Maragkoudakis P. A., Zoumpopoulou G., Miaris C., Kalantzopoulos G., Pot B., Tsakalidou E. (2006): Probiotic potential of *Lactobacillus* strains isolated from dairy products. *Int. Dairy J.*, 16: 189-199. doi:10.1016/j.idairyj.2005.02.009
- Markowiak P., Śliżewska K. (2017). Effects of Probiotics, Prebiotics, and Synbiotics on Human Health. *Nutrients* 9 (9): 1021. doi:10.3390/nu9091021
- Martín R., Langella P. (2019): Emerging health concepts in the probiotics field: Streamlining the definitions. *Front. Microbiol.* 10:1047. doi:10.3389/fmicb.2019.01047
- Martín R., Miquel S., Benevides L., Bridonneau C., Robert V., Hudault S., Chain F., Berteau O., Azevedo V., Chatel J. M., Sokol H., Bermúdez-Humarán L. G., Thomas M., Langella P. (2017): Functional characterization of novel *Faecalibacterium prausnitzii* strains isolated from healthy volunteers: A step forward in the use of *F. prausnitzii* as a next-generation probiotic. *Front. Microbiol.* 8: 1226. doi: 10.3389/fmicb.2017.01226
- Martín R., Olivares M., Marín M. L., Xaus J., Fernández L., Rodríguez J. M. (2005): Characterization of a reuterin-producing *Lactobacillus coryniformis* strain isolated from a goat's milk cheese. *Int. J. Food Microbiol.* 104: 267- 277. doi:10.1016/j.ijfoodmicro.2005.03.007
- Martín R., Suarez J. E. (2010): Biosynthesis and degradation of H₂O₂ by vaginal lactobacilli. *Appl. Environ. Microbiol.* 76 (2): 400-405. doi:10.1128/AEM.01631-09
- Martins E. M. F., Ramos A. M., Vanzela E. S. L., Stringheta P. C., de Oliveira Pinto C. L., Martins J. M. (2013): Products of vegetable origin: A new alternative for the consumption of probiotic bacteria. *Food Res. Int.* 51 (2): 764-770. doi:10.1016/j.foodres.2013.01.047
- Matsubara V. H., Wang Y., Bandara H. M. H. N., Mayer M. P. A., Samaranayake L.P. (2016): Probiotic lactobacilli inhibit early stages of *Candida albicans* biofilm development by reducing their growth, cell adhesion, and filamentation. *Appl. Microbiol. Biotechnol.* 100: 6415-6426. doi:10.1007/s00253-016-7527-3

- McAuliffe O., Cano R. J., Klaenhammer T. R. (2005): Genetic analysis of two bile salt hydrolase activities in *Lactobacillus acidophilus* NCFM. *App. Environ. Microbiol.* 71: 4925-4929. doi:10.1128/AEM.71.8.4925-4929.2005
- McSweeney P. L. H., Fox P. F., Ciocia F. (2017): Metabolism of residual lactose and of lactate and citrate. In: P. L. H. McSweeney, P. F. Fox, P. D. Cotter, D. W. Everett, editors. *Cheese: chemistry, physics, and microbiology*. 4th ed. San Diego: Academic Press. pp 411–23.
- Merritt M. E., Donaldson J. R. (2009). Review - Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *Journal of Medical Microbiology* 58: 1533-1541. doi:10.1099/jmm.0.014092-0
- Metchnikoff E. (1907): *The Prolongation of Life*, Heinemann, London.
- Meyers S. A., Cuppett S. L., Hutkins R. W. (1996): Lipase production by lactic acid bacteria and activity on butter oil. *Food Microbiol.* 13(5): 383-389. doi:10.1006/fmic.1996.0044
- Miao M., Ma Y., Huang C., Jiang B., Cui S. W., Zhang T. (2015): Physicochemical properties of a water soluble extracellular homopolysaccharide from *Lactobacillus reuteri* SK24.003. *Carbohydr. Polymers* 131: 377-383. doi:10.1016/j.carbpol.2015.05.066
- Mijač V. D., Đukića S. V., Opavskia N. Z., Đukić M. K., Ranina L. T. (2006): Hydrogen peroxide producing lactobacilli in women with vaginal infections. *Eur. J. Obstetr. Gynecol. Reprod. Biol.* 129 (1): 69-76. doi:10.1016/j.ejogrb.2005.11.036
- Minato T., Nirasawa S., Sato T., *et al.* (2020): B38-CAP is a bacteria-derived ACE2-like enzyme that suppresses hypertension and cardiac dysfunction. *Nat. Commun.* 11: 1058. doi:10.1038/s41467-020-14867-z
- Monteagudo-Mera A., Rastall R. A., Gibson G. R., Charalampopoulos D., Chatzifragkou A. (2019): Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. *App. Microbiol. Biotechnol.* 103 (16): 6463-6472. doi:10.1007/s00253-019-09978-7
- Montiela R., Martín-Cabrejas I., Langa S., El Aouad N., Arqués J. L., Reyes F., Medina M. (2014): Antimicrobial activity of reuterin produced by *Lactobacillus reuteri* on *Listeria monocytogenes* in cold-smoked salmon. *Food Microbiol.* 44: 1-5. doi:10.1016/j.fm.2014.05.006
- Mora-Villalobos J. A., Montero-Zamora J., Barboza N., Rojas-Garbanzo C., Usaga J., Redondo-Solano M., Schroedter L., Olszewska-Widdrat A., López-Gómez J. P. (2020): Multi-product lactic acid bacteria fermentations: A Review. *Fermentation* 6: 23. doi:10.3390/fermentation6010023
- Moreno-Montoro M., Navarro-Alarcón M., Bergillos-Meca T., Giménez-Martínez R., Sánchez-Hernández S., Olalla-Herrera M. (2018): Physicochemical, nutritional, and organoleptic characterization of a skimmed goat milk fermented with the probiotic strain *Lactobacillus plantarum* C4. *Nutrients* 10 (5): 1-5. doi:10.3390/nu10050633
- Moreno-Montoro M., Navarro-Alarcón M., Bergillos-Meca T., Giménez-Martínez R., Sánchez-Hernández S., Olalla-Herrera M. (2018): Physicochemical, nutritional, and organoleptic characterization of a skimmed goat milk fermented with the probiotic strain *Lactobacillus plantarum* C4. *Nutrients* 10 (5): 633. doi: 10.3390/nu10050633.
- Mortazavian A., Razavi S. H., Ehsani M. R., Sohrabvandi S. (2007): Principles and methods of microencapsulation of probiotic microorganisms. *Iran. J. Biotech.* 5 (1): 1-18.
- Moy T. I., Mylonakis E., Calderwood S. B., Ausubel F. M. (2004): Cytotoxicity of hydrogen peroxide produced by *Enterococcus faecium*. *Inf. Immun.* 72 (8): 4512-4520. doi:10.1128/IAI.72.8.4512-4520.2004
- Mroueh M., Issa D., Khawand J., Haraty B., Malek A., Kassaify Z., Toufeili I. (2008): Levels of benzoic and sorbic acid preservatives in commercially produced yoghurt in Lebanon. *J. Food, Agricul. Environm.* 6 (1): 62-66.

- Mu W., Yu S., Zhu L. *et al.* (2012): Recent research on 3-phenyllactic acid, a broad-spectrum antimicrobial compound. *Appl. Microbiol. Biotechnol.* 95: 1155-1163. doi:10.1007/s00253-012-4269-8
- Mühlhansová A., Horáčková Š., Plocková M. (2016): Stanovení životaschopných laktobacilů s využitím propidium monoazidu pomocí qPCR. *Mlékařské listy* 154, 9-13.
- Mühlhansová A., Zhexenbay N., Kozybayev A., Horáčková Š., Solichová K., Plocková M. (2016): Quantification of *Lactobacillus helveticus* in a mixture of lactic acid bacteria using qPCR in cheese. *Acta Aliment. Int. J. Food Sci.* 45 (4): 493-499. doi:10.1556/066.2016.45.4.5
- Nariadení komise EU č. 432/2012 ze dne 16.5.2012, kterým se zřizuje seznam schválených zdravotních tvrzení při označování potravin jiných než tvrzení o snížení rizika onemocnění a o vývoji zdraví dětí.
- Nataraj B. H., Ali S. A., Behare P. V., Yadav H. (2020): Postbiotics-parabiotics: the new horizons in microbial biotherapy and functional foods. *Microb. Cell Fact.* 19: 168. doi:10.1186/s12934-020-01426-w
- Nedovic V., Kalusevic A., Manojlovic V., Levic S., Bugarski B. (2011): An overview of encapsulation technologies for food applications. *Procedia Food Sci.* 1: 1806-1815. doi:10.1016/j.profoo.2011.09.265
- Neves A. R., Pool W. A., Kok J., Kuipers O. P., Santos H. (2005): Overview on sugar metabolism and its control in *Lactococcus lactis* – The input from in vivo NMR. *FEMS Microbiol. Rev.* 29: 531-554. doi: 10.1016/j.femsre.2005.04.005
- Nguyen B. T., Bujna E., Fekete N., Tran Anh T. M., Rezessy-Szabo J. M., Prasad R., Nguyen Q. D. (2019): Probiotic beverage from pineapple juice fermented with *Lactobacillus* and *Bifidobacterium* strains. *Front. Nutrit.* 6: Article 54. doi:10.3389/fnut.2019.00054
- Nielsen S. D., Beverly R. L., Qu Y., Dallas D. C. (2017): Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chemistry* 232: 673-682. doi:10.1016/j.foodchem.2017.04.056
- Ning Y., Aihong Yan A., Yang K., Wang Z., Li X., Jia Y. (2017): Antibacterial activity of phenyllactic acid against *Listeria monocytogenes* and *Escherichia coli* by dual mechanisms. *Food Chem.* 228: 533-540. doi:10.1016/j.foodchem.2017.01.112
- Nocker A., Cheung Ch.-Y., Camper A. K. (2006): Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Methods*, 67: 310-320. doi:10.1016/j.mimet.2006.04.015
- Noriega L., Gueimonde M., Sánchez B., Margolles A., de los Reyes-Gavilán C. G. (2004): Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in *Bifidobacterium*. *Int. J. Food Microbiol.* 94: 79-86. doi:10.1016/j.ijfoodmicro.2004.01.003
- Oberman H., Piatkiewicz A., Libudzi Z. (1982): Production of diacetyl and acetoin by lactic acid bacteria. *Die Nahrung* 26 (7/8): 615-623.
- Oelschlaeger T. A. (2010): Mechanisms of probiotic actions – A review. *Int. J. Med. Microbiol.* 300: 57-62. doi:10.1016/j.ijmm.2009.08.005.
- Olaimat A. N., Aolymat I., Al-Holy M. *et al.* (2020): The potential application of probiotics and prebiotics for the prevention and treatment of COVID-19. *npj Sci. Food* 4: 17 (2020). doi:10.1038/s41538-020-00078-9
- Oliveira P. M., Brosnan B., Furey A., Coffey A., Zannini E., Arendt E. K. (2015): Lactic acid bacteria bioprotection applied to the malting process. Part I: strain characterization and identification of antifungal compounds. *Food Control* 51: 433-443. doi:10.1016/j.foodcont.2014.11.011

- Oliveira P. M., Zannini E., Arendt E. K. (2014): Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: From crop farming to cereal products. *Food Microbiol.* 37: 78-95. doi:10.1016/j.fm.2013.06.003
- Ortiz-Rivera Y., Sánchez-Vega R., Gutiérrez-Méndez N., León-Félix J., Acosta-Muñoz C., Sepulveda D. R. (2017): Production of reuterin in a fermented milk product by *Lactobacillus reuteri*: Inhibition of pathogens, spoilage microorganisms, and lactic acid bacteria. *J. Dairy Sci.* 100: 4258-4268. doi:10.3168/jds.2016-11534
- O'Toole P., Marchesi J., Hill C. (2017): Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nat. Microbiol.* 2: 17057. doi:10.1038/nmicrobiol.2017.57
- Özcelika S., Kuleyb E., Özogulb F. (2016): Formation of lactic, acetic, succinic, propionic, formic and butyric acid by lactic acid bacteria. *LWT – Food Sci. Technol.* 73: 536-542. doi:10.1016/j.lwt.2016.06.066
- Pankasemsuk, T., Apichartsrangkoon, A., Worametrachanon, S., Techarang, J. (2016): Encapsulation of *Lactobacillus casei* 01 by alginate along with hi-maize starch for exposure to a simulated gut model. *Food Biosci.* 16: 32-36. doi:/10.1016/j.fbio.2016.07.001
- Park S.-Y., Yoo M.-Y., Paik H.-D., Lim S.-D. (2017): Production of benzoic acid as a natural compound in fermented skim milk using commercial cheese starter. *J. Dairy Sci.* 100: 4269-4275. doi:10.3168/jds.2016-12399
- Parker R. B. (1974): Probiotics, the other half of the antibiotics story. *Animal Nutr. Health* 29: 4-8.
- Partty A., Kalliomaki M., Wacklin P., Salminen S., Isolauri E. (2015): A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial. *Pediatr. Res.* 77: 823-828. doi:10.1038/pr.2015.51
- Patarroyo J. L., Florez-Rojas J. S., Pradilla D., Valderrama-Rincón J. D., Cruz J. C., Reyes L. H. (2020): Formulation and characterization of gelatin-based hydrogels for the encapsulation of *Kluyveromyces lactis* - Applications in packed-bed reactors and probiotics delivery in humans. *Polymers* 12: 1287. doi:10.3390/polym12061287
- Perez R. H., Zendo T., Sonomoto K. (2014): Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microb. Cell Fact.* 13: S3. doi:10.1186/1475-2859-13-S1-S3
- Perricone M., Bevilacqua A., Altieri C., Sinigaglia M., Corbo M. R. (2015): Challenges for the production of probiotic fruit juices. *Beverages*, 1: 95-103. doi:10.3390/beverages1020095
- Perricone M., Corbo M. R., Sinigaglia M., Speranza B., Bevilacqua A. (2014): Viability of *Lactobacillus reuteri* in fruit juices. *J. Funct. Foods*, 10: 421-426. doi.org/10.1016/j.jff.2014.07.020
- Pfeiler E. A., Klaenhammer T. R. (2007): The genomics of lactic acid bacteria. *Trends Microbiol.* 15: 546-53. doi:10.1016/j.tim.2007.09.010
- Picot A., Lacroix Ch. (2004): Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. *Int. Dairy J.* 14 (6): 505-515. doi:10.1016/j.idairyj.2003.10.008
- Pretorius N., Engelbrecht L., Du Toit M. (2019): Influence of sugars and pH on the citrate metabolism of different lactic acid bacteria strains in a synthetic wine matrix. *J. Appl. Microbiol.* 127 (5): 1490-1500. doi.org/10.1111/jam.14401
- Raveschot C., Cudennec B., Coutte F., Flahaut C., Fremont M., Drider D., Dhulster P. (2018): Production of bioactive peptides by *Lactobacillus* species: From gene to application. *Front. Microbiol.* 9: 2354. doi:10.3389/fmicb.2018.02354
- Reid G., Gadir A. A., Dhir R. (2019): Probiotics: Reiterating What They Are and What They Are Not. *Front Microbiol.* 10: 424. doi:10.3389/fmicb.2019.00424

- Reis J. A., Paula A. T., Casarotti S. N., Penna A. L. B. (2012): Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Eng. Rev.* 4: 124-140. doi:10.1007/s12393-012-9051-2
- Ren J., Sun K., Wu Z., Yao J., Guo B. (2011): All 4 bile salt hydrolase proteins are responsible for the hydrolysis activity in *Lactobacillus plantarum* ST-III. *J. Food Sci.* 76: 622-628. doi:10.1111/j.1750-3841.2011.02431.x
- Reyes-Nava L. A., Garduño-Siciliano L., Santos E. L. P., Hernández-Sánchez H., Arauz A. J., *et al.* (2016): Use of bile acids as a selection strategy for *Lactobacillus* strains with probiotic potential. *J. Food Nutr. Disor.* 5:1. doi:10.4172/2324-9323.1000187
- Reyes-Nava L. A., Rivera-Espinoza Y., Harold J. (2014): Isolation sources of bile salt hydrolase-microorganisms. *Herald J. Agri. Food Sci. Res.* 3: 49-54.
- Ridlon J. M., Kang D. J., Hylemon P. (2006): Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* 47, 241-259. doi:10.1194/jlr.R500013-JLR200
- Rodrigues D., Sousa S., Rocha-Santos T., *et al.* (2011): Influence of l-cysteine, oxygen and relative humidity upon survival throughout storage of probiotic bacteria in whey protein-based microcapsules *Int. Dairy J.* 21 (11): 869-876. doi:10.1016/j.idairyj.2011.05.005
- Rodriguez M., Salgado N. J., Cortés S., Domínguez J. M. (2012): Antimicrobial activity of D-3-phenyllactic acid produced by fed-batch process against *Salmonella enterica*. *Food Control* 25: 274-284. doi:10.1016/j.foodcont.2011.10.042
- Rokana N., Singh B., Thakur N., Sharma C., Gulhane R., Panwar H. (2018): Screening of cell surface properties of potential probiotic lactobacilli isolated from human milk. *J. Dairy Res.* 85 (3), 347-354. doi:10.1017/S0022029918000432
- Rossi F., Rizzotti L., Felis G. E., Torriani S. (2014): Horizontal gene transfer among microorganisms in food: Current knowledge and future perspectives. *Food Microbiol.* 42: 232-243. doi:10.1016/j.fm.2014.04.004.
- Ruas-Madiedo M. P., Gueimonde M., Margolles A., de Los Reyes-Gavila C. G., Salminen S. (2006): Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal. *J. Food Prot.* 69 (8): 2011-2015. doi:10.4315/0362-028x-69.8.2011
- Rubak Y. T., Nuraida L., Iswantini D., Prangdimurti E. (2020): Angiotensin-I-converting enzyme inhibitory peptides in milk fermented by indigenous lactic acid bacteria. *Veterin. World* 13(2): 345-353. doi:10.14202/vetworld.2020.345-353
- Russo P., de Chiara M.L.V., Capozzi V., Arena M. P., Amodio M. L., Rascón A., Dueñas M. T., López P., Spano G. (2016): *Lactobacillus plantarum* strains for multifunctional oatbased foods. *LWT - Food Sci. Technol.* 68: 288-294. doi:10.1016/j.lwt.2015.12.040
- Sadat-Mekmene L., Genay M., Atlan D., Lortal S., Gagnaire V (2011): Original features of cell-envelope proteinases of *Lactobacillus helveticus*. A review. *Int. J. Food Microbiol.* 146 (1): 1-13. doi:10.1016/j.ijfoodmicro.2011.01.039
- Sadiq F. A., Yan B., Tian F., Zhao J., Zhang H., Chen W. (2019): Lactic acid bacteria as antifungal and anti-mycotoxigenic agents: A Comprehensive review. *Compr. Rev. Food Sci. Food Saf.* 18: 1403-1436. doi:10.1111/1541-4337.12481
- Sakai T., Oishi K., Asahara T., Takada T., Yuki N., Matsumoto K., Nomoto K., Kushiro A. (2010): M-RTL agar, a novel selective medium to distinguish *Lactobacillus casei* and *Lactobacillus paracasei* from *Lactobacillus rhamnosus*. *Int. J. Food Microbiol.* 139: 154-160. doi:10.1016/j.ijfoodmicro.2010.03.019
- Salminen M. K., Rautelin H., Tynkkynen S., Poussa T., Saxelin M., Valtonen V., Järvinen A. (2004): *Lactobacillus* bacteremia, clinical significance, and patient outcome, with special focus on probiotic *L. rhamnosus* GG 38. *Clin. Infect. Dis* (1): 62-69. doi:10.1086/380455

- Sangmanee P., Hongpattarakere T. (2014): Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus* and *Aspergillus parasiticus*. Food Control 40: 224-233. doi:10.1016/j.foodcont.2013.12.005
- Santiago-Silva P., Soares N. F. F., Nóbrega J. E., Júnior M. A. W., Barbosa K. B. F., Volp A. C. P., Zerdas E. R. M. A., Würlitzer N. J. (2009): Antimicrobial efficiency of film incorporated with pediocin on preservation of sliced ham. Food Control 20: 85-89. doi:10.1016/j.foodcont.2008.02.006
- Sato H., Torimura M., Kitahara M., Ohkuma M., Hotta Y., Tamura H. (2012): Characterization of the *Lactobacillus casei* group based on the profiling of ribosomal proteins coded in S10-spc-alpha operons as observed by MALDI-TOF MS. Syst. App. Microbiol. 35: 447-454. doi:10.1016/j.syapm.2012.08.008
- Sauvageot N., Gouffi K., Laplace J.-M., Auffray Y. (2000): Glycerol metabolism in *Lactobacillus collinoides*: production of 3-hydroxypropionaldehyde, a precursor of acrolein. Int. J. Food Microbiol. 55: 167-170. doi:10.1016/S0168-1605(00)00191-4
- Sedláčková P., Horáčková Š., Shi T., Kosová M., Plocková M. (2015): Two different methods for screening of bile salt hydrolase activity in *Lactobacillus* strains. Czech J. Food Sci. 33: 13-18. doi:10.17221/299/2014-CJFS
- Shah N. P., Ravula R. R. (1998): Selective enumeration of *L. casei* from yogurt and fermented milk drinks. Biotechnol. Tech. 12: 819-822. doi:10.1023/A:1008829004888
- Shimakawa Y., Matsubara S., Yuki N., Ikeda M., Ishikawa F. (2003): Evaluation of *Bifidobacterium breve* strain Yakult-fermented soymilk as a probiotic food. Int. J. Food Microbiol. 81 (2): 131-136. doi:10.1016/s0168-1605(02)00224-6
- Shu G., He Y., Chen L., Song Y., Meng J., Chen H. (2017): Microencapsulation of *Lactobacillus acidophilus* by xanthan-chitosan and its stability in yoghurt. Polymers. 9 (12): 733. doi:10.3390/polym9120733
- Schwebke J. R. (2001): Role of vaginal flora as a barrier to HIV acquisition. Curr. Infect. Dis. Rep. 3: 152-155. doi:10.1007/s11908-996-0040-6
- Sidira M., Santarmaki V., Kiourtzidis M., Argyri A., Papadopoulou S., Chorianopoulos N., Kourkoutas Y. (2017): Evaluation of immobilized *Lactobacillus plantarum* 2035 on whey protein as adjunct probiotic culture in yoghurt production. LWT - Food Sci. Technol. 75: 137-146. doi.org/10.1016/j.lwt.2016.08.026
- Siezen R. J., van Enckevort F. H., Kleerebezem M., Teusink B. (2004): Genome data mining of lactic acid bacteria: the impact of bioinformatics. Curr. Opin. Biotechnol. 15: 105-115. doi:10.1016/j.copbio.2004.02.002
- Silva E. O., O., Nespolo C. R., Sehn C., P., Pinheiro F. C., Stefani L. M. (2020): Lactic acid bacteria with antimicrobial, proteolytic and lipolytic activities isolated from ovine dairy products. Food Sci. Technol. 40 (1): 293-299. doi:org/10.1590/fst.11019
- Sirilun S., Sivamaruthi B. S., Kesika P., Peerajan S., Chaiyasut C. (2017): Lactic acid bacteria mediated fermented soybean as a potent nutraceutical candidate. Asian Pac. J. Trop. Biomed. 7 (10): 930-936. doi:10.1016/j.apjtb.2017.09.007
- Sung W. Y., Yu J. W., Hwang J. T., et al. (2020): Insight into the bovine milk peptide LPcin-YK3 selection in the proteolytic system of *Lactobacillus* species. J. Pep. Sci. 26: e3268. doi:10.1002/psc.3268
- Suskovic J., Kos B., Beganovic J., Pavunc A. L., Habjanic K., Matosic S. (2010): Antimicrobial activity of lactic acid bacteria. Food Technol. Biotechnol. 48 (3): 296-307.

- Sutula J., Coulthwaite L., Verran J. (2012): Culture media for differential isolation of *Lactobacillus casei* Shirota from oral samples. J. Microbiol. Methods 90: 65-71.
doi:10.1016/j.mimet.2012.03.015
- Tachedjian G., O'Hanlon D. E., Ravel J. (2018): The implausible “*in vivo*” role of hydrogen peroxide as an antimicrobial factor produced by vaginal microbiota. Microbiome 6: 29.
doi:10.1186/s40168-018-0418-3
- Talarico T. L., Casas I. A., Chung T. C., Dobrogosz W. J. (1988): Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. 32 (12): 1854-1858. doi:10.1128/aac.32.12.1854
- Talwalkar A., Kailasapathy K. (2004): Comparison of selective and differential media for the accurate enumeration of strains of *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *Lactobacillus casei* complex from commercial yoghurts. Int. Dairy J. 14 (2): 143-149.
doi:10.1016/S09586946(03)00172-9
- Tanaka H., Doesburg K., Iwasaki T., Mierau I. (1999): Screening of lactic acid bacteria for bile salt hydrolase activity. J. Dairy Sci. 82 (12): 2530-2535. doi:10.3168/jds.S0022-0302(99)75506-2
- Tanaka H., Hashib H., Kok J., Mireau I. (2000): Bile salt hydrolase of *Bifidobacterium longum* – Biochemical and genetic characterization. Appl. Environ. Microbiol. 66: 2502-2512.
doi:10.1128/AEM.66.6.2502-2512.2000
- Tanasupawat S., Phoottosavako M., Keeratipibul S. (2015): Characterization and lipolytic activity of lactic acid bacteria isolated from Thai fermented meat. J. App. Pharm. Sci. 5(3): 6-12.
doi:10.7324/JAPS.2015.50302
- Tantratian S., Watanaprasert S., Suknaisilp S. (2018): Effect of partial substitution of milk-non-fat with xanthan gum on encapsulation of a probiotic *Lactobacillus*. J. Food Process. Preserv. 42: e13673. doi:10.1111/jfpp.13673
- Thomas E. L., Milligan T. W., Joyner R. E., Jefferson M. M. (1994): Antibacterial Activity of hydrogen peroxide and the lactoperoxidase-hydrogen peroxide-thiocyanate system against oral streptococci. Infect. Immun. 62 (2): 529-535.
- Todorov S. D., De Melo Franco B. D. G. (2010): *Lactobacillus plantarum*: Characterization of the species and application in food production. Food Rev. Int. 26 (3): 205-229.
doi:10.1080/87559129.2010.484113
- Toh H., Oshima K., Nakano A., Tahakata M., Murakami M., Takaki T., Nishiyama H., Igimi S., Hattori M., Morita H. (2013): Genomic Adaptation of the *Lactobacillus casei* Group. PloS ONE 8: 10. doi:10.1371/journal.pone.0075073
- Tommasi C., Equitani F., Masala M., *et al.* (2008): Diagnostic difficulties of *Lactobacillus casei* bacteraemia in immunocompetent patients: A case report. J. Med. Case Reports 2: 315.
doi:10.1186/1752-1947-2-315
- Tyssen D., Wang Y.-Y., Hayward J., Agius P. A., DeLong K., Aldunate M., Ravel J., Moench T. T., Cone R. A., Tachedjian G. (2018): Anti-HIV-1 activity of lactic acid in human cervicovaginal fluid. mSphere 3 (4): e00055-18. doi:10.1128/mSphere.00055-18
- Valerio F., Di Biase M., Lattanzio V. M., Lavermicocca P. (2016): Improvement of the antifungal activity of lactic acid bacteria by addition to the growth medium of phenylpyruvic acid, a precursor of phenyllactic acid. Int. J. Food Microbiol. 222: 1-7. doi:10.1016/j.ijfoodmicro.2016.01.011
- Valero-Cases E., Frutos M. J. (2015): Effect of different types of encapsulation on the survival of *Lactobacillus plantarum* during storage with inulin and in vitro digestion. LWT – Food Sci. Technol. 64: 824–828. doi:10.1016/j.lwt.2015.06.049
- Valladares R. B., Graves C., Wright K., Gardner C. L., Lorca G. L., Gonzalez C. F. (2015): H₂O₂ production rate in *Lactobacillus johnsonii* is modulated via the interplay of a hetero- dimeric flavin

- oxidoreductase with a soluble 28 Kd PAS domain containing protein. *Front. Microbiol.* 6: 1-14. doi:10.3389/fmicb.2015.00716
- Van de Castele S. T., Vanheuverzwijn T., Ruysen T., Van Assche P., Swings J., Huys G. (2006): Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yogurt or cheese starters. *Int. Dairy J.* 16: 1470-1476. doi:10.1016/j.idairyj.2005.12.002
- Vandeputte D., Falony G., Vieira-Silva S., *et al.* (2017): Prebiotic inulin-type fructans induce specific changes in the human gut microbiota. *Gut* 66: 1968-1974. doi:10.1136/gutjnl-2016-313271
- Vargas García C. E., Petrova M., Claes I. J. J., De Boeck I., Verhoeven T. A. L., Dilissen E., von Ossowski I., Palva A., Bullens D. M., Vanderleyden J., Lebeer S. (2015): Piliation of *Lactobacillus rhamnosus* GG promotes adhesion, phagocytosis, and cytokine modulation in macrophages. *Appl. Environ. Microbiol.* 81 (6): 2050-2062. doi:10.1128/AEM.03949-14
- Verraes C., Van Boxtael S., Van Meervenne E., Van Coillie E., Butaye P., Catry B., de Schaetzen M. A., Van Huffel X., Imberechts H., Dierick K., Daube G., Saegerman C., De Block J., Dewulf J., Herman L. (2013): Antimicrobial resistance in the food chain: a review. *Int. J. Environ. Res. Public Health* 10 (7): 2643-2669. doi:10.3390/ijerph10072643.
- Veselá K., Kumherová M., Kłodová I., Solichová K., Horácková Š., Plocková M. (2019): Selective culture medium for the enumeration of *Lactobacillus plantarum* in the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. *LWT – Food Sci. Technol.* 114: 108365. doi:10.1016/j.lwt.2019.108365
- Vinderola C. G., Reinheimer J. A. (2000): Enumeration of *Lactobacillus casei* in the presence of *L. acidophilus*, bifidobacteria and lactic starter bacteria fermented dairy products. *Int. Dairy J.* 10: 271-275. doi:10.1016/S0958-6946(00)00045-5
- Visser S., Exterkate F. A., Slangen C. J., de Veer G. J. C. M. (1986): Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine α S1-, β -, and κ -casein. *Appl. Environ. Microbiol.* 52: 1162-1166.
- Vitetta L., Coulson S., Thomsen M., Nguyen T., Hall S. (2017): Probiotics, D-lactic acidosis, oxidative stress and strain specificity. *Gut Microbes* 8 (4): 311-322. doi:10.1080/19490976.2017.1279379
- Volkert M., Ananata E., Luscher C., Knorr D. (2008): Effect of air freezing, spray freezing, and pressure shift freezing on membrane integrity and viability of *Labctobacillus rhamonosus* GG. *J. Food Eng.* 87: 532-540. doi:10.1016/j.jfoodeng.2008.01.008
- Vyhláška č. 274/2019 Sb.: Vyhláška, kterou se mění vyhláška č. 397/2016 Sb., o požadavcích na mléko a mléčné výrobky, mražené krémy a jedlé tuky a oleje.
- Wang Z., Chai W., Burwinkel M., Twardziok S., Wrede P., *et al.* (2013): Inhibitory influence of *Enterococcus faecium* on the propagation of swine influenza a virus *in vitro*. *PLOS ONE* 8(1): e53043. doi:10.1371/journal.pone.0053043
- Wegh C., Geerlings S. Y., Knol J., Roeselers G., Belzer, C. (2019): Postbiotics and their potential applications in early life nutrition and beyond. *Int. J. Mol. Sci.* 20 (19): 4673. doi:10.3390/ijms20194673
- Wessels S., Axelsson L., Bech Hansen E., De Vuyst L., Laulund S., Lähteenmäki L., *et al.* (2004): The lactic acid bacteria, the food chain, and their regulation. *Trends Food Sci. Technol.* 15: 498-505. doi:10.1016/j.tifs.2004.03.003
- WHO/FAO (2002): Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food; London, Ontario, Canada, April 30 and May 1.
- Worsztynowicz P., Białasa W., Grajeka W. (2020): Integrated approach for obtaining bioactive peptides from whey proteins hydrolysed using a new proteolytic lactic acid bacteria. *Food Chemistry* 312: 126035. doi:10.1016/j.foodchem.2019.126035

- Wu C., Huang J., Zhou R. (2017): Genomics of lactic acid bacteria: Current status and potential applications. *Crit. Rev. Microbiol.* 43 (4): 393-404. doi:10.1080/1040841X.2016.1179623
- Xu K., Cai H., Shen Y., Qin N. I., Chen Y., Shaohua H. U., Li J. Wang H., Yu L., Huang H., Qiu Y., Wei G., Fang Q., Zhou J., Sheng J., Liang T., Li L. (2020): Management of COVID-19: the Zhejiang experience. *J Zhejiang Univ (Med Sci)* 49 (2): 147-157. doi:10.3785/j.issn.1008-9292.2020.02.02
- Yang E. J., Chang H. C. (2010): Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. *Int. J. Food Microbiol.* 139: 56-63. doi:10.1016/j.ijfoodmicro.2010.02.012
- Zangl I., Pap I. J., Aspöck C., Schüller C. (2019): The role of *Lactobacillus* species in the control of *Candida* via biotrophic interactions. *Microb. Cell* 7 (1): 1-14. doi:10.15698/mic2020.01.702
- Zendeboodi F., Khorshidian N., Mortazavian A. M., da Cruz A. G. (2020): Probiotic: conceptualization from a new approach. *Curr. Opin. Food Sci.* 32: 103-123. doi:10.1016/j.cofs.2020.03.009.
- Zhan W. Y., Wu R. N., Sun Z. H., Sun T. S., Meng H., Zhang H. P. (2009): Molecular cloning and characterization of bile salt hydrolase in *Lactobacillus casei* Zhang. *Ann. Microbiol.* 59: 721-726. doi:10.1007/BF03179214
- Zhang T., Li Q., Cheng L., Buch H., Zhang F. (2019): *Akkermansia muciniphila* is a promising probiotic. *Microb. Biotechnol.* 12 (6): 1109-1125. doi:10.1111/1751-7915.13410
- Zheng J., Wittouck S., Salvetti E., Franz C. M. A. P., Harris H. M. B., Mattarelli P., O'Toole P. W., Pot B., Vandamme P., Walter J., Watanabe K., Wuyts S., Felis G. E., Gänzle M. G., Lebeer S. (2020): A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int. J. Syst. Evol. Microbiol.* 70 (4): 2782-2858. doi:10.1099/ijsem.0.004107.
- Zhou X. L., Kong X. F., Yang X. J., Yin Y. L. (2012): Soybean oligosaccharides alter colon short-chain fatty acid production and microbial population in vitro. *J. Anim. Sci.* 90 (Suppl 4): 37-39. doi:10.2527/jas.50269

7 Seznam zkratek

B. *Bifidobacterium*

BMK bakterie mléčného kvašení

E. *Enterococcus*

EMA ethidium monoazid

EPS exopolysacharidy

GIT gastrointestinální trakt

HPA hydroxypropanal

L. *Lactobacillus*

MRS de Man, Rogosa a Sharp (agar)

PMA propidium monoazid

8 Seznam příloh

Seznam příloh obsahuje vybrané autorské nebo spoluautorské publikace související s tematikou „Bakterie mléčného kvašení a jejich funkční vlastnosti“, jejich aplikace a stanovení, které jsou citovány v této habilitační práci.

Příloha I:

Horáčková Š., Sedláčková P., Sluková M., Plocková M. (2014): The influence of whey, whey component and malt on the growth and acids production of lactobacilli in milk. Czech J. Food Sci. 32 (6): 526-531. doi:10.17221/214/2014-CJFS

Příloha II:

Bialasová K., Němečková I., Kyselka J., Štětina J., Solichová K., **Horáčková Š.** (2018): Influence of flaxseed components on fermented dairy product properties. Czech J. Food Sci. 36, 51-56. doi:10.17221/411/2017-CJFS

Příloha III:

Horáčková Š., Nováková T., Sluková M., Bialasová K., Plocková M. (2018a): Vliv laktobacilů na růst plísně *Fusarium culmorum* a *Penicillium expansum*. Sborník Celostátní přehlídky sýrů 2018 - Výsledky přehlídek a sborník konference Mléko a sýry. (ed. Štětina J., Čurda L.), 171-175, VŠCHT Praha 2018 (ISBN 978-80-7592-030-0).

Příloha IV:

Kumherová M., Veselá K., Kosová M., Mašata J., **Horáčková Š.**, Šmidrkal J. (2020): Novel potential probiotic lactobacilli for prevention and treatment of vulvovaginal infections. Probiotics and Antimicrob. Proteins (2020). doi:10.1007/s12602-020-09675-2

Příloha V:

Feldeková E., Solichová K., **Horáčková Š.**, Kumherová M., Kyselka J. (2020): The impact of l-lanthionine supplementation on the production of nisin by lactococci. Eur. Food Res. Technol. 246: 845-851. doi:10.1007/s00217-020-03449-4

Příloha VI:

Horáčková Š., Nováková T., Sluková M., Bialasová K., Kumherová M., Plocková M. (2018b): Antifungal activity of selected lactobacilli intended for sourdough production. App. Food Biotechnol. 5 (4): 213-220. doi:10.22037/afb.v%vi%i.22173

Příloha VII:

Koval D., Plocková M., Kyselka J., Skřivan P., Sluková M., **Horáčková Š.** (2020): Buckwheat secondary metabolites: Potential antifungal agents. J. Agric. Food Chem. 68: 11631-11643. doi:10.1021/acs.jafc.0c04538

Příloha VIII:

Horáčková Š., Plocková M., Demnerová K. (2018c): Importance of microbial defence systems to bile salts and mechanisms of serum cholesterol reduction. Biotechnol. Adv. 36: 682-690. doi:10.1016/j.biotechadv.2017.12.005

Příloha IX:

Horáčková Š., Veselá K., Kloidová I., Berčíková M., Plocková M. (2020): Bile salt hydrolase activity, growth characteristics and surface properties in *Lactobacillus acidophilus*. Eur. Food Res. Technol. 246: 1627-1636. doi:10.1007/s00217-020-03518-8

Příloha X:

Sedláčková P., **Horáčková Š.**, Shi T., Kosová M., Plocková M. (2015): Two different methods for screening of bile salt hydrolase activity in *Lactobacillus* strains. Czech J. Food Sci. 33: 13-18. doi:10.17221/299/2014-CJFS

Příloha XI:

Lisová I., **Horáčková Š.**, Kováčová R., Rada V., Plocková M. (2013): Emulsion encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 with the addition of lecithin. Czech J. Food Sci. 31: 270-274. doi:10.17221/188/2012-CJFS

Příloha XII:

Kumherová M., Veselá K., Jokešová K., Kloidová I., **Horáčková Š.** (2020): Influence of co-encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 with inulin and ascorbic acid on its viability. Czech J. Food Sci. 38 (1): 57-62. doi:10.17221/292/2019-CJFS

Příloha XIII:

Horáčková Š., Mühlhansová A., Sluková M., Schulzová V., Plocková M. (2015): Fermentation of soymilk by yoghurt and bifidobacteria strains. Czech J. Food Sci. 33 (4): 313-319. doi:10.17221/115/2015-CJFS

Příloha XIV:

Horáčková Š., Rokytová K., Bialasová K., Kloidová I., Sluková M. (2018d): Fruit juices with probiotics – new type of functional foods. Czech J. Food Sci. 36: 284-288. doi:10.17221/39/2018-CJFS

Příloha XV:

Mühlhansová A., **Horáčková Š.**, Plocková M. (2016): Stanovení životaschopných laktobacilů s využitím propidium monoazidu pomocí qPCR. Mlékařské listy 154, 9-13.

Příloha XVI:

Mühlhansová A., Zhexenbay N., Kozybayev A., **Horáčková Š.**, Solichová K., Plocková M. (2016): Quantification of *Lactobacillus helveticus* in a mixture of lactic acid bacteria using qPCR in cheese. Acta Aliment. Int. J. Food Sci. 45 (4): 493-499. doi:10.1556/066.2016.45.4.5

Příloha XVII:

Veselá K., Kumherová M., Kloidová I., Solichová K., **Horáčková Š.**, Plocková M. (2019): Selective culture medium for the enumeration of *Lactobacillus plantarum* in the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. LWT – Food Sci. Technol. 114: 108365. doi:10.1016/j.lwt.2019.108365

9 Přílohy

Influence of Whey, Whey Component and Malt on the Growth and Acids Production of Lactobacilli in Milk

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Abstract

HORÁČKOVÁ Š., SEDLÁČKOVÁ P., SLUKOVÁ M., PLOCKOVÁ M. (2014): **Influence of whey, whey component and malt on the growth and acids production of lactobacilli in milk.** Czech J. Food Sci., 32: 526–531.

The effect of whey powder, whey protein concentrate, caseinomacropeptide, and malt addition into milk on the growth and acid production of lactobacilli (*Lactobacillus casei* Lafti L-26, *Lactobacillus acidophilus* CCDM 151, and *Lactobacillus casei* CCDM 198) was evaluated. The ability of these strains to use different types of saccharides from milk and plant sources was also tested. Glucose, galactose, fructose and maltose were utilised by all tested strains. The results showed that the addition of malt positively affected the growth of lactobacilli strains compared to the growth in milk enriched by whey ingredients. The addition of malt increased significantly the production of D(–) isomer of lactic acid by *Lactobacillus acidophilus* CCDM 151 and *Lactobacillus casei* CCDM 198 and the production of acetic acid by *Lactobacillus casei* CCDM 198.

Keywords: *Lactobacillus*; caseinomacropeptide; whey protein; malt; acetic acid; lactic acid

One of the most attractive alternatives of whey usage for human consumption is the manufacture of fermented or non-fermented whey or combined whey-milk products, namely when also some probiotic bacteria are used as further health-promoting component of these products. These kinds of beverages could be considered as a good carrier for the probiotics (ALMEIDA *et al.* 2009) as the whey proteins improve the viability of lactic acid bacteria (LAB) due to enhancing the buffering capacity of culture medium (KAILASAPATHY & SUPRIADI 1996). Whey proteins and peptides derived from these proteins were confirmed to possess many health benefits. Apart from being a balanced source of valuable and essential amino acids, whey proteins and their hydrolysates are associated with antihypertensive, antimicrobial, anticarcinogenic, and immunomodulatory activities (KORHONEN 2009).

Cereals are other useful fermented milk additional components. Some of them can act as prebiotics due to the content of non-digestible saccharides and also

can enhance the survival of LAB during the passage through the gastrointestinal tract (PATEL *et al.* 2004). The aim of the present paper was to investigate the growth of selected probiotic lactobacilli in milk with the addition of whey components and malt and also to test the influence of these substances on the production of lactic and acetic acids which possess the impact on the sensory quality of milk based products. As the vitality and viability of LAB are not only influenced by food matrix parameters but also are strain dependent, one commercial probiotic strain of *Lactobacillus casei* Lafti L-26 and two strains with certain probiotic properties formerly proved (HORÁČKOVÁ *et al.* 2011) were used.

MATERIAL AND METHODS

Microorganisms. *Lactobacillus casei* Lafti L-26 (DSM Food Specialties, Heerlen, Netherlands); *Lactobacillus acidophilus* CCDM 151; *L. casei* CCDM 198

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(Culture Collection of Dairy Microorganisms, Laktoflora[®], Milcom, Prague, Czech Republic).

Cultivation and determination of lactobacilli count. For the inoculation of tested media, the overnight lactobacilli cultures cultivated in 5% (v/v) CO₂ atmosphere at 37°C in MRS broth (Merck, Darmstadt, Germany), pH 5.6, were used.

For the count determination, lactobacilli were cultivated at 37°C in a CO₂ atmosphere (5% v/v) for 48 h and counted as colony forming units (CFU) per ml on MRS agar (Merck, Darmstadt, Germany), pH 5.6.

Lactobacilli growth in fermentation media with different saccharides. The fermentation medium (5 g yeast extract, 5 g peptone, 2 g sodium acetate, 1 ml Tween 80, 0.4 g MgSO₄·6H₂O, 0.2 g MnSO₄·4H₂O, 0.2 g FeSO₄·6H₂O, 0.2 g NaCl in 1 l of distilled water, pH adjusted to 5.8, sterilised at 121°C 15 min) enriched with different types of saccharides (lactose, glucose, galactose, maltose, arabinose, xylose, cellobiose, and fructose) in a concentration of 20 g/l was used. Cultivations were carried out in microtitre plates in Biotek (Bio Tek U.S., Seattle, USA). The wells were filled with 200 µl of fermentation media with different saccharides and 2 µl of overnight lactobacilli culture. The microtitre plates were incubated at 37°C in an aerobic atmosphere for 26 hours. The cell growth was monitored by the absorbance measurement at 600 nm. All samples were tested in triplicate.

Cultivation of strains in skim milk and in skim milk with the addition of whey or malt. The effect of the addition of whey powder (Spray dry whey; Moravia Lacto, Jihlava, Czech Republic), whey protein concentrate (WPC) (Whey Protein Concentrate 70; PML Protein Mléko Laktóza, Nový Bydžov, Czech Republic), caseinomacropeptide (CMP) (Ekomilk, Frýdek Místek, Czech Republic), Pilsner malt (Research Institute of Brewing and Malting, Prague, Czech Republic) and dry malt extract Sladovit (Sladovna Bruntál, Czech Republic) on the growth of selected lactobacilli in skim milk was tested during 24 h cultivation. The addition of 5 g into 95 g of skim milk was used for all ingredients. Malt grains were first ground in a KM4 laboratory mill (Labora, Prague, Czech Republic) with a sieve mesh size of 0.5 mm. Media were twice sterilised at 100°C for 15 min in the interval of 24 hours. Lactobacilli inoculum (1 ml) was used for subsequent cultivation at 37°C in CO₂ atmosphere. The samples were taken in 0, 3, 6, 18, and 24 h and the number of cells expressed as CFU/ml was counted by the plate method on MRS agar after the appropriate dilution. pH of samples was measured by digital pH meter 3020 (Jenway, Staffordshire, UK).

Titrateable acidity was measured by titrating 25 ml of the sample with 0.25 mol/l NaOH and expressed in °SH units.

The results are the means from two independent repetitions, both parallel samples were analysed twice ($n = 4$).

Determination of lactic and acetic acids. The amount of lactic and acetic acids was determined by Megazyme enzyme kits (Megazyme International, Bray, Ireland) and the results are expressed as a mean from two samples.

Statistical analyses. The significance of differences between the pH values was tested by Student's *t*-test ($\alpha = 0.01$).

RESULTS AND DISCUSSION

First, the lactobacilli were cultivated in a medium with different saccharides of milk and cereal origin as carbon sources (data not shown). It was found out that glucose, galactose, fructose, and maltose were utilised by all tested strains well. Fructose, glucose, and maltose represent the main saccharides in malt hydrolysates (ROZADA-SANCHÉZ *et al.* 2008); glucose and galactose are utilised after hydrolysis of lactose, present both in milk and whey, by β -galactosidase, enzyme present in all LAB. *L. casei* Lafti L-26 did not utilise xylose and cellobiose, *L. acidophilus* CCDM 151 did not utilise xylose and arabinose, and *L. casei* CCDM 198 did not utilise cellobiose. There were differences observed between strains, the best growth was proved generally for *L. casei* Lafti L-26.

Table 1 demonstrate the effect of addition of 5 g of whey, WPC, CMP, malt, and malt extract into 95 g of skim milk on the growth of lactobacilli during the 24 h cultivation at 37°C. When cultivated in milk and milk with the addition of whey, WPC and CMP, the final increase in the cell number by 1.5–2 log cycles was found out which was similar to the cultivation in pure milk. Results obtained for supplementation of milk by whey proteins, WPC and CMP are contradictory (BURY *et al.* 1998; DAVE & SHAH 1998; MARTÍN-DIANA *et al.* 2003; ANTUNES *et al.* 2005). Some authors did not prove a difference in the growth of probiotic lactobacilli in milk with added whey protein supplements (MARTÍN-DIANA *et al.* 2003), others proved a stimulation even by the application of 2% WPC into milk (BOŽANIČ *et al.* 2004). The growth promoting activity of WPC could be due to CMP and whey protein content (JANER *et al.* 2004). CMP contains not only available nitrogen for the bacterial growth but also amino-saccharides,

Table 1. Number of cells of *L. casei* Lafti L-26, *L. acidophilus* CCDM 151, and *L. casei* CCDM 198 (log CFU/ml) during 24 h cultivation at 37°C in different tested media

Time (h)	Milk	M+whey	M+WPC	M+CPM	M+malt	M+extract
<i>L. casei</i> Lafti L-26						
0	6.77	6.72	7.19	6.69	6.66	6.78
3	6.89	6.96	7.25	6.80	6.67	6.75
6	7.54	7.59	7.65	7.44	7.67	7.83
18	8.57	8.53	8.67	8.93	9.12	8.70
24	8.60 ^a	8.76 ^a	8.89 ^a	8.97 ^b	9.49 ^b	8.91 ^b
<i>L. acidophilus</i> CCDM 151						
0	6.47	6.42	6.75	6.34	6.53	6.54
3	6.59	6.79	6.84	6.46	6.64	6.75
6	6.61	7.01	7.27	6.75	6.97	7.08
18	8.63	8.84	8.89	8.27	8.84	8.83
24	8.64 ^a	8.88 ^a	9.01 ^b	8.42 ^a	9.84 ^b	8.93 ^b
<i>L. casei</i> CCDM 198						
0	6.47	6.42	6.75	6.34	6.53	6.54
3	6.59	6.79	6.84	6.46	6.64	6.75
6	6.61	7.01	7.27	6.75	6.97	7.08
18	8.63	8.84	8.89	8.27	8.84	8.83
24	8.64 ^a	8.88 ^a	9.01 ^b	8.42 ^a	9.84 ^b	8.93 ^b

M = milk; WPC = whey protein concentrate; CPM = caseinomacropetide; extract = malt extract; values in the same row followed by different lowercase letters are significantly different ($\alpha = 0.01$)

such as sialic acid and *N*-acetylgalactosamine, which could be utilised as prebiotics by some probiotics, namely bifidobacteria (AZUMA *et al.* 1984). It is evident from Table 1 that a significant increase in the number of viable lactobacilli cells was detected after the supplementation of 95 g milk by 5 g malt or malt extract. After 24 h cultivation the number of cells was significantly different for all three tested lactobacilli.

When using milk with malt, the number of viable cells increased by 3 log cycles in the case of

L. casei Lafti L-26 and by 3.3 log cycles in the case of *L. acidophilus* CCDM 151 after 24 h cultivation. Also, the specific growth rates of *L. casei* Lafti L-26 and *L. casei* CCDM 198 were significantly higher compared to those in milk (data not shown). There are not many papers documenting the effect of malt addition to milk on the growth of probiotic lactobacilli, but RANADHEERA *et al.* (2010) found also the stimulating effect of malt on the growth of several lactobacilli including *Lactobacillus acidophilus* due to the presence of fermentable saccharides and free

Table 2. pH values of different media at the beginning and after 24 h cultivation of tested lactobacilli at 37°C

Media	<i>L. casei</i> Lafti L-26		<i>L. acidophilus</i> CCDM 151		<i>L. casei</i> CCDM 198	
	0 h	24 h	0 h	24 h	0 h	24 h
Skim milk	6.57 ^a	4.81 ^a	6.67 ^a	4.68 ^a	6.58 ^a	4.68 ^a
Milk + whey powder	6.43 ^b	4.86 ^a	6.35 ^{b,c,d}	4.35 ^b	6.43 ^b	4.89 ^{a,c}
Milk + WPC	6.47 ^b	4.94 ^a	6.45 ^{b,c}	4.80 ^a	6.44 ^b	5.51 ^b
Milk + CMP	6.29 ^c	5.13 ^b	6.30 ^{b,d}	5.16 ^c	6.27 ^c	5.37 ^c
Milk + malt extract	6.49 ^b	3.85 ^c	6.39 ^{b,c}	3.72 ^d	6.52 ^{a,b}	3.72 ^d
Milk + malt	6.54 ^{a,b}	3.96 ^c	6.44 ^{b,c}	3.67 ^d	6.52 ^{a,b}	3.65 ^d

WPC = whey protein concentrate, CPM = caseinomacropetide; values in the same column followed by different lowercase letters are significantly different ($\alpha = 0.01$)

Table 3. Values of titratable acidity of different media at the beginning and after 24h cultivation of tested lactobacilli at 37°C and lactic/acetic acid ratio at 24 h

Media	<i>L. casei</i> Lafti L-26			<i>L. acidophilus</i> CCDM 151			<i>L. casei</i> CCDM 198		
	titratable acidity (°SH)		acid ratio*	titratable acidity (°SH)		acid ratio*	titratable acidity (°SH)		acid ratio*
	0 h	24 h		0 h	24 h		0 h	24 h	
Skim milk	6.8	27.6	0.5	7.4	28.6	10.3	7.4	28.2	8.9
Milk + whey powder	9.8	37.8	1.3	10.0	26.6	7.9	9.2	23.8	12.0
Milk + WPC	9.8	25.6	2.9	9.2	57.6	15.6	9.4	27.8	11.8
Milk + CMP	15.2	29.2	1.2	15.6	37.2	14.2	12.4	28.8	15.1
Milk + malt extract	8.4	51.8	1.9	10.8	66.6	19.0	8.2	66.4	3.3
Milk + malt	7.8	50.4	4.0	8.8	68.6	19.8	8.0	71.2	1.0

WPC = whey protein concentrate, CPM = caseinomacropeptide; *lactic/acetic acid molar ratio after 24-h cultivation

nitrogen. Attention was also paid to the effect of malt on the viability of probiotic lactobacilli in culture media. The positive effect of malt on the viability of several lactobacilli strains of different species including *L. acidophilus*, *L. plantarum*, and *L. reuteri* was proved (CHARALAMPOPOULOS *et al.* 2002; CHARALAMPOPOULOS & PANDIELLA 2010).

Significant differences in the final pH value between milk containing whey component and milk supplemented by malt or malt extract were found after 24 h cultivation (Table 2). At the beginning of cultivation, the pH values were influenced by different supplements and also by the strain inoculated into the media. While differences in pH value were from 0.9 to 2.0 in the case of milk with whey components, in malt supplemented milk they reached 2.6 to 2.9 after 24 hours. In general, all lactobacilli decreased pH in milk supplemented by whey components to a lesser extent and in the malt supplemented milk to a higher extent compared with the skim milk. OLIVEIRA *et al.* (2001), who compared the effect of milk supplementation and culture composition on milk acidification, concluded that the acidification activity was greatly improved with casein hydrolysate but not by the addition of whey. The positive effect of malt on the acidification activity of lactobacilli was also confirmed by CHARALAMPOPOULOS *et al.* (2002). The final pH of fermented products is influenced by the buffering capacity of the medium which is in relation to the amount and type of proteins present.

Lactic and acetic acids are the main products of carbohydrate fermentation in *Lactobacillus* genus. The content of lactic and acetic acids has an impact on sensory properties of fermented products. Furthermore, lactate and acetate as representatives of non-branched short chain fatty acids can play a positive role in human nutrition. The presence of

short chain fatty acids in the intestine lowers pH, increases bioavailability of calcium and magnesium and inhibits the growth of potentially harmful bacteria (CRAEYVELD *et al.* 2008). The content of both acids was analysed in all tested media after 24 h fermentation and the results are presented in Figure 1. All

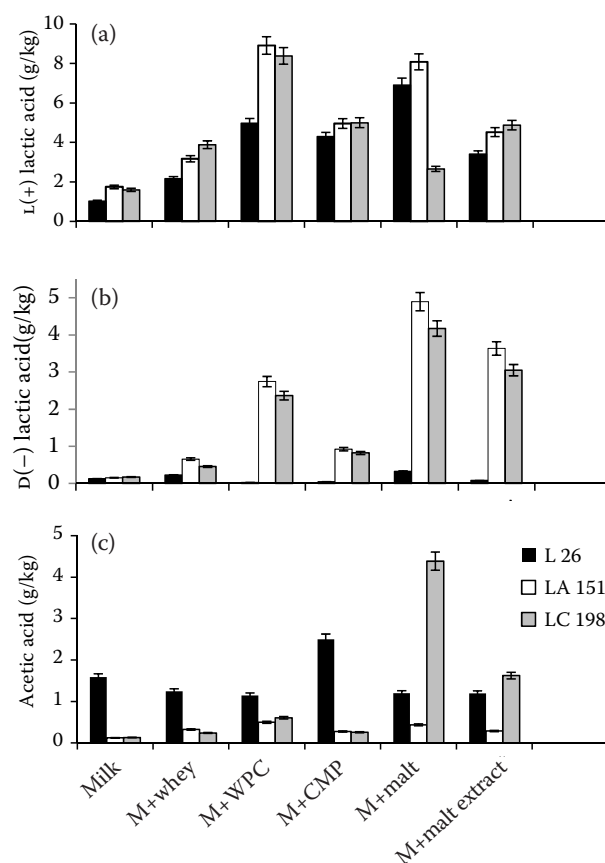


Figure 1. The content of (a) L(+)-lactic acid, (b) D(-)-lactic acid, and (c) acetic acid after 24 h cultivation of tested lactobacilli at 37°C

L 26 = *Lactobacillus casei* Lafti L-26; LA 151 = *Lactobacillus acidophilus* CCDM 151; LC 198 = *Lactobacillus casei* CCDM 198

added substances increased the amount of lactic acid. The highest content of L(+) lactic acid was detected in the case of *L. acidophilus* CCDM 151 and *L. casei* CCDM 198 in milk with whey protein concentrate (8.9 and 8.3 g/kg, respectively) and in the case of *L. casei* Lafti L-26 in milk with malt (6.9 g/kg). After cultivation of all three tested strains in milk, only traces of D(–)lactic acid were detected. The addition of malt and malt extract increased significantly the production of D(–) isomer of lactic acid at two out of the three tested lactobacilli strains (*L. acidophilus* CCDM 151 and *L. casei* CCDM 198; 4.9 and 4.2 g/kg, respectively). Malt and malt extract addition also increased the production of acetic acid by *L. casei* CCDM 198. CHARALAMPOPOULOS and PANDIELLA (2010) found a lower level of lactic acid production in malt-containing media after the cultivation of *L. plantarum* (2.1 g/l) similarly like HELLAND *et al.* (2004), who reported only 1.36–4.0 g/kg of lactic acid in maize porridge with added malted barley. However, some authors determined more than 15.0 g/kg of lactic acid in a malt medium after the cultivation of *Bifidobacterium breve* (ROZADA-SANCHEZ *et al.* 2009). Furthermore, the values of titratable acidity and lactic/acetic acid molar ratio were compared (Table 3). It is evident that titratable acidity is influenced not only by the production of lactic and acetic acids but also by acid reactive residues of amino acids contained in the proteins of culture media. Different lactic/acetic acid molar ratios were obtained between different species and also between two strains of *L. casei*. These results are in accordance with statements of other authors (ZALÁN *et al.* 2010) who proved that the real fermentation profile of LAB is intensively influenced by the composition of media, cultivation conditions and strain and that it can differ from the theoretical end products of metabolic pathways.

CONCLUSION

It was proved that the addition of 5 g of malt-based supplements into 95 g of milk increased the final number of probiotic lactobacilli used for fermentation. At the same time, it influenced more the amount and composition of produced metabolites compared with the supplementation of milk by whey products. Furthermore, the differences in growth and biochemical activity between the tested strains were proved in the same media. Careful selection of probiotic LAB strains and cultivation conditions for the new milk fermented products supplemented with whey or cereal-based additives is necessary.

References

- ALMEIDA K.E., TAMINE A.Y., OLIVIERA M.N. (2009): Influence of total solids content of milk whey on the acidifying profile and viability of various lactic acid bacteria. *Food Science and Technology*, **42**: 672–678.
- ANTUNES A., CAZETTO T., BOLINI H. (2005): Viability of probiotic micro-organisms during storage, postacidification and sensory analysis of fat-free yogurts with added whey protein concentrate. *International Journal of Dairy Technology*, **58**: 169–173.
- AZUMA N., YAMAUCHI K., MISTUOKA T. (1984): Bifidus growth-promoting activity of a glycomacropeptide derived from human κ -casein. *Agricultural and Biological Chemistry*, **48**: 2159–2162.
- BOŽANIČ R., TRATNIK L.J., HERCEG Z., MARIC O. (2004): The influence of milk powder, whey protein concentrate and inulin on the quality of cow and goat acidophilus milk. *Acta Alimentaria*, **33**: 337–346.
- BURY D., JELEN P., KIMURA K. (1998): Whey protein concentrate as a nutrient supplement for lactic acid bacteria. *International Dairy Journal*, **8**: 149–151.
- CHARALAMPOPOULOS D., PANDIELLA S.S. (2010): Survival of human derived *Lactobacillus plantarum* in fermented cereal extracts during refrigerated storage. *LWT-Food Science and Technology*, **43**: 431–435.
- CHARALAMPOPOULOS D., PANDIELLA S.S., WEBB C. (2002): Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. *Journal of Applied Microbiology*, **92**: 851–859.
- CRAEYVELD V., SWENNEN K., DORNEZ E., WIELE T., MARZORATI M., VERSTRAETE W., DELAEDT Y., ONAGBESAN O., DECUYPERE E., BUYSE J. (2008): Structurally different wheat-derived arabinoxyloligosaccharides have different prebiotic and fermentation properties in rats. *Journal of Nutrition*, **138**: 2348–2355.
- DAVE R., SHAH N.P. (1998): Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *Journal of Dairy Science*, **81**: 2804–2816.
- HELLAND M.H., WICKLUND T., NARVHUS J.A. (2004): Growth and metabolism of selected strains of probiotic bacteria in milk- and water-based cereal puddings. *International Dairy Journal*, **14**: 957–965.
- HORÁČKOVÁ Š., ŽALUDOVÁ K., PLOČKOVÁ M. (2011): Stability of selected lactobacilli in the condition simulating gastrointestinal tract. *Czech Journal of Food Sciences*, **29**: 30–35.
- JANER C., PELÁEZ C., REQUENA T. (2004): Caseinomacropeptide and whey protein concentrate enhance *Bifidobacterium lactis* growth in milk. *Food Chemistry*, **86**: 263–267.
- KAILASAPATHY K., SUPRIADI D. (1996): Effect of whey protein concentrate on the survival of *Lactobacillus aci-*

- dophilus* in lactose hydrolyzed yogurt during refrigerated storage. *Milchwissenschaft*, **51**: 566–569.
- KORHONEN H. (2009): Milk-derived bioactive peptides: from science to applications. *Journal of Functional Foods*, **1**: 177–187.
- MARTÍN-DIANA A.B., JANER C., PELÁEZ C., REQUENA T. (2003): Development of fermented goat's milk containing probiotic bacteria. *International Dairy Journal*, **13**: 827–833.
- MURAKAMI M., TOUNOUCHI H., TAKAHASHI R., KITAZAWA H., KAWAI Y., NEGISHI H. (2004): Structural analysis of a new anti-hypertensive peptide (β -lactosin B) isolated from a commercial whey product. *Journal of Dairy Science*, **87**: 1967–1974.
- OLIVEIRA M.N., SODINI I., REMEUF F., CORRIEU G. (2001): Effect of milk supplementation and culture composition on acidification, textural properties and microbiological stability of fermented milks containing probiotic bacteria. *International Dairy Journal*, **11**: 935–942.
- PATEL H.M., PANDIELLA S.S., WANG R.H., WEBB C. (2004): Influence of malt, wheat and barley extracts on the bile tolerance of selected strains of lactobacilli. *Food Microbiology*, **21**: 83–89.
- ROZADA-SANCHÉZ R., SATTUR A.P., THOMAS K., PANDIELLA S.S. (2008): Evaluation of *Bifidobacterium* spp. for the production of a potentially probiotic malt based beverages. *Process Biochemistry*, **43**: 848–854.
- RANADHEERA R.D.C.S., BAINES S.K., ADAMS M.C. (2010): Importance of food in probiotic efficacy. *Food Research International*, **43**: 1–7.
- ROZADA-SANCHEZ R., VÁZQUEZ J.A., CHARALAMPOPOULOS T., PANDIELLA S.S. (2009): Effect of storage temperature and media composition on the survivability of *Bifidobacterium breve* NCIMB 702257 in malt hydrolysate. *International Journal of Food Biochemistry*, **73**: 14–21.
- ZALÁN Z., HUDÁČEK J., ŠTĚTINA J., CHUMCHALOVÁ J., HALÁSZ A. (2010): Production of organic acids by *Lactobacillus* strains in three different media. *European Food Research and Technology*, **230**: 395–404.

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Influence of Flaxseed Components on Fermented Dairy Product Properties

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Abstract

Bialasová K., Němečková I., Kyselka J., Štětina J., Solichová K., Horáčková Š. (2018): Influence of flaxseed components on fermented dairy product properties. Czech J. Food Sci., 36: 00–00.

The addition of flaxseed meal and flaxseed oil on the growth and viability of *Lactobacillus acidophilus* CCDM 151 and yoghurt culture CCDM 21 during cold storage in fermented milk was tested. It was found that the oil addition in the amount of 0.6% w/w in milk did not influence the growth and acid production of *Lactobacillus acidophilus* CCDM 151, while the acidification activity of yoghurt culture was slightly lower compared to pure milk and connected with lower growth of *Streptococcus thermophilus*. On the contrary the addition of meal in amount of 7.6% w/w into milk stimulated the growth and acid production of *Lactobacillus acidophilus* CCDM 151. The viability of both tested cultures during one month storage of fermented milks at $5 \pm 1^\circ\text{C}$ was not influenced by the oil supplementation but the addition of meal decreased their viability significantly. The unusual volatile compounds acetone and butane-2-one were detected by SPME-GC in yoghurt with meal. Unlike oil, the addition of flaxseed meal increased the yoghurt firmness and influenced negatively yoghurt taste and flavour.

Keywords: flaxseed meal; flaxseed oil; *Lactobacillus acidophilus*; textural analysis; yoghurt culture

Currently, both researchers and consumers are paying great attention to so-called ‘functional foods’ containing components such as probiotic microorganisms, prebiotics and their combination (synbiotics), fibre, essential fatty acids, etc. Generally the increased consumption of fibre can help to control weight and serum cholesterol levels, reduce blood pressure and improve intestinal functions. Moreover certain types of fibre, mostly oligosaccharides, have a prebiotic effect defined as a selective stimulation of one or more beneficial bacteria in the colon (GIBSSON 2004). Fibre can also protect probiotic microorganisms during food processing and storage

as well as improve their stability both in products and the intestinal tract (SAARELA *et al.* 2006) as these bacteria are often sensitive to low pH, processing or storage temperature and other factors (TRIPATHI & GIRI 2014). Water soluble and insoluble fibres (galactooligosaccharides, fructooligosaccharides, lactose derivatives, inulin and polydextrose) have been suggested as potential probiotic protectants (CHARALAMPOPOULOS *et al.* 2002). Food fortification by flax seed components has been proven to have many health benefits (MERCIER *et al.* 2014). Flaxseed is an important source of ω -3-fatty acids, especially α -linolenic acid 50–65%, plant lignans,

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soluble/insoluble fibre, cyclic peptides and various minerals (BUSTAMANTE *et al.* 2015). Omega-3-fatty acids are considered to be associated with blood lipids improvements, may reduce the risk of cardiovascular disease, osteoporosis, diabetes or gastrointestinal disease (BLOEDON & SZAPÁRY 2004; SHIM *et al.* 2014). However, the effect of flaxseed components on lactic acid bacteria has not yet been studied in much detail. The aim of this study was to test the influence of addition of flaxseed meal (a source of fibre and lignans) and flaxseed oil (an important source of ω -3-fatty acids) to milk on the growth, acidification properties and storage stability of *Lactobacillus acidophilus* CCDM 151 and yoghurt culture CCDM 21 and to evaluate the texture and sensory properties of subsequent fermented products.

MATERIALS AND METHODS

Microorganisms. *Lactobacillus acidophilus* CCDM 151 and Yoghurt culture CCDM 21 (Culture Collection of Dairy Microorganisms, Laktoflora[®]; Milcom, Czech Republic) were used in this work.

Cultivation, determination of cell count and pH. For the inoculation of tested media, overnight cultures (1% v/v inoculum) cultivated in skimmed UHT milk (Madeta, Czech republic) at 37°C in 5% (v/v) CO₂ atmosphere for *L. acidophilus* CCDM 151 and aerobically at 30°C for yoghurt culture were used. The number of *L. acidophilus* CCDM 151 cells was determined according to ISO 20128:2006 and the number of yoghurt culture cells according to ISO 7889:2003. The pH values were measured using a pH meter 3020 (Jenway, UK) provided with a combined electrode.

Flaxseed components. Flaxseed meal (Organic Brown Flax Fibre 300–500 μ m; Functional Whole Food, New Zealand) and flaxseed oil (Functional Whole Food, New Zealand) were used in this study. Flaxseed meal was characterised as follows: proteins 31.5 w/w% (Kjeldahl method), lipids 20.6 w/w% (Soxhlet extraction), water 10.3 w/w% (halogen moisture analyser Mettler Toledo HR73, Switzerland), insoluble fibre 38 w/w% and soluble fibre 7 w/w% (manufacturer's specification). Fatty acid composition was determined according to AOCS Official Methods Ce 1f-96 (2002). Analysis was performed on an Agilent 6890N Gas Chromatograph (Agilent Technologies, USA) and SPTM 2560 capillary column (Supelco, USA) 0.25 mm $\times$ 100 m; film thickness

0.2 μ m was used. The average composition was: palmitic acid 5.4%, stearic acid 4.9%, oleic acid 20.1%, linoleic acid 15.1% and linolenic acid 54.5%.

Cultivation of strains. The effect of the addition of flaxseed meal in the ratio of 7.6 g to 92.4 g of skimmed UHT milk (milk + meal) and flaxseed oil in the ratio of 0.6 g with 0.06 g soy lecithin (Mogador, Czech Republic) to 99.34 g of skimmed UHT milk (milk + oil) on the growth of selected dairy cultures was tested compared to the growth in pure skimmed UHT milk (milk). Flaxseed oil was first stirred (stirrer RZR 2021; Heidolph, Germany) with pre-heated (60°C) milk and lecithin at 200 g for 5 min and further homogenised (T-25 basic Ultra-Turrax[®]; IKA, Germany) at 13 500 g for 4 min followed by another stirring (200 g, 10 min). All media used were heat treated at 90°C for 10 min, inoculated after cooling either by 1% (v/v) *L. acidophilus* CCDM 151 or 0.1% (v/v) yoghurt culture CCDM 21 to get a starter concentration 10⁶ CFU/g and cultivated at the appropriate temperature for 16 hours. Samples were analysed after fermentation and after 14 and 28 days storage at 5 $\pm$ 1°C. The results are the means from two independent fermentations, both parallel samples were analysed twice ($n = 4$).

HPLC analysis of organic acids. Before the analysis the samples with flax meal were diluted in 1 : 10 ratio with distilled water. To a 250 μ l sample, 1.6 ml of ethanol was added, allowed to stay for 30 minutes. The mixture was then centrifuged (13 000 g, 10 min and 4°C) and filtered through a 0.22 μ m membrane prior to injection of 20 μ l into the chromatographic system. Separation was performed using HPLC system (Agilent 1260 Infinity; USA) with precolumn 50 $\times$ 8 mm and polymer column IEX H, 250 $\times$ 8 mm (both Watrex, Czech Republic) connected to UV/VIS detector (210 nm). Aqueous solution of sulfuric acid (9 mmol/l) was used as mobile phase at 0.6 ml/min flow rate, the column temperature was 60°C.

Analysis of volatile compounds. Volatile compounds were isolated from the sample headspace by solid phase microextraction (SPME) and determined by gas chromatograph Agilent Technologies 7890 (USA) coupled with mass spectrometry detector 5975C (Agilent Technologies, USA). Five grams of sample were tempered in a glass vial at 40°C for 30 min and then conditioned SPME fibre was exposed to the sample headspace for 1 min. The analyses were performed on HP5 column (Agilent Technologies, USA), particle size 0.32 mm $\times$ 30 m, 0.25 μ m; with temperature mode: initial temperature

40°C for 7 min followed by an increase of 5°C/min up to 240°C. Helium was used as a mobile phase with 0.9 ml/min flow rate.

Textural analysis. Texture Analyser TA.XT plus (Stable Micro Systems Ltd., UK) was used to measure texture profile analysis. A cylindrical aluminium probe (P/20) was repeatedly punctured into sample to a defined depth (25 mm) at fixed speed 5 mm/s and sample temperature 5°C. Three textural characteristics, firmness, adhesiveness and cohesiveness, were evaluated (BOURNE 2002). The results indicate the average of three determinations.

Sensory evaluation. Ten previously trained laboratory members were chosen as panellists. The samples were evaluated according to six attributes – appearance, flavour, taste, texture, overall rating and purchase intention. The rating of individual attributes was carried out using 0–10 scale (0 = unacceptable, 10 = excellent). Samples were fermented one day before evaluation and tempered to $15 \pm 1^\circ\text{C}$. Each sample was coded by a random four digit number.

RESULTS AND DISCUSSION

First, the prepared media were cooled to fermentation temperature, 37°C for *L. acidophilus* CCDM 151 and to 30°C for yoghurt culture CCDM 21. Before fermentation, the average pH of milk was 6.60 ± 0.05 ; milk + oil 6.62 ± 0.01 and milk + meal 6.33 ± 0.04 . Table 1 summarises the number of cells, pH changes and lactic and acetic acid concentration after 16 h cultivation. *L. acidophilus* CCDM 151 showed less growth activity in milk and in milk with oil. The pH values reached were only 5.51 ± 0.12 and 5.68 ± 0.06 , respectively, and were not sufficient for fermented dairy products. On the contrary, the addition of flaxseed meal into

milk stimulated its growth (by 0.8 log cycle) including acidification ($\text{pH } 4.22 \pm 0.14$). Flaxseed components slightly decreased the number of *S. thermophilus* cells. Yoghurt culture produced a significantly higher amount of lactic acid and consequently pH reached after fermentation was around 4.42 ± 0.14 in all media tested. However, compared to milk, the decrease in pH was lower in milk with oil. Acetic acid, which can cause an undesirable off-flavour of fermented products, was not detected in media with *L. acidophilus* CCDM 151 and only in traces in media with yoghurt culture. The influence of plant components on growth and acidification ability has also been proved by other authors (CHARALAMPOPOULOS *et al.* 2002). SAH *et al.* (2016) confirmed that milk supplementation by pineapple peel powder reduced fermentation time inoculated by *L. acidophilus* ATCC 4356 or *L. casei* ATCC 393, but in the study of SENDRA *et al.* (2008) citrus fiber had inhibitory effect on *B. bifidum* CECT 870. Slow acidification of fermented products can bring the risk of potential contamination growth and therefore careful selection of culture, cultivation conditions and functional components is necessary for industrial applications.

Further, all prepared fermented products were stored at $5 \pm 1^\circ\text{C}$ for 1 month to investigate the influence of flaxseed components on cell viability. The cell number found after fermentation (1 day) and after 14, respectively 28 days storage is shown on Figure 1. No significant changes in pH were detected during the storage of fermented products (data not shown). Although the flaxseed meal promoted the growth of *L. acidophilus* CCDM 151 during fermentation, Figure 1 shows that there was a significant decrease in cell viability during storage compared to milk. Oil addition did not influence the cell viability. After 28 days of storage, the cell number

Table 1. The number of cells, pH changes and lactic and acetic acid concentrations

Culture	Medium	Cell count (log CFU/g)		Δ pH	Lactic acid (g/l)	Acetic acid (g/l)
<i>L. acidophilus</i> CCDM 151	milk	8.18 ± 0.14		1.07 ± 0.07	0.25 ± 0.02	nd
	milk & oil	8.19 ± 0.03		0.93 ± 0.05	0.17 ± 0.03	nd
	milk & meal	8.98 ± 0.20		2.11 ± 0.09	0.89 ± 0.07	nd
		<i>L. delbrueckii</i> ssp. <i>bulgaricus</i>	<i>S. thermophilus</i>			
Yoghurt culture CCDM 21	milk	8.23 ± 0.15	8.76 ± 0.32	2.21 ± 0.07	0.91 ± 0.06	traces
	milk & oil	8.32 ± 0.14	8.32 ± 0.14	1.59 ± 0.09	1.00 ± 0.12	traces
	milk & meal	8.20 ± 0.20	8.31 ± 0.23	1.87 ± 0.04	0.97 ± 0.11	traces

Cultivation of *L. acidophilus* (CCDM 151) and yoghurt culture (CCDM 21) 16 h in tested media; nd – not detected

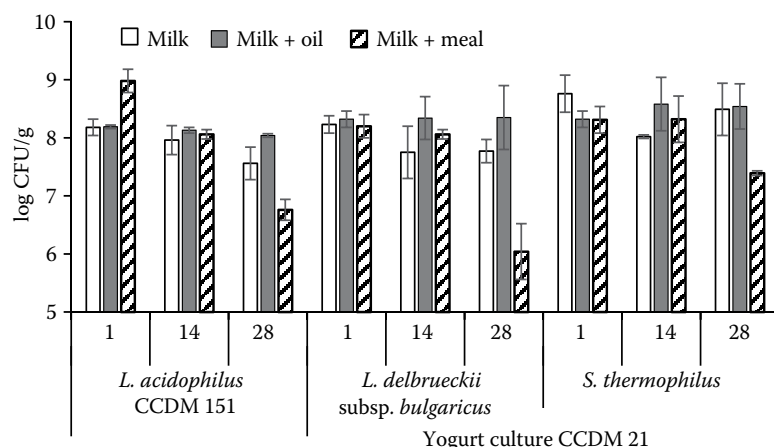


Figure 1. The number of cells after fermentation (1 day) and after 14 and 28 days storage at $5 \pm 1^\circ\text{C}$ in tested products

in fermented milk and oil-added milk was only by 0.61 and 0.65 log cycle CFU/g respectively lower than immediately after fermentation, whereas in milk with meal it was by 1.45 log cycle CFU/g. The same trend was observed for both microorganisms of yoghurt culture. The cell number in yoghurt with oil did not decrease during storage but the loss of cells in yoghurt with flaxseed meal was 0.98 log cycle CFU/g for *S. thermophilus* and 1.80 log cycle CFU/g for *L. delbrueckii* subsp. *bulgaricus*. There are very few studies documenting the effect of flaxseed components on growth or stability of lactic acid bacteria in milk and fermented milk. VESTERLUND *et al.* (2012) demonstrated the increase of viability of probiotic bacteria *L. rhamnosus* GG during storage at 22°C for 14 months when milled flaxseeds were added to dried matrices. Also, HADINEZHAD *et al.* (2013), who tested the influence of soluble flax fibre on the stability of kefir culture in combination with *L. acidophilus* B-4495 and *B. animalis* subsp. *lactis* 41405 during storage (28 days, 4°C), discovered its positive effect. In this study, the negative effect of flaxseed meal can be explained by high concentration of plant oil. Since the meal contains about 20% w/w of fat, the majority of it consists of long fatty acids. These acids were proved to have the antimicrobial and antifungal activities (ZHENG *et al.* 2005; CALCE *et al.* 2014). Likewise, other components of plant fibre, for example polyphenols, can influence the microorganisms' growth (HERVERT-HERNÁNDEZ *et al.* 2009). Further, the microbial contamination of flaxseed meal, which was detected in heat treated media with meal, can act antagonistically against lactic acid bacteria. The raw meal contained 1.8×10^3 CFU/g of total microorganisms count; the heat treated medium (90°C , 10 min) less than 10 CFU/g. When this medium was cultivated (30°C , 16 h) without

dairy culture addition the total count of microorganisms increased to 6.6×10^4 CFU/g and after storage ($5 \pm 1^\circ\text{C}$, 28 days) to 4.4×10^5 CFU/g. This suggests that the flax fibre used may exacerbate potential contamination in fermented products.

Furthermore, due to the off-flavour found (after bitter almonds), the comparison of volatile compounds of yoghurt and yoghurt with flaxseed meal was done using the SPME-GC method. An example of a chromatogram for yoghurt with meal is shown on Figure 2. Unlike yoghurt alone, these samples contained acetone and butane-2-one. Flax (*Linum usitatissimum*) and flaxseed meal contain cyanogenic glycosides (linamarin, linustatin, and neolinustatin) (RUSSO & REGGIANI 2014) which can be degraded to cyanide hydrogen, ketones and aldehydes (MØLLER 2010).

The addition of flaxseed meal to yoghurt, in a quantity consistent with the nutritional claim 'source of fibre' (Regulation 1924/2006), also significantly affected the yoghurt texture (Table 2) and sensory properties

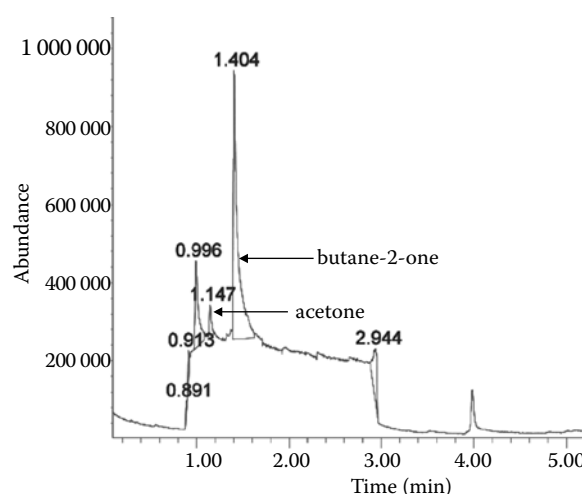


Figure 2. SPME-GC chromatogram of volatile compounds in yoghurt with flaxseed meal

Table 2. Textural properties of yoghurt and yoghurt with flaxseed meal

	Firmness (N)	Adhesiveness (N·s)	Cohesiveness	Firmness (N)	Adhesiveness (N·s)	Cohesiveness
	1 day			28 days		
Yoghurt	0.28 ± 0.02	0.41 ± 0.04	0.59 ± 0.01	0.27 ± 0.03	0.36 ± 0.07	0.62 ± 0.05
Yoghurt & meal	1.36 ± 0.08	0.56 ± 0.04	0.47 ± 0.02	1.60 ± 0.15	0.85 ± 0.12	0.59 ± 0.07

Samples after 16 h fermentation at 30°C and after 28 days storage at 5 ± 1°C

Table 3. Sensory evaluation and purchase intention of fermented milk products

Culture	Medium	Appearance	Aroma	Flavour	Consistency	Overall impression	Purchase interaction
<i>L. acidophilus</i> CCDM 151	milk	5.1 ± 0.99	3.4 ± 1.17	4.8 ± 0.63	2.9 ± 0.74	2.1 ± 0.74	2.6 ± 0.51
	milk & oil	5.2 ± 0.92	2.9 ± 0.88	2.1 ± 0.74	2.7 ± 0.67	2.0 ± 0.67	1.7 ± 0.48
	milk & meal	2.0 ± 0.82	1.8 ± 0.63	1.4 ± 0.52	1.8 ± 0.63	1.7 ± 0.82	1.4 ± 0.52
Yoghurt culture CCDM 21	milk	7.6 ± 0.84	8.0 ± 0.94	4.9 ± 0.74	7.9 ± 0.74	6.7 ± 0.82	5.6 ± 0.84
	milk & oil	7.5 ± 0.85	7.1 ± 0.99	4.1 ± 0.88	8.0 ± 0.67	6.2 ± 0.79	5.4 ± 0.70
	milk & meal	2.1 ± 0.87	3.0 ± 1.05	1.8 ± 0.63	4.1 ± 0.99	2.0 ± 0.67	1.7 ± 0.82

(Table 3). A claim that a food is a source of fibre, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 3 g of fibre per 100 g or at least 1.5 g of fibre per 100 kcal (Regulation 1924/2006). In this work, the total content of fibre was 3.4 g in 100 g of product. The texture of the yoghurt was strongly dependent on milk supplementation. Flaxseed meal increased the firmness by around five times in yoghurt after fermentation and by around six times after 28 day storage due to a higher solid content and a high viscosity of flaxseed polysaccharides in milk (VELEZ-RUIZ *et al.* 2013). At the same time, although flax oil has a characteristic aroma, the yoghurt with oil addition was not evaluated negatively due to off-flavour (Table 3). The addition of flaxseed oil would be an important source of ω -3-fatty acids in consumers' diets. A claim that a food is a source of ω -3 fatty acids may be made where the product contains at least 0.3 g α -linolenic acid per 100 g (Regulation 1924/2006); in this case it was 0.33 g per 100 g. Milk and milk with oil fermented by *L. acidophilus* CCDM 151 reached a pH only 5.51 ± 0.12 and 5.68 ± 0.06 respectively after cultivation. This fact was judged by the panellists to be insufficient and therefore negative.

CONCLUSION

Flaxseed components may be a source of bioactive compounds that positively affect human health but

can also negatively influence the sensory parameters of foods and the growth and stability of lactic acid bacteria in fermented foods. Interactions should be examined and an alternative solution could lie in a suitable combination of different types of lactic acid bacteria. Based on the results of this study, the addition of flaxseed oil to milk fermented by *L. acidophilus* CCDM 151 or yogurt culture CCDM 21 could be a promising option. Milk supplementation by oil did not influence the growth of dairy cultures and had a positive effect on their viability during storage.

References

- Bloedon L.T., Szapary P.O. (2004): Flaxseed and cardiovascular risk. *Nutrition Reviews*, 62: 18–27.
- Bourne M.C. (2002): *Food Texture and Viscosity*. 2nd Ed. London, Academic Press: 182–186.
- Bustamante M., Villarroel M., Rubilar M., Shene C. (2015): *Lactobacillus acidophilus* La-05 encapsulated by spray drying: Effect of mucilage and protein from flaxseed (*Linum usitatissimum*). *LWT-Food Science and Technology*, 62: 1162–1168.
- Calce E., Mignogna E., Bugatti V., Galdiero M., Vittoria V., De Luca S. (2014): Pectin functionalized with natural fatty acids as antimicrobial agent. *International Journal of Biological Macromolecules*, 68: 28–32.
- Charalampopoulos D., Wang R., Pandiella S.S., Webb C. (2002): Application of cereals and cereal components in functional foods: a review. *International Journal of Food Microbiology*, 79: 131–141.

- Gibson G.R. (2004): Prebiotics. *Best Practice & Research Clinical Gastroenterology*, 2: 287–298.
- Hadinezhad M., Duc C., Han N.F., Hosseini F. (2013): Flaxseed soluble dietary fibre enhances lactic acid bacterial survival and growth in kefir and possesses high antioxidant capacity. *Journal of Food Research*, 2: 152–163.
- Hervet-Hernández D., Pintado C., Rotger R., Goñi I. (2009): Stimulatory role of grape pomace polyphenols on *Lactobacillus acidophilus* growth. *International Journal of Food Microbiology*, 136: 119–122.
- Mercier S., Villeneuve S., Moresoli Ch., Mondor M., Marcos B., Power K.A. (2014): Flaxseed-enriched cereal-based products. A review of the impact of processing conditions. *Comprehensive Reviews in Food Science and Food Safety*, 13: 400–412.
- Møller B.L. (2010): Functional diversifications of cyanogenic glucosides. *Current Opinion in Plant Biology*, 13: 338–347.
- Russo R., Reggiani R. (2014): Variation in the content of cyanogenic glycosides in flaxseed meal from twenty-one varieties. *Food and Nutrition Sciences*, 5: 1456–1462.
- Saarela M., Virkajärvi I., Nohynek L., Vaari A., Mättö J. (2006): Fibres as carriers for *Lactobacillus rhamnosus* during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals. *International Journal of Food Microbiology*, 112: 171–178.
- Sah B.N.P., Vasiljevic T., McKechnie S., Donkor O.N. (2016): Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage. *LWT- Food Science and Technology*, 65: 978–986.
- Sendra E., Fayos P., Lario Y., Fernández-López J., Sayas-Barberá E., Pérez-Alvarez J.A. (2008): Incorporation of citrus fibers in fermented milk containing probiotic bacteria. *Food Microbiology*, 25: 13–21.
- Shim Y.Y., Gui B., Arnison P.G., Wang Y., Reaney M.J.T. (2014): Flaxseed (*Linum usitatissimum* L.) bioactive compounds and peptide nomenclature: a review. *Trends in Food Science & Technology*, 38: 5–20.
- Tripathi M.K., Giri S.K. (2014): Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, 9: 225–241.
- Velez-Ruiz J.F., Hernandez-Carranza P., Sosa-Morales M. (2013): Physicochemical and flow properties of low-fat yogurt fortified with calcium and fiber. *Journal of Food Processing and Preservation*, 37: 210–221.
- Vesterlund S., Salminen K., Salminen S. (2012): Water activity in dry foods containing live probiotic bacteria should be carefully considered: A case study with *Lactobacillus rhamnosus* GG in flaxseed. *International Journal of Food Microbiology*, 80: 319–321.
- Zheng Ch.J., Yoo J.-S., Lee T.-G., Choc H.-Y., Kim Y.-H., Kim W.-G. (2005): Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *LEBS Letters*, 579: 5157–5162.

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ANTIFUNGÁLNÍ AKTIVITA LAKTOBACILŮ

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Antifungal activity of lactobacilli

Summary:

In this work, agar diffusion method on malt agar and milk agar plate's method were used for screening of antifungal activity of *Lactobacillus acidophilus* CCDM 151, *L. plantarum* MP3, *L. zymae* CCDM 361, *L. panis* CCDM 471 and *L. sanfranciscensis* CCDM 827 against strains *Fusarium culmorum* DMF1 and *Penicillium expansum* DMF 0004, which are common contaminants of food and environment. Lactobacilli were characterized by growth in MRS broth (cell's count, pH) and by the production of lactic and acetic acids (HPLC). *L. plantarum* MP3 showed the biggest inhibitory effect on the growth of both fungi species during 10 days of observation. Strain *F. culmorum* DMF1 was more sensitive to antifungal activity of growing lactobacilli cells. The antifungal activity of heat-treated cell-free supernatants was detectable only after 3 days of cultivation with *F. culmorum* DMF1. Synergetic effect of lactic and acetic acid was also proved. The size of colonies of *F. culmorum* DMF1 on agar supplemented with acetic acid (0.1g/100 g of agar) was 7.9 cm, while on agar whose pH was initially adjusted by the addition of 10% wt. lactic acid to 4.6 and then acetic acid was added (0.1g/100 g) the average colony diameter was only 0.55 cm. Use of lactobacilli cultures with antifungal activity when preparing for example the leaven for the baking industry can lead to suppression of mould growth on bread.

Vláknité houby a kvasinky jsou často se vyskytující mikroorganismy způsobující kažení potravin. Plísně mohou růst na celé řadě potravinářských produktů jako je máslo, jogurty, pekárenské výrobky, ovoce a zelenina, obiloviny apod. Mají schopnost produkovat lipolytické, proteolytické a sacharolytické enzymy, které mohou způsobovat zhoršení sensorických vlastností výrobků. Z hlediska zdravotního je důležitá jejich tvorba sekundárních metabolitů – mykotoxinů, které jsou pro obratlovce toxické již v malé koncentraci. Jednou z možností, jak prodloužit trvanlivost potravin a zároveň zabránit růstu nežádoucích plísní, je použití přírodní konzervace potravin pomocí činnosti bakterií mléčného kvašení, které produkují celou řadu antimikrobiálních metabolitů. Mezi metabolity s nejvyšší antifungální aktivitou lze zařadit hlavně organické kyseliny (kyselina mléčná, octová, fenylmléčná, p-hydroxyfenylmléčná, kyselina propionová)¹, peroxid vodíku², reuterin³, vyšší mastné kyseliny a hydroxylované mastné kyseliny⁴ či cyklické peptidy⁵.

Je prokázáno, že z bakterií mléčného kvašení vykazují antifungální aktivitu především kmeny rodu *Lactobacillus*, méně kmeny rodu *Lactococcus*.⁶ Rod *Lactobacillus* je řazen mezi grampozitivní, nesporulující, katalasa-negativní, tyčinkovité bakterie, jejichž hlavním konečným produktem fermentace sacharidů je kyselina mléčná. Ke svému růstu potřebují na živiny bohatá média obsahující např. sacharidy, vitaminy, soli, mastné kyseliny nebo estery mastných kyselin či deriváty nukleových kyselin. Jedná se o rozmanitou skupinu bakterií, což dokazuje široká škála poměrů C+G a malá homologie DNA-DNA mezi mnoha druhy.⁷ Antifungální aktivita byla prokázána u zástupců všech fermentačních skupin laktobacilů, tj. u homofermentativních, ale zvláště u fakultativně heterofermentativních a obligátně heterofermentativních druhů.^{8,9} Účinek různých druhů rodu *Lactobacillus* byl zjištěn u plísní rodu *Penicillium*, *Aspergillus*, *Fusarium*.^{10,11} Z tohoto důvodu se také stále hledají nové kmeny laktobacilů, které by mohly být použity jako protektivní kultury v mlékárenském, masném i pekařském průmyslu.^{12,13,14}

Cílem této práce bylo otestovat účinek vybraných druhů laktobacilů na růst plísní *Penicillium expansum* a *Fusarium culmorum*, které jsou častými kontaminanty prostředí i potravin.

Použité metody

Použité mikroorganismy:

Lactobacillus acidophilus CCDM 151; *L. zymae* CCDM 361; *L. panis* CCDM 471; *L. sanfranciscensis* CCDM 827 – všechny Laktoflora[®], Milcom, a.s.; *Lactobacillus plantarum* MP3 – Ústav mléka, tuků a kosmetiky, VŠCHT Praha

Fusarium culmorum DMF1; *Penicillium expansum* DMF0004 - Ústav mléka, tuků a kosmetiky, VŠCHT Praha

Kmeny laktobacilů byly kultivovány v MRS bujónu (pH 6) při 37 °C po dobu 16 – 18 h s 2 % obj. inokulem. Po kultivaci byl zjišťován počet buněk (KTJ/ml) plotnovou metodou za použití MRS agaru (Oxoid, Velká Británie; pH 6, 37 °C, 72 h, atmosféra 5 % obj. CO₂), pH (pH metr Cyberscan PC 5500, Eutech, Velká Británie) a obsah kyseliny mléčné a octové metodou HPLC.

Použité plísně byly uchovávány na agaru s maltosovým výtažkem po kultivaci 5–10 dnů při 22 °C.

Agarová difusní metoda pro stanovení antifungální aktivity¹⁵

Ke stanovení bylo použito 100 µl příslušného ředění suspenze laktobacilů ve fyziologickém roztoku, které bylo zalito 10 ml modifikovaného MRS agaru (bez octanu sodného). Po zatuhnutí byl agar převrstven 5 ml agaru s maltosovým výtažkem (konc. agaru 0,75 % hm.) a následně zaočkován 5 µl suspenze spor plísni (cca 10⁵ KTJ/ml). V průběhu 10 dnů byly měřeny průměry kolonií narostlých plísni.

Metoda mléčných agarových ploten pro stanovení antifungální aktivity bezbuněčných supernatantů¹⁶

Sterilní odstředěné mléko (5 ml) s 1 ml supernatantu bylo smícháno s 10 ml BCP agaru (15 g agar, 100 mg bromkresolové červeně v 1 l), po zatuhnutí převrstveno 5 ml agaru s maltosovým výtažkem (0,75 % hm. agaru), a zaočkováno suspenzí spor (cca 10⁵ KTJ/ml). Průměry kolonií plísni byly měřeny v průběhu 10 dnů. Supernatanty byly získány po kultivaci laktobacilů při 37 °C, 18 h a následném odstředění kultury při 9000 ot./min po dobu 10 min při 20 °C. Takto připravené supernatanty byly tepelně ošetřeny ve vodní lázni při 100 °C po dobu 5 min.

Sledování antifungální aktivity média s různým poměrem kyseliny mléčné a octové¹⁷

Byl použit agar s maltosovým výtažkem s různými přídávky koncentrované kyseliny octové – 0; 0,05 a 0,10 g/100 g agaru a agar s maltosovým výtažkem se stejnými přídávky kyseliny octové, jehož pH bylo na začátku upraveno 10 % obj. kyselinou mléčnou na pH 4,65. Po zatuhnutí agaru bylo do středu misky vpichem naočkováno 5 µl suspenze spor plísni. Kultivace probíhala při pokojové teplotě, kdy byly v intervalu 2, 7 a 10 dnů měřeny průměry kolonií plísni.

Uvedené výsledky u všech metod jsou aritmetické průměry z 2 – 3 paralelních stanovení.

Výsledky a diskuse

Základní charakteristika použitých kmenů laktobacilů je uvedena v Tab. 1. Je patrné, že menší růst a tím i tvorbu organických kyselin v MRS bujónu vykazovaly kmeny *L. panis* CCDM 471 a *L. sanfranciscensis* CCDM 827, což může být způsobeno specifickými nároky těchto druhů na živiny, které byly izolovány z kvasů. U kmenů 361 a MP3 byla prokázána tvorba kyseliny fenylmléčné.

Tabulka I

Charakteristika použitých kmenů laktobacilů – růst v MRS bujónu a tvorba organických kyselin

Kmen	počet buněk (KTJ/ml)	pH	glukosa (g/l)	k. mléčná (g/l)	k. octová (g/l)	k. fenylmléčná
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<i>L. acidophilus</i> CCDM 151	2,2 x 10 ⁹	3,82	6,7	15,1	2,9	ND*
<i>L. plantarum</i> MP3	2,5 x 10 ⁹	3,78	4,1	17,7	3,5	stopy
<i>L. zymae</i> CCDM 361	1,9 x 10 ⁹	3,75	1,9	18,2	3,6	stopy
<i>L. panis</i> CCDM 471	4,5 x 10 ⁷	5,04	16,2	2,6	3	ND*
<i>L. sanfranciscensis</i> CCDM 827	4,7 x 10 ⁷	4,94	14,3	3,7	3,1	ND*
		MRS	19,5	0,8	2,8	ND*

ND* nedetekováno

Pro sledování antifungální aktivity byla nejprve použita agarová difusní metoda. V první fázi práce bylo u testovaných kmenů laktobacilů použito do misek 100 µl suspenze buněk o koncentraci 10⁹ KTJ/ml. Tato koncentrace způsobovala naprostou inhibici růstu obou plísni, růst nenastal ani po 10 dnech kultivace. Proto byla pro následující měření použita ředění nakultivovaných laktobacilů, byla dávkována koncentrace buněk 10³ KTJ/ml. V tabulkách II a III jsou uvedeny průměrné velikosti kolonií plísni během 10denní kultivace při pokojové teplotě.

Tabulka II

Průměr kolonií (cm) plísně *F. culmorum* DMF1 při kultivaci s testovanými laktobacily agarovou difusní metodou

Kmen	151	MP3	361	471	827	Kontrola
Den						
4	2,3	1,4	1,4	2,6	3,0	3,3
6	2,9	1,4	1,5	4,9	4,9	5,5
10	4,7	1,8	2,0	7,7	7,6	CM*

CM* = plíseň porostla celou miskou (průměr misky činil 9,0 cm)

Tabulka III

Průměr kolonií (cm) plísně *P. expansum* DMF 0004 při kultivaci s testovanými laktobacily agarovou difusní metodou

Kmen	151	MP3	361	471	827	Kontrola
Den						
4	0,8	0,7	0,8	0,8	0,8	0,7
6	0,9	0,8	0,9	0,8	0,9	0,8
10	1,0	0,8	1,0	1,1	1,1	1,3

CM* = plíseň porostla celou miskou (průměr misky činil 9,0 cm)

Z tab. II a III je patrné, že nejvyšší inhibiční aktivitu vykazoval kmen *L. plantarum* MP3 a *L. zymae* CCDM 361. V porovnání s kontrolním stanovením pouze nepatrně inhibovaly růst plísni kmeny *L. panis* CCDM 471 a *L. sanfranciscensis* CCDM 827, což může být způsobeno jejich nízkou růstovou aktivitou a nízkou tvorbou kyselin za podmínek této metody. K účinkům laktobacilů byl daleko citlivější kmen *F. culmorum* DMF1. Při kontrolním růstu porostl tento kmen po 10 dnech kultivace celou miskou (9,0 cm), při inhibičním působení laktobacilů byl např. u kmene *L. plantarum* MP3 po této době průměr kolonie pouze 1,8 cm. Kmen *P. expansum* DMF0004 vykazoval pomalý růst na agaru s maltosovým výtažkem (kontrola po 10 dnech 1,3 cm). Bylo by vhodné pro tuto metodu otestovat i jiné druhy kultivačních médií pro plísně, jako je agar s bramborovým extraktem či Czapek-Dox agar, aby se zajistil větší nárůst této plísni, a tím i lepší porovnání antifungální aktivity laktobacilů. Mezi kmeny laktobacilů nebyly zjištěny výrazné rozdíly v inhibici *P. expansum*. Po 10 dnech kultivace vykazoval vyšší aktivitu opět kmen MP3. Podobné závěry byly publikovány i jinými autory. Dal Bello a kol.⁸ zjistili, že *L. plantarum* FST má silné inhibiční účinky vůči plísni *Fusarium culmorum* TMW, zatímco *L. sanfranciscensis* LTH 2581 nevykazoval žádnou inhibici růstu plísni. Antifungální aktivitu kmene *L. rhamnosus* VT1

ověřili také Stiles a kol.¹⁵ vůči plísním rodu *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp. a *Rhizopus* sp. Došli k závěru, že octan sodný jakožto jedna ze složek MRS agaru má antifungální účinek a v kombinaci s kmenem *L. rhamnosus* VT1 projevuje synergický efekt (v naší studii byl použit MRS agar bez octanu sodného). Nejvíce citlivý rod z testovaných plísní byl také *Fusarium* sp.¹⁵

Pro použití v potravinářství je důležité zjišťovat rovněž aktivitu samotných metabolitů laktobacilů, neboť v některých technologiích mohou být živé buňky inaktivovány tepelným záhřevem. Proto byla antifungální aktivita testována i u tepelně ošetřených bezbuněčných supernatantů metodou mléčných agarových ploten. Výsledky jsou uvedeny v tab. IV.

Tabulka IV

Průměr kolonií (cm) plísně *F. culmorum* DMF1 při stanovení antifungální aktivity bezbuněčných tepelně ošetřených supernatantů testovaných laktobacilů

Kmen	151	MP3	361	471	827	Kontrola
Den						
3	3,1	3,2	3,2	3,8	4,4	4,3
6	6,7	6,6	6,1	7,0	7,2	7,3
10	CM*	CM*	CM*	CM*	CM*	CM*

CM* = plíseň porostla celou miskou (průměr misky činil 9,0 cm)

Po 3 dnech kultivace nebyly mezi inhibičními účinky supernatantů kmenů 151, MP3 a 361 významné rozdíly, slabší inhibiční aktivitu vykazovaly opět kmeny 471 a 827. Antifungální aktivita byla potvrzena ještě po 6 dnech kultivace u kmenů 151, MP3 a 361, ale po 10 dnech kultivace byl inhibiční účinek všech supernatantů zcela potlačen a mycelium plísně porostlo celou miskou. V porovnání s aktivitou rostoucích buněk měly supernatanty slabší antifungální aktivitu. Antifungální aktivita supernatantů byla prokázána i jinými autory.^{6,18}

Bylo prokázáno, že za antifungální aktivitu laktobacilů jsou zodpovědné především organické kyseliny.¹ Vzhledem k tomu, že se produkce kyseliny mléčné a octové může u různých kmenů laktobacilů značně lišit, byl v dalším experimentu ověřen antifungální efekt obou kyselin samostatně i antifungální efekt jejich směsi. Jako kultivační médium byl použit agar s maltosovým výtažkem bez úpravy pH (pH 6,6) nebo s přidavkem 10 % obj. kyseliny mléčné (pH upraveno na 4,6) a to buď s nebo bez přidavku kyseliny octové (0,05 a 0,10 g/100 g agaru). Výsledky růstu plísně *F. culmorum* DMF1 jsou uvedeny v tab. V a na obr. 1.

Tabulka V

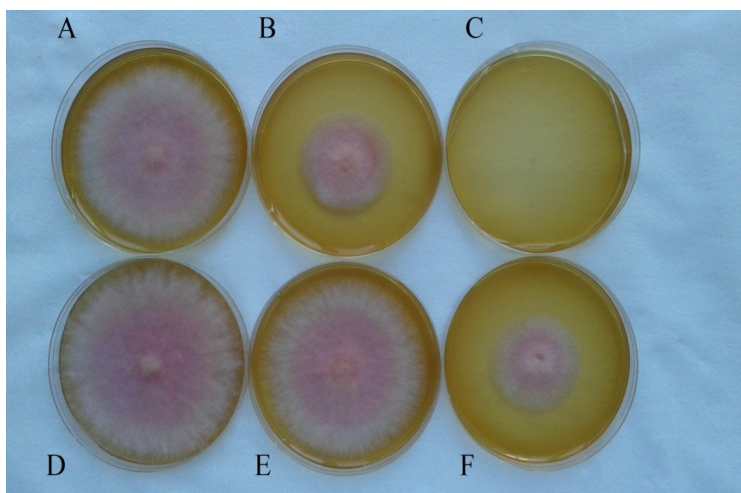
Průměr kolonií (cm) plísně *F. culmorum* DMF1 při stanovení antifungálního účinku kyseliny mléčné a octové

	Agar s přidavkem k. mléčné (pH upraveno na 4,6)			Agar bez přidavku k. mléčné (pH 6,6)		
	A	B	C	D	E	F
	0	0,05	0,1	0	0,05	0,1
k. octová (g/100 g agaru)						
konečné pH	4,63	4,45	4,17	6,65	5,81	4,62
Den						
2	0,8	0,5	0,5	1,1	0,8	0,5
7	7,4	4,3	0,5	CM*	7,4	4,2
10	CM*	7,9	0,5	CM*	CM*	7,9

CM* = plíseň porostla celou miskou (průměr misky činil 9,0 cm)

Z naměřených výsledků vyplývá, že obě kyseliny vykazovaly v prvních dnech růstu inhibiční účinky. Po 10 dnech kultivace však byla účinnost již značně snížena, kyselina octová přidaná do agaru v koncentraci 0,05 g/100g způsobila pouze malý rozdíl v růstu plísně vzhledem ke

kontrole. Přídavek samotné kyseliny mléčné (pH agarů upraveno na 4,6) byl již po 7 dnech málo účinný (kolonie se rozrostla na celou misku (9 cm) oproti kontrole (průměr kolonie 7,4 cm). Naopak při současném použití obou kyselin byl i po 10 dnech růst plísně potlačen, při okyselení agarů kyselinou mléčnou na pH 4,6 a následném přidavku kyseliny octové (0,1 g/100 g) agarů dosáhl průměr kolonie plísně pouze 0,5 cm. Synergický efekt obou kyselin potvrdil vhodnost použití heterofermentativních kmenů laktobacilů, které tyto kyseliny produkují, jako protektivních kultur.



Obr. 1. Růst plísně *F. culmorum* DMF1 na agaru s maltosovým výtažkem o různém pH upraveném kyselinou mléčnou a různé koncentraci přidané kyseliny octové po 7 dnech kultivace; legenda A – F – viz tab. V.

Závěrem je možno konstatovat, že testované druhy laktobacilů vykazovaly různý stupeň inhibiční aktivity vůči plísním *F. culmorum* DMF1 a *P. expansum* DMF0004. Nejvyšší antifungální aktivita byla zjištěna u kmene *L. plantarum* MP3. Bezbuňčné tepelně ošetřené supernatanty vykazovaly nižší antifungální aktivitu v porovnání s živými rostoucími buňkami. K působení laktobacilů byl ve všech případech citlivější druh *F. culmorum*. Byl prokázán synergický efekt kyseliny mléčné a octové na potlačení růstu plísně *F. culmorum* DMF1. Použití laktobacilových kultur s antifungální aktivitou, např. při přípravě kvasů pro pekárenský průmysl, může vést k potlačení plesnivění pekárenských výrobků.

Použitá literatura:

1. Gerez L. C., Torino M. I., Rollán G., Font de Valdez G.: Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control* 20, 144-148 (2009).
2. Venturini M. E., Blanco D., Oria R.: *In vitro* antifungal activity of several compounds against *Penicillium expansum*. *J. Food Prot.* 65, 934-939 (2002).
3. Dalié D. K. D., Deschamps A. M., Richard-Forget F.: Lactic acid bacteria – Potential for control of mould growth and mycotoxins. *Food Control* 21, 370-380 (2010).
4. Belguesmia Y., Rabesona H., Mounier J., Pawtowsky A., Le Blay G., Barbier G., Haertlé T., Chobert J. M.: Characterization of antifungal organic acids produced by *Lactobacillus harbinensis* K.V9.3.1Np immobilized in gellan-xanthan beads during batch fermentation. *Food Control* 36, 205-211 (2014).
5. Ryan L. A. M., Zannini E., Dal Bello F., Pawlowska A., Koehler P., Arendt E. K.: *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. *Int. J. Food Microbiol.* 146, 276-283 (2011).
6. Plocková M., Chumchalová J., Stiles J.: Hodnocení antifungální aktivity bakterií mléčného kvašení metodou mléčných agarových ploten. *Mlék. Listy* 62, 16-19 (2000).
7. Curry B., Crow V.: General Characteristics. V knize: *Encyclopedia of dairy science* (Roginski H., ed.), sv. III, str. 1479. Academic Press, Londýn 2003.

8. Dal Bello F., Clarke C. I., Ryan L. A. M., Ulmer H., Schober T. J., Ström K., Sjögren J., Van Sinderen D., Schnürer J., Arendt E. K.: Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1,7. *J. Cereal Sci.* 45, 309-318 (2007).
9. Corsetti A., Settanni L.: Lactobacilli in sourdough fermentation. *Food Res. Int.* 40, 539-558 (2007).
10. Sangmanee P., Hongpattarakere T.: Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K 35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus* and *Aspergillus parasiticus*. *Food Control* 40, 224-233 (2014).
11. Schillinger U., Villarreal J. V.: Inhibition of *Penicillium nordicum* in MRS medium by lactic acid bacteria isolated from foods. *Food Control* 21, 107-111 (2010).
12. Comi G., Tirloni E., Andyanto D., Manzano M., Iacumin L.: Use of bio-protective cultures to improve the shelf-life and the sensorial characteristics of commercial hamburgers. *Food Sci. Technol.* 62, 1198-1202 (2015).
13. Delavenne E., Ismail R., Pawtowski A., Mounier J., Barbier G., Le Blay G.: Assessment of lactobacilli strains as yogurt bioprotective cultures. *Food Control* 30, 206-213 (2013).
14. Coda R., Cassone A., Rizzello C. G., Nionelli L., Cardinali G., Gobbetti M.: Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during sourdough fermentation: identification of novel compounds and long term effect during storage of wheat bread. *Appl. Environ. Microbiol.* 77, 3484-3492 (2011).
15. Stiles J., Penkar S., Plocková M., Chumchalová J., Bullerman L. B.: Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. *J. Food Prot.* 65, 1188-1191 (2002).
16. Suzuki I., Nomura M., Morichi T.: Isolation of lactic acid bacteria which suppress mould growth and show antifungal action. *Milchwiss.* 46, 635-639 (1991).
17. Aunbjerg S. D., Honoré A. H., Marcussen J., Ebrahimi P., Vogensen F. K., Benfeldt C., Skov T., Knøchel S.: Contribution of volatiles to the antifungal effect of *Lactobacillus paracasei* in defined medium and yogurt. *Int. J. Food Microbiol.* 194, 46-53 (2015).
18. Ström K., Sjögren J., Broberg A., Schnürer J.: *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides and phenyllactic acid. *Appl. Environ. Microbiol.* 68, 4322-4327 (2002).

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Novel Potential Probiotic Lactobacilli for Prevention and Treatment of Vulvovaginal Infections

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Abstract

Lactobacilli in the vaginal tract are essential to protect against microbial infections. We therefore focused on isolating vaginal lactobacilli from pregnant women and testing their functional properties. Lactobacilli were isolated from 50 vaginal swabs and the purified isolates were identified by MALDI-TOF MS. Functional properties (antimicrobial activity, organic acids and hydrogen peroxide production, antibiotic susceptibility, auto-aggregation, and hydrophobicity) of selected isolates were tested. Lactobacilli (41 strains) were identified in 58% of swabs with a predominance of *Lactobacillus crispatus* (48%) followed by *L. jensenii* (21%), *L. rhamnosus* (14%), *L. fermentum* (10%), and *L. gasseri* (7%). The highest antibacterial activity was determined for *L. fermentum* and *L. rhamnosus*. Strong anti-*Candida* activity was observed for strains *L. crispatus*, *L. fermentum*, and *L. rhamnosus*. Strain *L. jensenii* 58C possessed the highest production of hydrogen peroxide (6.32 ± 0.60 mg/l). The best lactic acid producer was strain *L. rhamnosus* 72A (11.6 ± 0.2 g/l). All strains were resistant to fluconazole and metronidazole. The highest auto-aggregation was observed for strain *L. crispatus* 51A ($98.8 \pm 0.1\%$ after 24 h). Strain *L. rhamnosus* 68A showed the highest hydrophobicity ($69.1 \pm 1.4\%$). Strains *L. fermentum* and *L. rhamnosus* showed high antibacterial activity and hydrophobicity, and strains *L. crispatus* possessed high auto-aggregation and anti-*Candida* activity. Thus, these strains alone or in a mix could be used for the preparation of probiotic products for treatment and prevention of vulvovaginal infections of pregnant and non-pregnant women.

Keywords Lactobacilli · Probiotics · Vulvovaginal infections · Vaginal microbiome

Introduction

The vaginal microbiome of healthy women is a dynamic ecosystem, and it is colonized by a variety of microorganisms. The composition of the vaginal microbiome is influenced by a number of factors such as age, hormonal levels, sexual activity, hygiene, phase of menstrual cycle, or diet [1, 2]. In healthy premenopausal women, bacteria of the genus *Lactobacillus* are dominant in the vaginal microbiome at 10^7 – 10^8 CFU/g of vaginal fluid [3]. The most frequently found are species of *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, but this

depends on ethnic group or geographic location. There are also differences between pregnant and non-pregnant women; lower strain diversity was found in pregnant women. On the other hand, lactobacilli in pregnant women are more stable than in non-pregnant women [2, 4, 5].

The healthy urogenital tract plays a significant role in protecting against vaginal infections, and vaginal lactobacilli are important because of their protective functions (adhesion to the vaginal tissue and production of antimicrobial substances) [3]. An abnormal vaginal microbiome may lead to vaginal infections, and production of pro-inflammatory cytokines and prostaglandins, which can cause uterine contractions and weaken fetal membranes at pregnant women. Thus, the presence of abnormal microbiome in early pregnancy is recognized as a risk factor for preterm delivery and low birth weight. Preterm birth is defined as delivery before 37 completed weeks of gestation and it is responsible for neonatal morbidity and mortality almost in 80% [6–9]. Usually, preterm birth is caused by microbial attack in the amniotic cavity, especially by *Streptococcus agalactiae*. This pathogenic microorganism colonizes the lower vaginal tract and can also

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cause neonatal infections [4, 10, 11]. Preterm birth or amniotic fluid infection is also associated with bacterial vaginosis, which can be caused by a variety of microorganisms such as *Gardnerella vaginalis*, *Mycoplasma hominis*, or *Atopobium vaginae* [4, 12].

The protective role of vaginal lactobacilli is based on two main mechanisms. The first protective mechanism is specific adhesion of lactobacilli to the vaginal tissue, where formation of a biofilm can inhibit adhesion of pathogenic vaginal microorganisms by several mechanisms (e.g., competition for receptors, displacement of adhered pathogenic microorganisms, and avoiding their re-adhesion) [2, 13, 14]. The second protective mechanism is the production of antimicrobial substances (e.g., organic acids, hydrogen peroxide, bacteriocins) [15]. Organic acids, mainly lactic acid, are produced as the final product of carbohydrate fermentation and acidify the vaginal tract to pH 3.5–4.5. However, the pH is influenced by many factors (age, vaginal infection, and phase of menstrual cycle) [2, 9, 16, 17]. Some strains of vaginal lactobacilli, including *L. acidophilus*, *L. crispatus*, *L. fermentum*, and *L. jensenii*, are able to produce hydrogen peroxide. These lactobacilli are important because the hydrogen peroxide can reduce the development of bacterial vaginosis, as well as infections caused by HIV-1 and herpes simplex viruses [2, 18]. Other antimicrobial substances produced by vaginal lactobacilli are bacteriocins (proteins with antimicrobial activity), reuterin, and biosurfactants [2, 16, 18].

Lactobacilli, therefore, are able to inhibit the growth of pathogenic microorganisms through various mechanisms and may help in the reduction of vaginal infections. Knowledge of the biology and metabolic activities of vaginal lactobacilli is therefore important for the prevention and treatment of vaginal infections.

The aim of this study was to isolate vaginal lactobacilli from healthy pregnant women in the 36th week of pregnancy. We focused on functional properties testing, especially on antimicrobial activity testing, to find out strains suitable for the treatment of pregnant women's vaginal infections which induce preterm birth or neonatal infections.

Materials and Methods

Samples

Fifty swab samples were obtained from healthy pregnant women (36th week of pregnancy) from the Czech Republic. Samples were collected at the Department of Obstetrics and Gynecology of the First Faculty of Medicine, General University Hospital, Charles University, Prague, Czech Republic, and stored at 6 °C for 48–72 h. Sample collecting was approved by the Ethics Committee of the General University Hospital.

Isolation and Identification

Swabs were vortexed for 1 min at maximum speed (2800 min⁻¹) in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany). The suspension was diluted 3 times with physiological saline. Each dilution (100 µl) was seeded on MRS agar (Merck, Germany) and blood agar (Columbia blood agar base with 5% defibrinated sheep blood) (Oxoid, UK) plates. The plates were incubated at 37 °C for 48 h, under a modified atmosphere containing 5% CO₂. After incubation, the colonies were identified by colony morphology, catalase test, and Gram staining. Selected colonies were identified by MALDI-TOF MS. Identified isolates were stored at –21 °C in MRS broth supplemented with glycerol (Penta, Czech Republic) (50% v/v).

Indicator Microorganisms

As indicator microorganisms for antimicrobial activity testing, we used the following pathogenic microorganisms. Five strains (*Escherichia coli* CCM 4517, *Gardnerella vaginalis* CCM 6221, *Staphylococcus aureus* CCM 4516, *Staphylococcus aureus* CCM 7719, *Candida albicans* CCM 8215) were obtained from the Czech collection of microorganisms (CCM, Czech Republic). *Gardnerella vaginalis* DSMZ 104275 was obtained from the German collection of microorganisms and cell cultures (DSMZ, Germany). Two strains (*Candida glabrata* ATCC 2001, *Candida parapsilosis* ATCC 22019) were obtained from the American type culture collection (ATCC, USA). Three strains (*Escherichia coli* MK 57B, *Streptococcus agalactiae* MK 14E, *Streptococcus agalactiae* MK 31B) were obtained from the Collection of microorganisms of the Department of Dairy, Fat and Cosmetics, both isolates originally from the vaginal tract.

Cultivation

Lactobacilli were cultivated in MRS broth at 37 °C, for 18 h, under a modified atmosphere containing 5% CO₂ (overnight grown culture). Pathogenic bacteria were cultivated aerobically in Brain heart infusion (BHI) broth (Himedia, India) at 37 °C, for 24 h. Pathogenic yeasts were cultivated aerobically in Malt extract broth (Oxoid, UK) at 30 °C, for 24 h.

Antibacterial Activity

Antibacterial activity of selected *Lactobacillus* spp. isolates was tested by agar spot-diffusion method against eight pathogenic microorganisms (*E. coli* CCM 4517, *E. coli* MK 57B, *G. vaginalis* DSMZ 104275, *G. vaginalis* CCM 6221, *S. aureus* CCM 4516, *S. aureus* CCM 7719, *St. agalactiae* MK 14E, *St. agalactiae* MK 31B). The BHI soft agar (pH 5.6 before sterilization) was inoculated with the indicator

pathogenic microorganisms at a density of 10^5 CFU/ml. The inoculated BHI soft agar was poured into a Petri dish and left to solidify. Live cells of each *Lactobacillus* strain (10 µl) were injected into the inoculated BHI soft agar. These plates were incubated aerobically at 37 °C for 24 h. Antibacterial activity was evaluated by the diameter of the zones of inhibition ($\leq$ 5 mm, low inhibition; 5–10 mm, medium inhibition, $\geq$ 10 mm, high inhibition).

Anti-*Candida* Activity

Anti-*Candida* activity of lactobacilli isolates was tested against three pathogenic yeasts (*C. albicans* CCM 8215, *C. glabrata* ATCC 2001, and *C. parapsilosis* ATCC 22019). Bacterial cells of an overnight grown culture were harvested by centrifugation at 8600g, for 5 min, at 4 °C. Cells were washed twice in physiological saline under the same conditions. Washed cells were adjusted to McFarland 2 in physiological saline. This suspension (1 ml) was inoculated onto a Petri dish and overlaid with MRS agar. Plates were incubated at 37 °C, for 24 h, under a modified atmosphere containing 5% CO₂. After 24 h, Malt extract agar was poured onto the inoculated plates. Yeast cells from an overnight grown culture were harvested by centrifugation at 8600g, for 5 min, at 4 °C. Cells were washed twice in physiological saline and then adjusted to McFarland 0.5 with the same solution. This suspension (100 µl) was inoculated onto the Malt extract agar layer. Plates were incubated at 37 °C, for 24 h, under a modified atmosphere containing 5% CO₂. These plates were subsequently incubated aerobically at 25 °C, for 24 h. The anti-*Candida* activity was assessed by yeast growth and inhibition activity was evaluated as follows: +++, high anti-*Candida* activity (total inhibition of *Candida* spp.); ++, medium anti-*Candida* activity; +, low anti-*Candida* activity; 0, no anti-*Candida* activity (high growth of *Candida* spp.).

Production of Organic Acids

Production of organic acids was detected by HPLC (Agilent 1260 Infinity, USA). Bacterial cells from an overnight grown culture were centrifuged at 17,000g, for 10 min, at 4 °C. The supernatant (250 µl) was transferred to an Eppendorf tube and 1600 µl of ethanol (96%) were added and mixed. This mixture was incubated for 30 min at room temperature and then centrifuged at 17,000g, for 10 min, at 4 °C. After centrifugation, the mixture was filtered through a 0.22 µm PVDF filter. HPLC was performed using a Polymer IEX H column (Watrex, Czech Republic) (350 × 8 mm) with a pre-column (50 × 8 mm). The column and pre-column were preheated to 60 °C. The mobile phase was H₂SO₄ (9 mmol/l) with a flow of 0.6 ml/min. Detection was by UV detector (210 nm; 55 °C; 500 mV). The concentration of lactic acid was evaluated using a calibration curve.

Hydrogen Peroxide Production—Plate Method

Determination of hydrogen peroxide produced by lactobacilli was tested on MRS agar supplemented with 3,3',5,5'-tetramethylbenzidine (250 µl/ml) (Sigma-Aldrich, USA) and horseradish peroxidase (0.01 mg/ml) (Sigma-Aldrich, USA). Plates were inoculated with selected *Lactobacillus* spp. and incubated anaerobically at 37 °C, for 48 h, using an anaerobic jar containing AnaeroGen™ (Oxoid, UK). After incubation, plates were exposed to aerobic conditions for 30 min. Colonies of hydrogen peroxide producing strains form a blue pigment.

Detection of Hydrogen Peroxide Concentration—Spectrophotometric Method

Bacterial cells from an overnight grown culture were centrifuged at 8600 g, for 5 min, at 4 °C. Supernatant was removed and the cells were washed twice by phosphate buffer (1 mol/l; pH 6.8). Cooled phosphate buffer (20 ml) was added to washed cells and mixed. This suspension was cultivated at 5 °C, for 48 h. After cultivation, the suspension was centrifuged at 8600g, for 5 min, at 4 °C. Obtained supernatant was used for the quantification of H₂O₂. The supernatant (5 ml) was mixed with water solution of horse radish peroxidase (1 ml; 0.001% w/v) and methanolic solution of o-dianisidine (0.1 ml; 1% w/v) (Sigma-Aldrich, USA), and this mixture was cultivated at 37 °C, for 10 min. Then HCl solution (0.2 ml; 4 mol/l) was added and the absorbance of samples was measured at 400 nm. Concentration of H₂O₂ produced by lactobacilli was evaluated according to the calibration curve of H₂O₂, which was measured by the same procedure with H₂O₂ solutions in phosphate buffer instead of supernatant.

Hemolytic Activity

Lactobacilli were streaked onto blood agar with 5% of defibrinated sheep blood. Inoculated plates were incubated at 37 °C, for 48 h, under a modified atmosphere with 5% of CO₂. The result was evaluated visually according to zones of hemolysis. The *Staphylococcus aureus* CCM 3953 was used as a positive control, which showed β-hemolysis.

Antibiotic Resistance

Antibiotic resistance was tested by disk diffusion method with 15 antibiotics (Oxoid, UK): ampicillin (AMP; 10 µg), cefotaxime (CTX; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), clindamycin (DA; 2 µg), erythromycin (E; 15 µg), fluconazole (FCA; 25 µg), gentamicin (CN; 10 µg), kanamycin (K; 30 µg), metronidazole (MTZ;

5 µg), nitrofurantoin (F; 300 µg), ofloxacin (OFX; 5 µg), streptomycin (S; 10 µg), tetracycline (TE; 30 µg), and vancomycin (VA; 30 µg). Bacterial cells of an overnight grown culture were centrifuged at 8600g, for 5 min, at 4 °C. Cells were washed in physiological saline under the same conditions. Washed cells were adjusted to McFarland 1 in physiological saline. This suspension (100 µl) was inoculated onto Mueller-Hinton agar (Oxoid, UK) plates and MRS agar plates. Antibiotic discs were placed on the surface of the agar and the plates were incubated at 37 °C, for 48 h, under a modified atmosphere containing 5% of CO₂. Diameters of inhibition zones were measured after incubation.

Auto-Aggregation

Bacterial cells from an overnight grown culture were harvested by centrifugation at 8600g, for 5 min, at 4 °C. Cells were washed twice in phosphate buffered saline (PBS) (pH 7.2 before sterilization) under the same conditions. Cells were adjusted to an absorbance (650 nm) of 0.45–0.55. Samples were incubated aerobically at 37 °C, for 24 h. At times 0, 6, and 24 h, 1 ml of the upper layer was transferred to a cuvette and the A₆₅₀ was measured. The percentage of auto-aggregation was calculated using the following equation. As a blank, pure PBS was used.

$$\text{Auto-aggregation (\%)} = \left[1 - \left(\frac{A_0}{A_x} \right) \right] 100$$

A₀...A₆₅₀ at 0 h

A_x...A₆₅₀ at times 6 or 24 h

Hydrophobicity

Bacterial cells from an overnight grown culture were harvested by centrifugation at 8600g, for 5 min, at 4 °C. Cells were washed twice in PBS (pH 7.2 before sterilization) under the same conditions. The suspension was adjusted to an A₆₅₀ of 0.45–0.55 and 3 ml were added to a glass tube. Hexane (0.5 ml) was added, vortexed for 1 min and incubated for 20 min at room temperature. The water layer was then removed and the A₆₅₀ was measured. The percentage of hydrophobicity was calculated using the following equation. As a blank, pure PBS was used.

$$\text{Hydrophobicity (\%)} = \left[\frac{(A_{\text{before}} - A_{\text{after}})}{A_{\text{before}}} \right] 100$$

A_{before}...A₆₅₀ adjusted before extraction

A_{after}...A₆₅₀ after extraction

Results

Identification of Vaginal Lactobacilli

Bacteria from the genus *Lactobacillus* occurred in 29 (58%) out of 50 samples of vaginal swabs of pregnant women and 41 strains of lactobacilli were isolated. The most frequent species was *L. crispatus* (48%). The next most frequent were *L. jensenii* (21%), *L. rhamnosus* (14%), *L. fermentum* (10%), and *L. gasseri* (7%). Pre-screening of all isolates was focused especially on growth ability and afterwards 21 isolates were selected for further testing of functional properties.

Antibacterial Activity

Antibacterial activity of lactobacilli was tested by the agar spot-diffusion method against five indicator pathogenic microorganisms. The antibacterial activity was measured with live lactobacilli. Results are summarized in Table 1. The major proportion of isolates was inhibitory against all pathogenic bacteria tested. All isolates inhibited growth of *G. vaginalis* DSMZ 104275 and *Str. agalactiae* MK 14E. Strains *L. rhamnosus* 10A, *L. fermentum* 74A, and *L. fermentum* 74B were strongly inhibitory against all pathogenic bacteria tested.

Anti-Candida Activity

Lactobacillus spp. isolates were tested against three pathogenic yeasts and results are presented in Table 2. The highest anti-*Candida* activity was observed against *C. albicans* CCM 8215 by all lactobacilli tested. Isolates of *L. crispatus*, *L. fermentum*, and *L. rhamnosus* showed very good inhibitory activity against *Candida* spp. whereas most strains of *L. gasseri* and *L. jensenii* showed poor or no anti-*Candida* activity.

Production of Hydrogen Peroxide and Organic Acids

Lactobacilli are able to produce antimicrobially active substances. The majority are organic acids (mainly lactic acid) and hydrogen peroxide. All isolates were tested for hydrogen peroxide production and this was determined by pigment changes in colonies. It was found that 71% (Table 3) of isolates were able to produce hydrogen peroxide. Production was divided into two groups, low production of H₂O₂ (light blue pigmented colonies) and high production of H₂O₂ (dark blue pigmented colonies). Color changes of colonies were detected for all strains of *L. crispatus*, *L. gasseri*, and *L. jensenii*. On the other hand, detection of hydrogen peroxide by spectrophotometry method showed all strains are able to produce hydrogen peroxide in different concentrations. The highest concentration of H₂O₂ was detected for *L. jensenii* 58C (6.32 ±

Table 1 Antibacterial activity of *Lactobacillus* spp. (live cells) against pathogenic bacteria

<i>Lactobacillus</i> spp.		Vaginal strains			Commercial culture strains				
		<i>St. agalactiae</i> MK ^a 14E	<i>St. agalactiae</i> MK ^a 31B	<i>E. coli</i> MK ^a 57B	<i>E. coli</i> CCM ^b 4517	<i>G. vaginalis</i> DSMZ ^c 104,275	<i>G. vaginalis</i> CCM ^b 6221	<i>S. aureus</i> CCM ^b 4516	<i>S. aureus</i> CCM ^b 7719
<i>L. crispatus</i>	2A	+++	+++	++	++	+++	+++	++	++
	46B	+++	+++	++	++	+++	+++	++	++
	49C	+++	++	++	++	++	++	++	++
	51A	+++	+++	++	++	+++	+++	++	++
	55B	++	++	++	+	+++	+++	+	++
	69E	++	++	++	++	+++	+++	++	++
<i>L. gasseri</i>	8D	+++	+++	++	++	+++	+++	+	++
	71A	++	+	+	+	++	++	0	+
	71B	++	+	+	++	++	+	0	0
	71C	++	++	0	0	++	++	0	+
	71D	+++	++	+	++	+++	++	++	0
<i>L. jensenii</i>	49E	+++	++	++	+	++	++	0	0
	51E	++	++	++	++	++	++	++	+
	58C	++	++	+	+	++	++	++	++
	62A	+++	++	++	+	+++	+++	+	+
<i>L. rhamnosus</i>	10A	+++	+++	+++	+++	+++	+++	+++	+++
	68A	++	++	++	+++	+++	+++	++	++
	72A	+++	+++	+++	+++	+++	+++	++	++
<i>L. fermentum</i>	43B	+++	+++	+++	++	+++	+++	+++	+++
	74A	+++	+++	+++	+++	+++	+++	+++	+++
	74B	+++	+++	+++	+++	+++	+++	+++	+++

0, without inhibitory activity; +, low inhibitory activity (diameter of zone ≤ 5 mm); ++, medium inhibitory activity (diameter of zone 5–10 mm); +++, high inhibitory activity (diameter of zone ≥ 10 mm)

^a Strains isolated from swabs of non-pregnant women with vaginal infection (MK)

^b Strains obtained from the Czech collection of microorganisms (CCM)

^c Strain obtained from the German collection of microorganisms and cell cultures (DSMZ)

0.60 mg/l). The lowest concentration of H₂O₂ was detected for strain *L. rhamnosus* 72A (0.33 ± 0.05 mg/l).

Production of organic acids was tested by HPLC and the concentration of lactic acid was calculated (Table 3). The concentration of lactic acid ranged from 4.6 ± 0.1 g/l to 11.6 ± 0.2 g/l and was strain specific. The best lactic acid producer was strain *L. rhamnosus* 72A (11.6 ± 0.2 g/l), followed by strains *L. crispatus* 2A and *L. crispatus* 69E (10.4 ± 0.0 g/l for both of them). The lowest concentration of lactic acid was detected for strain *L. crispatus* 51A (4.6 ± 0.1 g/l).

Hemolytic Activity

No strain showed the β -hemolysis (complete lysis of erythrocytes) on blood agar with defibrinated sheep blood. Strains *L. gasseri* (71 B, 71C) showed α -hemolysis (partial decomposition of hemoglobin). No hemolysis was detected for other tested lactobacilli.

Antibiotic Resistance

Antibiotic resistance was tested for all 21 *Lactobacillus* spp. isolates on Mueller-Hinton agar and MRS agar plates. Differences between each tested media were detected in 28% cases without significant variance. Table 4 shows the sensitivity of each strain to antibiotics on Mueller-Hinton agar. All isolates were resistant to fluconazole and metronidazole and sensitive to ampicillin, erythromycin, chloramphenicol, and tetracycline. When antibiotic susceptibility was tested on MRS agar, lactobacilli possessed higher resistance to gentamicin, kanamycin, and streptomycin. Sensitivity to other antibiotics tested was strain specific.

Auto-Aggregation and Hydrophobicity

The results of auto-aggregation and hydrophobicity testing are summarized in Table 3. These results represent strain diversity between isolates. Our isolates showed auto-aggregation

Table 2 Anti-*Candida* activity of *Lactobacillus* spp. isolates

<i>Lactobacillus</i> spp.		<i>C. albicans</i> CCM ^a 8215	<i>C. glabrata</i> ATCC ^b 2001	<i>C. parapsilosis</i> ATCC ^b 22,019
<i>L. crispatus</i>	2A	++	++	+
	46B	+++	++	++
	49C	++	+	+
	51A	++	+	+
	55B	+++	+++	++
	69E	++	++	++
<i>L. gasseri</i>	8D	++	+	+
	71A	+	+	0
	71B	+	0	0
	71C	0	0	0
	71D	0	0	0
<i>L. jensenii</i>	49E	0	0	0
	51E	0	0	0
	58C	+	+	+
	62A	+	+	0
<i>L. rhamnosus</i>	10A	+++	++	++
	68A	+++	++	+
	72A	+++	++	++
<i>L. fermentum</i>	43B	+++	++	+
	74A	+++	+++	++
	74B	+++	+++	++

+++; high anti-*Candida* activity (total inhibition of *Candida* spp.); ++, medium anti-*Candida* activity; +, low anti-*Candida* activity; 0, no anti-*Candida* activity (high growth of *Candida* spp.)

^a Strain obtained from the Czech collection of microorganisms (CCM)

^b Strains obtained from American type culture collection (ATCC)

between 12.1–89.9% after 6 h and 32.5–98.8% after 24 h. A significant proportion of *L. crispatus* strains tested showed very strong auto-aggregation, more than 90% after 24 h. The highest auto-aggregation was determined for strain *L. crispatus* 51A ($98.8 \pm 0.1\%$ after 24 h) and the lowest was observed for strain *L. rhamnosus* 10A ($32.5 \pm 3.8\%$ after 24 h).

Hydrophobicity of our isolates was in the range 19.1–69.1%. The highest hydrophobicity was exhibited by strain *L. rhamnosus* 68A, which achieved $69.1 \pm 1.4\%$ and the lowest was shown by strain *L. jensenii* 51E ($19.4 \pm 2.4\%$). In this study, no correlation between auto-aggregation and hydrophobicity was observed.

Discussion

Lactobacilli were isolated from vaginal swabs obtained from healthy pregnant women in the third trimester. All swabs were collected in the same time in the 36th week of pregnancy to obtain relevant results. During this period, estrogen levels are high, as well as lactobacilli abundance. Romero et al. [19] found out that bacterial microbiota is more stable during

normal pregnancy than in the non-pregnant state. Also, they identified four lactobacilli (*L. crispatus*, *L. gasseri*, *L. jensenii*, *L. vaginalis*) as species with higher relative abundance in pregnant women. In our study, the most frequent species were *L. crispatus*, followed by *L. jensenii*, *L. rhamnosus*, *L. fermentum*, and *L. gasseri*. These results are typical for European woman and they correspond with several studies. For example, Kiss et al. [20] observed lactobacilli in the vaginal microbiome of pregnant women of Caucasian origin, with *L. crispatus* and *L. gasseri* being the most frequently found species, followed by *L. jensenii* and *L. rhamnosus*. Petricevic et al. [6] identified five leading *Lactobacillus* spp. in the vaginal microbiome of pregnant women: *L. crispatus*, *L. iners*, *L. gasseri*, *L. johnsonii*, and *L. jensenii*. In our study *L. iners* was not identified. *Lactobacillus iners* grows on blood agar and is uncultivable on MRS agar, in contrast with other lactobacilli that grow readily on MRS agar. In our study, lactobacilli were isolated only by cultivation, in contrast with other studies where genetic methods, independent of cultivation, were used for identification purposes. This may explain why *L. iners* was not detected in our study.

Antimicrobial activity of isolates is considered as a significant criterion for probiotic strain selection. The majority of

Table 3 Functional properties of vaginal lactobacilli

<i>Lactobacillus</i> spp.		H ₂ O ₂		Lactic acid [g/l]	Auto-aggregation [%]		Hydrophobicity [%]
		Colony pigment	[mg/l]		6 h	24 h	
<i>L. crispatus</i>	2A	++	1.32 ± 0.06	10.4 ± 0.0	56.3 ± 0.2	72.2 ± 4.5	25.7 ± 0.9
	46B	+	1.86 ± 0.33	9.1 ± 0.2	35.9 ± 1.3	96.5 ± 2.4	39.5 ± 0.6
	49C	+	0.97 ± 0.01	6.7 ± 0.0	89.9 ± 4.9	96.1 ± 1.6	24.8 ± 0.2
	51A	+	2.27 ± 0.01	4.6 ± 0.1	73.2 ± 3.6	98.8 ± 0.1	62.8 ± 1.7
	55B	+	0.71 ± 0.08	7.6 ± 0.0	29.0 ± 2.7	87.3 ± 2.5	19.1 ± 0.6
	69E	++	1.69 ± 0.05	10.4 ± 0.0	82.6 ± 2.1	93.2 ± 8.7	49.2 ± 0.6
<i>L. gasseri</i>	8D	++	0.29 ± 0.09	10.2 ± 0.2	61.2 ± 1.0	80.2 ± 3.4	62.1 ± 0.4
	71A	++	1.88 ± 0.23	6.5 ± 0.0	30.3 ± 3.2	70.4 ± 1.9	40.9 ± 0.9
	71B	++	4.22 ± 0.2	7.9 ± 0.0	36.3 ± 3.2	40.6 ± 1.4	24.6 ± 0.2
	71C	++	1.95 ± 0.12	6.5 ± 0.1	37.9 ± 0.6	53.6 ± 2.6	35.1 ± 1.8
	71D	++	4.17 ± 0.10	6.9 ± 0.1	41.2 ± 2.7	52.7 ± 1.8	29.1 ± 0.5
<i>L. jensenii</i>	49E	+	5.05 ± 0.17	6.5 ± 0.1	26.1 ± 0.1	45.5 ± 3.6	25.4 ± 0.2
	51E	+	1.58 ± 0.37	7.7 ± 0.2	27.4 ± 1.9	58.9 ± 1.9	19.4 ± 2.4
	58C	+	6.32 ± 0.60	8.3 ± 0.2	41.2 ± 3.9	73.3 ± 1.9	42.0 ± 0.1
	62A	++	0.67 ± 0.00	5.9 ± 0.1	39.7 ± 1.0	64.2 ± 0.8	64.8 ± 4.2
<i>L. rhamnosus</i>	10A	–	0.90 ± 0.08	8.5 ± 0.3	16.1 ± 2.2	32.5 ± 3.8	50.4 ± 0.8
	68A	–	0.53 ± 0.03	10.3 ± 0.1	36.8 ± 0.1	51.0 ± 0.2	69.1 ± 1.4
	72A	–	0.33 ± 0.05	11.6 ± 0.2	36.4 ± 0.5	53.5 ± 0.1	23.8 ± 0.2
<i>L. fermentum</i>	43B	–	1.13 ± 0.07	7.2 ± 0.1	12.1 ± 2.1	42.3 ± 0.6	53.4 ± 0.9
	74A	–	0.55 ± 0.06	6.9 ± 0.0	23.5 ± 1.2	47.0 ± 0.1	36.3 ± 1.0
	74B	–	0.74 ± 0.07	6.5 ± 0.1	28.8 ± 1.4	51.9 ± 1.9	50.9 ± 0.5

–, without production of H₂O₂; +, production of H₂O₂ (light blue pigment); ++, high production of H₂O₂ (dark blue pigment)

isolates showed medium or high inhibitory activity against the pathogenic bacteria tested. All strains inhibited growth of *Str. agalactiae* MK 14E. *Streptococcus agalactiae* can colonize newborns during delivery and can cause fetal sepsis [6]. Our vaginal isolates therefore have the potential to decrease the number of *Str. agalactiae* in pregnant women and to decrease the risk of fetal sepsis. All isolates were able to inhibit *G. vaginalis* DSMZ 104275, the bacteria associated with bacterial vaginosis, and almost all isolates also showed inhibitory activity against bacteria that are usually associated with aerobic vaginitis, such as *E. coli* and *S. aureus*. Aerobic vaginitis and bacterial vaginosis appear to be linked with spontaneous miscarriage, premature rupture of membranes, and increased risk of preterm birth [21, 22].

Some strains of vaginal lactobacilli inhibit the growth of *Candida* spp. [23]. Our results show that the highest inhibitory activity occurred with strains of *L. crispatus*, *L. fermentum*, and *L. rhamnosus*. These results are in accordance with the study of Strus et al. [23], where strains of *L. fermentum* and *L. rhamnosus* showed partial or total inhibition of *Candida* growth. Hütt et al. [24] tested antagonistic activity of *L. crispatus*, *L. gasseri*, and *L. jensenii* against *C. albicans* and *C. glabrata*. In that study, *L. crispatus* showed substantially higher antagonistic activity than *L. gasseri* and

L. jensenii. In general, inhibitory activity of lactobacilli decreased in the order *C. albicans*, *C. glabrata*, and *C. parapsilosis*.

Hydrogen peroxide is an antimicrobial substance produced by certain strains of vaginal lactobacilli. These lactobacilli play a significant role, especially in the vaginal tract of pregnant women. Hydrogen peroxide has a protective effect against catalase-negative microorganisms such as *G. vaginalis*, which cause bacterial vaginosis [4, 25]. Hydrogen peroxide producing strains are responsible for maintaining microbial balance and their absence is associated with the development of bacterial vaginosis [2, 18]. In this study, we found that 71% of isolates were able to produce hydrogen peroxide, especially strains of *L. crispatus*, *L. gasseri*, and *L. jensenii*. Rabe and Hillier [26] also observed production of hydrogen peroxide by the same vaginal lactobacilli. Bouridane et al. [25] tested 70 isolates and 57 of them (81.42%) were able to produce hydrogen peroxide.

Lactic acid is an antimicrobial compound produced by lactobacilli as the main product of carbohydrate fermentation. Lactic acid maintains vaginal pH at values 3.5–4.5 and inhibits growth of pathogenic microorganisms [17]. Production of lactic acid is strain specific. In a previous study, the best lactic acid producers were strains of *L. gasseri* [24]. In

Table 4 Antibiotic susceptibility of vaginal isolates on Mueller-Hinton agar

<i>Lactobacillus</i> spp.		AMP	CTX	CN	E	C	TE	OFX	S	DA	FCA	K	MTZ	CIP	VA	F
<i>L. crispatus</i>	2A	S	S	R/S	S	S	S	R	R/S	S	R	R/S	R	R	S	S
	46B	S	S	S	S	S	S	S	S	S	R	R/S	R	R	S	S
	49C	S	S	S	S	S	S	R	R/S	S	R	S	R	R/S	S	S
	51A	S	S	S	S	S	S	R	S	S	R	R	R	R	S	R
	55B	S	S	S	S	S	S	R	R/S	S	R	R/S	R	R	S	R/S
	69E	S	S	R/S	S	S	S	R	R	S	R	R	R	R	S	R
<i>L. gasseri</i>	8D	S	S	S	S	S	S	R	S	R	R	R/S	R	R	S	R
	71A	S	S	S	S	S	S	R	S	S	R	R/S	R	S	R	R
	71B	S	S	S	S	S	S	R	S	R/S	R	S	R	R	S	S
	71C	S	S	S	S	S	S	R	R/S	S	R	R/S	R	R	S	S
	71D	S	S	S	S	S	S	R	S	R	R	R/S	R	R	S	S
<i>L. jensenii</i>	49E	S	S	R/S	S	S	S	R	S	R/S	R	R/S	R	R	S	R
	51E	S	S	R	S	S	S	S	S	S	R	S	R	R	S	R
	58C	S	S	S	S	S	S	R	R/S	S	R	S	R	R	S	R
	62A	S	S	S	S	S	S	R	S	S	R	S	R	R	S	S
<i>L. rhamnosus</i>	10A	S	S	S	S	S	S	R/S	R/S	S	R	R/S	R	R/S	R	S
	68A	S	S	S	S	S	S	R	R/S	S	R	R/S	R	R/S	R	S
	72A	S	S	S	S	S	S	S	R/S	S	R	R/S	R	S	R	S
<i>L. fermentum</i>	43B	S	S	R	S	S	S	S	R/S	S	R	R	R	R	R	S
	74A	S	R	S	S	S	S	R	R/S	S	R	S	R	R	R	S
	74B	S	R	S	S	S	S	R	R/S	S	R	S	R	R	R	S

R, resistant (diameter of zone < 12 mm); R/S, intermediate (diameter of zone 12–16 mm); S, sensitive (diameter of zone > 16 mm)

our study, the highest concentration of lactic acid was detected for strain *L. rhamnosus* 72A, which was followed by strains of *L. crispatus*.

It is very important to know the antibiotic susceptibility of vaginal lactobacilli, because resistance to an antibiotic can be considered as advantageous. A resistant strain can be administered together with a vaginal drug and the strain can start colonizing the urogenital mucosa [25, 27]. In our study, it was determined that all strains were resistant to metronidazole and fluconazole. Usually, metronidazole is used to treat bacterial vaginosis [16, 28]. Thus, these strains could be co-administrated with these antibiotics during treatment of bacterial vaginosis.

Adherence to vaginal cells is one antimicrobial mechanism. A previous study showed that there was a correlation between adhesion to the vaginal tissue and auto-aggregation [29]. Auto-aggregation can correlate with hydrophobicity [27, 30], but in our study, this correlation was not proven. Generally, strain diversity was detected for each property tested.

For future testing, six strains with appropriate functional properties were selected. Strains *L. crispatus* 46B and *L. crispatus* 69E were capable of substantial production of hydrogen peroxide, strong auto-aggregation, and high or medium anti-*Candida* activity. High antibacterial activity against all indicator pathogenic microorganisms was proven for

strains *L. rhamnosus* 10A, *L. fermentum* 74A, and *L. fermentum* 74B. Relatively satisfactory properties were determined for strain *L. gasseri* 8D, which showed high hydrogen peroxide production, strong auto-aggregation, and high inhibitory activity against *G. vaginalis* and *Str. agalactiae*. Thus, these lactobacilli isolates could be appropriate candidates on the base of their stability and properties according to Romero et al. [19], where higher stability of lactobacilli from pregnant women was detected. On the other hand, Romero et al. [19] compared isolates from pregnant women with isolates from non-pregnant women. This comparison has not been done in our study, so it could be little limitation of our research. Also, weaknesses of this study may be that all tested properties were determined only in vitro and lactobacilli may show a little bit different property in the real system of vaginal tract. Therefore, it is necessary to subject selected strains for clinical trials.

In conclusion, lactobacilli are important for maintaining the normal environment of the vaginal tract. Lactobacilli isolated in our study have a promising potential for future use and six selected strains (*L. crispatus* 46B and 69E, *L. fermentum* 74A and 74B, *L. gasseri* 8D, *L. rhamnosus* 10A) could be potential probiotic candidates for clinical trials. They could be used for preparation of probiotic products for treatment and prevention of vulvovaginal infections, especially for pregnant women with abnormal microbiome to reduce risk of preterm birth or

neonatal infection causes by *Streptococcus agalactiae*. The advantage of lactobacilli isolated from the vaginal tract of pregnant women is the increased chance of their later adhesion and colonization of the tract since this represents a transfer back to their natural environment. They can also be potentially used in non-pregnant women as a replacement for antibiotic therapy of vulvovaginal infections.

Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Sample collecting was approved by the Ethics Committee of the General University Hospital, Prague.

Conflict of Interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

- Granato P (2006) Microbial flora of humans and microbial virulence factors. In: Wooley D, Byers K (eds) Biological safety: principles and practices. ASM Press, Washington, pp 3–17
- Borges S, Silva J, Teixeira P (2014) The role of lactobacilli and probiotics in maintaining vaginal health. Arch Gynecol Obstet 289: 479–489. <https://doi.org/10.1007/s00404-013-3064-9>
- Boris S, Barbés C (2000) Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes Infect 2:543–546. [https://doi.org/10.1016/S1286-4579\(00\)00313-0](https://doi.org/10.1016/S1286-4579(00)00313-0)
- DiGiulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, Sun CL, Goltsman DSA, Wong RJ, Shaw G, Stevenson DK, Holmes SP, Relman DA (2015) Temporal and spatial variation of the human microbiota during pregnancy. Proc Natl Acad Sci U S A 112:11060–11065. <https://doi.org/10.1073/pnas.1502875112>
- Walther-António MRS, Jeraldo P, Berg Miller ME, Yeoman CJ, Nelson KE, Wilson BA, White BA, Chia N, Creedon DJ (2014) Pregnancy's stronghold on the vaginal microbiome. PLoS One 9:1–10. <https://doi.org/10.1371/journal.pone.0098514>
- Petricevic L, Domig KJ, Nierscher FJ, Sandhofer MJ, Fidesser M, Krondorfer I, Husslein P, Kneifel W, Kiss H (2014) Characterisation of the vaginal *Lactobacillus* microbiota associated with preterm delivery. Sci Rep 4:1–6. <https://doi.org/10.1038/srep05136>
- Farr A, Kiss H, Hagmann M, Machal S, Holzer I, Kueronya V, Husslein PW, Petricevic L (2015) Role of *Lactobacillus* species in the intermediate vaginal flora in early pregnancy: a retrospective cohort study. PLoS One 10:1–12. <https://doi.org/10.1371/journal.pone.0144181>
- Park JS, Park CW, Lockwood CJ, Norwitz ER (2005) Role of cytokines in preterm labor and birth. Minerva Ginecol 57:349–366
- Nuriel-Ohayon M, Neuman H, Koren O (2016) Microbial changes during pregnancy, birth, and infancy. Front Microbiol 7:1–13. <https://doi.org/10.3389/fmicb.2016.01031>
- Boldenow E, Gendrin C, Ngo L, Bierle C, Vornhagen J, Coleman M, Merillat S, Armistead B, Whidbey C, Alishetti V, Santana-Ufret V, Ogle J, Gough M, Srinouanprachanh S, MacDonald JW, Bammler TK, Bansal A, Liggitt HD, Rajagopal L, Adams Waldorf KM (2016) Group B *Streptococcus* circumvents neutrophils and neutrophil extracellular traps during amniotic cavity invasion and preterm labor. Sci Immunol 1:1–13. <https://doi.org/10.1126/sciimmunol.aah4576>
- Borges S, Silva J, Teixeira P (2012) Survival and biofilm formation by group B streptococci in simulated vaginal fluid at different pHs. Antonie Leeuwenhoek 101:677–682. <https://doi.org/10.1007/s10482-011-9666-y>
- Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, Frances Coach M, Edelman R, Pastorek JG, Vijaya Rao A, McNellis D, Regan JA, Carey C, Klebanoff MA (1995) Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. N Engl J Med 333:1737–1742. <https://doi.org/10.1056/NEJM199512283332604>
- Borges S, Barbosa J, Teixeira P (2016) Gynecological health and probiotics. In: Watson RR, Preedy VR (eds) Probiotics, prebiotics, and synbiotics - bioactive foods in health promotion. Elsevier, London, pp 741–752
- Boris S, Suárez JE, Vázquez F, Barbés C (1998) Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect Immun 66:1985–1989
- Verdenelli MC, Coman MM, Cecchini C, Silvi S, Orpianesi C, Cresci A (2014) Evaluation of antipathogenic activity and adherence properties of human *Lactobacillus* strains for vaginal formulations. J Appl Microbiol 116:1297–1307. <https://doi.org/10.1111/jam.12459>
- Reid G (2001) Probiotic agents to protect the urogenital tract against infection. Am J Clin Nutr 78:437–443. <https://doi.org/10.1093/ajcn/73.2.437s>
- Lalan MS, Misra A (2011) Applications of polymers in vaginal drug delivery. In: Misra A, Shahiwal A (eds) Applications of polymers in drug delivery. Smithers Rapra, Shawbury, pp 351–377
- Amin M, Goodarzi H, Orang Z, Farsi S, Jorfi M (2011) Isolation and identification of *Lactobacillus* species from the vagina and their antimicrobial properties. Afr J Microbiol Res 5:3300–3304. <https://doi.org/10.5897/AJMR11.481>
- Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Nikita L, Galuppi M, Lamont RL, Chaemsaihong P, Miranda J, Chaiworapongsa T, Ravel J (2014) The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. Microbiome 4:1–19. <https://doi.org/10.1186/2049-2618-2-4>
- Kiss H, Kögler B, Petricevic L, Sauerzapf I, Klayraung S, Domig K, Viernstein H, Kneifel W (2007) Vaginal *Lactobacillus* microbiota of healthy women in the late first trimester of pregnancy. BJOG 114:1402–1407. <https://doi.org/10.1111/j.1471-0528.2007.01412.x>
- Rosenstein IJ, Morgan DJ, Sheehan M, Lamont RF, Taylor-Robinson D (1996) Bacterial vaginosis in pregnancy: distribution of bacterial species in different gram-stain categories of the vaginal flora. J Med Microbiol 45:120–126. <https://doi.org/10.1099/00222615-45-2-120>
- Donders GGG, Bellen G, Grinceviciene S, Ruban K, Vieira-Baptista P (2017) Aerobic vaginitis: no longer a stranger. Res Microbiol 168:845–858. <https://doi.org/10.1016/j.resmic.2017.04.004>
- Strus M, Kucharska A, Kukla G, Brzychczy-Wloch M, Maresz K, Heczko PB (2005) The *in vitro* activity of vaginal *Lactobacillus* with probiotic properties against *Candida*. Infect Dis Obstet Gynecol 13:69–75. <https://doi.org/10.1080/10647440400028136>
- Hütt P, Lapp E, Štšepetova J, Smidt I, Taelma H, Borovkova N, Oopkaup H, Ahelik A, Rööp T, Hoidmets D, Samuel K, Salumets A, Mändar R (2016) Characterisation of probiotic properties in

- human vaginal lactobacilli strains. *Microb Ecol Health Dis* 27:1–9. <https://doi.org/10.3402/mehd.v27.30484>
25. Bouridane H, Sifour M, Idoui T, Annick L, Thonard P (2016) Technological and probiotic traits of the lactobacilli isolated from vaginal tract of the healthy women for probiotic use. *Iran J Biotech* 14:192–201. <https://doi.org/10.15171/ijb.1432>
 26. Rabe LK, Hillier SL (2003) Optimization of media for detection of hydrogen peroxide production by *Lactobacillus* species. *J Clin Microbiol* 41:3260–3264. <https://doi.org/10.1128/JCM.41.7.3260-3264.2003>
 27. Kaewnopparat S, Dangmanee N, Kaewnopparat N, Srichana T, Chulasiri M, Settharaksa S (2013) *In vitro* probiotic properties of *Lactobacillus fermentum* SK5 isolated from vagina of a healthy woman. *Anaerobe* 22:6–13. <https://doi.org/10.1016/j.anaerobe.2013.04.009>
 28. Anukam KC, Reid G (2008) Effects of metronidazole on growth of *Gardnerella vaginalis* ATCC 14018, probiotic *Lactobacillus rhamnosus* GR-1 and vaginal isolate *Lactobacillus plantarum* KCA. *Microb Ecol Health Dis* 20:48–52. <https://doi.org/10.1080/08910600701837964>
 29. Malik S, Petrova MI, Claes IJJ, Verhoeven TLA, Busschaert P, Vanechoutte M, Lievens B, Lambrechts I, Siezen RJ, Balzarini J, Vanderleyden J, Lebeer S (2013) The highly autoaggregative and adhesive phenotype of the vaginal *Lactobacillus plantarum* strain CMPG5300 is sortase dependent. *J Appl Environ Microbiol* 79:4576–4585. <https://doi.org/10.1128/AEM.00926-13>
 30. Kos B, Šušćković J, Vuković S, Šimpraga M, Frece J, Matošić S (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J Appl Microbiol* 94:981–987. <https://doi.org/10.1046/j.1365-2672.2003.01915.x>

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The impact of L-lanthionine supplementation on the production of nisin by lactococci

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Abstract

The combination of cost-effective synthesis of L-lanthionine in conjunction with a fermentation process could be considered as an original approach. Innovative biosynthesis of nisin is easy to scale-up. After the selective organic synthesis of the thioether building block, cultivation medium M17 broth with glucose (0.5% w/w) was supplemented with L-lanthionine (0.78, 3.91 and 7.82 mg ml⁻¹) to stimulate nisin biosynthesis by lactococci. The viability of nisin-producing *Lactococcus lactis* subsp. *lactis* strains (CCDM 731, NIZO R5, LTM 32, CCDM 416) and nisin production were evaluated. Nisin synthesis was significantly influenced by L-lanthionine supplementation and it increased for all the strains tested in comparison with a control. The most significant increase in nisin concentration was observed for strains CCDM 731 and LTM 32, where up to 88.6% and 90.0%, respectively, were achieved. The influence of L-lanthionine concentration was shown to be strain specific.

Keywords *Lactococcus lactis* subsp. *lactis* · Nisin · L-Lanthionine synthesis · Sulfur extrusion · Lantibiotics · Bacteriocins

Introduction

Lantibiotics are unique class of antimicrobial peptides produced on ribosomes, containing post-translationally modified amino acids such as (2S,6R)-lanthionine (Lan), (2S,3S,6R)-methyllanthionine (MetLan), dehydrobutyrine and dehydroalanine. The presence of thioether bridges (Lan, MetLan) is a characteristic structural motif forming polycyclic lanthipeptides [1–3]. The lantibiotics are effective bacteriocins with broad antimicrobial activity against foodborne pathogens (*Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium* spp., *Bacillus* spp.) and food spoilage bacteria (*Micrococcus* spp., *Streptococcus* spp., *Pediococcus* spp., *Lactobacillus* spp.) and also prevents spore germination [1]. Therefore, lantibiotics such as nisin were commercialized either as food biopreservatives (e.g., Nisaplin®, Valisin®) or veterinary drugs (WipeOut®) for the treatment of bovine

mastitis [2, 4, 5]. Nisin is also categorized as a food additive (E234), is generally recognized as safe (GRAS), and is decomposed by digestive enzymes [5].

Although commercial nisin products have been routinely used for over 5 decades, microbial resistance has not been documented [2, 6, 7]. The primary reason is the unique mode of nisin action, targeting lipid II, which does not allow the evolution of bacterial resistance [7]. Sequestration of lipid II, an essential precursor of the bacterial cell wall, inhibits murein biosynthesis and bacterial cell growth [3, 6, 7]. Moreover, nisin-lipid II complexes induce pore formation, accompanied by the breakdown of membrane potential [3, 7]. The pentacyclic structure of nisin (ring A-ring B cage) is an essential criterion for targeted recognition of the lipid II pyrophosphate moiety [7]. Thus, post-translational modifications catalyzed by LanB dehydratase and stereoselective LanC cyclase are key steps in lanthipeptide biosynthesis [1, 2]. In recent research, it was shown that intramolecular Michael-type addition may be under thermodynamic control [8, 9]. Yang et al. [9] have provided clear evidence about the reversibility of conjugated addition (thioether ring opening).

Industrial production of food-grade nisin by strains of *Lactococcus lactis* subsp. *lactis* is a batch fermentation process. Nisin producers are cultivated in whey or milk media (pH 6.0, 30 °C) for 8–10 h after which nisin yield is maximized by purification steps such as foaming, precipitation

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(NaCl solution, acetone) and drying [5]. Food-grade nisin preparations were standardized to 10^6 IU g^{-1} [2, 5]. Large-scale nisin production is limited by several factors: low biomass formation, lactate accumulation in the medium, nisin feedback inhibition of *L. lactis* producers and proteolytic degradation of nisin after cell autolysis. Mixed culture fermentation and lactate removal (extraction, alkaline neutralization) are promising technological improvements [2, 4, 5]. Chemical synthesis of nisin is an alternative approach, completely independent of biosynthetic pathways. Nevertheless, stereochemical problems (Lan, MetLan) and low yields ($\approx 0.003\%$) made it unprofitable [2, 4].

In the present study, we tried to combine the fermentation process with cost-effective organic synthesis of L-lanthionine. To the best of our knowledge, the impact of simple L-lanthionine supplementation of the cultivation medium on nisin production by lactococci has not been reported.

Experimental

Reagents and instrumentation

Cystine dimethyl ester dihydrochloride, DL-lanthionine, trifluoroacetic acid, trifluoroacetic anhydride, tris(diethylamino) phosphine, dioxane and benzene were purchased from Sigma Aldrich Company. All other reagents and solvents were of analytical grade. ^1H NMR and ^{13}C NMR APT spectra of the L-lanthionine dissolved in deuteriochloroform ($\text{D}_2\text{O} + \text{DCI}$) were recorded on a Bruker Avance 600 and a Bruker Avance 500 (Bruker, Inc., Billerica, MA, USA). Working frequencies were 600.1 MHz and 499.8 MHz for ^1H , 150.9 MHz and 125.7 MHz for ^{13}C , respectively. APCI-MS spectra were measured with LC-MS LTQ-Orbitrap Velos (ThermoScientific, Waltham, MA, USA). Merck precoated silica gel F254 plates were used for thin-layer chromatography (TLC). Spots were detected by heating after spraying with 5% phosphomolybdic acid in EtOH. Melting points were measured on a Boetius hot-stage microscope and were not corrected.

Selective synthesis of L-lanthionine

Selective desulfurization of cystine dimethyl ester dihydrochloride was done according to Harpp and Gleason [10]. In brief, to a stirred solution of cystine dimethyl ester dihydrochloride (13.2 mmol, 4.5 g) and trifluoroacetic acid (15.0 ml) were added dropwise 10 ml of trifluoroacetic anhydride (71.9 mmol). After the mixture had been stirred for 1 h at -5°C and 1 h at 20°C , it was poured over 200 ml of ice- H_2O . Filtration yielded white crystals of *N,N'*-bis(trifluoroacetyl)-L-cystine dimethyl ester with a yield of 43.18% (5.7 mmol, 3.04 g), which was directly used for subsequent sulfur extrusion. To a stirred solution

of disulfide (5.7 mmol, 3.04 g), 33 ml of anhydrous benzene was added slowly 1.6 g of tris(diethylamino)phosphine (6.5 mmol). The reaction mixture was stirred for 10 min under N_2 . Afterward, it was taken up in hexane (50 ml) and the resulting suspension was filtered to afford white crystals in a molar yield of 37.88% (5.0 mmol, 2.5 g). Resulting *N,N'*-bis(trifluoroacetyl)-L-lanthionine dimethyl ester was dissolved in 30 ml of dioxane in an ice-water bath. At this point, 50 ml of 1.0 mol l^{-1} NaOH was added slowly. After 30 min, the pH was adjusted to 6.0 with 2.0 mol l^{-1} solution of HCl. The solvent was evaporated under reduced pressure to yield a lipophilic residue, which was poured into 30 ml of H_2O and crystalline L-lanthionine (28.8%, 3.8 mmol, 0.79 g) was collected by filtration.

Characterization of L-lanthionine was as follows: the product was obtained in 28.8% yield as a white solid. $\text{Mp} = 293\text{--}295^\circ\text{C}$; $R_f = 0.19\text{--}0.21$ (50 mM phosphate buffer (pH 5.8)/ethanol (96%), 7:3); ^1H NMR (500 MHz, $\text{D}_2\text{O} + \text{DCI}$) δ 4.40/4.27 (2H, dd, $J = 7.8$ and 4.2 Hz, 2 CH), 3.38 (2H, dd, $J = 15.2$ and 4.2 Hz, 2 CHHS), 3.22 (2H, dd, $J = 15.2$ and 7.4 Hz, 2 CHHS); ^{13}C NMR (126 MHz, $\text{D}_2\text{O} + \text{DCI}$) δ 170.4/170.2 (CO), 52.1/51.6 (C- α), 36.1/31.4 (CH $_2$); APCI-MS m/z 209.05904 $[\text{M} + \text{H}]^+$ (calculated for $\text{C}_6\text{H}_{13}\text{N}_2\text{O}_4\text{S}_1^+ = 209.05905$).

Cultivation of strains

Four *L. lactis* subsp. *lactis* strains (CCDM 416, CCDM 731, LTM 32 and NIZO R5) were routinely cultivated aerobically in M17 broth (Oxoid, Basingstoke, UK) with glucose (0.5% w/w) (GM17) at 30°C for 18 h and strain *Lactobacillus helveticus* CH1 was cultivated in modified atmosphere (5% v/v CO_2) in MRS broth with pH 5.2 (Merck, Darmstadt, DE) at 37°C for 18 h. Characteristics of strains used are summarized in Table 1.

Influence of L-lanthionine addition to cultivation medium on nisin production

Lactococcal strains were inoculated (1% v/v inoculum) into GM17 broth without L-lanthionine (control) or in GM17 broth with L-lanthionine at three different concentrations (0.78 mg ml^{-1} , 3.91 mg ml^{-1} and 7.82 mg ml^{-1}) and cultivated aerobically at 30°C for 15 h with agitation. After cultivation, the number of cells (plate count method, GM17 agar, 30°C , 48 h) and nisin production were determined.

A modified agar diffusion method was used to evaluate nisin production [11, 12]. After lactococci cultivation, 1 ml of each suspension was acidified with 0.02 mol l^{-1} HCl to reach $\text{pH } 2.5 \pm 0.1$, heated (98°C , 5 min) and centrifuged (18,000g, 5 min, 4°C). Supernatants were further used for nisin determination. A suspension of nisin-sensitive strain *L. helveticus* CH1 was mixed with MRS agar enriched with 1%

Table 1 Characteristics of used strains

Strain	Properties	Source
<i>Lc. lactis</i> subsp. <i>lactis</i> CCDM 416	Nisin production	CCDM
<i>Lc. lactis</i> subsp. <i>lactis</i> CCDM 731	Nisin production	CCDM
<i>Lc. lactis</i> subsp. <i>lactis</i> NIZO R5	Nisin production, contain transposone Tn5276	NIZO
<i>Lc. lactis</i> subsp. <i>lactis</i> LTM 32	Nisin production, origin Vietnamese fermented milk	UCT
<i>Lbc. helveticus</i> CH1	Nisin sensitive	Ch. Hansen

Lc. = *Lactococcus*; *Lbc.* = *Lactobacillus*; CCDM = Culture Collection of Dairy Microorganism Laktoflora®, Milcom, Prague, Czech Rep.; NIZO = Netherlands Institute for Dairy Research, Department of Biophysical Chemistry and Genetics, Ede, Netherlands; UCT = University of Chemistry and Technology, Prague, Czech Rep.; Ch. Hansen, Copenhagen, Denmark

(v/v) Tween 20 and poured onto plates; the final concentration of lactobacilli was approx. 5×10^6 CFU ml⁻¹ of agar. After solidification, wells were cut using a 9-mm diameter steel borer and 150 µl of treated lactococci supernatants were placed into each well. Plates were incubated at 4 °C for 6 h and afterwards cultivated at 37 °C for 18 h in a modified atmosphere (5% v/v CO₂). The diameter of the inhibition zones was measured in two different directions using a digital caliper. The growth of *L. helveticus* CH1 was not affected by pure GM17 broth (nisin free) acidified with HCl (pH 2.5 ± 0.1). The results were expressed as mean $\pm$ SD from three independent trials in two parallels ($n = 6$). Standard statistical methods (t test) were used to evaluate the results. Differences were considered significant at $p \leq 0.05$.

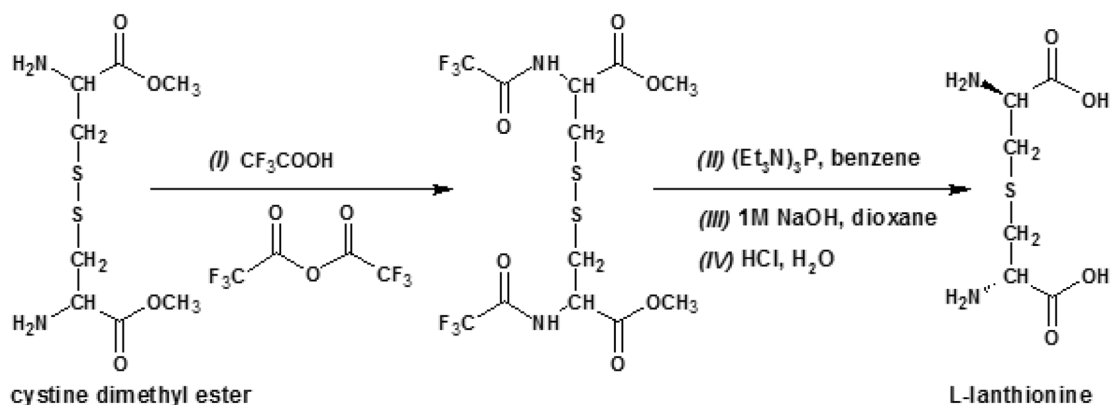
The calibration curve was prepared with a commercial product Valisin® (nisin, 1000 IU mg⁻¹, Mayasan Food Industries a.s., Istanbul, TR) which was diluted to 50, 100, 500 and 1000 IU ml⁻¹ with 0.02 mol l⁻¹ HCl and 150 µl of

each concentration was applied, in duplicate, into wells in the same MRS agar as lactococci supernatants. Log nisin concentrations were plotted against average diameters of inhibition zones.

Results and discussion

Selective synthesis of L-lanthionine

L-Lanthionine is an essential component of peptide lantibiotics including nisin, with a great potential in the food industry as well as in veterinary medicine. Therefore, the focus of current research is the development of a more efficient method of nisin production. The approach selected in our laboratory was a combination of L-lanthionine chemical synthesis and further enrichment of lactococcal cultivation medium with L-lanthionine. Chemical synthesis of both protected and unprotected lanthionine derivatives suffers from undesirable side reactions (e.g. eliminations, Michael addition, racemization, formation of thioesters and hydrolyses of synthons), low yields and difficult isolation of key compounds [13, 14]. In our study, we have employed selective extrusion of sulfur from a protected cystine molecule according to Harpp and Gleason [10]; this was an attractive approach due to the low cost of starting material and high selectivity of reaction sequence, with final yield of 28.8% (Fig. 1). Moreover, sulfur extrusion from cystine can be easily scaled-up. The exact structural elucidation of synthesized product was based on the comparison of retention characteristics, EI-MS, APCI-MS, and 1D NMR spectra with a commercial standard. A positive APCI-MS spectrum provided the characteristic molecular ion adduct m/z 209.05904 $[M + H]^+$ as shown in Fig. 2. ¹H and ¹³C APT spectra showed intense CH and CH₂ signals at α and β positions typical only for L-lanthionine as shown in supplementary Fig. 1, 2 and the “Selective synthesis of L-lanthionine”

**Fig. 1** Selective extrusion of sulfur from protected cystine resulting in L-lanthionine

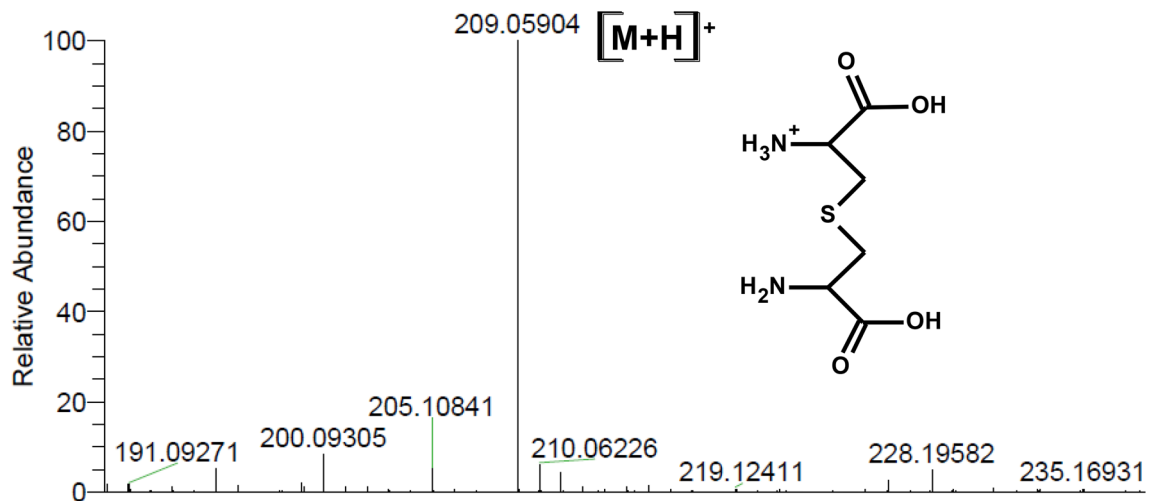


Fig. 2 Positive APCI-MS spectra of L-lanthionine molecular ion adduct $[M+H]^+$

section. Moreover, supplementary section was provided with 1D-NMR spectra of commercial DL-lanthionine standard for comparative purposes.

Influence of L-lanthionine addition to cultivation medium on nisin production

L-Lanthionine was used to supplement cultivation medium (GM17 broth). As seen from Table 2, the addition of L-lanthionine did not significantly influence the growth and the final cell concentration of lactococcal strains. Cell counts ranged from 10^8 to 10^9 CFU ml⁻¹ in all samples tested.

Enrichment of GM17 broth with L-lanthionine substantially influenced nisin production. An increase in nisin production in comparison with the control was observed for all lactococci tested (Figs. 3, 4) but the L-lanthionine concentration-dependent synthesis of nisin was not confirmed. The influence of L-lanthionine concentration on nisin production seemed to be strain specific. Based on the results obtained, an optimal concentration of L-lanthionine in medium for nisin production 0.78 mg ml⁻¹ for the LCC 416 and NIZO R5 strains, and 3.91 mg ml⁻¹ for LTM 32 and LCC 731 strains can be suggested.

The results showing the change in nisin production (in percentage) with L-lanthionine in comparison with control are summarized in Table 3. The most significant increase in nisin concentration compared to the control was observed for strains CCDM 731 and LTM 32; it reached 88.6% in GM17 broth with 7.82 mg ml⁻¹ of L-lanthionine for strain CCDM 731 and 90.0% in GM17 broth with 3.91 mg ml⁻¹ of L-lanthionine for strain LTM 32. The increase in nisin production for strain LTM 32 and CCDM 731 ranged from 74.4 to 90.0% and 47.0 to 88.6%, respectively. Strain NIZO R5 showed the highest nisin production (70.3%) in GM17 broth with 0.78 mg ml⁻¹ of L-lanthionine. The addition of L-lanthionine increased nisin concentration by 34.4–70.3%. There was a negligible impact of L-lanthionine enrichment on nisin production by strain CCDM 416. Nevertheless, an increase in nisin production was demonstrated and it ranged from 3.9 to 15.0%. The highest influence (15.0%) was observed for cultivation in GM17 broth with 0.78 mg ml⁻¹ of L-lanthionine. It can be concluded that the highest L-lanthionine concentration used (7.82 mg ml⁻¹) did not significantly increase the diameter of inhibition zones, i.e. nisin production. But when using the agar diffusion method, the results depend on a number of factors such as the type of nisin-sensitive strain, the addition of surfactant and also the amount of agar and

Table 2 Counts of *L. lactis* subsp. *lactis* strains [log CFU ml⁻¹] after cultivation at 30 °C for 18 h in GM17 broth with and without the addition of L-lanthionine

Strain	Counts of lactococci [log CFU ml ⁻¹]			
	Control	0.78 mg ml ⁻¹	3.91 mg ml ⁻¹	7.82 mg ml ⁻¹
LTM 32	8.76 ± 0.16 ^a	8.51 ± 0.24 ^a	9.10 ± 0.17 ^a	8.42 ± 0.19 ^a
CCDM 731	8.80 ± 0.31 ^a	8.40 ± 0.11 ^a	8.90 ± 0.21 ^a	8.69 ± 0.23 ^a
CCDM 416	9.06 ± 0.22 ^a	8.76 ± 0.32 ^a	9.10 ± 0.16 ^a	8.90 ± 0.26 ^a
NIZO R5	8.83 ± 0.18 ^a	9.03 ± 0.27 ^a	8.93 ± 0.27 ^a	8.86 ± 0.17 ^a

Values in the same row followed by different lowercase letters are significantly different ($p \leq 0.05$)

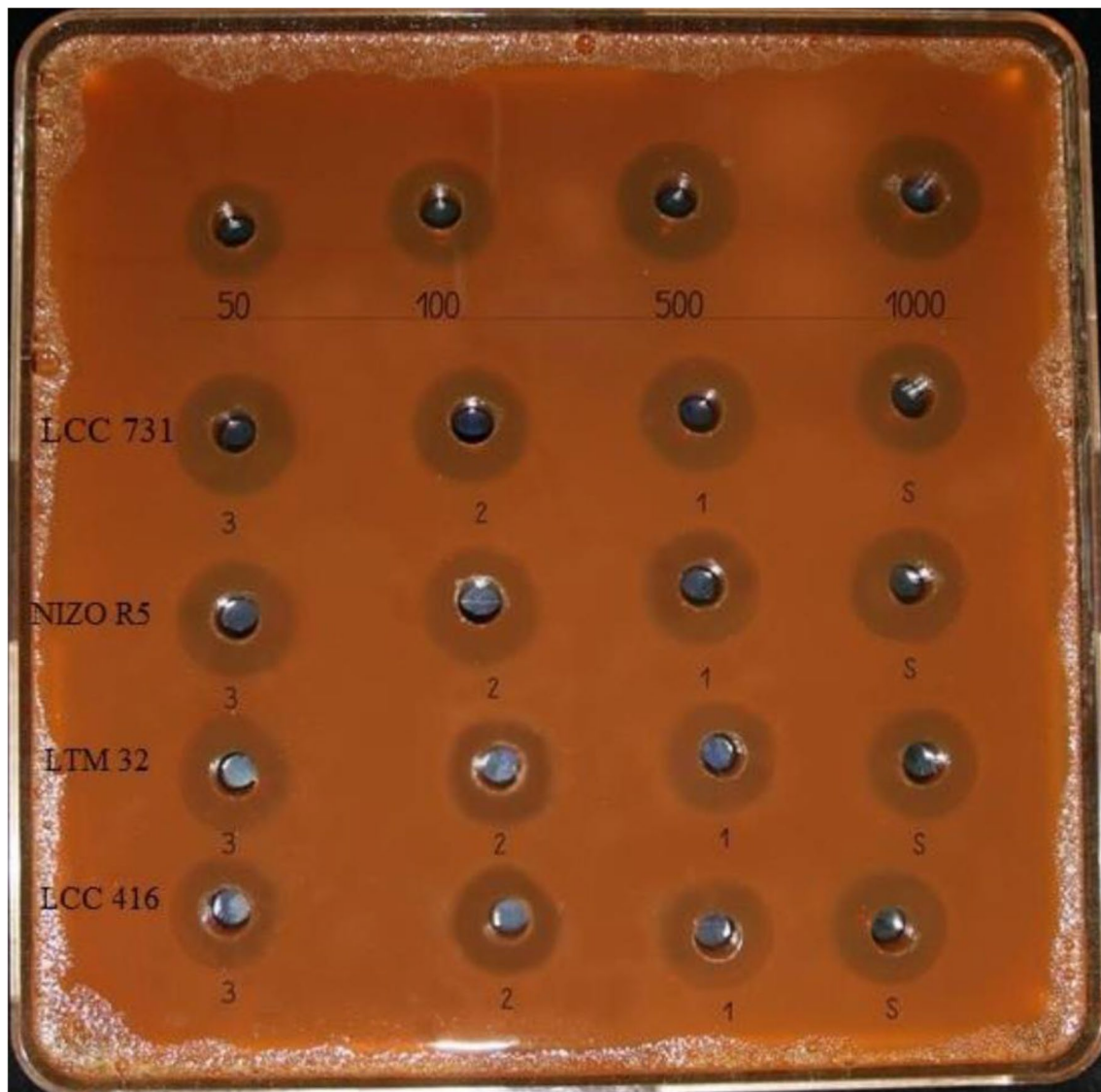


Fig. 3 Nisin calibration concentration [IU ml^{-1}] and inhibition zones for strains tested (S=control; 1=0.78 $\text{mg}\cdot\text{ml}^{-1}$; 2=3.91 $\text{mg}\cdot\text{ml}^{-1}$; 3=7.82 $\text{mg}\cdot\text{ml}^{-1}$ of L-lanthionine)

pre-incubation (pre-diffusion) step. Probably, the measurement of inhibition in liquid media could, in case of higher nisin concentrations, be more accurate and reliable [15].

Nisin biosynthesis is strongly dependent on the presence of a sulfur source, an inorganic salt (magnesium sulfate or sodium thiosulfate) or the amino acids methionine, cysteine or cystathionine. The amino acids, serine, threonine and cysteine, greatly stimulate nisin production without affecting the final cell yield, indicating their precursor role during nisin biosynthesis [16]. Nisin production can be enhanced by increasing the concentrations of peptone and yeast extract powder, because serine, threonine and cysteine are abundant in these sources [17]. The

influence of L-lanthionine on nisin production has not been published in the literature until now. Thus, our research brings new information, which may be beneficial in nisin production.

There is a constant search for ways to increase nisin production and reduce its cost, as it is the only bacteriocin so far to be approved for food production. Leaving aside the GMO approach, other options may be to increase biomass production by continuous neutralization of the lactic acid produced, by co-cultivating with yeasts or by supplementation of the growth medium. We have shown that the addition of lanthionine can effectively increase the production of nisin in selected *L. lactis* strains.

Fig. 4 Nisin concentration [IU ml⁻¹] produced by of *L. lactis* subsp. *lactis* strains in GM17 broth with and without the addition of L-lanthionine. Different letters in the columns of particular nisin concentrations indicate significant differences ($p \leq 0.05$) among samples

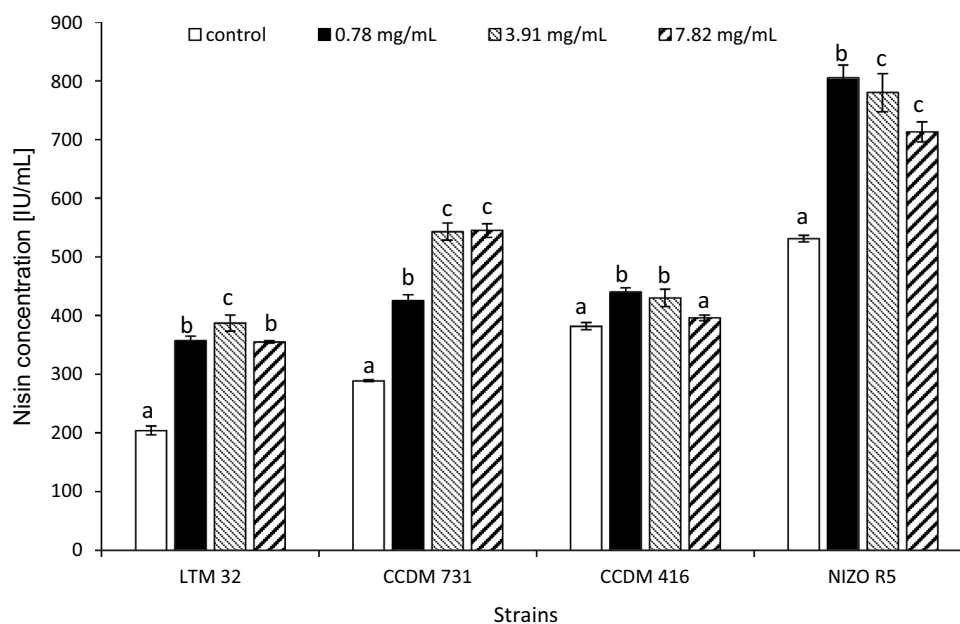


Table 3 The change in nisin production (%) in GM17 with L-lanthionine in comparison with control

Strain	Concentration of L-lanthionine		
	0.78 mg ml ⁻¹	3.91 mg ml ⁻¹	7.82 mg ml ⁻¹
LTM 32	75.5 ± 2.2	90.0 ± 1.0	74.4 ± 2.3
LCC 731	47.0 ± 3.4	88.0 ± 4.4	88.6 ± 4.2
LCC 416	15.0 ± 1.3	12.2 ± 3.0	3.9 ± 0.6
NIZO R5	70.3 ± 5.0	46.9 ± 4.7	34.4 ± 4.7

Conclusion

L-lanthionine was successfully synthesized and used to enhance nisin production by *L. lactis* subsp. *lactis* strains (CCDM 731, NIZO R5, LTM 32, CCDM 416). Growth of tested lactococci was not affected by the addition of L-lanthionine into GM17 broth. Nisin production increased in GM17 broth enriched with L-lanthionine in comparison to controls (GM17 broth without L-lanthionine) for all four strains tested. A general concentration-dependent effect of L-lanthionine concentration was not proven, although it seems to be strain specific. Consider the positive impact of L-lanthionine addition on nisin production tested lactococci probably possess a system for L-lanthionine transport into the cell, and its use for nisin production, and it is a possibility how to increase the efficiency of nisin production. The cultivation of lactococci in milk/whey, as a traditional medium used for industrial scale nisin manufacture, with addition of L-lanthionine will be tested in the further experiments.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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References

- Kakkar N, Prez JG, Liu RW, Jewett MC, van der Donk WA (2018) Incorporation of nonproteinogenic amino acids in class I and II lantibiotics. *ACS Chem Biol* 13:951–957
- Ongey EL, Neubauer P (2016) Lanthipeptides: chemical synthesis versus in vivo biosynthesis as tools for pharmaceutical production. *Microb Cell Factories* 15:97
- Ebner P, Reichert S, Luqman A, Krismer B, Popella P, Götz F (2018) Lantibiotic production is a burden for the producing staphylococci. *Sci Rep* 8:7471
- Mesa-Pereira B, Rea MC, Cotter PD, Hill C, Ross RP (2018) Heterologous expression of biopreservative bacteriocins with a view to low cost production. *Front Microbiol* 9:1654
- Özel B, Şimşek Ö, Akçelik M, Saris PEJ (2018) Innovative approaches to nisin production. *Appl Microbiol Biotechnol* 102:6299–6307
- Hudson GA, Mitchell DA (2018) RiPP antibiotics: biosynthesis and engineering potential. *Curr Opin Chem Biol* 45:61–69
- Dickman R, Mitchell SA, Figueiredo AM, Hansen DF, Tabor AB (2019) Molecular recognition of lipid II by lantibiotics: synthesis and conformational studies of analogues of nisin and mutacin rings A and B. *J Org Chem* 84(18):11493–11512

8. Goto Y, Ayşe Ö, van der Donk WA (2011) Mechanistic studies of Ser/Thr dehydration catalysed by a member of the LanL lanthionine synthetase family. *Biochemistry* 50(5):891–898
9. Yang X, van der Donk WA (2015) Michael-type cyclizations in lantibiotic biosynthesis are reversible. *ACS Chem Biol* 10(5):1234–1238
10. Harpp DN, Gleason JG (1971) Preparation and mass spectral properties of cystine and lanthionine derivatives. A novel synthesis of L-lanthionine by selective desulfurization. *J Org Chem* 36(1):73–80
11. Tramer J, Fowler GG (1964) Estimation of nisin in foods. *J Sci Food Agric* 15:522–528
12. Pongtharangkul T, Demirci A (2004) Evaluation of agar diffusion bioassay for nisin quantification. *Appl Microbiol Biotechnol* 65:268–272
13. Denoël T, Zervosen A, Gerards T, Lemaire C, Joris B, Blanot D, Luxen A (2014) Stereoselective synthesis of lanthionine derivatives in aqueous solution and their incorporation into the peptidoglycan of *Escherichia coli*. *Bioorg Med Chem* 22:4621–4628
14. Denoël T, Lemaire C, Luxen A (2018) Progress in lanthionine and protected lanthionine synthesis. *Chem Eur J* 24:15421–15440
15. Papagianni M, Avramidis N, Filioussis G, Dasiou D, Ambrosiadis I (2006) Determination of bacteriocin activity with bioassays carried out on solid and liquid substrates: assessing the factor “indicator microorganism”. *Microb Cell Factories* 5:30
16. De Vuyst L (1995) Nutritional factors affecting nisin production by *Lactococcus lactis* subsp. *lactis* NIZO 22186 in a synthetic medium. *J Appl Bacteriol* 78:28–33
17. Guo W, Zhang Y, Lu J, Jiang L, Teng L, Wang Y, Liang Y (2010) Optimization of fermentation medium for nisin production from *Lactococcus lactis* subsp. *lactis* using response surface methodology (RSM) combined with artificial neural network-genetic algorithm (ANN-GA). *Afr J Biotechnol* 9(38):6264–6272

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Antifungal Activity of Selected Lactobacilli Intended for Sourdough Production

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Abstract

Background and objective: Presently, there is a growing interest in food produced without the addition of chemical preservatives. Lactic acid bacteria have a high potential to control the growth of undesirable microorganisms. In this work, the antifungal activity of eight strains of lactobacilli isolated from plant sources and foodstuffs (tartar sauce, wheat flour, barley flour, sourdough) were tested against *Fusarium culmorum* DMF301 and *Penicillium expansum* DMF04.

Materials and methods: Antifungal activity of live cells of lactobacilli and their heat-treated supernatants was determined by agar diffusion method at 30°C, the radial growth of fungi was measured. Organic acid production of tested strains was examined by HPLC.

Results and conclusion: Live cells of three *Lactobacillus plantarum* strains showed the highest antifungal activities. *Fusarium culmorum* DMF301 was more sensitive to the activity of lactobacilli. Heat-treated bacterial supernatants (100°C, 10 min) were also tested, being added at 10 or 20% v v⁻¹ to the culture medium; growth of *Fusarium culmorum* DMF301 was inhibited by range of 80-90% compared to controls at 10% supernatant concentration and fully at 20%. However, after neutralization, only the heat treated supernatant from *Lactobacillus plantarum* CCDM 583 had partially effective antifungal activity. The mutual inhibition of lactobacilli strains reduced their antifungal activity. Statistically significant differences in activity against *Fusarium culmorum* DMF 301 were found using individual strains of *Lactobacillus plantarum* CCDM 583 and MP2 or their combination. For use in combination with other cultures, it is therefore necessary to verify the compatibility of various strains of lactobacilli.

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1. Introduction

Functional properties of lactic acid bacteria (LAB) have been traditionally applied not only in fermented dairy products but also in fermented vegetables, in sourdough and subsequently in bakery products. During fermentation, LAB produce a variety of metabolites (organic acids, bacteriocins, reuterin, hydrogen peroxide, cyclic peptides, ethanol, diacetyl etc.) showing both antibacterial and antifungal activity and these have been used successfully in the natural preservation of foods as a partial replacement of chemical preservatives [1,2].

The number of LAB isolates from sourdough includes more than 80 species of bacteria, with a significant number of strains from the families Lactobacillaceae and Leuconostocaceae, but lactococci, streptococci and

enterococci are also present [3]. *Lactobacillus* (*L.*) *plantarum* and *L. sanfranciscensis* have been found in 50% of sourdoughs tested [4]. However, microbial metabolic activity, in combination with the enzymatic activity of the cereal substrates, has a significant effect on the quality of the resulting bakery product. The basic biochemical processes that take place during the preparation of sourdoughs are alcoholic and lactic fermentation, but different LAB present may also differ with other metabolic activities such as the ability to effectively utilize maltose or the formation of exopolysaccharides from sucrose [3,5,6].

Organic acids occur in foods as additives or as products of carbohydrates or protein metabolism. Lactic acid is the main product of carbohydrate metabolism by LAB; acetic

acid is produced in relatively large amounts only in the hetero-fermentative strains, and is a very important component involved in the taste and smell of sourdough [7,8]. The conversion of free fatty acids to antifungal-active hydroxy-fatty acids is a strain specific property of some LAB [9]. Phenylalanine and tyrosine may be degraded to phenyllactic acid and its p-hydroxy-derivative which inhibits mould growth at low concentrations [10].

The mechanism of antimicrobial action of organic acids is mainly acidification of the cytoplasm. The undissociated forms of acids diffuse through the microbial cell wall due to their hydrophobic character and then dissociate inside the cell. This phenomenon leads to inhibition of metabolic activities and the collapse of proton motive force. This mechanism is also likely to affect some types of fungi [11]. Using scanning and transmission electron microscopy has shown that *L. plantarum* K35 supernatant causes damage to the fungal cell wall and cytoplasmic membrane, as well as destruction of organelles including mitochondria and the nucleus [12]. Antifungal effects of cyclic peptides, hydrogen peroxide and the synergistic effects of various agents have also been investigated [1,2,10,13].

Selecting suitable protective cultures can have a number of positive effects since filamentous fungi are widespread food spoilage microorganisms responsible for economic losses and safety concerns due to the potential production of mycotoxins. This study focused on antifungal activities of selected lactobacilli against *Fusarium* (*F.*) *culmorum* DMF301 and *Penicillium* (*P.*) *expansum* DMF04 isolated from bakery products. *Fusarium* spp. and *Penicillium* spp. were identified as frequent fungal contaminants in food, causing spoilage and synthesizing highly toxic mycotoxins [14]. The aim of this work was also to verify the stability of the antifungal activity at baking temperatures. Additionally, the antifungal activity of the individual strains and their combinations was compared.

2. Materials and methods

Microorganisms

Lactobacilli were obtained from Laktoflora® Milcom collection of microorganisms (Prague, Czech Republic). *L. acidophilus* CCDM 151 (original culture); *L. plantarum* CCDM 181 (source silage); *L. zymae* CCDM 361 (isolated from wheat); *L. sanfranciscensis* CCDM 451; *L. panis* CCDM 471; *L. sanfranciscensis* CCDM 827 (all isolated from sourdough); *L. plantarum* CCDM 583 (isolated from barley flour); and *F. culmorum* DMF301; *P. expansum* DMF04 and *L. plantarum* MP2 (isolated from tartar sauce) were obtained from the collection of the Department of Dairy, Fat and Cosmetics, University of Chemistry and Technology (Prague, Czech Rep.).

Cultivation of microorganisms

Lactobacilli strains were routinely cultivated in MRS br-

oth (Merck, Germany) at 37°C for 18 h in an atmosphere of 5% v v⁻¹ CO₂. After cultivation, the numbers of lactobacilli were determined by plating on MRS agar (Merck, Germany), pH 5.8 at 37°C in an atmosphere of 5% v v⁻¹ CO₂.

Both fungi were plated on yeast extract sucrose slant agar (YESA) containing yeast extract 20 g (Oxoid, Altrincham, UK), sucrose 150 g (VWR, Oud-Heverlee, Belgium) magnesium sulphate heptahydrate 0.5 g (Lachema, Czech Rep.), zinc sulphate heptahydrate 0.01 g (Penta, Prague, Czech Rep.), copper sulphate pentahydrate 0.005 g (Lachema, Czech Rep.), agar 20 g (Oxoid, Altrincham, UK) and 885 ml water, pH 5.7, and incubated at room temperature aerobically for 10 days.

Preparation of spore suspension

A test-tube with slant agar was washed with 5 ml of saline solution containing 0.1% w v⁻¹ peptone and 0.1% v v⁻¹ Tween 80. The spore concentration in this solution was determined by cultivation method and subsequently adjusted by dilution with saline solution to approximately 10⁵ per ml and stored at 8°C until further use.

HPLC analysis of organic acids

Determination of organic acids was performed by HPLC (Agilent 1260 Infinity, USA): Polymer IEX H column (Watrex, Prague, Czech Rep.) 250 × 8 mm and pre-column 40 × 8 mm (Watrex) both with particle size 8 µm, temperature 60°C, mobile phase sulfuric acid (9 mmol l⁻¹), flow rate 0.6 ml min⁻¹; sample volume 20 µl; detection was by UV detector at 210 nm.

Antifungal activity experiment

Antifungal activity was determined using an agar diffusion method [15]. 100 µl of appropriate dilution of lactobacilli suspension were mixed with 10 ml of modified (acetate-free) MRS agar to a final concentration of 10²-5×10² CFU ml⁻¹ and poured on Petri dishes (with a diameter of 8.5 cm). After solidification, the plates were overlaid with 5 ml of YESA soft agar (0.75% agar), dried and 5 µl of spore suspension of *F. culmorum* DMF301 or *P. expansum* DMF04 were spotted in the middle of the plate. Radial growth of moulds was measured regularly in two different directions during aerobic cultivation at 30°C. In order to evaluate the activity of cell free supernatants, the lactobacilli cells after cultivation were centrifuged (9000 ×g, 4°C, 5 min), supernatants were heat-treated (100°C, 10 min) and then either neutralized to pH 7 with 10% w w⁻¹ NaOH, or used without neutralization. Both types of supernatants (heat-treated and heat-treated with neutralization) were mixed with YESA to achieve a final concentration of 10 or 20% v v⁻¹ in agar. Subsequent fungal cultivation was carried out for 10 days at 25°C and radial growth of the mould was monitored by measuring the diameter of the colony using a digital sliding scale. As

a control, growth of mould on agar without the addition of lactobacilli or supernatants was used.

Statistical analysis

All measurements were means from three independent trials with three parallel repetitions ($n=9$). Standard statistic methods in Excel (Microsoft® Office 2010) were used to evaluate the results: F-test to assess the equality of variances and student's paired t-test to evaluate the means significant differences. Differences were considered significant at $p \leq 0.05$.

3. Results and discussion

Tested strains of lactobacilli were characterized by growth in MRS broth, acid production and final pH after 18 h cultivation. The results are shown in Table 1. All strains showed final cell concentrations of 10^8 - 10^9 CFU ml⁻¹ except strains 451 and 471 (only 10^5 - 10^6 CFU ml⁻¹). These strains differed in acid production. Strains 361 and 583 produced the largest amount of lactic and acetic acids. In case of the strain 361, it was 38.6 ± 1.7 and 4.0 ± 0.2 g l⁻¹ and in the case of the strain 583 it was 38.0 ± 2.4 and 3.7 ± 0.1 g l⁻¹ respectively. Although strain 151 belongs to the homo-fermentative group of lactobacilli, it was found to produce little acetic acid. Production of organic acids (lactic, acetic, phenyllactic) is the main reason for inhibition of mould growth [16]. Trace amounts of phenyllactic acid were only found in MP2 and 361. Its production could however be increased by the addition of phenylalanine to the cultivating medium as a precursor [17]. Surprisingly, low levels of acetic acid were found in the obligatory hetero-fermentative strain CCDM 827. The formation of antifungal compounds depends on the growth and metabolic activity of LAB. Basic factors influencing LAB growth and the level of antifungal metabolites include temperature, incubation time, nutritional factors and the pH of the culture medium [12,14,18].

The radial growth of fungi in the presence of a growing culture of lactobacilli during 10 days cultivation at 30°C is

summarized in Fig. 1 and Fig. 2, being compared to control growth (without lactobacilli). *Fusarium* spp. and *Penicillium* spp. and their mycotoxins are of the most common contaminants of cereals [2]. They pose not only a health risk for humans and animals but also influence technological and baking properties of dough [19].

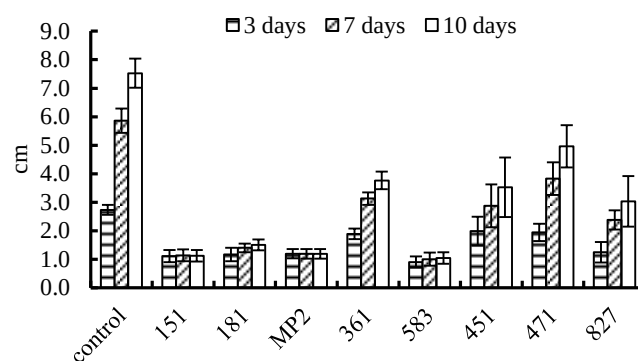


Figure 1. The radial growth of *Fusarium culmorum* DMF01 in the presence of growing cells of lactobacilli at 30°C

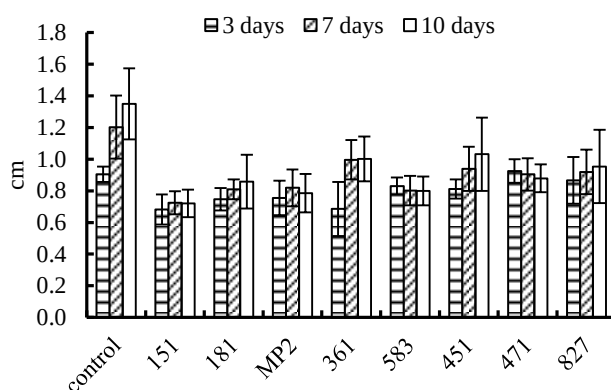


Figure 2. The radial growth of *Penicillium expansum* DMF01 in the presence of growing cells of lactobacilli at 30°C

Table 1. Growth characteristics and acid production of tested lactobacilli after cultivation in MRS broth at 37°C, 18 h, 5% v v⁻¹ CO₂

Strain	Cell count [CFU ml ⁻¹]	pH	Lactic acid [g l ⁻¹]	Acetic acid [g l ⁻¹]	Phenyllactic acid
<i>Lactobacillus acidophilus</i> CCDM 151	$4.6 \pm 0.2 \cdot 10^9$	4.52	28.5 ± 0.3	0.3 ± 0.0	ND
<i>Lactobacillus plantarum</i> CCDM 181	$2.9 \pm 0.1 \cdot 10^9$	4.01	36.3 ± 2.1	2.8 ± 0.1	ND
<i>Lactobacillus plantarum</i> MP2	$3.1 \pm 0.3 \cdot 10^9$	3.78	34.1 ± 2.8	2.7 ± 0.1	Traces
<i>Lactobacillus zymae</i> CCDM 361	$8.1 \pm 0.3 \cdot 10^8$	3.75	38.6 ± 1.7	4.0 ± 0.2	Traces
<i>Lactobacillus sanfranciscensis</i> CCDM 451	$3.8 \pm 0.5 \cdot 10^5$	4.25	13.5 ± 0.7	2.9 ± 0.1	ND
<i>Lactobacillus panis</i> CCDM 471	$2.3 \pm 0.4 \cdot 10^6$	5.04	29.1 ± 1.1	0.9 ± 0.0	ND
<i>Lactobacillus plantarum</i> CCDM 583	$6.5 \pm 0.2 \cdot 10^8$	3.81	38.0 ± 2.4	3.7 ± 0.1	ND
<i>Lactobacillus sanfranciscensis</i> CCDM 827	$8.6 \pm 0.1 \cdot 10^8$	4.94	29.8 ± 1.7	0.8 ± 0.0	ND

Growing cells of lactobacilli were able to inhibit the growth *F. culmorum* DMF301 much more than *P. expansum* DMF04. The results shown in Fig. 1 and Fig. 2 demonstrate that *L. plantarum* strains CCDM 181, CCDM 583, MP2 and *L. acidophilus* CCDM 151 had the greatest inhibitory effects (about 80-86% inhibition when compared to the control growth after 10 days) on growth of *F. culmorum* DMF301, whereas the inhibitory activity against *P. expansum* DMF04 was lower and reached only 36-46%. On the other hand, the weakest antifungal activity against moulds was observed using strains 361, 451 and 471 (about 35-53% inhibition activity against *F. culmorum* DMF301 and 23-34% against *P. expansum* DMF04). The antifungal effect of lactobacilli is described in a number of studies. Stiles et al. [15] verified the antifungal effect of *L. rhamnosus* VT1 against *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp. and *Rhizopus* sp. They found that sodium acetate, which is one of the components of MRS agar, exhibits antifungal activity. They noted that this strain showed a synergistic effect with sodium acetate. Also, Schillinger and Villarreal [20] proved the antifungal effect of MRS medium on *P. nordicum* using different concentrations of sodium acetate. For this reason, modified MRS agar without sodium acetate was used in this work. Yang and Chang [21] recorded the ability of *L. plantarum* AF1 to inhibit the growth of *Aspergillus* sp., *Cladosporium* sp., *Epicoccum* sp. and *Penicillium* sp. Demirba et al. [22] confirmed the antifungal effect of *L. paraplantarum* N-15 and *L. paralimentarius* E-106 on *A. niger* and *P. chrysogenum*.

Bread could also be an important non-dairy vehicle for LAB since some of the bacteria can survive the high baking temperatures, as was proven in several studies. After baking, Zhang et al. [23] found 10^{4-5} CFU g⁻¹ live cells of *L. plantarum* P8, the number of which increased up to 10^8 CFU g⁻¹ in the crust and 10^6 CFU g⁻¹ in the crumb during storage. Nevertheless, during the baking process, live lactobacilli are mostly inactivated. Antifungal activity is then caused by stable antifungal metabolites. The temperature inside the crumb usually does not exceed 90-100°C and therefore heating at 100°C was chosen to produce heat-treated supernatants [24]. The plates of YESA were supplemented with 10 or 20% v v⁻¹ heat-treated cell free supernatant (CFS) of strains with good growth in MRS broth (151, 181, MP2, 361, 583, 827) to test their activity against *F. culmorum* DMF301. A 10% addition of CFS meant an approximate acid concentration (lactic and acetic) produced by LAB of about 0.37-0.43 g to 100 g of agar and a 20% addition about 0.74-0.85 g to 100 g of agar. Strong inhibitory activity (80-90% inhibition) was observed when the medium was supplemented with either 10% or 20% v v⁻¹ heat-treated CFS of strains 361, 583 and 827. A concentration of 20% v v⁻¹ was required to observe a reduction in growth of the mould

(approximately 90%) for strains 151, 181 and MP2. In the case of 10% supplementation with CFS, only 18%, 46% and 58% reduction respectively was detected after 7 days cultivation. The conclusion that the cell-free heat treated supernatants of four LAB (80°C for 1 h or 121°C for 15 min) were inhibitory against *P. notatum* was also published by Rouse et al. [25]. Similar results were confirmed for heat treated supernatants (exposure to a temperature of 100°C for 10 min) of different strains of *Lactobacillus* against *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp [26]. Heating, refrigeration or freezing did not change the supernatant inhibitory activity of lactobacilli against different moulds [27]. In the present study, no inhibition was detected when neutralized heat-treated cell free supernatants were added to the agar at a final concentration of either 10% v v⁻¹ or 20% v v⁻¹ except for strain 583. As is shown in Fig. 3 and Fig. 4, neutralized supernatant (20%) of strain 583 was able to significantly inhibit the growth of *F. culmorum* DMF301 but not *P. expansum* DMF04 at 25°C.

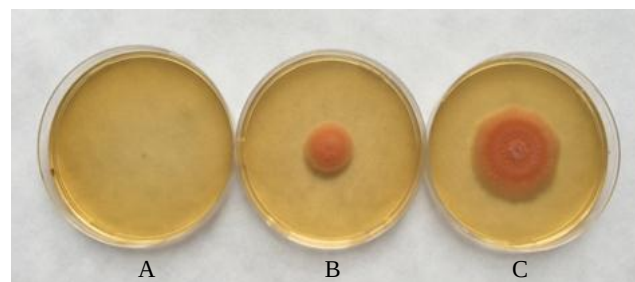


Figure 3. Growth of *Fusarium culmorum* DMF301 on YESA supplemented with 20% v v⁻¹ heat-treated CFS (A) and neutralized heat-treated CFS (B) of *Lactobacillus plantarum* CCDM 583 and the control growth without cell free supernatant (C) of the mould after 5 days at 25°C.

The ability of heat-treated cell-free supernatants to inhibit fungal growth has been described in a number of studies. Organic acids are responsible for the antimicrobial effects of non-neutralized cell-free supernatants. The same conclusion was drawn by Gerez et al. [28] who found that the cell-free supernatants of strains of lactobacilli had lost their antifungal activities after neutralization. De Muynck et al. [29] noted that strains belonging to the species *P. paneum* and *P. roqueforti* were very susceptible to the action of the cell-free supernatant of the *L. acidophilus* LMG9433. They further demonstrated that after neutralization cell-free supernatants of *L. acidophilus* LMG9433, *L. amylovorus* DSM20532, *L. brevis* LMG6906 and *L. coryniformis* subsp. *coryniformis* LMG9196 lost their antifungal effects. Le Lay et al. [30] noted that the cell-free supernatants of *L. brevis* Lu35 and *L. citreum* L123 showed strong growth inhibition of *A. niger* UBOCC-A-112064 and *P. corylophilum* UBOCC-A-112081.

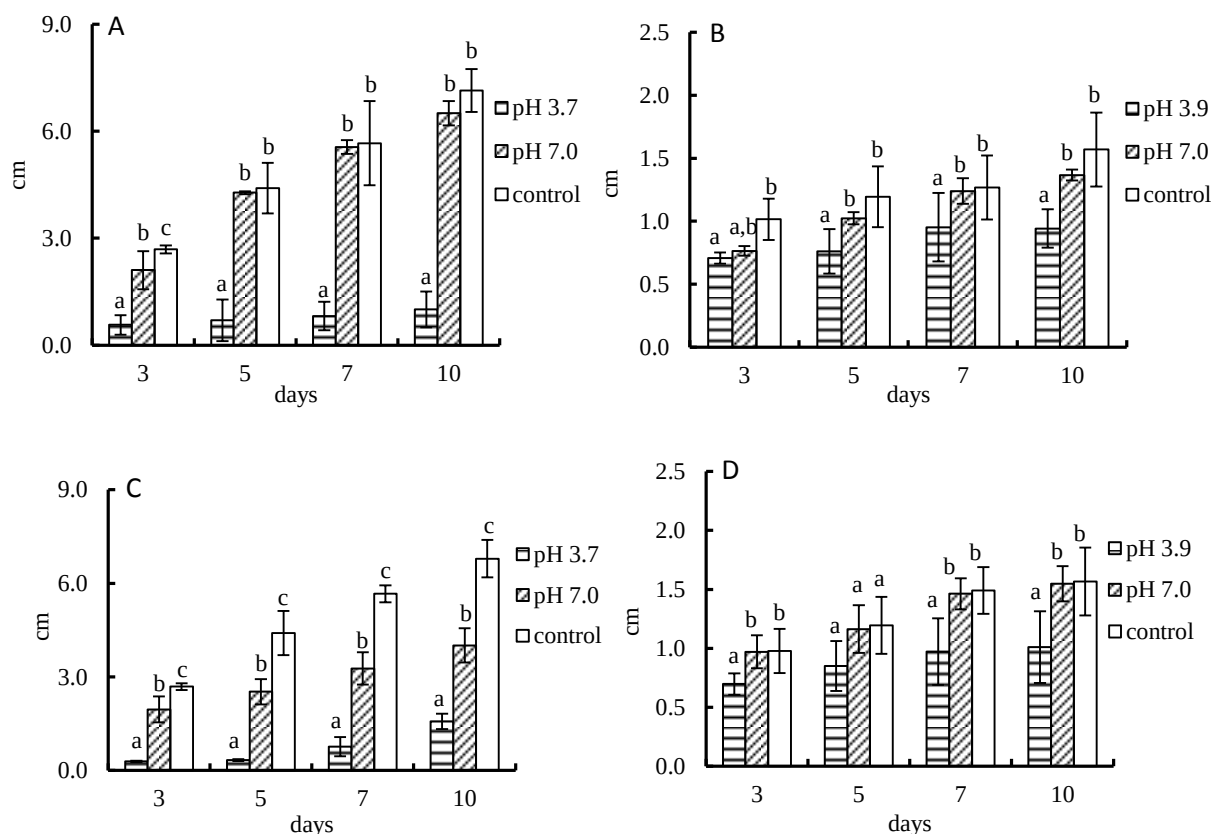


Figure 4. The radial growth of mould *Fusarium culmorum* DMF301 (A,C) and *Penicillium expansum* DMF04 (B,D) on YESA supplemented with 10% v/v heat-treated CFS (pH 3.7 or 3.9) or neutralized heat-treated CFS (pH 7) of *Lactobacillus zymae* CCDM 361 (A,B) and *Lactobacillus plantarum* CCDM 583 (C,D) at 25°C.

Different letters in the columns of particular days indicate significant differences ($p \leq 0.05$) among samples.

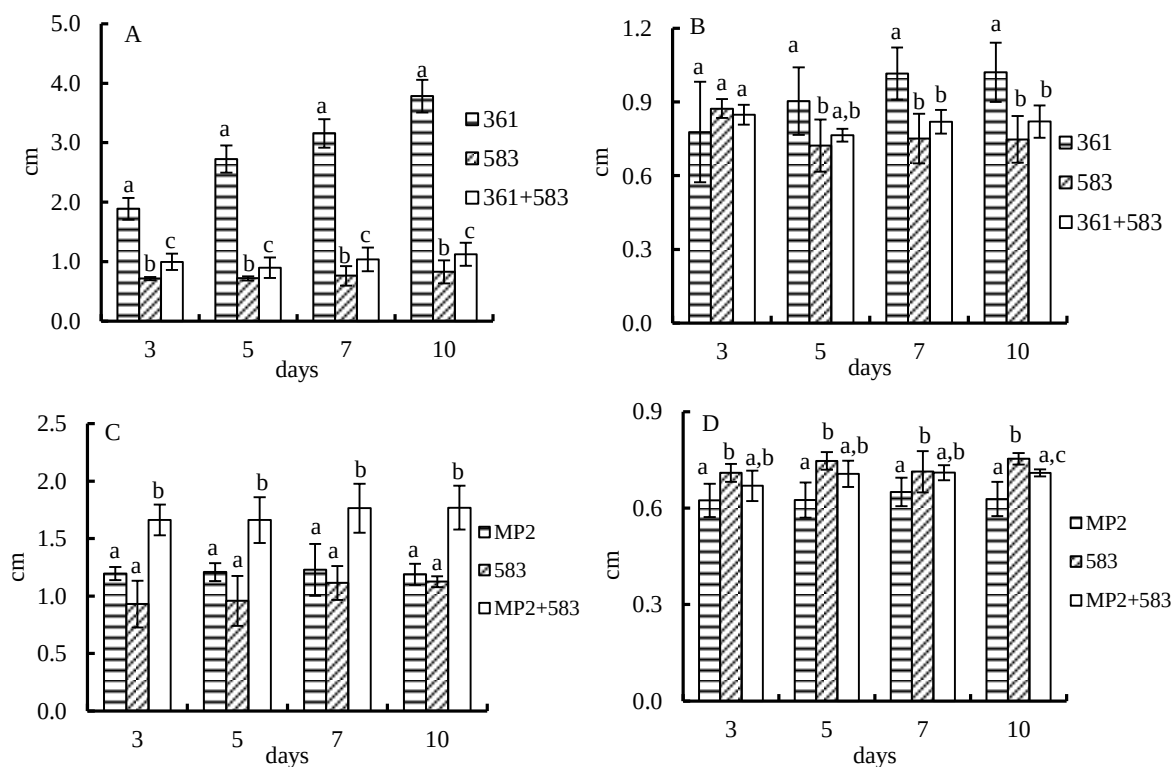


Figure 5. The radial growth of *Fusarium culmorum* DMF301 (A,C) and *Penicillium expansum* DMF04 (B,D) in the presence of single strains *Lactobacillus zymae* CCDM 361, *Lactobacillus plantarum* MP2 and *Lactobacillus plantarum* CCDM 583 and their combination at 30°C.

Different letters in the columns of particular days indicate significant differences ($p \leq 0.05$) among samples.

Since protective adjunct cultures can often be used in mixtures, the antifungal effect of selected combinations of lactobacilli strains, i.e. combination of 361 and 583 and combination of MP2 and 583, were investigated in further work. The final concentration of live cells was again 10^2 - 5×10^2 CFU ml⁻¹ with the use of a 50% inoculum of each strain. Synergistic effects of the strains were expected but, as shown in Fig. 5, the efficiency of *L. plantarum* CCDM 583 against *F. culmorum* DMF301 was reduced when grown together with strains 361 and MP2. This phenomenon was not observed in tests of antifungal activity against *P. expansum* DMF04. Mutual inhibition was confirmed in all three strains of lactobacilli tested against each other. The zones of inhibition, determined by agar diffusion method, are shown in Table 2 and Fig. 6. The results show that careful selection of mutually compatible strains is necessary to ensure their desired properties. It is important especially in the cases where other dairy cultures are used for food production. The antifungal activity of lactobacilli can successfully be utilized not only to suppress mould growth in sourdough breads, but in a variety of other applications as well [31]. Antifungal LAB were tested for prevention of mould on fresh vegetables or apples and *L. plantarum* strains were able to inhibit spoilage yeast in refrigerated foods or to inhibit fumonisin producing *F. proliferatum* in poultry feeds [18,25,32,33]. Antifungal cultures have also been introduced for yoghurt production and can be a good choice to increase the shelf life of cereal-based beverages [34,35].

Table 2. Inhibition zones (mm) found by agar diffusion method after common cultivation of lactobacilli strains *Lactobacillus zymae* CCDM 361, *Lactobacillus plantarum* MP2 and *Lactobacillus plantarum* CCDM 583 at 37°C, 48 h, 5% v v⁻¹ CO₂ atmosphere

Inhibited by strain	361	583	MP2
361	-	15.33±0.31	15.55±0.18
583	14.33±0.33	-	18.15±0.38
MP2	15.30±0.18	18.10±0.21	-

361= *Lactobacillus zymae* CCDM 361, 583= *Lactobacillus plantarum* CCDM 583, MP2= *Lactobacillus plantarum* MP2

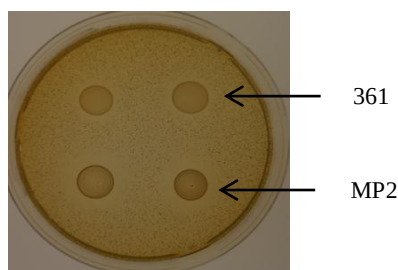


Figure 6. Inhibition zones of the strain *Lactobacillus zymae* CCDM 361 and *Lactobacillus plantarum* MP2 against the strain *Lactobacillus plantarum* CCDM 583; cultivation at 37°C, 48 h, 5% v v⁻¹ CO₂

4. Conclusion

The use of antimicrobial properties of LAB is an interesting alternative and could replace artificial chemical preservatives, as is frequently demanded by many consumers. Based on the results obtained in this work, *L. plantarum* strains CCDM 181, CCDM 583 and MP2 can be recommended as potential antifungal agents for further application because their activity against selected mould was confirmed. When used, their compatibility with regard to antifungal activity must be taken into account. Different lactobacilli strains may inhibit each other in mixed cultures, which may lead to a subsequent reduction of their antifungal effect.

5. Acknowledgement

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6. Conflict of interest

The authors declare no conflict of interest.

References

- Muhialdin BJ, Hassan Z, Sadon SKH. Antifungal activity of *Lactobacillus fermentum* Te007, *Pediococcus pentosaceus* Te010, *Lactobacillus pentosus* G004, and *L. paracasei* D5 on selected foods. J Food Sci. 2011; 76(7):493-499. doi:10.1111/j.1750-3841.2011.02292.
- Oliveira PM, Zannini E, Arendt EK. Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: From crop farming to cereal products. Food Microbiol. 2014; 37:78-95. doi:10.1016/j.fm.2013.06.003.
- Ganzle M, Ripari V. Composition and function of sourdough microbiota: From ecological theory to bread quality. Int J Food Microbiol. 2016; 239:19-25. doi:10.1016/j.ijfoodmicro.2016.05.004.
- De Vuyst L, Van Kerrebroeck S, Harth H, Huys G, Daniel HM, Weckx S. Microbial ecology of sourdough fermentations: diverse or uniform? Food Microbiol. 2014; 37:11-29. doi: 10.1016/j.fm.2013.06.002.
- Ganzle MG. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol. 2014; 37:2-10. doi:10.1016/j.fm.2013.04.007.
- Galle S, Arendt EK. Exopolysaccharides from sourdough lactic acid bacteria. Crit Rev Food Sci Nutr. 2014; 54(7):891-901. doi:10.1080/10408398.2011.617474.
- De Vuyst L, Neysens P. The sourdough microflora: biodiversity and metabolic interactions. Trends Food Sci Technol. 2005; 16:43-56. doi:10.1016/j.tifs.2004.02.012.
- Gul H, Özçelik S, Sagdıç O, Certel M. Sourdough bread production with lactobacilli and *S. cerevisiae* isolated from sourdoughs. Process Biochem. 2005; 40(2): 691-697. doi:10.1016/j.procbio.2004.01.044
- Black BA, Zannini E, Curtis JC, Ganzle MG. Antifungal hydroxy fatty acids produced during sourdough

- fermentation: Microbial and enzymatic pathways, and antifungal activity in bread. *Appl Environ Microbiol.* 2013; 79(6):1866-1873.
doi:10.1128/AEM.03784-12.
10. Cortes-Zavaleta O, Lopez-Malo A, Hernandez-Mendoza A, Garcia HS. Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *Int J Food Microbiol.* 2014; 173:30-35.
doi:10.1016/j.ijfoodmicro.2013.12.016.
 11. Reis JA, Paula AT, Casarotti SN, Penna ALB. Lactic acid bacteria antimicrobial compounds: Characteristics and applications. *Food Eng Rev.* 2012; 4(2):124-140.
doi:10.1007/s12393-012-9051-2.
 12. Sangmanee P, Hongpattarakere T. Inhibitory of multiple antifungal components produced by *Lactobacillus plantarum* K35 on growth, aflatoxin production and ultrastructure alterations of *Aspergillus flavus* and *Aspergillus parasiticus*. *Food Control.* 2014; 40:224-233.
doi:10.1016/j.foodcont.2013.12.005.
 13. Dagnas S, Gauvry E, Onno B, Membre JM. Quantifying effect of lactic, acetic, and propionic acids on growth of molds isolated from spoiled bakery products. *J Food Prot.* 2015; 78(9):1689-1698.
doi.org/10.4315/0362-028X.JFP-15-046.
 14. Dalie DKD, Deschamps AM, Richard-Forget F. Lactic acid bacteria-Potential for control of mould growth and mycotoxins: A Review. *Food Control.* 2010; 21:370-380.
doi:10.1016/j.foodcont.2009.07.011.
 15. Stiles J, Penkar S, Plockova M, Chumchalova J, Bullerman LB. Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. *J Food Prot.* 2002; 65(7):1188-1191.
 16. Ventimiglia G, Alfonzo A, Galluzzo P, Corona O, Francesca N, Caracappa S, Moschetti G, Settanni L. Codominance of *Lactobacillus plantarum* and obligate heterofermentative lactic acid bacteria during sourdough fermentation. *Food Microbiol.* 2015; 51:57-68.
doi:10.1016/j.fm.2015.04.011
 17. Naz S, Gueguen-Minerbe M, Cretenet M, Vernoux JP. Aromatic amino acids as precursors of antimicrobial metabolites in *Geotrichum candidum*. *FEMS Microbiol Lett.* 2013; 344(1):39-47.
doi:10.1111/1574-6968.
 18. Sathe SJ, Nawani NN, Dhakephalkar PK, Kapadnis BP. Antifungal lactic acid bacteria with potential to prolong shelf-life of fresh vegetables. *J. Appl. Microbiol.* 2007; 103(6):2622-2628.
doi:10.1111/j.1365-2672.2007.03525.x.
 19. Prange A, Modrow H, Hormes J, Kramer J, Kohler P. Influence of mycotoxin producing fungi (*Fusarium*, *Aspergillus*, *Penicillium*) on gluten proteins during suboptimal storage of wheat after harvest and competitive interactions between field and storage fungi. *J Agric Food Chem.* 2005; 53(17):6930-6938.
doi: 10.1021/jf050821t.
 20. Schillinger U, Villarreal JV. Inhibition of *Penicillium nordicum* in MRS medium by lactic acid bacteria isolated from foods. *Food Control.* 2010; 21(2):107-111.
doi:10.1016/j.foodcont.2008.11.010.
 21. Yang EJ, Chang HC. Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. *Int J Food Microbiol.* 2010; 139(1-2):56-63.
doi:10.1016/j.ijfoodmicro.2010.02.012.
 22. Demirba F, Ispirli H, Kurnaz AA, Yilmaz MT, Dertli E. Antimicrobial and functional properties of lactic acid bacteria isolated from sourdoughs. *Lwt Food Sci Technol.* 2017; 79:361-366.
doi:10.1016/j.lwt.2017.01.067.
 23. Zhang L, Taal MA, Boom RM, Chen XD, Schutyser MAI. Effect of baking conditions and storage on the viability of *Lactobacillus plantarum* supplemented to bread. *LWT-Food Sci Technol.* 2018; 87:318-325.
doi:10.1016/j.lwt.2017.09.005.
 24. Besbes E, Jury V, Monteau JY, Le Bail A. Effect of baking conditions and storage with crust on the moisture profile, local textural properties and staling kinetics of pan bread. *LWT-Food Sci Technol.* 2014; 58(2):658-666.
doi:10.1016/j.lwt.2014.02.037.
 25. Rouse S, Harnett D, Vaughan A, Van Sinderen D. Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *J Appl Microbiol.* 2008; 104(3):915-923.
doi:10.1111/j.1365-2672.2007.03619.x.
 26. Gerez CL, Torino MI, Rollan G, Font de Valdez G. Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control.* 2009; 20(2):144-148.
doi:10.1016/j.foodcont.2008.03.005.
 27. Laref N, Guessas B. Antifungal activity of newly isolates of lactic acid bacteria. *Innov Rom Food Biotechnol.* 2013; 13:80-88.
 28. Gerez CL, Torres MJ, Font de Valdez G, Rollan G. Control of spoilage fungi by lactic acid bacteria. *Biol Control.* 2013; 64(3):231-237.
doi:10.1016/j.biocontrol.2012.10.009.
 29. De Muynck C, Leroy AIJ, De Maeseneire S, Arnaut F, Soetaert W, Vandamme EJ (2004) Potential of selected lactic acid bacteria to produce food compatible antifungal metabolites. *Microbiol Res.* 2004; 159(4): 339-346.
doi:10.1016/j.micres.2004.07.002.
 30. Le Lay C, Mounier J, Vasseur V, Weill A, Le Blay G, Barbier G. In vitro and In situ screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds. *Food Control.* 2016; 60:247-255.
doi:10.1016/j.foodcont.2015.07.034.
 31. Axel C, Rocker B, Brosnan B, Zannini E, Furey A, Coffey A, Arendt EK. Application of *Lactobacillus amylovorus* DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. *Food Microbiol.* 2015; 47:36-44.
doi:10.1016/j.fm.2014.10.005.
 32. Crowley S, Mahony J, van Sinderen D. Comparative analysis of two antifungal *Lactobacillus plantarum* isolates and their application as bioprotectants in refrigerated foods. *J Appl Microbiol.* 2012; 113(6):1417-1427.
doi:10.1111/jam.12012.
 33. Deepthi BV, Poornachandra Rao K, Chennapa G, Naik MK, Chandrashekhara KT, Sreenivasa MY. Antifungal attributes of *Lactobacillus plantarum* MYS6 against *Fumonisin* producing *Fusarium proliferatum* associated with poultry feeds. *Plos One* 2016; 11(6):e0155122.
doi: 10.1371/journal.pone.0155122.
 34. Delavenne E, Trunet C, Barbier G, Mounier J, Le Blay G. Characterization of the antifungal activity of *Lactobacillus harbinensis* K.V9.3.1Np and *Lactobacillus rhamnosus* K.C8.3.1I in yogurt. *Food Microbiol.* 2015; 45:10-17.
doi: 10.1016/j.fm.2014.04.017.
 35. Russo P, Arena MP, Fiocco D, Capozzi V, Drider D, Spano G. *Lactobacillus plantarum* with broad antifungal activity: A promising approach to increase safety and shelf-life of cereal-based products. *Int J Food Microbiol.* 2017; 247:48-54.
doi: 10.1016/j.ijfoodmicro.2016.04.027.

فعالیت ضدقارچی لاکتوباسیل‌های انتخابی برای تولید خمیر ترش

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چکیده

سابقه و هدف: اخیراً علاقه روزافزونی در زمینه تولید مواد غذایی بدون نگهدارنده‌های شیمیایی ایجاد شده است. باکتری‌های اسید لاکتیک توانایی زیادی در کنترل رشد ریزاندامگان ناخواسته دارند. در این کار، فعالیت ضدقارچی هشت سویه لاکتوباسیل جدا شده از منابع گیاهی و مواد غذایی (سس تارتار، آرد گندم، آرد جو، خمیر ترش) در برابر فوزاریوم کلاموروم DMF301 و پنی‌سیلیوم اکسپانسونوم بررسی شد.

مواد و روش‌ها: فعالیت ضدقارچی سلول‌های زنده لاکتوباسیل و مایع رویی حرارت دیده آنها با روش انتشار در آگار در درجه حرارت ۳۰ درجه سلسیوس تعیین و هاله رشد اندازه‌گیری شد. تولید اسید آلی سویه‌های مورد آزمون با HPLC بررسی شد.

یافته‌ها و نتیجه‌گیری: سلول‌های زنده سه سویه لاکتوباسیلوس پلانتاروم بیشترین فعالیت ضدقارچی را از خود نشان دادند. فوزاریوم کلاموروم DMF301 به فعالیت لاکتوباسیل‌ها بیشتر حساس بود. مایع رویی باکتری‌های حرارت دیده (۱۰۰°C، ۱۰ دقیقه) نیز مورد آزمون قرار گرفت و به میزان ۱۰ یا ۲۰ درصد حجمی-حجمی به محیط کشت اضافه شد؛ رشد فوزاریوم کلاموروم DMF301 به میزان ۸۰ تا ۹۰ درصد در غلظت ۱۰ درصد مایع رویی و به طور کامل در غلظت ۲۰ درصد آن مهار شد. هرچند، پس از خنثی‌سازی فقط مایع رویی حرارت دیده لاکتوباسیلوس پلانتاروم CCDM 583 فعالیت ضدقارچی نسبتاً مؤثری داشت. اثر مهارکنندگی مشترک سویه‌های لاکتوباسیل فعالیت ضدقارچی آنها را کاهش داد. در فعالیت سویه‌های لاکتوباسیلوس پلانتاروم CCDM 583 و MP2 به تنهایی یا در ترکیب با هم در برابر فوزاریوم کلاموروم DMF301 تفاوت آماری معنی دار یافت شد. برای استفاده در ترکیب با سایر کشت‌ها لازم است سازگاری سویه‌های گوناگون لاکتوباسیل مورد تایید قرار گیرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافع مرتبط با انتشار این مقاله ندارند.

Buckwheat Secondary Metabolites: Potential Antifungal Agents

Daniel Koval,* Milada Plocková, Jan Kyselka, Pavel Skřivan, Marcela Sluková, and Šárka Horáčková



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ABSTRACT: Research groups have put significant emphasis on the evaluation of nutritional, health-promoting, and other biological activities of secondary metabolites from buckwheat. Among these phytochemicals, phenolic and lipophilic antioxidants, particularly, phenolic acids, flavonoids, and tocopherols, have been the focus of the latest studies since antioxidant activity has recently been associated with the possibility of inhibiting fungal growth and mycotoxin biosynthesis. The mycotoxin contamination of cereal and pseudocereal grains caused primarily by *Fusarium*, *Penicillium*, and *Aspergillus* species poses a significant hazard to human health. Therefore, efforts to examine the involvement of plant antioxidants in the biosynthesis of mycotoxins at the transcriptional level have emerged. In addition, hydrophobic interactions of buckwheat phenolics with cell membranes could also explain their capacity to reduce fungal development. Eventually, possibilities of enhancing the biological activity of cereal and pseudocereal phytochemicals have been studied, and sourdough fermentation has been proposed as an efficient method to increase antioxidant activities. This effect could result in an increased antifungal effects of sourdough and bakery products. This review reports the main advances in research on buckwheat phenolics and other antioxidant phytochemicals, highlighting possible mechanisms of action and processes that could improve their biological activities.

KEYWORDS: phenolic compounds, buckwheat, antifungal activity, mycotoxin, lactic acid bacteria

INTRODUCTION

Buckwheat is a gluten-free plant belonging to the group of pseudocereals that are defined as crops of various species not related to cereals, but their seeds share several similarities with cereal grains.¹ Buckwheat (genus *Fagopyrum*) is placed in the Polygonaceae family, and more than 20 species have been identified and characterized. Nonetheless, only nine species have agricultural, nutritional, or medicinal value, and only two species are cultivated globally for food production: *Fagopyrum (Fag.) esculentum* known as common buckwheat and *Fag. tataricum* known as Tartary buckwheat.² Although both species have strong adaptability to adverse environmental conditions and can grow in soils of low fertility, only Tartary buckwheat exhibits frost resistance and is mainly cultivated in the mountainous areas.³ Buckwheat is widely distributed in the world but grows for the most part in the northern hemisphere. The largest producing countries of buckwheat are China, Russian Federation, France, and Ukraine, with annual production of more than 100 000 tons each, followed by Poland, the United States of America, and Kazakhstan which grow more than 80 000 tons of buckwheat per year each. Overall, almost 3 million tons of buckwheat were harvested worldwide in 2018.⁴

Two buckwheat products are mainly used for food consumption: flour and groats. These products are considered to have high nutritional value as they contain high levels of nutritionally valuable proteins, minerals, polyphenols, and phytosterols.^{1,5–7} Numerous studies have reported the health benefits of buckwheat consumption including anticancer, anti-inflammatory, hypoglycemic, and hypocholesterolemic activities, which are presumably associated with health-promoting

compounds such as buckwheat proteins and phenolic compounds.^{8–11} These specific compounds and their mechanisms of action are the subject of recent studies. Some of the beneficial effects of phenolic compounds are possibly related to their antioxidant activities.^{12–14} The latest advances in research on buckwheat health-promoting effects are well described in the work of Zhu et al. (2020)¹⁵ or Yilmaz et al. (2020).¹⁶ Among the buckwheat secondary metabolites with antioxidant properties, lipophilic compounds such as tocopherols must also be mentioned.¹⁷

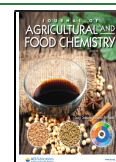
Furthermore, phenolic and lipophilic compounds of plants, including cereals and pseudocereals, exhibit antifungal activities resulting from their antioxidant and hydrophobic properties. Oxidative stress and exposure to reactive oxygen species (ROS) have been associated with the stimulation of mycotoxin production, and buckwheat antioxidants are considered as potential ROS scavengers.¹⁸ Consequently, buckwheat phytochemicals with antioxidant activity could modulate and decrease mycotoxin biosynthesis.¹⁹ Furthermore, the association of phenolics with cell membranes has been reported. These interactions could cause ion leakage and inhibit fungal growth.²⁰ This is an important feature in the baking industry where fungal spoilage of the final products is the most frequent cause of economic losses.^{21,22} Furthermore,

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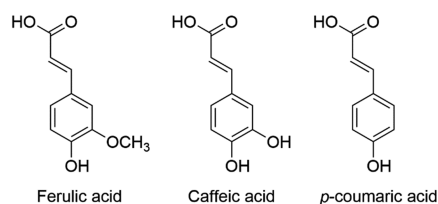
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Hydroxycinnamic derivatives in buckwheat



Hydroxybenzoic derivatives in buckwheat

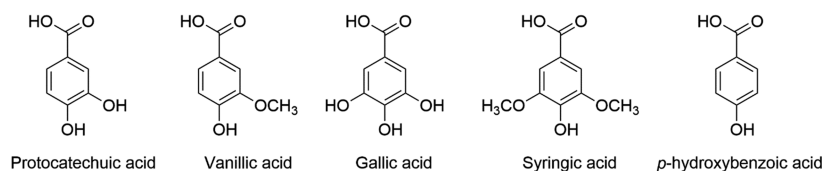


Figure 1. Frequently reported phenolics acids present in buckwheat.

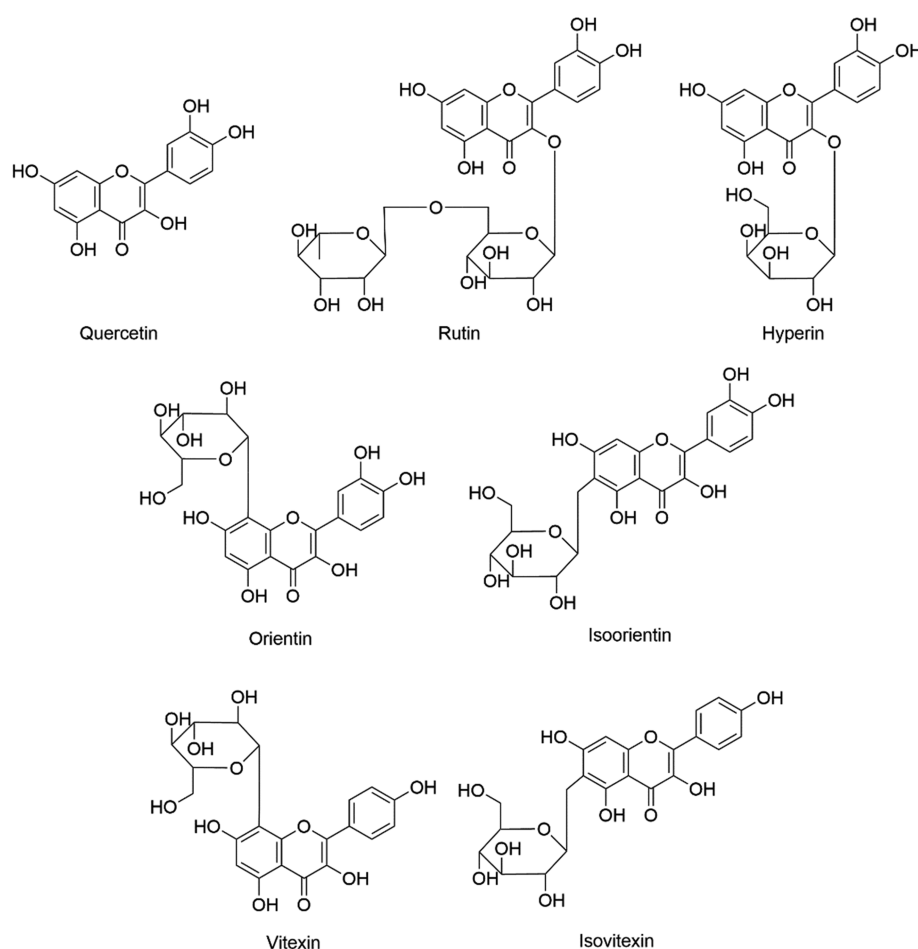


Figure 2. Frequently reported flavonoids in buckwheat.

mycotoxins are a group of dangerous secondary metabolites with severe harmful health effects, and the prevention of mycotoxin contamination of cereal-based products is one of the most important issues discussed.²³ The most common fungi found in cereal and pseudocereal commodities include *Penicillium* (P.), *Aspergillus* (A.), *Fusarium* (F.), and *Eurotium*.^{21,24}

Over recent years, the potential enhancement of antioxidant activity by sourdough fermentation has also gained increasing

attention.²⁵ However, very few studies have focused precisely on improving the bioavailability of buckwheat phenolic compounds, and their contribution to overall inhibitory activities against fungal development and mycotoxin biosynthesis has not yet been studied.

This paper aims to characterize phenolic and lipophilic compounds with antioxidative potential present in buckwheat seeds and describes their potential effect on fungal growth and mycotoxin production. Minimal inhibitory concentrations and

Table 1. Total Phenolic Content and Total Flavonoid Content in Wholegrain and Light Buckwheat Flour

species	source	total phenolic content	total flavonoid content	references
<i>Fag. esculentum</i> Moench	light buckwheat flour	105 mg GAE/100 g		Sedej et al. (2010) ³⁵
<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour	191 GAE/100 g		Sedej et al. (2010) ³⁵
<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour		245–288 mg CE/100 g	Klepacka et al. (2011) ⁴²
<i>Fag. esculentum</i> Moench	light buckwheat flour	179 GAE/100 g		Sensoy et al. (2006) ³⁶
<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour	1047 GAE/100 g		Sensoy et al. (2006) ³⁶
<i>Fag. esculentum</i> Moenchcv. Mankan	whole buckwheat flour	273 mg FAE/100 g	218 mg RE/100 g	Hung et al. (2008) ⁷
<i>Fag. tataricum</i>	light buckwheat flour	876–1643 GAE/100 g	1268–1923 mg RE/100 g	Guo et al. (2011) ⁴³

^aGAE: Gallic acid equivalents, FAE: ferulic acid equivalents, CE: catechin equivalents, RE: rutin equivalents.

possible mechanisms of action are also addressed with a focus on the relationship between lipophilic properties of phenolics and their antimycotic effects. The effect of extraction conditions on the yield of phenolic compounds is discussed. A brief description of changes in phenolic content induced by sourdough fermentation is also included.

■ PHENOLIC COMPOUNDS

Phenolic compounds are secondary metabolites produced by the majority of plants, particularly berries, fruits, vegetables, and cereals, and comprise at least one hydroxyl group attached to one or more benzene rings. The number of hydroxyl groups and benzene rings may differ greatly; therefore, phenolic compounds represent a diverse group of phytochemical substances that can be divided into different types based on their chemical structure.^{26,27} The main groups of buckwheat polyphenols are phenolic acids, flavonoids, tannins, lignans, and stilbenes.^{28–32} Phenolic compounds are classified as phytoanticipins, constitutive plant secondary metabolites with antioxidant and protective effect against both abiotic (UV radiation) and biotic stresses (pathogens).³³

The most abundant phenolic compounds found in buckwheat are phenolic acids (Figure 1) including ferulic, gallic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, syringic and caffeic acids, and flavonoids (Figure 2), mainly rutin, quercetin, hyperin, orientin, isoorientin, vitexin, and isovitexin.^{14,34} Buckwheat phenolic acids predominantly occur in free forms and are unevenly distributed in the pericarp, coat, and grains (endosperm, embryo, two cotyledons), whereas the phenolics of cereal grains are to a great extent bound to cell walls in the hull. On the other hand, buckwheat flavonoids are reported to be linked to cell wall components, with the exception of rutin, which is mainly present in the free form and can be extracted using an ethanol solution.⁷ Consequently, buckwheat extracts exhibit higher antioxidant activities compared to cereal extracts.³⁵ Buckwheat phenolic compounds are concentrated in the outer layers of grains.⁷

The total phenolic content (TPC) in buckwheat flours is within the range from 105 to 1643 mg of gallic acid equivalents (GAE)/100 g (Table 1) depending on the buckwheat cultivar, the type of buckwheat flour, and extraction conditions, particularly the temperature and the solvent.^{28,36,37} Sensoy et al. (2006)³⁶ investigated the effect of roasting (200 °C, 10 min) on TPC in white and dark buckwheat flours and found that changes in TPC induced by roasting were statistically insignificant. However, the antioxidant activity of dark buckwheat flour slightly decreased. Inglett et al. (2010)³⁷ examined the impact of extraction parameters, namely, temperature, type of solvent, and heating method on TPC and antioxidant activity of whole buckwheat flour. The yield of total phenolics generally increased with higher temperatures,

but antioxidant activity did not follow this trend. It was suggested that higher temperature possibly causes degradation of phenolics with high antioxidant activity. Furthermore, 50% ethanol extracts showed the highest yield of TPC, whereas 100% ethanol extracts had the highest antioxidant activities. In addition, Inglett et al. (2011)²⁸ compared the content of free, bound, and total phenolics in commercially available buckwheat flours. It was confirmed that a 50% ethanol solution is the most effective solvent for the extraction of free phenolic acids. The use of absolute ethanol resulted in higher yields of bound phenolics, representing 38–76% of TPC. Hung et al. (2008)⁷ reported that the total phenolic content of buckwheat flour was 273 mg of ferulic acid/100 g of buckwheat flour.

Extraction conditions also affect total flavonoid content (TFC), which varied from 39 to 107 mg of rutin/100 g of buckwheat grains.^{7,28} It was observed that the most effective concentration of ethanol for the extraction of free and bound flavonoids was 50 and 100%, respectively. Temperatures above 75 °C decreased the TFC, possibly caused by the oxidation of flavonoids; therefore, the optimal temperature was recommended to be 75 °C. Finally, the optimal solid to solvent ratio was proposed to be 1:50. Qin et al. (2010)³⁸ reported a higher TFC in Tartary buckwheat flour (665–2274 mg/100 g) compared to common buckwheat flour (67–225 mg/100 g). The experiment was carried out using 70% ethanol solution and a temperature of 70 °C.

Overall, the comparison of TPC and TFC from buckwheat flours should be approached with caution because the various studies used different extraction methodologies and conditions, and the results obtained are expressed in different units either as GAE or FAE for TPC and RE or CE for TFC (Table 1). The most common solvents included ethanol, methanol, or acetone at various concentrations and temperatures. The extraction time and extraction steps varied greatly from a simple, two-step extraction for 15 min up to 24 h repeated four times.

Phenolic Acids. Phenolic acids are primarily phenylpropanoid derivatives of hydroxybenzoic or hydroxycinnamic acid.^{35,39,40} Among the benzoic acid derivatives in buckwheat flours, protocatechuic acid is the most abundant (1.3–7.4 mg/100 g), followed by syringic acid (1.0–1.6 mg/100 g), gallic acid (0.5–2.6 mg/100 g), and vanillic acid (0.2–2.6 mg/100 g).⁴¹ Klepacka et al. (2011)⁴² reported the presence of syringic acid in higher concentrations (7.3–19.1 mg/100 g) and vanillic acid in trace amounts. The concentration of *p*-coumaric acid was also determined (2.1–2.8 mg/100 g). Inglett et al. (2011)²⁸ found free and bound *p*-coumaric, gallic, and caffeic acids in whole buckwheat flour. With regard to bound forms of phenolics acids, 2-hydroxy-3-*O*- β -D-glucopyranosyl-benzoic acid and caffeic acid hexose were identified. Karamać et al.

Table 2. Phenolic Acid and Flavonoid Content in Wholegrain and Light Common and Tartary Buckwheat

	compound	species	source	content [mg/100 g]	refs
hydroxycinnamic acid derivatives	ferulic acid	<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	1.1	Sedej et al. (2010) ³⁵
		<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	1.4	Sedej et al. (2010) ³⁵
		<i>Fag. tataricum</i>	light buckwheat flour ^a	1.9–7.3	Guo et al. (2011) ⁴³
	caffeic acid	<i>Fag. tataricum</i>	light buckwheat flour ^a	0.1–0.5	Guo et al. (2011) ⁴³
	coumaric acid	<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	2.1–2.8	Klepacka et al. (2011) ⁴²
hydroxybenzoic acid derivatives	protocatechuic acid	<i>Fag. tataricum</i>	light buckwheat flour ^a	0.3–1.0	Guo et al. (2011) ⁴³
		<i>Fag. esculentum</i> Moench	Light buckwheat flour ^a	1.3–3.3	Mikulajová et al. (2016) ⁴¹
		<i>Fag. tataricum</i>	light buckwheat flour ^a	4.0–7.4	Mikulajová et al. (2016) ⁴¹
	vanillic acid	<i>Fag. tataricum</i>	light buckwheat flour ^a	2.1–4.6	Guo et al. (2011) ⁴³
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.2–1.2	Mikulajová et al. (2016) ⁴¹
		<i>Fag. tataricum</i>	light buckwheat flour ^a	0.5–2.7	Mikulajová et al. (2016) ⁴¹
		<i>Fag. tataricum</i>	light buckwheat flour ^a	0.2–1.6	Guo et al. (2011) ⁴³
	gallic acid	<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.5–1.3	Mikulajová et al. (2016) ⁴¹
		<i>Fag. tataricum</i>	light buckwheat flour ^a	1.0–2.6	Mikulajová et al. (2016) ⁴¹
		<i>Fag. tataricum</i>	light buckwheat flour ^a	0.5–0.6	Guo et al. (2011) ⁴³
	syringic acid	<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	1.0–1.6	Mikulajová et al. (2016) ⁴¹
		<i>Fag. tataricum</i>	light buckwheat flour ^a	1.3–1.5	Mikulajová et al. (2016) ⁴¹
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	7.3–19.1	Klepacka et al. (2011) ⁴²
		<i>Fag. tataricum</i>	light buckwheat flour ^a	0.1–0.2	Guo et al. (2011) ⁴³
	hydroxybenzoic acid	<i>Fag. tataricum</i>	light buckwheat flour ^a	3.1–5.7	Guo et al. (2011) ⁴³
		<i>Fag. esculentum</i> Moench	Light buckwheat flour ^a	8.2	Sedej et al. (2010) ³⁵
flavones	rutin	<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	17.9	Sedej et al. (2010) ³⁵
		<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	12.6–35.9	Kitabayashi et al. (1995) ⁵⁰
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	79.2	Tian et al. (2002) ⁵¹
		<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	11.6–18.2	Kreft et al. (2006) ⁴⁷
		<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	21.9	Kreft et al. (2006) ⁴⁷
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	11.3	Kreft et al. (2006) ⁴⁷
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	14.7	Lee et al. (2016) ⁵²
		<i>Fag. tataricum</i>	light buckwheat flour ^a	1077.0	Lee et al. (2016) ⁵²
		<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	15.0–168.0	Qin et al. (2010) ³⁸
		<i>Fag. tataricum</i>	wholegrain buckwheat flour ^b	606.0–1867.0	Qin et al. (2010) ³⁸
flavones	rutin	<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	6.0–30.5	Mikulajová et al. (2016) ⁴¹
		<i>Fag. tataricum</i>	light buckwheat flour ^a	16.2–373.7	Mikulajová et al. (2016) ⁴¹
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.1	Sedej et al. (2010) ³⁵
		<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	0.4	Sedej et al. (2010) ³⁵
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	21.2	Tian et al. (2002) ⁵¹
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	ND	Lee et al. (2016) ⁵²
	quercetin	<i>Fag. tataricum</i>	light buckwheat flour ^a	127.0	Lee et al. (2016) ⁵²
		<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	5.0–9.0	Qin et al. (2010) ³⁸
		<i>Fag. tataricum</i>	wholegrain buckwheat flour ^b	31.0–238.0	Qin et al. (2010) ³⁸
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.1–2.7	Mikulajová et al. (2016) ⁴¹
		<i>Fag. tataricum</i>	light buckwheat flour ^a	1.2–31.4	Mikulajová et al. (2016) ⁴¹
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.27	Sedej et al. (2010) ³⁵
lipophilic compounds	α -tocopherol	<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	0.86	Sedej et al. (2010) ³⁵
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.09	Zieliński et al. (2006) ¹⁰⁶
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	1.72	Sedej et al. (2010) ³⁵
	γ -tocopherol	<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	2.53	Sedej et al. (2010) ³⁵
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	5.14	Zieliński et al. (2006) ¹⁰⁶
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.01	Sedej et al. (2010) ³⁵
	δ -tocopherol	<i>Fag. esculentum</i> Moench	wholegrain buckwheat flour ^b	0.05	Sedej et al. (2010) ³⁵
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.24	Zieliński et al. (2006) ¹⁰⁶
		<i>Fag. esculentum</i> Moench	light buckwheat flour ^a	0.24	Zieliński et al. (2006) ¹⁰⁶

^aDehulled buckwheat seeds were ground into flour. ^bWhole buckwheat seeds were ground into flour.

(2015)⁴⁰ determined the concentration of phenolic acids in buckwheat seed fractions and gallic, *p*-coumaric, caffeic, and 5-*O*-caffeoylquinic acids were discovered.

In conclusion, numerous studies have focused on the composition of phenolic acids in buckwheat grains and flours,

but significant differences between published data were observed. This may be due to the use of different extraction procedures, analytical methods, and laboratory equipment. Additionally, the levels of phenolic acids in buckwheat are dependent on species and cultivar. For example, in comparison

Table 3. IC₅₀, IC₁₀₀, and MIC for *Fusarium*, *Aspergillus* and *Penicillium* Species

compound	fungus species (strains)	IC ₅₀ (mmol/L or kg)	IC ₁₀₀ (mmol/L or kg)	MIC (mmol/L or kg)	refs
ferulic acid	<i>F. graminearum</i> (CBS185.32; INRA 202; 245AP4; INRA159)	0.7–2.2			Ponts et al. (2011) ⁵⁶
	<i>F. graminearum</i> (Z3639)	1.8			Bollina et al. (2011) ⁷⁷
	<i>F. graminearum</i> Schwabe (15–35)	2.4			Kumaraswamy et al. (2011) ⁵⁸
	<i>F. graminearum</i> (Butte 86; Roblin)	2.3–3.4			McKeehen et al. (1999) ¹⁰⁷
	<i>F. culmorum</i> (89.4)	1.7			McKeehen et al. (1999) ¹⁰⁷
	<i>F. coeruleum</i> , <i>F. moniliforme</i> , <i>F. solani</i> (not specified)	5.2			Guiraud et al. (1995) ⁶⁵
	<i>F. oxysporum</i> (not specified)	0.2	>51.5		Zabka et al. (2013) ²⁷
	<i>F. verticillodes</i> (not specified)	0.2	>51.5		Zabka et al. (2013) ²⁷
	<i>P. brevicompactum</i> (not specified)	0.4	>51.5		Zabka et al. (2013) ²⁷
	<i>P. expansum</i> (not specified)	0.2	>51.5		Zabka et al. (2013) ²⁷
	<i>A. flavus</i> (not specified)	0.5	>51.5		Zabka et al. (2013) ²⁷
	<i>A. fumigatus</i> (not specified)	0.3	>51.5		Zabka et al. (2013) ²⁷
<i>p</i> -coumaric acid	<i>F. graminearum</i> (CBS185.32; INRA 202; 245AP4; INRA159)	1.0–4.2			Ponts et al. (2011) ⁵⁶
	<i>F. graminearum</i> (Z3639)	1.2			Bollina et al. (2011) ⁷⁷
	<i>F. graminearum</i> (Butte 86; Roblin)	1.9–4.8			McKeehen et al. (1999) ¹⁰⁷
	<i>F. culmorum</i> (89.4)	3.4			McKeehen et al. (1999) ¹⁰⁷
caffeic acid	<i>F. graminearum</i> (CBS185.32; INRA 202; 245AP4; INRA159)	4.0–7.1			Ponts et al. (2011) ⁵⁶
	<i>F. graminearum</i> Schwabe (15–35)	2.5			Kumaraswamy et al. (2011) ⁵⁸
	<i>F. graminearum</i> (CBS 185.32; Fg 156; Fg 605; 245AP4; Fg91; Fg183)	6.7–10.1			Gauthier et al. (2016) ⁸⁷
	<i>F. culmorum</i> (Fc 130; Fc 337; Fc 319; MCf 21; FC124; Fc233; Fc305)	8.8–10.0			Gauthier et al. (2016) ⁸⁷
	<i>F. oxysporum</i> , <i>F. verticillodes</i> , <i>P. brevicompactum</i> , <i>P. expansum</i> , <i>A. flavus</i> , <i>A. fumigatus</i> (not specified)	>51.5			Zabka et al. (2013) ²⁷
	<i>A. flavus</i> , <i>A. parasiticus</i> (not specified)		11.1		Aziz et al. (1998) ⁶³
gallic acid	<i>F. oxysporum</i> , <i>F. verticillodes</i> , <i>P. brevicompactum</i> , <i>P. expansum</i> , <i>A. flavus</i> , <i>A. fumigatus</i> (not specified)	>51.5			Zabka et al. (2013) ²⁷
syringic acid	<i>F. graminearum</i> (CBS185.32; INRA 202; 245AP4; INRA159)	3.5–6.2			Ponts et al. (2011) ⁵⁶
	<i>F. coeruleum</i> , <i>F. moniliforme</i> , <i>F. solani</i> (not specified)	>5,0			Guiraud et al. (1995) ⁶⁵
	<i>F. oxysporum</i> , <i>F. verticillodes</i> , <i>P. brevicompactum</i> , <i>P. expansum</i> , <i>A. flavus</i> , <i>A. fumigatus</i> (not specified)	>51.5			Zabka et al. (2013) ²⁷
	<i>A. flavus</i> , <i>A. parasiticus</i> (not specified)		15.1		Aziz et al. (1998) ⁶³
hydroxybenzoic acid	<i>F. oxysporum</i> f. sp. <i>Niveum</i> (not specified)			1.5	Wu et al. (2009) ¹⁰⁸
protocatechuic acid	<i>F. graminearum</i> (CBS185.32; INRA 202; 245AP4; INRA159)	8.4–10.3			Ponts et al. (2011) ⁵⁶
	<i>F. coeruleum</i> , <i>F. moniliforme</i> , <i>F. solani</i> (not specified)	>6.5			Guiraud et al. (1995) ⁶⁵
vanillic acid	<i>A. flavus</i> , <i>A. parasiticus</i> (not specified)		19.5		Aziz et al. (1998) ⁶³
	<i>F. coeruleum</i> , <i>F. moniliforme</i> , <i>F. solani</i> (not specified)	>6.0			Guiraud et al. (1995) ⁶⁵
rutin	<i>A. flavus</i> , <i>A. parasiticus</i> (not specified)		11.9		Aziz et al. (1998) ⁶³
	<i>Candida albicans</i> (PC31)			3.6	Tempesti et al. (2012) ⁶⁶
	<i>Fusarium graminearum</i> (FG 13192), <i>F. poae</i> (FP 13045)	>1.6			Schöneberg et al. (2018) ²⁴
	<i>F. langsethiae</i> (FL 13005; FL 13014; FL 14001)			0.002	Schöneberg et al. (2018) ²⁴
quercetin	<i>Fusarium graminearum</i> . (FG 13192), <i>F. poae</i> (FP 13045)	>3.3			Schöneberg et al. (2018) ²⁴
	<i>F. langsethiae</i> (FL 13005; FL 13014; FL 14001)			0.003	Schöneberg et al. (2018) ²⁴
	<i>F. graminearum</i> (Z3639)				Bollina et al. (2011) ⁷⁷
	<i>A. flavus</i> (CGMCC3.6434)			1.7	Li et al. (2019) ¹⁰⁹

^aIC₅₀: Half maximal inhibitory concentration; IC₁₀₀: Maximal inhibitory concentration; MIC: Minimal inhibitory concentration.

with common buckwheat, Tartary buckwheat generally contains larger amounts of ferulic, caffeic, protocatechuic, and gallic acids, although in the work of Guo et al. (2011),⁴³ the concentration of gallic acid found in Tartary buckwheat (0.5–0.6 mg/100 g) was lower than the gallic acid content (0.5–1.4 mg/100 g) of common buckwheat as reported by Mikulajová et al. (2016).⁴¹ Nonetheless, many reports recognize buckwheat as an important source of phenolic acids and confirm that the levels of phenolic acids in buckwheat grains are considerably higher compared to other cereals, also resulting in higher levels of antioxidant activity.^{35,42,44}

Flavonoids. The basic structural backbone of a flavonoid is characterized by the presence of two aromatic rings linked by three carbon rings, also called the “flavan nucleus”. According to hydroxylation patterns and modifications in the chromane ring, flavonoids are divided into different groups, namely, flavonols, flavones, flavanols, and anthocyanins.⁴⁵ Numerous studies focused on the identification and quantification of buckwheat flavonoids, and rutin, quercetin, anthocyanins, orientin, isoorientin, vitexin, and isovitexin were frequently reported.⁴⁶ Buckwheat flavonoids are generally bound to the cell wall components of the seed with the exception of rutin, which is found mainly in the free form.⁷ Over the last decades, beneficial health effects of buckwheat have been shown to be associated with the high content of rutin and quercetin, and recently, efforts to study the antifungal activity of these flavonoids have emerged.^{17,47–49}

The rutin content in common buckwheat groats was reported to be in the range of 6.0–168.0 mg/100 g (Table 2), whereas the concentration of rutin in Tartary buckwheat groats was 10 times higher.^{47,50–52} Qin et al. (2010)³⁸ compared the rutin content of common and Tartary buckwheat flours of 39 buckwheat cultivars and confirmed that a Tartary buckwheat flour is a significantly better source of flavonoids, as the concentration differed greatly for common buckwheat flour (15.0–168.0 mg/100 g) and Tartary buckwheat flour (606.0–1867.0 mg/100 g). The aglycon derived from rutin, known as quercetin, was also found both in common and Tartary buckwheat flours, but the concentrations are significantly lower (common buckwheat: 0.1–21.2 mg/100 g; Tartary buckwheat: 1.2–238 mg/100 g).

Lipophilic Compounds. The main lipophilic compounds that exhibit significant antioxidant effects in buckwheat are tocopherols characterized by the presence of a chromanol ring attached to the C16 phytol chain. The total concentration of these secondary metabolites in whole buckwheat flour can reach 5.47 mg/100 g (Table 2).^{35,44} Both studies also revealed the presence of the γ -form in the highest concentration (0.17–5.14 mg/100 g) followed by α - (0.09–0.86 mg/100 g) and δ -forms (0.01–0.24 mg/100 g). In addition, Sedej et al. (2010) compared the tocopherol levels of wheat and buckwheat flours and reported a higher total tocopherol content in buckwheat flour, although whole wheat flour contained a higher concentration of α -tocopherol.

■ ANTIFUNGAL AND ANTIMYCOTOXIN ACTIVITIES

In recent years, efforts have been made to understand the role of antioxidants in plant defense against fungal contamination. Numerous studies have focused on the characterization of secondary metabolites of plants (such as cereals and pseudocereals) including polyphenols and lipophilic compounds and their potential use against fungal development and

mycotoxin production.^{17,19,53} Polyphenols and lipophilic compounds reduce oxidative stress, which is an important factor in the stimulation of mycotoxin biosynthesis, thus potentially modulating the levels of these harmful substances.^{48,54}

Phenolic Acids. Sardi et al. (2016)⁵⁵ assessed the antifungal efficacy of caffeic acid and its derivatives against 10 strains of *Candida spp.*; however, the minimal inhibitory concentrations (MIC) for caffeic acid were not reported. In addition, Zabka et al. (2013)²⁷ reported that the half maximal inhibitory concentration (IC₅₀) of caffeic acid, syringic acid, and gallic acid against *F. oxysporum*, *F. verticillioides*, *P. brevicompactum*, *P. expansum*, *A. flavus*, and *A. fumigatus* was higher than 51.5 mmol/L and the IC₅₀ of ferulic acid varied from 0.2 to 0.5 mmol/L depending on the fungus (Table 3), but the number of strains was not mentioned. The effect of caffeic acid against *F. graminearum* and *F. culmorum* was also studied by Ponts et al. (2011)⁵⁶ (four strains of *F. graminearum*), Gauthier et al. (2016)⁵⁷ (six strains of *F. graminearum* and seven strains *F. culmorum*), and Kumaraswamy et al. (2011)⁵⁸ (the number of *F. graminearum* strains was not specified). Several studies described the antimicrobial potential of protocatechuic acid against both Gram-positive and Gram-negative bacteria and the antifungal effect of various extracts from trees, almonds, and winery byproducts, but very few studies have focused on the evaluation of the antifungal activity of protocatechuic acid alone.^{59–61} For example, the activity of protocatechuic acid derived from onion scales against *Colletotrichum circinans* was reported.⁶² Aziz et al. (1998)⁶³ obtained full inhibition of *A. flavus* and *A. parasiticus* growth and aflatoxin production with 19.5 mmol/L of protocatechuic and syringic acids and 15.1 mmol/L of vanillic and caffeic acids. With emphasis on phenolic acids, extensive research has been conducted to explain the mechanism of action against *Candida* species.⁶⁴

Because of the variability in reported data associated with the application of different methodologies and fungal strains, the interpretation and comparison of results should be undertaken with caution. For example, according to Kumaraswamy et al. (2011),⁵⁸ Ponts et al. (2011),⁵⁶ and Gauthier et al. (2016),⁵⁷ caffeic acid affected the growth of *F. graminearum* with IC₅₀ values of 2.5–10.1 mmol/L but had no impact on the development of *F. oxysporum* and *F. verticillioides* when concentrations up to 51.5 mmol/L were used in the work of Zabka et al. (2013).²⁷ Furthermore, the actual levels of phenolic acids should be considered when assessing the potential antifungal properties of buckwheat. For instance, in the work of Sedej et al. (2010),³⁵ whole buckwheat flour contained 1.4 mg of ferulic acid/100 g of flour; however, the IC₅₀ values against *F. coeruleum* reported by Guiraud et al. (1995)⁶⁵ were 5.2 mmol/L (101.0 mg/100 mL of medium). When comparing the results for *F. graminearum*, ferulic acid displayed the highest antifungal activity followed by caffeic, syringic, and protocatechuic acids. Moreover, ferulic and protocatechuic acids successfully inhibited the growth of *Aspergillus* strains (Table 3). These results support the hypothesis that the antifungal properties of phenolic acids correlate with their lipophilic character as ferulic acid displays the highest fungicidal efficiency.^{56,65}

Overall, the inhibitory effect of single buckwheat phenolic acids against fungi varies greatly, and full inhibition cannot be achieved with concentrations present in buckwheat flours. However, when the antifungal effects of phenolic acids are

combined, they possibly contribute to the overall antifungal activity of buckwheat resulting in enhanced shelf life of bakery products.^{27,40,42}

Flavonoids. The antifungal activity of rutin against *Candida albicans* was demonstrated with an IC₅₀ value of 3.6 mmol/L of medium.⁶⁶ Schöneberg et al. (2018)²⁴ discovered that rutin at concentrations from 0.0002 to 0.1638 mmol/L of media stimulated the growth of *F. graminearum* and *F. poae*, and inhibited the growth of *F. langsethiae*. The same trend was observed also for quercetin. In addition, inhibition of *A. flavus* was achieved with a quercetin MIC value of 1.7 mmol/L of medium. Regarding the question of how flavonoids interact with fungi, some studies attribute the antifungal properties of flavonoids to their ability to incorporate into the lipid membrane followed by depolarization of the cell membranes causing cell death.^{67,68} In conclusion, the IC values of flavonoids against fungi are also dependent on the method and fungal strains used, and further research is required to fully understand the mechanism of their action.

■ INHIBITION OF MYCOTOXIN SYNTHESIS

As mentioned above, fungal growth is in many cases associated with the production of harmful secondary metabolites called mycotoxins. The crucial factor that stimulates the biosynthesis of these compounds is oxidative stress, e.g., the presence of hydrogen peroxide and other (ROS). Therefore, naturally occurring antioxidants in buckwheat may potentially modulate the synthesis of mycotoxins.^{54,69}

Phenolic Acids. The successful inhibition of Type B trichothecene (TBTC) synthesis in *F. graminearum* and *F. culmorum* was observed when hydroxycinnamic derivatives such as caffeic, sinapic, and ferulic acids along with syringic acid were applied. In contrast, benzoic acid derivatives promoted mycotoxin formation.⁷⁰ The inhibitory effect of caffeic and chlorogenic acid at a concentration of 0.5 mM against *F. graminearum* and *F. culmorum* was confirmed in the report of Gauthier et al. (2016)⁵⁷ who also found that the impact on fungal growth and mycotoxin production depended on the fungal strain. The concentration of TBTC was reduced in 10 out of 13 strains.⁵⁷ The study of Boutigny et al. (2009)⁷¹ reported a positive correlation between the inhibitory effect of ferulic acid and its increasing concentration (0–5 mM). Full inhibition of mycotoxin synthesis was achieved at 2.5 mM of ferulic acid.⁷¹ When addressing the topic of inhibition of mycotoxin metabolism, TBTC is not the sole subject of interest. Various studies also focused on the biosynthetic modulation of other mycotoxins such as fumonisins, ochratoxins, and aflatoxins.^{53,72–75}

Flavonoids. Among flavonoids, the potential effect of rutin, quercetin, and kaempferol against mycotoxin production in *Aspergillus* and *Penicillium* species was demonstrated. For instance, Chitarrini et al. (2014)¹⁹ compared the antimycotoxin impact of rutin (3.28 mM) *in vivo* and suggested that a Tartary buckwheat rich in antioxidants was more resistant to *A. flavus* contamination. Furthermore, the metabolic conversion of rutin to quercetin and a higher antifungal efficiency of quercetin were observed.¹⁹ Kaempferol was found to be efficient inhibitor of aflatoxin B1 synthesis by *A. flavus*.⁷⁵ In addition, quercetin was also proved to negatively impact patulin production in *P. expansum* (42% reduction of mycotoxin at 16.5 mM)⁷⁶ and trichothecene biosynthesis in *F. culmorum* (full inhibition at 2.5 mM).⁷⁷

Tocopherols. Tocopherols are another group of antioxidants in buckwheat with the potential ability to restrain inhibit mycotoxin production. The promising inhibitory effect on fumonisin synthesis in *F. verticillioides* was obtained when α -tocopherol was added in the medium at sublethal doses (0.1 mmol/L).⁷⁸

In conclusion, there are only a few studies that have focused on the inhibitory effect of cereal and pseudocereal antioxidants on mycotoxin synthesis, especially the effects of tocopherols.

■ MECHANISM OF MYCOTOXICITY

Despite the fact that numerous papers examined the antimycotic impact of buckwheat secondary metabolites with antioxidant properties, particularly, phenolics and tocopherols, the precise mechanisms of action of these naturally occurring substances are still unclear. Over recent years, several hypotheses have been proposed suggesting that one of the most important factors is the hydrophobic character of phenolic compounds, although other potential mechanisms are likely to contribute to overall antifungal activity.^{27,79} For instance, Ultee et al. (2002)⁸⁰ suggested that the presence of the hydroxyl group was the crucial factor for the toxicity of phenolic compounds. Detachment of the H⁺ ion from the –OH group enables carvacrol to transfer H⁺ and other cations such as K⁺ through the membrane, causing disruption of the proton gradient and eventually cell death. In addition, Rao et al. (2010)⁶⁷ found that the presence of carvacrol induced a stress response similar to calcium stress. The inhibitory mechanisms of phenolic acids has also been studied and according to Morales et al. (2017),⁷⁹ *p*-coumaric acid possibly modulates oxidative phosphorylation. Numerous studies have focused on inhibitory effects against *Candida* species, which is the main group of human pathogenic fungi (for review, see Teodoro et al. (2015)⁶⁴).

The effect of phenolic acids against plant fungi has also been the subject of study. The inhibitory activities of phenolic acids are associated with their lipophilic properties, and this hypothesis was confirmed in many works where ferulic acid exhibited the highest antifungal activity against *Fusarium*, *Penicillium*, and *Aspergillus* species.^{27,71,73} In addition, antioxidant properties of phenolic acids might affect fungal development and mycotoxin production that is induced predominantly by oxidative stress. This theory was supported by Chitarrini et al. (2014)¹⁹ for *A. flavus* and by Pani et al. (2014)⁸¹ for *F. culmorum*, although no linear correlations between antioxidant properties and inhibition of fungal development or mycotoxin biosynthesis were discovered. This suggests that the mechanism of action is a complex process and that further research is required to fully understand it. Moreover, Palumbo et al. (2007) tested the inhibitory effect of gallic, vanillic, caffeic, protocatechuic, chlorogenic, and *p*-hydroxybenzoic acids against *A. alliaceus*, *A. lanosus*, *A. ochraceus*, *A. albertensis*, *A. melleus*, *A. sulphureus*, *A. carbonarius*, and *A. elegans*, and discovered that vanillic and *p*-hydroxybenzoic acid were the most effective inhibitors. They proposed that the antimycotic effect of phenolic acids may be linked to primary (phenolic acids affecting the fungal growth) or secondary metabolism or both.

Montibus et al. (2015)⁵⁴ reviewed discoveries related to a possible linkage between oxidative stress and secondary metabolism of fungi and further supported the hypothesis that the biosynthesis of secondary metabolites such as mycotoxins is connected to oxidative stress. The mechanism

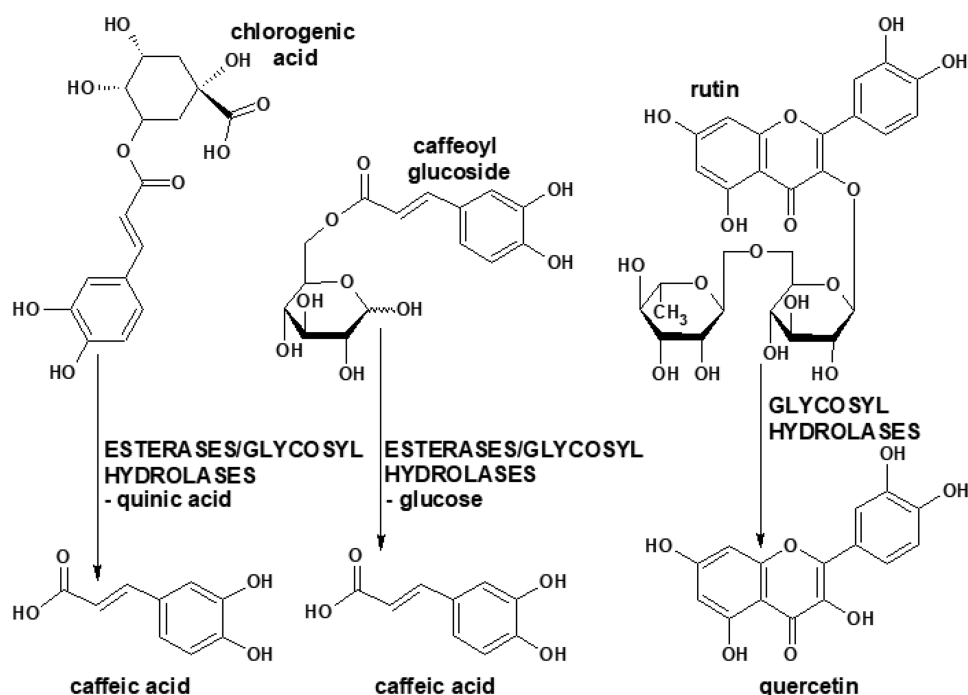


Figure 3. Metabolism of glycosylated polyphenols by LAB.^{90–93}

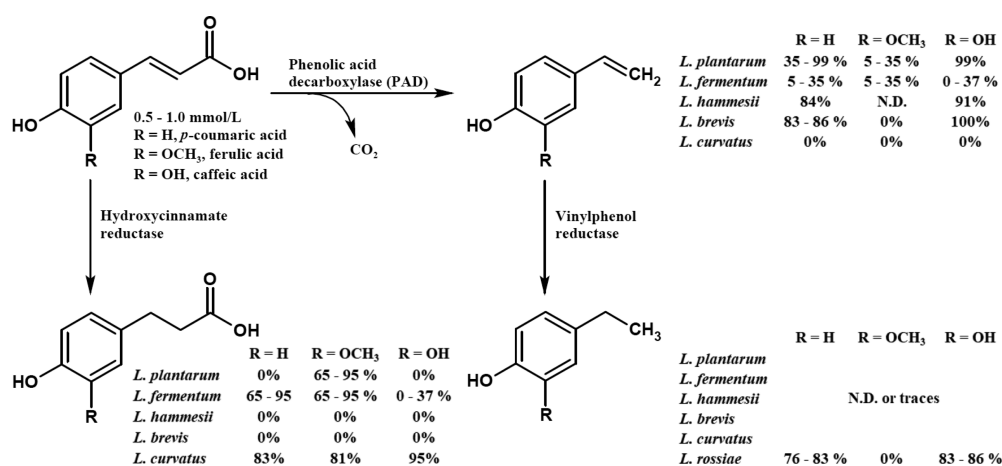


Figure 4. Metabolism of Free Polyphenols by LAB.^{89,93–96}

remains unknown, however, the study of Reverberi et al. (2008)⁶⁹ revealed that oxidative stress modulates aflatoxin synthesis at the transcription level. When *Aspergillus* was exposed to ROS, the antioxidant-producing gene *ApyapA* was activated, leading to the production of antioxidant enzymes. Once the concentration of ROS exceeded the antioxidant capacity of the enzymes, aflatoxin production was triggered. Similar findings were reported by other researchers, indicating that phenolic acids and flavonoids regulate gene expression responsible for mycotoxin synthesis.^{76,82} Recently, an *A. flavus* mutant without a core protein of the catalase enzyme (CTA) was generated by Zhu et al. (2020).¹⁵ This mutant showed significantly higher levels of ROS in the cells, and a decrease in aflatoxin synthesis was observed. Zhu et al. (2020)¹⁵ suggested that CTA could be involved in maintaining ROS at concentrations that are suitable for effective mycotoxin synthesis. Moreover, Guan et al. (2019)⁸³ generated *Afap*-deletion mutants of *A. flavus* and confirmed that *Afap*, a leucine zipper transcription factor, is involved in the oxidative

stress response and aflatoxin synthesis. These mutants showed increased sensitivity to H₂O₂ and a significant (approximately 75%) decrease in aflatoxin production.

The hypothesis that certain types of phytochemicals could reduce fungal development and mycotoxin production seems valid. The assumption that buckwheat phenolics inhibit fungal growth is based on the evidence that phenolic acids and flavonoids display hydrophobic properties and are likely to interact with the lipid membranes causing ion leakage and possibly disruption of the cell membrane. In addition, oxidative stress and ROS stimulate mycotoxin production at the transcriptional level, and buckwheat phenolics and tocopherols have ability to scavenge ROS. On the one hand, this effect is beneficial considering the adverse effects of mycotoxins on human health. On the other hand, because of the scavenging abilities of buckwheat phytochemicals, reduced oxidative stress supports the fungal development. Nevertheless, the link between secondary metabolites with antioxidant properties and oxidative stress is far from being clarified, and extensive

research on gene expression during oxidative stress and the potential role of plant antioxidants in this complex process must be conducted.

■ BUCKWHEAT PHENOLICS AND LACTIC ACID BACTERIA

One of the major issues discussed in recent papers is how to improve the bioavailability of phytochemical substances in cereals and pseudocereals. In the baking industry, milling, malting, and fermentation are the main processes that remarkably affect the biological activity of phenolics, and sourdough fermentation has proven to be the most effective.²⁵ Sourdough is a mixture of flour, water, and possibly other ingredients fermented by lactic acid bacteria (LAB) and yeasts. LAB have the capacity to metabolize phenolics including phenolic acids and flavonoids that exhibit antibacterial activities.⁸⁴ The metabolism of these compounds is a primary tool for detoxification and a prerequisite to survive in plant matrices under difficult environmental conditions.^{85–87} The survivability of LAB is dependent on the enzymatic arsenal of particular species and strains.⁸⁸ While some LAB have the ability to release phenolic acids or flavonoids from their glycosylated forms, certain LAB are capable of further degradation of these phytochemicals into vinyl or ethyl derivatives (Figure 3 and Figure 4). For instance, in the work of Ripari et al. (2019),⁸⁹ 18 out of 144 strains of *Lactobacillus* (*L.*) *plantarum* possessed a phenolic acid decarboxylase gene (*pdc*); however, only one strain was found to metabolize ferulic acid into ethyl-guaiacol. The most common metabolic products included vinyl-guaiacol and dihydroferulic acid, which resulted from ferulic acid decarboxylase activity.⁸⁹ The genes encoding an esterase,^{90–93} a decarboxylase,^{89,93,94} and a reductase^{89,94–96} responsible for degradation of hydroxycinnamic acids in *L. plantarum* were recently identified as well.

The potential enhancement of biological activities of phenolics has been the subject of interest in many studies. In the study of Rizzello et al. (2012),⁹⁷ the total phenolic content of sourdough wheat dough was higher compared to a standard wheat dough without sourdough, although the antioxidant activity did not follow the trend. Moreover, Katina et al. (2007)⁹⁸ reported significantly higher levels of free phenolic acids (up to 11-fold) in germinated and fermented wholemeal rye flour compared to native rye wholemeal flour. Đorđević et al. (2010)⁹⁹ described the fermentation of buckwheat that resulted in an increased extractability of phenolic compounds resulting in higher total phenolic content and antioxidant activity. With emphasis on bakery products, rye and wheat sourdough bread exhibited significantly higher antioxidant properties compared to yeast bread.¹⁰⁰ Similarly, the antioxidant activity of sourdough buckwheat muffins was remarkably higher in comparison with nonfermented analogs.¹⁰¹

Numerous studies have also devoted their attention to the metabolism of specific phenolic compounds such as ferulic acid or quercetin.^{93,100,102–104} For example, Konopka et al. (2014)¹⁰⁰ evaluated rye and wheat ferulic acid content during sourdough fermentation, and the release of bound ferulic acid was observed, resulting in an increase in free ferulic acid up to 13.5%. Contrary to these findings, reports focusing on the metabolism of LAB have described the further degradation of phenolics by LAB with esterase, reductase, and decarboxylase activities.⁹³ As a result, a decrease in free phenolic acids has

also been associated with sourdough fermentation.^{103,104} Nonetheless, according to Svensson et al. (2010),⁹³ the antimicrobial activity of sorghum flour extracts remained unchanged after fermentation despite the fact that phenolic acids and flavonoids were metabolized. In contrast, some studies reported either enhancement or a decline in antimicrobial activity.^{84,105} It should be noted that these studies focused solely on the antibacterial activities of phenolics in sourdough fermentation, and antifungal properties of these compounds should still be investigated.

In conclusion, sourdough fermentation represents a potential tool to increase the antioxidant properties and consequently the antifungal activity of buckwheat sourdough, which could result in an increased shelf life of bakery products. Nonetheless, more data concerning the inhibitory effect of phenolics against plant fungi in sourdough technology should be sought as no published data on this subject has been found. One possible explanation is the complexity of the sourdough antifungal effect. LAB produce organic acids including lactic and acetic acid along with other antifungal agents such as hydrogen peroxide, ethanol, azelaic acid, and others.²¹ Therefore, evaluating the inhibitory impact of phenolics against fungal development is a complicated task. Limited studies have been carried out on the enhancement of antioxidant activity of buckwheat phenolics during sourdough fermentation. Future efforts should be focused on the optimization of this process to ensure a maximum yield of biologically active phenolic compounds and to understand their contribution to the antifungal effect of sourdough.

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ABBREVIATIONS USED

Fag, *Fagopyrum*; ROS, reactive oxygen species; F, *Fusarium*; P., *Penicillium*; A., *Aspergillus*; TPC, total phenolic content; TFC, total flavonoid content; GAE, gallic acid equivalents; FAE, ferulic acid equivalents; CE, catechin equivalents; RE, rutin equivalents; LAB, lactic acid bacteria; L., *Lactobacillus*

REFERENCES

- (1) Ahmed, A.; Khalid, N.; Ahmad, A.; Abbasi, N. A.; Latif, M. S. Z.; Randhawa, M. A. Phytochemicals and Biofunctional Properties of Buckwheat: A Review. *J. Agric. Sci.* **2014**, *152* (3), 349–369.
- (2) Ohsako, T.; Li, C. Classification and Systematics of the *Fagopyrum* Species. *Breed. Sci.* **2020**, *70* (1), 93–100.
- (3) Li, S. Q.; Zhang, Q. H. Advances in the Development of Functional Foods from Buckwheat. *Crit. Rev. Food Sci. Nutr.* **2001**, *41* (6), 451–464.
- (4) FAOSTAT. Buckwheat production. <http://www.fao.org/faostat/en/#data/QC/visualize> (accessed Jun 9, 2020).
- (5) Bonafaccia, G.; Marocchini, M.; Kreft, I. Composition and Technological Properties of the Flour and Bran from Common and Tartary Buckwheat. *Food Chem.* **2003**, *80* (1), 9–15.
- (6) Pongrac, P.; Vogel-Mikuš, K.; Jeromel, L.; Vavpetič, P.; Pelicon, P.; Kaulich, B.; Gianoncelli, A.; Eichert, D.; Regvar, M.; Kreft, I. Spatially Resolved Distributions of the Mineral Elements in the Grain of Tartary Buckwheat (*Fagopyrum Tataricum*). *Food Res. Int.* **2013**, *54* (1), 125–131.
- (7) Van Hung, P.; Morita, N. Distribution of Phenolic Compounds in the Graded Flours Milled from Whole Buckwheat Grains and Their Antioxidant Capacities. *Food Chem.* **2008**, *109* (2), 325–331.
- (8) Zheng, C.; Hu, C.; Ma, X.; Peng, C.; Zhang, H.; Qin, L. Cytotoxic Phenylpropanoid Glycosides from *Fagopyrum Tataricum* (L.) Gaertn. *Food Chem.* **2012**, *132* (1), 433–438.
- (9) Karki, R.; Park, C.-H.; Kim, D.-W. Extract of Buckwheat Sprouts Scavenges Oxidation and Inhibits Pro-Inflammatory Mediators in Lipopolysaccharide-Stimulated Macrophages (RAW264.7). *J. Integr. Med.* **2013**, *11* (4), 246–252.
- (10) Yang, N.; Li, Y. M.; Zhang, K.; Jiao, R.; Ma, K. Y.; Zhang, R.; Ren, G.; Chen, Z.-Y. Hypocholesterolemic Activity of Buckwheat Flour Is Mediated by Increasing Sterol Excretion and Down-Regulation of Intestinal NPC1L1 and ACAT2. *J. Funct. Foods* **2014**, *6*, 311–318.
- (11) Yao, Y.; Shan, F.; Bian, J.; Chen, F.; Wang, M.; Ren, G. D-Chiro-Inositol-Enriched Tartary Buckwheat Bran Extract Lowers the Blood Glucose Level in KK-Ay Mice. *J. Agric. Food Chem.* **2008**, *56* (21), 10027–10031.
- (12) Giménez-Bastida, J. A.; Zieliński, H. Buckwheat as a Functional Food and Its Effects on Health. *J. Agric. Food Chem.* **2015**, *63* (36), 7896–7913.
- (13) Lee, M. T.; Lin, W. C.; Yu, B.; Lee, T. T. Antioxidant Capacity of Phytochemicals and Their Potential Effects on Oxidative Status in Animals - A Review. *Asian-Australas. J. Anim. Sci.* **2017**, *30* (3), 299–308.
- (14) Zhang, Z.-L.; Zhou, M.-L.; Tang, Y. Y.-X.; Li, F.-L.; Tang, Y. Y.-X.; Shao, J.-R.; Xue, W.-T.; Wu, Y.-M. Bioactive Compounds in Functional Buckwheat Food. *Food Res. Int.* **2012**, *49* (1), 389–395.
- (15) Zhu, Z.; Yang, M.; Bai, Y.; Ge, F.; Wang, S. Antioxidant-Related Catalase CTA1 Regulates Development, Aflatoxin Biosynthesis, and Virulence in Pathogenic Fungus *Aspergillus Flavus*. *Environ. Microbiol.* **2020**, *22* (7), 2792–2810.
- (16) Yılmaz, H.; Ayhan, N.; Meriç, Ç. Buckwheat: A Useful Food and Its Effects on Human Health. *Curr. Nutr. Food Sci.* **2020**, *16*, 29–34.
- (17) Nobili, C.; de Acutis, A.; Reverberi, M.; Bello, C.; Leone, G. P.; Palumbo, D.; Natella, F.; Procacci, S.; Zjalic, S.; Brunori, A. Buckwheat Hull Extracts Inhibit *Aspergillus Flavus* Growth and AFB1 Biosynthesis. *Front. Microbiol.* **2019**, *10*, 1997.
- (18) Masisi, K.; Beta, T.; Moghadasian, M. H. Antioxidant Properties of Diverse Cereal Grains: A Review on in Vitro and in Vivo Studies. *Food Chem.* **2016**, *196*, 90–97.
- (19) Chitarrini, G.; Nobili, C.; Pinzari, F.; Antonini, A.; de Rossi, P.; del Fiore, A.; Procacci, S.; Tolaini, V.; Scala, V.; Scarpini, M.; Reverberi, M. Buckwheat Achenes Antioxidant Profile Modulates *Aspergillus Flavus* Growth and Aflatoxin Production. *Int. J. Food Microbiol.* **2014**, *189*, 1–10.
- (20) Phan, H. T. T.; Yoda, T.; Chahal, B.; Morita, M.; Takagi, M.; Vestergaard, M. C. Structure-Dependent Interactions of Polyphenols with a Biomimetic Membrane System. *Biochim. Biophys. Acta, Biomembr.* **2014**, *1838* (10), 2670–2677.
- (21) le Lay, C.; Coton, E.; le Blay, G.; Chobert, J.-M.; Haertlé, T.; Choiset, Y.; van Long, N. N.; Meslet-Cladière, L.; Mounier, J. Identification and Quantification of Antifungal Compounds Produced by Lactic Acid Bacteria and Propionibacteria. *Int. J. Food Microbiol.* **2016**, *239*, 79–85.
- (22) Leung, E.; Ng, T. A Relatively Stable Antifungal Peptide from Buckwheat Seeds with Antiproliferative Activity toward Cancer Cells. *J. Pept. Sci.* **2007**, *13*, 762–767.
- (23) Jard, G.; Liboz, T.; Mathieu, F.; Guyonvarc'h, A.; Lebrhi, A. Review of Mycotoxin Reduction in Food and Feed: From Prevention in the Field to Detoxification by Adsorption or Transformation. *Food Addit. Contam., Part A* **2011**, *28* (11), 1590–1609.
- (24) Schöneberg, T.; Kibler, K.; Sulyok, M.; Musa, T.; Bucheli, T. D.; Mascher, F.; Bertossa, M.; Voegelé, R. T.; Vogelsgang, S. Can Plant Phenolic Compounds Reduce *Fusarium* Growth and Mycotoxin Production in Cereals? *Food Addit. Contam., Part A* **2018**, *35* (12), 2455–2470.
- (25) Gobbetti, M.; De Angelis, M.; Di Cagno, R.; Calasso, M.; Archetti, G.; Rizzello, C. G. Novel Insights on the Functional/Nutritional Features of the Sourdough Fermentation. *Int. J. Food Microbiol.* **2019**, *302* (0), 103–113.
- (26) Rodríguez, H.; Curiel, J. A.; Landete, J. M.; de las Rivas, B.; de Felipe, F. L.; Gómez-Cordovés, C.; Mancheño, J. M.; Muñoz, R. Food Phenolics and Lactic Acid Bacteria. *Int. J. Food Microbiol.* **2009**, *132* (2), 79–90.
- (27) Zabka, M.; Pavela, R. Antifungal Efficacy of Some Natural Phenolic Compounds against Significant Pathogenic and Toxinogenic Filamentous Fungi. *Chemosphere* **2013**, *93* (6), 1051–1056.
- (28) Inglett, G. E.; Chen, D.; Berhow, M.; Lee, S. Antioxidant Activity of Commercial Buckwheat Flours and Their Free and Bound Phenolic Compositions. *Food Chem.* **2011**, *125* (3), 923–929.
- (29) Liu, B.; Zhu, Y. Extraction of Flavonoids from Flavonoid-Rich Parts in Tartary Buckwheat and Identification of the Main Flavonoids. *J. Food Eng.* **2007**, *78* (2), 584–587.
- (30) Steadman, K. J.; Burgoon, M. S.; Lewis, B. A.; Edwardson, S. E.; Obendorf, R. L. Minerals, Phytic Acid, Tannin and Rutin in Buckwheat Seed Milling Fractions. *J. Sci. Food Agric.* **2001**, *81* (11), 1094–1100.
- (31) Durazzo, A.; Zaccaria, M.; Polito, A.; Maiani, G.; Carcea. Lignan Content in Cereals, Buckwheat and Derived Foods. *Foods* **2013**, *2*, 53–63.
- (32) Reinisalo, M.; Kärklund, A.; Koskela, A.; Kaarniranta, K.; Karjalainen, R. O. Polyphenol Stilbenes: Molecular Mechanisms of Defence against Oxidative Stress and Aging-Related Diseases. *Oxid. Med. Cell. Longevity* **2015**, *2015*, 340520.
- (33) Csepregi, K.; Hideg, É. Phenolic Compound Diversity Explored in the Context of Photo-Oxidative Stress Protection. *Phytochem. Anal.* **2018**, *29* (2), 129–136.

- (34) Morishita, T.; Yamaguchi, H.; Degi, K. The Contribution of Polyphenols To Antioxidative Activity In Common Buckwheat and Tartary Buckwheat Grain. *Plant Prod. Sci.* **2007**, *10* (1), 99–104.
- (35) Sedej, I.; Mandić, A.; Sakač, M.; Mišan, A.; Tumbas Šaponjac, V. Comparison of Antioxidant Components and Activity of Buckwheat and Wheat Flours. *Cereal Chem.* **2010**, *87*, 387.
- (36) Sensoy, I.; Rosen, R.; Ho, C.-T.; Karwe, M. Effect of Processing on Buckwheat Phenolics and Antioxidant Activity. *Food Chem.* **2006**, *99*, 388–393.
- (37) Inglett, G. E.; Rose, D. J.; Chen, D.; Stevenson, D. G.; Biswas, A. Phenolic Content and Antioxidant Activity of Extracts from Whole Buckwheat (*Fagopyrum Esculentum* Moench) with or without Microwave Irradiation. *Food Chem.* **2010**, *119* (3), 1216–1219.
- (38) Qin, P.; Wang, Q.; Shan, F.; Hou, Z.; Ren, G. Nutritional Composition and Flavonoids Content of Flour from Different Buckwheat Cultivars. *Int. J. Food Sci. Technol.* **2010**, *45* (5), 951–958.
- (39) Kumar, N.; Goel, N. Phenolic Acids: Natural Versatile Molecules with Promising Therapeutic Applications. *Biotechnology Reports* **2019**, *24*, No. e00370.
- (40) Karamać, M.; Biskup, I.; Kulczyk, A. Fractionation of Buckwheat Seed Phenolics and Analysis of Their Antioxidant Activity. *Pol. J. Food Nutr. Sci.* **2015**, *65* (4), 243–249.
- (41) Mikulajová, A.; Šedivá, D.; Hybenová, E.; Mošovská, S. Buckwheat Cultivars — Phenolic Compounds Profiles and Antioxidant Properties. *Acta Chimica Slovaca* **2016**, *9*, 124.
- (42) Klepacka, J.; Gujska, E.; Michalak, J. Phenolic Compounds as Cultivar- and Variety-Distinguishing Factors in Some Plant Products. *Plant Foods Hum. Nutr.* **2011**, *66* (1), 64–69.
- (43) Guo, X.-D.; Ma, Y.-J.; Parry, J.; Gao, J.-M.; Yu, L.-L.; Wang, M. Phenolics Content and Antioxidant Activity of Tartary Buckwheat from Different Locations. *Molecules* **2011**, *16* (12), 9850–9867.
- (44) Zieliński, H.; Ciska, E.; Kozłowska, H. The Cereal Grains: Focus on Vitamin E. *Czech J. Food Sci.* **2013**, *19*, 182–188.
- (45) Heim, K. E.; Tagliaferro, A. R.; Bobilya, D. J. Flavonoid Antioxidants: Chemistry, Metabolism and Structure-Activity Relationships. *J. Nutr. Biochem.* **2002**, *13* (10), 572–584.
- (46) Giménez-Bastida, J. A.; Laparra-Llopis, J. M.; Baczek, N.; Zieliński, H. Buckwheat and Buckwheat Enriched Products Exert an Anti-Inflammatory Effect on the Myofibroblasts of Colon CCD-18Co. *Food Funct.* **2018**, *9* (6), 3387–3397.
- (47) Kreft, I.; Fabjan, N.; Yasumoto, K. Rutin Content in Buckwheat (*Fagopyrum Esculentum* Moench) Food Materials and Products. *Food Chem.* **2006**, *98* (3), 508–512.
- (48) Bojnanska, T.; Francaková, H.; Chlebo, P.; Vollmannova, A. Rutin Content in Buckwheat Enriched Bread and Influence of Its Consumption on Plasma Total Antioxidant Status. *Czech J. Food Sci.* **2009**, *27*, S236–S240.
- (49) Bai, C. Z.; Feng, M. L.; Hao, X. L.; Zhong, Q. M.; Tong, L. G.; Wang, Z. H. Rutin, Quercetin, and Free Amino Acid Analysis in Buckwheat (*Fagopyrum*) Seeds from Different Locations. *GMR, Genet. Mol. Res.* **2015**, *14* (4), 19040–19048.
- (50) Kitabayashi, H.; Ujihara, A.; Hirose, T.; Minami, M. Varietal Differences and Heritability for Rutin Content in Common Buckwheat, *Fagopyrum Esculentum* Moench. *Ikushugaku Zasshi* **1995**, *45* (1), 75–79.
- (51) Tian, Q.; Li, D.; Patil, B. S. Identification and Determination of Flavonoids in Buckwheat (*Fagopyrum Esculentum* Moench, Polygonoaceae) by High-Performance Liquid Chromatography with Electrospray Ionisation Mass Spectrometry and Photodiode Array Ultraviolet Detection. *Phytochem. Anal.* **2002**, *13* (5), 251–256.
- (52) Lee, L.-S. S.; Choi, E.-J. J.; Kim, C.-H. H.; Sung, J.-M. M.; Kim, Y.-B. B.; Seo, D.-H. H.; Choi, H.-W. W.; Choi, Y.-S. S.; Kum, J.-S. S.; Park, J.-D. D. Contribution of Flavonoids to the Antioxidant Properties of Common and Tartary Buckwheat. *J. Cereal Sci.* **2016**, *68*, 181–186.
- (53) Atanasova-Penichon, V.; Barreau, C.; Richard-Forget, F. Antioxidant Secondary Metabolites in Cereals: Potential Involvement in Resistance to Fusarium and Mycotoxin Accumulation. *Front. Microbiol.* **2016**, *7*, 566.
- (54) Montibus, M.; Pinson-Gadais, L.; Richard-Forget, F.; Barreau, C.; Ponts, N. Coupling of Transcriptional Response to Oxidative Stress and Secondary Metabolism Regulation in Filamentous Fungi. *Crit. Rev. Microbiol.* **2015**, *41* (3), 295–308.
- (55) Sardi, J. de C. O.; Gullo, F. P.; Freires, I. A.; Pitangui, N. de S.; Segalla, M. P.; Fusco-Almeida, A. M.; Rosalen, P. L.; Regasini, L. O.; Mendes-Giannini, M. J. S. Synthesis, Antifungal Activity of Caffeic Acid Derivative Esters, and Their Synergism with Fluconazole and Nystatin against *Candida* Spp. *Diagn. Microbiol. Infect. Dis.* **2016**, *86* (4), 387–391.
- (56) Ponts, N.; Pinson-Gadais, L.; Boutigny, A. L.; Barreau, C.; Richard-Forget, F. Cinnamic-Derived Acids Significantly Affect *Fusarium Graminearum* Growth and in Vitro Synthesis of Type B Trichothecenes. *Phytopathology* **2011**, *101* (8), 929–934.
- (57) Gauthier, L.; Bonnin-Verdal, M. N.; Marchegay, G.; Pinson-Gadais, L.; Ducos, C.; Richard-Forget, F.; Atanasova-Penichon, V. Fungal Biotransformation of Chlorogenic and Caffeic Acids by *Fusarium Graminearum*: New Insights in the Contribution of Phenolic Acids to Resistance to Deoxynivalenol Accumulation in Cereals. *Int. J. Food Microbiol.* **2016**, *221* (856), 61–68.
- (58) Kumaraswamy, G. K.; Bollina, V.; Kushalappa, A. C.; Choo, T. M.; Dion, Y.; Rioux, S.; Mamer, O.; Faubert, D. Metabolomics Technology to Phenotype Resistance in Barley against *Gibberella Zeae*. *Eur. J. Plant Pathol.* **2011**, *130* (1), 29–43.
- (59) Gutiérrez-Larrazar, M.; Rúa, J.; Caro, I.; de Castro, C.; de Arriaga, D.; García-Armesto, M. R.; del Valle, P. Evaluation of Antimicrobial and Antioxidant Activities of Natural Phenolic Compounds against Foodborne Pathogens and Spoilage Bacteria. *Food Control* **2012**, *26* (2), 555–563.
- (60) Mandalari, G.; Bisignano, C.; D'Arrigo, M.; Ginestra, G.; Arena, A.; Tomaino, A.; Wickham, M. S. J. Antimicrobial Potential of Polyphenols Extracted from Almond Skins. *Lett. Appl. Microbiol.* **2010**, *51* (1), 83–89.
- (61) Mendoza, L.; Yañez, K.; Vivanco, M.; Melo, R.; Cotoras, M. Characterization of Extracts from Winery By-Products with Antifungal Activity against *Botrytis Cinerea*. *Ind. Crops Prod.* **2013**, *43*, 360–364.
- (62) Kakkar, S.; Bais, S. A Review on Protocatechuic Acid and Its Pharmacological Potential. *Int. Scholarly Res. Notices* **2014**, *2014*, No. 952943, DOI: 10.1155/2014/952943.
- (63) Aziz, N. H.; Farag, S. E.; Mousa, L. A.; Abo-Zaid, M. A. Comparative Antibacterial and Antifungal Effects of Some Phenolic Compounds. *Microbios* **1998**, *93* (374), 43–54.
- (64) Teodoro, G. R.; Ellepola, K.; Seneviratne, C. J.; Koga-Ito, C. Y. Potential Use of Phenolic Acids as Anti-*Candida* Agents: A Review. *Front. Microbiol.* **2015**, *6* (DEC), 1–11.
- (65) Guiraud, P.; Steiman, R.; Seiglemurandi, F.; Benoît-guyod, J. L. Comparison of the Toxicity of Various Lignin-Related Phenolic Compounds toward Selected Fungi Perfecti and Fungi Imperfecti. *Ecotoxicol. Environ. Saf.* **1995**, *32*, 29–33.
- (66) Tempesti, T. C.; Alvarez, M. G.; de Araújo, M. F.; Catunda Júnior, F. E. A.; de Carvalho, M. G.; Durantini, E. N. Antifungal Activity of a Novel Quercetin Derivative Bearing a Trifluoromethyl Group on *Candida Albicans*. *Med. Chem. Res.* **2012**, *21* (9), 2217–2222.
- (67) Rao, A.; Zhang, Y.; Muend, S.; Rao, R. Mechanism of Antifungal Activity of Terpenoid Phenols Resembles Calcium Stress and Inhibition of the TOR Pathway. *Antimicrob. Agents Chemother.* **2010**, *54* (12), 5062–5069.
- (68) Xu, J.; Zhou, F.; Ji, B.-P.; Pei, R.-S.; Xu, N. The Antibacterial Mechanism of Carvacrol and Thymol against *Escherichia Coli*. *Lett. Appl. Microbiol.* **2008**, *47* (3), 174–179.
- (69) Reverberi, M.; Zjalic, S.; Ricelli, A.; Punelli, F.; Camera, E.; Fabbri, C.; Picardo, M.; Fanelli, C.; Fabbri, A. A. Modulation of Antioxidant Defense in *Aspergillus Parasiticus* Is Involved in Aflatoxin Biosynthesis: A Role for the ApyapA Gene. *Eukaryotic Cell* **2008**, *7* (6), 988–1000.
- (70) Boutigny, A. Etude de l'effet de Composés Du Grain de Blé Dur Sur La Régulation de La Voie de Biosynthèse Des

Trichothécènes B Purification de Composés Inhibiteurs, Analyse Des Mécanismes Impliqués. Thèse de doctorat, 2007.

(71) Boutigny, A.-L.; Barreau, C.; Atanasova-Penichon, V.; Verdal-Bonnin, M.-N.; Pinson-Gadais, L.; Richard-Forget, F. Ferulic Acid, an Efficient Inhibitor of Type B Trichothecene Biosynthesis and Tri Gene Expression in Fusarium Liquid Cultures. *Mycol. Res.* **2009**, *113* (6), 746–753.

(72) Samapundo, S.; de Meulenaer, B.; Osei-Nimoh, D.; Lamboni, Y.; Debevere, J.; Devlieghere, F. Can Phenolic Compounds Be Used for the Protection of Corn from Fungal Invasion and Mycotoxin Contamination during Storage? *Food Microbiol.* **2007**, *24* (5), 465–473.

(73) Palumbo, J. D.; O'Keeffe, T. L.; Mahoney, N. E. Inhibition of Ochratoxin A Production and Growth of Aspergillus Species by Phenolic Antioxidant Compounds. *Mycopathologia* **2007**, *164* (5), 241–248.

(74) Nesci, A.; Gsponer, N.; Etcheverry, M. Natural Maize Phenolic Acids for Control of Aflatoxigenic Fungi on Maize. *J. Food Sci.* **2007**, *72* (5), M180–M185.

(75) Norton, R. A. Inhibition of Aflatoxin B1 Biosynthesis in Aspergillus Flavus by Anthocyanidins and Related Flavonoids. *J. Agric. Food Chem.* **1999**, *47* (3), 1230–1235.

(76) Sanzani, S. M.; Schena, L.; Nigro, F.; de Girolamo, A.; Ippolito, A. Effect of Quercetin and Umbelliferone on the Transcript Level of Penicillium Expansum Genes Involved in Patulin Biosynthesis. *Eur. J. Plant Pathol.* **2009**, *125* (2), 223–233.

(77) Bollina, V.; Kushalappa, A. C. In Vitro Inhibition of Trichothecene Biosynthesis in Fusarium Graminearum by Resistance-Related Endogenous Metabolites Identified in Barley. *Mycology* **2011**, *2* (4), 291–296.

(78) Picot, A.; Atanasova-Pénichon, V.; Pons, S.; Marchegay, G.; Barreau, C.; Pinson-Gadais, L.; Roucolle, J.; Daveau, F.; Caron, D.; Richard-Forget, F. Maize Kernel Antioxidants and Their Potential Involvement in Fusarium Ear Rot Resistance. *J. Agric. Food Chem.* **2013**, *61* (14), 3389–3395.

(79) Morales, J.; Mendoza, L.; Cotoras, M. Alteration of Oxidative Phosphorylation as a Possible Mechanism of the Antifungal Action of p-Coumaric Acid against Botrytis Cinerea. *J. Appl. Microbiol.* **2017**, *123* (4), 969–976.

(80) Ultee, A.; Bennik, M. H. J.; Moezelaar, R. The Phenolic Hydroxyl Group of Carvacrol Is Essential for Action against the Food-Borne Pathogen Bacillus Cereus. *Appl. Environ. Microbiol.* **2002**, *68* (4), 1561–1568.

(81) Pani, G.; Scherm, B.; Azara, E.; Balmas, V.; Jahanshahi, Z.; Carta, P.; Fabbri, D.; Dettori, M. A.; Fadda, A.; Dessi, A.; Dallochio, R.; Migheli, Q.; Delogo, G. Natural and Natural-like Phenolic Inhibitors of Type B Trichothecene in Vitro Production by the Wheat (Triticum Sp.) Pathogen Fusarium Culmorum. *J. Agric. Food Chem.* **2014**, *62* (22), 4969–4978.

(82) Etzerodt, T.; Maeda, K.; Nakajima, Y.; Laursen, B.; Fomsgaard, I. S.; Kimura, M. 2,4-Dihydroxy-7-Methoxy-2H-1,4-Benzoxazin-3(4H)-One (DIMBOA) Inhibits Trichothecene Production by Fusarium Graminearum through Suppression of Tri6 Expression. *Int. J. Food Microbiol.* **2015**, *214*, 123–128.

(83) Guan, X.; Zhao, Y.; Liu, X.; Shang, B.; Xing, F.; Zhou, L.; Wang, Y.; Zhang, C.; Bhatnagar, D.; Liu, Y. The BZIP Transcription Factor Afap1 Mediates the Oxidative Stress Response and Aflatoxin Biosynthesis in Aspergillus Flavus. *Rev. Argent. Microbiol.* **2019**, *51* (4), 292–301.

(84) Sánchez-Maldonado, A. F.; Schieber, A.; Gänzle, M. G. Structure–Function Relationships of the Antibacterial Activity of Phenolic Acids and Their Metabolism by Lactic Acid Bacteria. *J. Appl. Microbiol.* **2011**, *111* (5), 1176–1184.

(85) Reverón, I.; de las Rivas, B.; Muñoz, R.; López de Felipe, F. Genome-Wide Transcriptomic Responses of a Human Isolate of Lactobacillus Plantarum Exposed to p-Coumaric Acid Stress. *Mol. Nutr. Food Res.* **2012**, *56* (12), 1848–1859.

(86) Jimenez, N.; Esteban-Torres, M.; Mancheno, J. M.; de las Rivas, B.; Munoz, R. Tannin Degradation by a Novel Tannase Enzyme

Present in Some Lactobacillus Plantarum Strains. *Appl. Environ. Microbiol.* **2014**, *80* (10), 2991–2997.

(87) Lamont, J. R.; Wilkins, O.; Bywater-Ekegård, M.; Smith, D. L. From Yogurt to Yield: Potential Applications of Lactic Acid Bacteria in Plant Production. *Soil Biol. Biochem.* **2017**, *111*, 1–9.

(88) Filannino, P.; di Cagno, R.; Gobbetti, M. Metabolic and Functional Paths of Lactic Acid Bacteria in Plant Foods: Get out of the Labyrinth. *Curr. Opin. Biotechnol.* **2018**, *49*, 64–72.

(89) Ripari, V.; Bai, Y.; Gänzle, M. G. Metabolism of Phenolic Acids in Whole Wheat and Rye Malt Sourdoughs. *Food Microbiol.* **2019**, *77*, 43–51.

(90) Esteban-Torres, M.; Landete, J. M.; Reverón, I.; Santamaría, L.; de las Rivas, B.; Muñoz, R. A Lactobacillus Plantarum Esterase Active on a Broad Range of Phenolic Esters. *Appl. Environ. Microbiol.* **2015**, *81* (9), 3235LP–3242.

(91) Lee, Y.; Oh, J.; Jeong, Y.-S. Lactobacillus Plantarum-Mediated Conversion of Flavonoid Glycosides into Flavonols, Quercetin, and Kaempferol in Cudrania Tricuspidata Leaves. *Food Sci. Biotechnol.* **2015**, *24* (5), 1817–1821.

(92) Bel-Rhliid, R.; Thapa, D.; Kraehenbuehl, K.; Hansen, C. E.; Fischer, L. Biotransformation of Caffeoyl Quinic Acids from Green Coffee Extracts by Lactobacillus Johnsonii NCC 533. *AMB Express* **2013**, *3*, 28.

(93) Svensson, L.; Sekwati-Monang, B.; Lutz, D. L.; Schieber, A.; Gänzle, M. G. Phenolic Acids and Flavonoids in Nonfermented and Fermented Red Sorghum (Sorghum Bicolor (L.) Moench). *J. Agric. Food Chem.* **2010**, *58* (16), 9214–9220.

(94) Santamaría, L.; Reverón, I.; López de Felipe, F.; de las Rivas, B.; Muñoz, R. Unravelling the Reduction Pathway as an Alternative Metabolic Route to Hydroxycinnamate Decarboxylation in Lactobacillus Plantarum. *Appl. Environ. Microbiol.* **2018**, *84* (15), No. e01123-18.

(95) Santamaría, L.; Reverón, I.; de Felipe, F. L.; de las Rivas, B.; Muñoz, R. Ethylphenol Formation by Lactobacillus Plantarum: Identification of the Enzyme Involved in the Reduction of Vinylphenols. *Appl. Environ. Microbiol.* **2018**, *84* (17), No. e01064-18.

(96) Filannino, P.; Bai, Y.; di Cagno, R.; Gobbetti, M.; Gänzle, M. G. Metabolism of Phenolic Compounds by Lactobacillus Spp. during Fermentation of Cherry Juice and Broccoli Puree. *Food Microbiol.* **2015**, *46*, 272–279.

(97) Rizzello, C. G.; Coda, R.; Mazzacane, F.; Minervini, D.; Gobbetti, M. Micronized By-Products from Debranned Durum Wheat and Sourdough Fermentation Enhanced the Nutritional, Textural and Sensory Features of Bread. *Food Res. Int.* **2012**, *46* (1), 304–313.

(98) Katina, K.; Liukkonen, K.-H.; Kaukovirta-Norja, A.; Adlercreutz, H.; Heinonen, S.-M.; Lampi, A.-M.; Pihlava, J.-M.; Poutanen, K. Fermentation-Induced Changes in the Nutritional Value of Native or Germinated Rye. *J. Cereal Sci.* **2007**, *46* (3), 348–355.

(99) Đorđević, T. M.; Šiler-Marinković, S. S.; Dimitrijević-Branković, S. I. Effect of Fermentation on Antioxidant Properties of Some Cereals and Pseudo Cereals. *Food Chem.* **2010**, *119* (3), 957–963.

(100) Konopka, I.; Tanska, M.; Faron, A.; Czaplicki, S. Release of Free Ferulic Acid and Changes in Antioxidant Properties during the Wheat and Rye Bread Making Process. *Food Sci. Biotechnol.* **2014**, *23* (3), 831–840.

(101) Zieliński, H.; Ciesarova, Z.; Kukurova, K.; Zielinska, D.; Szawara-Nowak, D.; Starowicz, M.; Wronkowska, M. Effect of Fermented and Unfermented Buckwheat Flour on Functional Properties of Gluten-Free Muffins. *J. Food Sci. Technol.* **2017**, *54* (6), 1425–1432.

(102) Lukšić, L.; Bonafaccia, G.; Timoracka, M.; Vollmannova, A.; Trček, J.; Nyambe, T. K.; Melini, V.; Acquistucci, R.; Germ, M.; Kreft, I. Rutin and Quercetin Transformation during Preparation of Buckwheat Sourdough Bread. *J. Cereal Sci.* **2016**, *69*, 71–76.

(103) Adebo, O. A.; Gabriela Medina-Meza, I. Impact of Fermentation on the Phenolic Compounds and Antioxidant Activity of Whole Cereal Grains: A Mini Review. *Molecules* **2020**, *25* (4), 927.

- (104) Bhanja Dey, T.; Kuhad, R. C. Upgrading the Antioxidant Potential of Cereals by Their Fungal Fermentation under Solid-State Cultivation Conditions. *Lett. Appl. Microbiol.* **2014**, *59* (5), 493–499.
- (105) Gänzle, M. G. Enzymatic and Bacterial Conversions during Sourdough Fermentation. *Food Microbiol.* **2014**, *37*, 2–10.
- (106) Zieliński, H.; Michalska, A.; Piskula, M. K.; Kozłowska, H. Antioxidants in Thermally Treated Buckwheat Groats. *Mol. Nutr. Food Res.* **2006**, *50* (9), 824–832.
- (107) McKeehen, J. D.; Busch, R. H.; Fulcher, R. G. Evaluation of Wheat (*Triticum Aestivum* L.) Phenolic Acids during Grain Development and Their Contribution to Fusarium Resistance. *J. Agric. Food Chem.* **1999**, *47* (4), 1476–1482.
- (108) Wu, H.; Shen, S.-H.; Han, J.; Liu, Y.; Liu, S. The Effect in Vitro of Exogenously Applied P-Hydroxybenzoic Acid on Fusarium Oxysporum f. Sp Niveum. *Phytopathol. Mediterranea* **2009**, *48*, 439–446.
- (109) Li, X.-M.; Li, Z.-Y.; Wang, Y.-D.; Wang, J.-Q.; Yang, P.-L. Quercetin Inhibits the Proliferation and Aflatoxins Biosynthesis of *Aspergillus Flavus*. *Toxins* **2019**, *11* (3), 154.



Research review paper

Importance of microbial defence systems to bile salts and mechanisms of serum cholesterol reduction

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ABSTRACT

An important feature of the intestinal microbiota, particularly in the case of administered probiotic microorganisms, is their resistance to conditions in the gastrointestinal tract, particularly tolerance to and growth in the presence of bile salts. Bacteria can use several defence mechanisms against bile, including special transport mechanisms, the synthesis of various types of surface proteins and fatty acids or the production of exopolysaccharides. The ability to enzymatically hydrolyse bile salts occurs in a variety of bacteria. Choloylglycine hydrolase (EC 3.5.1.24), a bile salt hydrolase, is a constitutive intracellular enzyme responsible for the hydrolysis of an amide bond between glycine or taurine and the steroid nucleus of bile acids. Its presence was demonstrated in specific microorganisms from several bacterial genera (*Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* spp., *Bacteroides* spp.). Occurrence and gene arrangement encoding this enzyme are highly variable in probiotic microorganisms. Bile salt hydrolase activity may provide the possibility to use the released amino acids by bacteria as sources of carbon and nitrogen, to facilitate detoxification of bile or to support the incorporation of cholesterol into the cell wall. Deconjugation of bile salts may be directly related to a lowering of serum cholesterol levels, from which conjugated bile salts are synthesized *de novo*. Furthermore, the ability of microorganisms to assimilate or to bind ingested cholesterol to the cell wall or to eliminate it by co-precipitation with released cholic acid was also documented. Some intestinal microflora produce cholesterol reductase that catalyses the conversion of cholesterol to insoluble coprostanol, which is subsequently excreted in faeces, thereby also reducing the amount of exogenous cholesterol.

1. Introduction

The commensal intestinal microbiome has an important role in the area of immune and metabolic functions, preventing the growth of undesirable microorganisms by the formation of antimicrobial agents or by direct competition for binding sites and nutrients. According to recent research, our psycho-behavioural functions could also be affected (Hemarajata and Versalovic, 2013).

Probiotic microorganisms are used not only in the food industry but also as a supplement during various medical treatments (Kotal et al., 2014). The term was first introduced by Lilly and Stillwell (1965); since then the definition has been changed several times. Currently the term probiotics is used to denote microorganisms associated with beneficial health effects for humans and animals if they are consumed in sufficient amounts (FAO/WHO, 2002). However, no legal definition of probiotics exists in EU or other countries. A wide variety of species and genera can be probiotics and are usually chosen from those bacteria located in the gastrointestinal tract (GIT). The most important and commercially used

are lactobacilli, which belong to the group of lactic acid bacteria, and bifidobacteria. In addition, attention is now also given to other microorganisms such as *Saccharomyces cerevisiae* var. *boulardii*, *Enterococcus* spp., *Pediococcus* spp., *Bacillus* spp. or microbes isolated from the GIT such as *Escherichia coli* or clostridia.

Probiotic properties are always strain specific and cannot be extrapolated to other strains. Experimental studies on probiotics concern the selection of strains and confirmation that they exhibit specific properties. To be classified as a probiotic, a microorganism has to fulfil numerous criteria: these include safety and requested properties, technological features (technological adequacy, storage stability) as well as documented health benefits. Probiotic microorganism should be of human origin and accurately characterized. One of the most important criteria for the selection is their ability to survive passage through the GIT; this means to be tolerant to very low pH of the stomach, to digestive enzyme activity and to the normal concentration of bile salts in the small intestine (Horáčková et al., 2011; Vasiljevic and Shah, 2008).

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Both colonization of the human tract with commensal microbiota and interactions in which probiotic bacteria provide numerous health advantages are influenced and controlled by host bile secretion since resistance to bile is the main selective factor affecting the composition of the microbiome in the GIT (Horáčková et al., 2017; Yokota et al., 2012).

2. Bile and bile acids

Bile is an important product of hepatocytes. It is a yellow-green liquid containing bile acids, cholesterol, phospholipids (mainly phosphatidylcholines), biliverdin pigment and steroid hormones. It is transported either directly into the duodenum, or temporarily stored and concentrated in the gallbladder. Bile serves as bio-surfactant in the form of aggregates that allow the digestion and transport of lipids and lipid soluble compounds, but also have an effect on protein denaturation and proteolysis (Maldonado-Valderrama et al., 2011). Bile acids have important signalling and metabolic regulator functions (Chiang, 2013; Li and Chiang, 2015). Under normal physiological conditions, the concentration of bile salts in the human small intestine ranges from $40 \text{ mmol} \cdot \text{l}^{-1}$ to $< 1 \text{ mmol} \cdot \text{l}^{-1}$ (2–0.05%) (Islam et al., 2011). Some authors reported values between only 2–1.5% in the first hour of digestion, followed by a decrease to 0.3% (Noriega et al., 2004). Bile acids have been studied since the early 19th century, but their structure was only identified after 1932, when the structure of cholesterol was elucidated by means of X-rays (Mukhopadhyay and Maitra, 2004). Mammalian bile acids are synthesized *de novo* from cholesterol (typically from LDL particles) by a multienzymatic process with cholesterol-7 α -hydroxylase (CYP7A1) as the key enzyme, which catalyses the conversion of cholesterol to 7 α -hydroxy-cholesterol (Fig. 1). Cytochrome P450 enzymes located on the smooth endoplasmic reticulum of hepatocytes participate in many of subsequent 14 reactions (Norlin and Wikvall, 2007). Via this classic pathway sterol 12 α -hydroxylase (CYP8B1) then regulates the cholic acid synthesis. An alternative pathway using mitochondrial sterol 27-hydroxylase (CYP27A1) followed by 25-hydroxycholesterol 7 α -hydroxylase (CYP7B1) and producing mostly chenodeoxycholic acid is also documented (de Aguiar Vallim and Tarling, 2013; Ridlon et al., 2013). The regulation of genes encoding these enzymes is important for maintaining normal bile acid synthesis but information is scarce. Bile acids serve as inter-organ signalling molecules, controlling metabolism and inflammation (Cháver-Talavera et al., 2017) and a number of diseases can result from mutations in genes encoding their synthesis (de Aguiar Vallim et al., 2015).

Bile acids and salts are C24 steroids carrying one carboxyl group and several hydroxyl groups. The steroid nucleus consists of three six-membered rings (A, B and C) and a five-membered ring (D) with methyl groups in positions C-18 and C-19 and the molecule is terminated by a short aliphatic chain (Fig. 2) (Hofmann et al., 1992).

In the synthesis of bile acids, a change in the arrangement of A and B-rings from a trans to a cis configuration is important, as this causes all of the hydrophilic groups on one side of the molecule to be present in the bile acids. Unlike cholesterol, they are then far more amphipathic. Primary bile acids - cholic and chenodeoxycholic acids - are quantitatively the most important cholesterol metabolites. After their biosynthesis and prior to secretion to the intestine, they are mostly activated by coenzyme A and bonded to glycine or the non-proteinogenic amino acid taurine (derived from cysteine), resulting in the formation

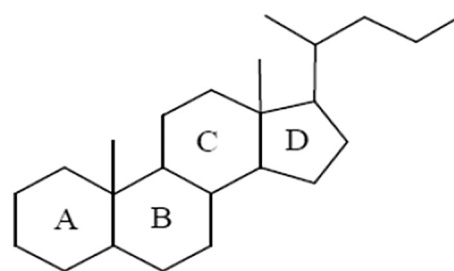


Fig. 2. Structure of bile acids.

of the major conjugated bile acid molecules, glycocholic, glychenodeoxycholic, taurocholic and taurochenodeoxycholic acids. When crosslinked with another amino acid, these conjugates are rapidly hydrolysed by pancreatic carboxypeptidases. The ratio of glyco-conjugated and tauro-conjugated acids is about 3:1 in human bile (de Aguiar Vallim and Tarling, 2013). Conjugation decreases the pK_A value of glycocholic and taurocholic acids to 4.4 and 1.8, resp. compared to pK_A of cholic acid (pK_A = 6.4) and therefore they occur in a substantially dissociated form as bile salts (Philipp, 2011). The secretion of bile salts from hepatocytes requires the bile salt export protein (BSEP, encoded by *ABCB11*) (de Aguiar Vallim and Tarling, 2013). The human liver produces about one litre of bile per day, but only a small amount is removed from the body (Patel et al., 2010). Up to 95% of bile acids are reabsorbed via the ileal bile acid transporter (IBAT) and taken up by hepatocytes. About 0.3 to 0.6 g a day is excreted in the faeces or urine and must be resynthesized. Bile acids are returned to the liver under normal conditions via the enterohepatic circulation, which is a process of bile acid secretion from the liver to the bile and intestine followed by reabsorption. Reabsorption can take place both through passive diffusion and active transport in the ileum (Lubanda and Vecka, 2009). Furthermore, bile salts can be subject to microbial conversion in the intestine to form secondary bile salts. The first step, catalysed by bile salts hydrolase, is a deconjugation (cleavage of glycine or taurine) to form primary bile acids. Their subsequent transformation by 7 α -dehydroxylation (a multistep pathway best characterized in *Clostridium scindens* (Studer et al., 2016)) creates deoxycholic or lithocholic acid (Ridlon and Hylemon, 2012). Another option is 7 α -dehydrogenation by 7 α -hydroxysteroid dehydrogenase (EC 1.1.1.159) to 7-oxo-deoxycholic or 7-oxo-lithocholic acids (Eggert et al., 2014; Fukiya et al., 2009), of which the other can be converted to ursodeoxycholic acid by epimerization (Lepercq et al., 2004). These secondary bile salts are often discussed for their possible negative impact on human health (Ridlon et al., 2016). Their increased concentration in the intestine may promote the development of carcinomas (Bernstein et al., 2011; Payne et al., 2011; Yoshimoto et al., 2013). Gall stones may be formed when a change in the ratio of bile salts to cholesterol leads to cholesterol precipitation with calcium salts and bile pigments (Noriega et al., 2006), or may induce non-alcoholic fatty liver disease (Park et al., 2016). Bile toxic compounds may disrupt the intestinal microflora, which can cause diarrhoea, intestinal mucosal inflammation and activate carcinogenesis in the gut. Another negative characteristic is the lower emulsifying ability of deconjugated bile salts compared to conjugates, which may result in impaired lipid digestion and disruption of the absorption of fatty acids and monoglycerides (Ahn et al., 2003). Only ursodeoxycholic acid is used for oral administration as a therapeutic drug to

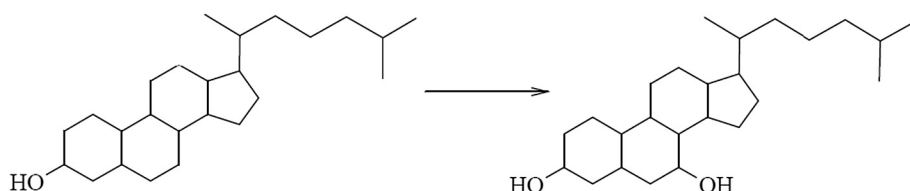


Fig. 1. Cholesterol conversion to 7-hydroxy-cholesterol involving the enzyme 7 α -hydroxylase (CYP7A1).

dissolve gallstones (Eggert et al., 2014).

Bile acids are able to form supramolecules. Their chain, with the cis configuration of the A and B rings of the steroid nucleus, has a curved character that allows the connection of several bile acids to the circle. The connection proceeds via a spacer to create organized units with canals in the middle, where a hydrophilic substance can be connected for transport through the membrane lipid bilayer (Davis, 2007). Structures of bile acids and their various derivatives have been studied intensively (Valkonen et al., 2008) due to their possible use in pharmacological applications as antimicrobial agents (Huang et al., 2009; Nascimento et al., 2015; Rasras et al., 2010; Ye et al., 2013) or in other options as summarized by Virtanen and Kolehmainen (2004).

3. Ability of microorganisms to grow in the presence of bile salts

Most ingested microorganisms are usually killed during exposure to the acidic conditions in the stomach, and later to the luminal media (bile and pancreatic secretions). Both conjugated and deconjugated forms of bile acids have been shown to have antibacterial properties with proven higher sensitivity to Gram-positive compared to Gram-negative bacteria (Hofmann and Eckmann, 2006). There are several known mechanisms of bile acid antimicrobial activity. Since bile acts as a detergent it could directly interact with cell membranes, dissipate transmembrane electrical potential and reduce the internal pH, as shown by Kurdi et al. (2006) for *Lactobacillus salivarius* subsp. *salicinius* JCM 1044 and *Bifidobacterium breve* JCM 1192. Ruiz et al. (2007) demonstrated that bile changed the ratio between cell membrane phospholipids and proteins, as well as the fatty acid composition in *Bifidobacterium animalis* subsp. *lactis*. Cell surface deformities were also recorded by electron microscopy. Alterations to the inner membrane can contribute to the sensitivity of Gram-negative bacteria to bile (López-Garrido et al., 2010). Cytoplasm acidification was also proven by Bustos et al. (2012) for several lactobacilli strains and *Enterococcus faecium* CRL183. Rodríguez-Betrán et al. (2012) suggested that bile contributes to oxidative stress in bacteria as was demonstrated for *Escherichia coli*. Additionally, direct DNA damage by bile has been demonstrated. Although enteric bacteria are very resistant to bile toxicity, Prieto et al. (2004) described that bile salts increased the frequency of point mutations and caused DNA damage in *Salmonella enterica*. The effect of bile on enteric and food-borne pathogens such as *E. coli*, *Salmonella* spp., *Listeria monocytogenes* and *Bacillus cereus* is summarized in the review by Merritt and Donaldson (2009).

Bile secretion is one of the mechanisms that maintain equilibrium between human host and intestinal microbiota. As will be described later, some bacteria have developed strategies to survive in this unfavourable environment. The ability of bacteria to reproduce and grow in different concentrations of bile salts has been intensively studied both for pathogenic bacteria and specifically for desirable (probiotic) types that can deliver health benefits in the intestine only when they act in live form. Bile tolerance allows better colonization in the human GIT. Generally, strains isolated from GIT are believed to be more resistant to the influence of bile salts than those from other sources. However, in recent years efforts to find new probiotic strains have led to tests of various types of bacteria (mainly lactic acid bacteria) that originate from various fermented dairy and non-dairy products or animal GIT. Examples of such studies are summarized in Table 1.

4. Defence mechanisms of microbial cells against bile

The ability to tolerate and grow in the presence of bile salts is a strain-specific property. Several mechanisms have been described to explain this feature.

4.1. Alterations in the cell wall

The first such mechanism is alteration in the physicochemical

properties of the cell wall, especially changes in the composition of bacterial proteins and membrane lipids, which may contribute to reducing the diffusion of bile salts (Ruiz et al., 2007; Taranto et al., 2003). Suzuki et al. (2014) described a significant increase in the relative content of oleic acid in cellular lipids in several *Lactobacillus brevis* strains. Induction of genes encoding the integrity or functionality of the cell wall and cytoplasmic membrane is not the only example of an effect of bile salts. Multiple genomic studies identifying overexpressed or underexpressed genes and transcriptional analysis during bile exposure have been completed for *L. reuteri* ATCC 55730 (Whitehead et al., 2008), *L. acidophilus* NCFM (Pfeiler et al., 2007) and *Enterococcus faecalis* V583 (Solheim et al., 2007). A large number of differentially transcribed genes and expressed proteins were found in *L. salivarius* LI01, together with modifications in the structure of the peptidoglycan layer by increasing the ratio of hydrophobic lipids to unsaturated fatty acids when cultivated with 0.15% of ox-bile (Lv et al., 2017). In *L. johnsonii* PF01, among proteins exhibiting changes during bile exposure, Lee et al. (2013) found those connected with cell division, carbohydrate transport and metabolism and cell wall biosynthesis. Furthermore, alternative synthesis of proteins responsible for nucleotide and lipid metabolism and stress response has been demonstrated in *L. reuteri* CRL1098 (Bustos et al., 2015). An et al. (2014) also reached similar conclusions for the strain *Bifidobacterium longum* BBMN68, where transcription of 236 genes was changed and the fatty acid composition of the cell membrane was modified.

The fact that cultivation of bacteria in the presence of bile salts influences cell surface properties was also shown by autoaggregation and hydrophobicity measurements (Pumbwe et al., 2007). These values have been used to assess the adhesion ability of probiotic microorganisms and the ability to co-aggregate with potential pathogens (Collado et al., 2008; Kotzamanidis et al., 2010; Tareb et al., 2013). In individual studies, bile effects on these properties differ. In bifidobacteria, the effect of sodium taurocholate, deoxycholic and cholic acids on autoaggregation and zeta-potential of hydrophobic and non-hydrophobic strains was found to be different (Kociubinski et al., 2002). In 3 isolates of *L. delbrueckii* subsp. *bulgaricus*, the ability to auto-aggregate and hydrophobicity in the presence of bile were reduced, but in one strain they were increased (Aslim et al., 2007). A protective function can also be met by other cell surface structures. High exopolysaccharide producing strains of yogurt culture, both *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, showed a significant protective effect against bile (Boke et al., 2010). The same conclusion was reached by Suzuki et al. (2013, 2014) for *L. brevis* strains. Bile also induced the production of exopolysaccharides (EPS) in *Bifidobacterium animalis* subsp. *lactis* 4549dOx, a bile-adapted strain, and this effect was related to the expression of *gtf01207* for glycosyltransferase involved in EPS synthesis (Madieto et al., 2009). Alp and Aslim (2010) found a positive correlation between bile resistance and EPS production in 31 bifidobacteria strains isolated from infant faeces or breast-milk. The presence of an S-layer may also play an important role. It was confirmed that the gene responsible for S-layer production in *L. acidophilus* ATCC 4356, *slpA*, was induced in the presence of bile within a concentration range of 0.01–0.05% (Khaleghi et al., 2010).

Outer membrane proteins and lipopolysaccharides are directly involved in the resistance of Gram-negative bacteria to bile. Bile salts have been shown to play an important role in changes in surface structures and surface protein gene expression in pathogenic GIT microorganisms such as *Listeria monocytogenes* (Melo et al., 2013), *Campylobacter jejuni* (Dzieciol et al., 2011), *Salmonella enterica* (López-Garrido et al., 2010), *Shigella* spp. (Nickerson et al., 2017), *Helicobacter pylori* (Shao et al., 2008) or *Bacteroides fragilis* (Boente et al., 2016).

4.2. Efflux pumps

Active efflux pumps and transporters are another way in which cells can protect themselves against toxic compounds such as bile salts, other

Table 1
Examples of studies on bile tolerance in microorganisms of non-human origin.

Microorganisms	Origin	Bile (% w/v)	References
Lactic acid bacteria	Fermented olives	0.5	Argyri et al., 2013
<i>Lactobacillus</i> spp.	Cheeses	0.4–2.0	Caggia et al., 2015
<i>Lactobacillus</i> spp., <i>Weissella</i> spp.	Fermented vegetable	0.3–0.5	Anandharaj et al., 2015
<i>Lactobacillus</i> spp.	Dairy samples	0.15–0.45	Kumar and Kumar, 2015
<i>Lactobacillus</i> spp., <i>Pediococcus</i> spp., <i>Oenococcus oeni</i>	Red wine	0.06–1.0	García-Ruiz et al., 2014
<i>L. brevis</i> , <i>Pediococcus pentosaceus</i>	Barley, olive, dry meat	0.3–1.0	Taheur et al., 2016
<i>Lactobacillus fermentum</i>	Swine, poultry GIT	0.3	Lin et al., 2007
<i>Lactobacillus plantarum</i> WCFS1		0.05–0.15	Bron et al., 2004
<i>Lactobacillus</i> spp., <i>Leuconostoc</i> spp.	Fruit drink	0.15–0.30	Sagdic et al., 2014
<i>Enterococcus durans</i>	Soft cheese	0.1–1.5	Pieniz et al., 2014
<i>Propionibacterium</i> spp.	DSM, ATCC	0.06–0.30	Campaniello et al., 2015
<i>Propionibacterium</i> spp.	Cheeses	0.06–0.30	Darilmaz and Beyatli, 2012
<i>Bacillus</i> spp.	Soy sauce	0.3	Lee et al., 2017
<i>Bacillus polyfermenticus</i>	Kimchi	0.3	Lee et al., 2015
Yeasts	Green olives	0.3	Oliveira et al., 2017

detergents, antibiotics, metals or different stress factors. Efflux pumps are ubiquitous systems described for both Gram-positive and Gram-negative bacteria. Based on their structure, mechanisms and energy systems, the pumps were divided into five groups, as reviewed by Sun et al. (2014). These include ATP-binding cassette (ABC) transporters, small multidrug resistance, resistance-nodulation-division (RDN), major facilitator superfamily (MFS) and MATE family pumps. Their influence has been monitored particularly in relation to the antibiotic resistance of pathogenic and nosocomial bacteria (Hernando-Amado et al., 2016; Pos, 2009). Pathogens can successfully activate virulence factors through bile efflux and subsequently repair damage. Quillin et al. (2011) investigated multidrug efflux pumps MdrM and MdrT in *Listeria monocytogenes* and concluded that MdrT can export cholic acid and that both systems are induced by bile. Despite the fact that *Bacteroides* spp. are common representatives in the GIT, outside of their normal niches they can also cause serious infections with high mortalities. The RDN type efflux pump was studied in *Bacteroides fragilis* NCTC9343, where changes in the pump gene transcription level after exposure to 0.15% conjugated or non-conjugated bile salts were confirmed (Wexler, 2012). Modulation and expression of efflux pump genes was also studied extensively in other pathogenic and conditionally pathogenic microorganisms (Alvarez-Ortega et al., 2013), however very few studies have been dedicated to this phenomenon in lactobacilli and bifidobacteria. ABC-type efflux pumps were studied in relation to antibiotic resistance in certain lactic acid bacteria (*Lactococcus lactis*, *Lactobacillus plantarum*) (Wacher-Rodarte et al., 2016). ATP dependent active efflux of taurocholic and cholic acids was demonstrated for *Lactobacillus reuteri* CRL 1098 by Bustos et al. (2011). Probably the first bile-inducible efflux transporter in bifidobacteria (*B. longum* NCIMB 702259^T) was described by Price et al. (2006) for cholate. The coding *ctr* gene was transferred into a negative strain, *E. coli* KAM3, to confirm bile resistance. Also, in other strains such as *Bifidobacterium longum* subsp. *longum* NCC2705 and *B. breve* UCC2003, bile induced the expression of two genes, encoding proteins BL0920 and Bbr0838 resp. which were shown to be responsible for multidrug resistance transporters from a major facilitator superfamily (Gueimonde et al., 2009). Ruiz et al. (2012) studied genes for transporters from MFS and ABC-type transporters in *B. breve* UCC2003. Their induction was shown to be substrate specific (ox-gall, cholate resp.).

4.3. Bile salt hydrolase

Attention has also been paid to the activity of bile salt hydrolase (BSH) cholesteryl glycolate hydrolase (EC 3.5.1.24), which can not only contribute to a reduction in bile toxicity, but is also associated with the therapeutic effect of lowering serum cholesterol. The presence of BSH was found in several bacterial genera originating from the GIT, such as

Lactobacillus spp., *Bifidobacterium* spp. (Jarocki et al., 2014; Kim and Lee, 2008; Kumar et al., 2013; Ren et al., 2011), *Enterococcus* spp. (Chand et al., 2016), *Listeria monocytogenes* (Dussurget et al., 2002), *Clostridium* spp., *Bacteroides* spp. (Gopal-Srivastava and Hylemon, 1988; Masuda, 1981; Rossocha et al., 2005; Yoon et al., 2017). The total distribution of BSH among gut microbiota, as determined by metagenomic analysis, was described by Jones et al. (2008). Although most authors have reported the presence of this enzyme especially in Gram-positive bacteria isolated from the GIT, the enzyme has also been demonstrated in microorganisms obtained from fermented milk products, raw cow's milk, hot springs or fermented vegetables (Reyes-Nava and Rivera-Espinoza, 2014).

Bile salt hydrolase is a constitutive, intracellular enzyme, that catalyses the hydrolysis of amide bonds. Amino acids (glycine or taurine) are released from the bile acid steroid nucleus. In microbial cells, BSH activity could be easily screened by several methods (Sedláčková et al., 2015). The enzyme is classified as an N-terminal nucleophilic hydrolase, with a cysteine residue at the N-terminus, the thiol group of which is a catalytically active site (Begley et al., 2006; Rossocha et al., 2005). In addition to cysteine 2, amino acids arginine 18, aspartic acid 21, asparagine 175 and arginine 228 are also involved in the active centre. Bile salt hydrolase shows high similarity in amino acid sequence with penicillin V amidase isolated from *Bacillus sphaericus* (Ridlon et al., 2006). The optimal conditions for this enzyme might differ slightly depending on its source, ranging from 37 °C to 45 °C in lactobacilli (Sridevi et al., 2009) or up to 50 °C in *Enterococcus faecalis* (Chand et al., 2016). Some additives used in the feed industry, e.g. salts (KIO₃, NaIO₃, CuSO₄, CuCl₂), riboflavin or certain antibiotics, were identified as significant (up to 100%) inhibitors of BSH activity in *L. acidophilus* PF01 (Lin et al., 2014). Many authors agree that this enzyme exhibits higher substrate specificity for glyco-conjugated acids compared to tauro-conjugates and that its production is higher in stationary than in exponential growth phases (Nguyen et al., 2007; Ramasamay et al., 2010).

The presence and arrangements of genes encoding BSH are highly variable in lactobacilli and bifidobacteria. Gene analysis in selected strains of the *Bifidobacterium* genus was described, for example, by Kim and Lee (2008) or Kim et al. (2004). In the case of some lactobacilli species - *L. fermentum* (Kumar et al., 2013), *L. casei* (Zhang et al., 2009), *L. gasseri* (Jiang et al., 2010; Rani et al., 2017) - only one gene was found. In contrast, two genes have been confirmed in *L. acidophilus* NCFM (*bshA* and *bshB*) (McAuliffe et al., 2005) and *L. johnsonii* (Elkins et al., 2001) or three also in *L. johnsonii* PF01 (Chae et al., 2012). *L. salivarius* possesses two genes (Bi et al., 2013), but Jiang et al. (2010) indicated only one gene. In *L. plantarum* the authors concurred on the presence of four different genes for BSH activity (Ren et al., 2011; Lambert et al., 2008). The exact function of BSH has not been fully

explained to date. The first hypothesis was that it assisted in the detoxification of bile and thus allowed better survival under GIT conditions (De Smet et al., 1995; Tanaka et al., 2000). The fact that deconjugation products at low pH (caused for example by the activity of lactic acid bacteria) can precipitate and leave the GIT also supports the detoxification theory (Begley et al., 2006). However, deconjugated bile salts also show strong antimicrobial activity. Free bile acids (cholic and deoxycholic acids) may be more toxic than their conjugated derivatives. Cholic acid itself is more hydrophobic and can enter cells freely (Oelschlaeger, 2010). Therefore, some authors tend to argue that deconjugation is a hostile response supporting autochthonous GIT microorganisms against pathogens (Reyes-Nava and Rivera-Espinoza, 2014). Studies that do not confirm a correlation between BSH activity and bile tolerance have also been published (Ahn et al., 2003; Moser and Savage, 2001). Moreover, not all microorganisms of the GIT exhibit BSH activity but they are still able to survive the passage through the GIT and show growth (Vinderola and Reinheimer, 2003).

However, bile salt deconjugation may have side effects. If this hydrolysis is more extensive in the host's gut, it could lead to a disease called a steatosis. In addition, increased concentrations of secondary bile acids are attributed to colorectal carcinoma-promoting effects (Ajouz et al., 2014).

5. Lowering of serum cholesterol level by probiotic bacteria

Increased levels of blood cholesterol are considered to be major contributors to cardiovascular disease in humans (Chien et al., 2010). Hypercholesterolaemia is the main cause of atherosclerosis, accumulation of cholesterol in coronary arteries and subsequent atheroma, which can cause ischemic heart disease. It has been shown that a 1% decrease in plasma cholesterol reduces the risk of coronary events by 2–3% (Jeun et al., 2010).

Different strategies for a reduction in blood cholesterol levels are used: changes to lifestyle, an adjustment in dietary habits and the application of medication (Dunn-Emke et al., 2001). However, routine pharmacological treatments with statins is expensive and often results in deleterious side effects (Backes et al., 2017). For this reason, other strategies are being sought. Intestinal bacteria, particularly lactobacilli and bifidobacteria, have attracted attention as potential cholesterol-lowering agents (Miremadi et al., 2016); however the exact mechanisms of this effect remain unclear and have to be clarified. Several hypotheses have been proposed regarding the hypocholesterolaemic effect of bacteria and the majority are from *in vitro* or animal experiments. Hypotheses include: deconjugation of bile, which is the most profound mechanism; cholesterol assimilation; coprecipitation of cholesterol with deconjugated bile; cholesterol conversion to coprostanol; production of short fatty acids (Ishimwe et al., 2015; Li, 2012; Michael et al., 2017) and down-regulation of Niemann-Pick C1-like 1 protein (NPC1L1) (Huang and Zheng, 2010).

Deconjugated bile acids are less soluble and less resorbable in the intestinal tract compared to conjugated bile salts (Begley et al., 2006; Nguyen et al., 2007). As already described, approx. 5% of these acids escape reabsorption and have to be subsequently synthesized *de novo* from cholesterol, thereby reducing its concentration in the blood serum. Immobilized BSH from *Lactobacillus buchneri* ATCC 4005 was administered to two groups of laboratory rats with artificially triggered hypercholesterolaemia, and an effect on the levels of total and HDL cholesterol was demonstrated by Sridevi et al. (2009).

Hydrolysed bile salts can also directly co-precipitate with cholesterol under acidic conditions ($\text{pH} \leq 5.5$) and allow it to be removed from the body in faeces. While the physiological pH in the intestine is more or less neutral or slightly alkaline, anaerobic bacteria produce short-chain fatty acids by fermenting dietary fibre and thereby lower the pH locally and create conditions for co-precipitation (Begley et al., 2006). The effect of eleven strains of lactobacilli were tested for co-precipitation of cholesterol with individual bile salts ($6 \text{ mmol} \cdot \text{l}^{-1}$

glycocholate or taurocholate), or their mixture ($2.8 \text{ mmol} \cdot \text{l}^{-1}$ glycocholate and $1.2 \text{ mmol} \cdot \text{l}^{-1}$ taurocholate). It was found that the highest level of co-precipitation occurred with glycocholate and *L. acidophilus* strains compared to *L. casei* (Liong and Shah, 2005a). Liong and Shah (2005b) also measured the co-precipitation of cholesterol with cholic acid, glycocholate and taurocholate at different pH ranging from 7 to 1 for bifidobacteria strains.

Lactobacilli can assimilate cholesterol from the medium during growth as well by absorption or adsorption; the mechanisms of that need to be further supported. As opposed to coprecipitation, cholesterol assimilation by bacteria is not pH-dependent. It has been shown that the absorbed cholesterol is not degraded, but stored in cells in the form of inclusions that can be used later as metabolic precursors (Dora et al., 2002). Nevertheless, Ahire et al. (2012, 2014) proved that cholesterol assimilated by *L. helveticus* was at the same time transformed by cholesterol oxidase. Furthermore, cholesterol may, to some extent, be assimilated both by non-growing and dead cells. Kimoto et al. (2002) observed that dead *Lactococcus* spp. cells were able to remove cholesterol from media and attributed this phenomenon to interactions between the cell wall and cholesterol, later specified and associated with the peptidoglycan cell wall composition (Ooi and Liong, 2010). This ability is species and strain specific, as shown by Lye et al. (2010a) for *L. acidophilus*, *L. bulgaricus* and *L. casei* strains. The best results in cholesterol assimilation were achieved in the presence of ox-bile. The presence of bile salts is probably a prerequisite for cholesterol assimilation by bacteria (Liong and Shah, 2005b). An increased amount of bound cholesterol on the cell surface inhibits the formation of cholesterol micelles which play a major role in the transport of lipids to enterocytes. A 25% reduction in micelle formation compared to a control sample for *L. acidophilus* ATCC 0291 and other lactobacilli in the presence of ox-bile was observed by Lye et al. (2010a), who also documented cholesterol attachment to lactobacilli (*L. bulgaricus* FTDC 1311) using scanning electron microscopy.

Bacteria are also able to remove cholesterol from the environment by incorporating it into their cytoplasmic membranes. One of the first suggestions of cholesterol being taken up by bacterial membranes was presented by Razin, 1975, for *Micrococcus lysodeikticus*, *Bacillus megatherium* and *Proteus mirabilis*. Since then, a few papers have suggested similar findings for probiotics. Noh et al. (1997) demonstrated incorporation of cholesterol into the cellular membrane of *Lactobacillus acidophilus* ATCC 43121. Part of the cholesterol that was assimilated was recovered in the membrane fractions of cells grown both at pH 6 as well as during growth without pH control. Using of the same experimental design, incorporation of cholesterol from the growth medium into the cellular membrane of *Bifidobacterium longum* was also proven (Dambekodi and Gilliland, 1998). In more recent papers, based on the determination of membrane cholesterol and phospholipids contents and membrane fluidity after ultrasound treatment of bacteria, it is stated that cholesterol is incorporated specifically into the polar part of the phospholipid bilayer (Lye et al., 2012).

The phenomenon leads to a change in the distribution of saturated and unsaturated fatty acids, resulting in increased cell membrane firmness (Ooi and Liong, 2010). Changes in composition of membrane fatty acids, particularly myristic, palmitic, stearic, stearoleic and oleic acids in 14 probiotic strains were also confirmed by Miremadi et al. (2014).

An important way to reduce the cholesterol pool, from both endogenous and exogenous sources, is its microbial conversion to coprostanol via hydrogenation, including the creation of 4-cholesten-3-one as an intermediate product (Gérard, 2014; Norin, 2008). Coprostanol represents approx. 50% of total sterols that accumulate in human faeces (Koppel et al., 2017) but this conversion is not unique only to humans (Tyagi et al., 2008). Coprostanol is not absorbed in the intestine and is directly excreted in faeces, thereby reducing cholesterol absorption (Ooi and Liong, 2010). Many strictly anaerobic cholesterol-reducing bacteria were isolated from the GIT and were found to belong

to the genus *Eubacterium* (*E. coprostanoligenes*) (Ismail and Chiang, 2011; Ren et al., 1996) or *Bacteroides* spp. (Gérard et al., 2007). Antharam et al. (2016) measured cholesterol and coprostanol levels in fecal samples of healthy volunteers and subjects with *Clostridium difficile* infection and identified a majority of phylotypes associated with high coprostanol levels as bacteria belonged to the Lachnospiraceae and Ruminococcaceae families of the order Clostridiales. The synthesis of cholesterol reductase and the conversion of cholesterol to coprostanol were also confirmed in lactobacilli strains (Lye et al., 2010b). Olejníková et al. (2017) compared the levels of cholesterol, coprostanol and cholestenone in samples of two groups with different dietary habits (vegetarians and omnivores) and observed higher amounts of lactobacilli and bifidobacteria in samples with higher levels of sterol conversion.

Previously, an interesting theory was explored by Favier et al. (1995) on the contribution of short chain fatty acids (acetic, propionic), produced by colonic microbiota from non-digestible fibre, to *de novo* lipogenesis. Acetate is a main potential substrate for cholesterol synthesis in the liver, where 3-hydroxy-3-methylglutaryl coenzyme A reductase is a limiting enzyme. This enzyme can be inhibited by propionate, which is better absorbed through the mucosa. Consequently, the ratio of acetate to propionate in the colon plays a significant role in the regulation of cholesterol and lipid metabolism in humans (Conterno et al., 2011).

An important role in cholesterol absorption is played by Niemann-Pick C1-Like 1 protein (NPC1L1), which is expressed in the membrane of enterocytes of the small intestine. This transmembrane protein has been identified as a specific transporter for cholesterol uptake (Davis et al., 2004; Jia et al., 2011). It has been reported that *Lactobacillus acidophilus* ATCC 4356 and *L. plantarum* Lp27 may lower cholesterol absorption in Caco-2 cells or hypercholesterolaemic rats by down-regulating NPC1L1 (Huang et al., 2010; Huang et al., 2013; Huang and Zheng, 2010) in the same way as *L. plantarum* NR74, *L. rhamnosus* BFE5264 (Yoon et al., 2013), *L. plantarum* PCS 20 and *L. plantarum* PCS 26 (Gorenjak et al., 2014). Additionally, Michael et al. (2016) suggested that reduced transcript levels of NPC1L1 by *L. plantarum* CUL66 may be able to inhibit the uptake of cholesterol by intestinal epithelial cells.

Many studies have been carried out demonstrating the ability of lactic acid bacteria and bifidobacteria to reduce the cholesterol content *in vitro* in cultivation media, or under simulated intestinal conditions (Lye et al., 2010a; Öner et al., 2014; Tomaro-Duchesneau et al., 2014). However, studies *in vivo*, both in humans and animals, have been less persuasive, and need more evidence in meta-analyses to provide better information (Jones et al., 2013). Ivey et al. (2015) found no effect in a randomized study with 156 volunteers administered with *L. acidophilus* La5 and *Bifidobacterium animalis* subsp. *lactis* Bb 12. In contrast, a study of 114 hypercholesterolaemic adults who consumed encapsulated *Lactobacillus reuteri* NCIMB 30242 in yogurt observed a significant reduction in total and LDL-cholesterol (Jones et al., 2012).

6. Conclusions

The interactions between bile salts and intestinal microbiota are of ongoing research interest, specifically concerning probiotic microorganisms. This review has concentrated on two interrelated aspects of this topic – mechanisms of defence of some bacteria against the toxic effects of the bile acids, including their ability to grow; and the effectiveness of these bile-tolerant bacteria in functioning as cholesterol-reducing agents. Several proposed mechanisms that could explain this ability include BSH activity of these bacteria, leading to deconjugation of bile acids and their coprecipitation with cholesterol. Alternatively, some lactobacilli seem to be able to assimilate cholesterol in their cells and/or on cell walls, probably aided by the presence of bile salts. Unfortunately, these proposed mechanisms have been based largely on *in vitro* studies and still await confirmation by studies carried out *in vivo*. It can be assumed that there are still many unanswered questions.

Nevertheless, strains with well-defined properties associated with proven cholesterol reduction could be a choice for food supplements or even drugs used for treatment of metabolic diseases.

References

- de Aguiar Vallim, T.Q., Tarling, E.J., 2013. Pleiotropic roles of bile acids in metabolism. *Cell Metab.* 17, 657–669.
- de Aguiar Vallim, T.Q., Tarling, E.J., Ahn, H., Hagey, L.R., Romanoski, E.E., Lee, R.G., Graham, M.J., Motohashi, H., Yamamoto, M., Edwards, P.A., 2015. MAFG is a transcriptional repressor of bile acid synthesis and metabolism. *Cell Metab.* 21, 298–310.
- Ahire, J.J., Bhat, A.A., Thakare, J.M., Pawar, P.B., Zope, D.G., Jain, R.J., Chaudhari, B.L., 2012. Cholesterol assimilation and biotransformation by *Lactobacillus helveticus*. *Biotechnol. Lett.* 34, 103–107.
- Ahire, J.J., Mokashe, N.U., Chaudhari, B.L., 2014. Cholesterol biotransformation of cholesta-4,6-die-3-ol and effect of assimilation on adhesion properties of *Lactobacillus helveticus* CD6. *J. Microbiol. Biotech. Food Sci.* 3, 398–401.
- Ahn, Y.T., Kim, G.B., Lim, Y.S., Baek, Y.J., Kim, Y.U., 2003. Deconjugation of bile salts by *Lactobacillus acidophilus* isolates. *Int. Dairy J.* 13, 303–311.
- Ajouz, H., Mukherji, D., Shamseddine, A., 2014. Secondary bile acids: an underrecognized cause of colon cancer. *World J. Surg. Oncol.* 12, 164. <http://dx.doi.org/10.1186/1477-7819-12-164>.
- Alp, G., Aslim, B., 2010. Relationship between the resistance to bile salts and low pH with exopolysaccharide (EPS) production of *Bifidobacterium* spp. isolated from infants feces and breast milk. *Anaerobe* 16, 101–105.
- Alvarez-Ortega, C., Olivares, J., Martínez, J.L., 2013. RND multidrug efflux pumps: what are they good for? *Front. Microbiol.* 4, 1–11.
- An, H., Douillard, F.P., Wang, G., Zhai, Z., Yang, J., Song, S., Cui, J., Ren, F., Luo, Y., Zhang, B., Hao, Y., 2014. Integrated transcriptomic and proteomic analysis of the bile stress response in a centenarian-originated probiotic *Bifidobacterium longum* BBMN68. *Mol. Cell. Proteomics* 13, 2558–2572.
- Anandharaj, M., Sivasankari, B., Santhanakaruppu, R., Manimaran, M., Rani, R.P., Sivakumar, S., 2015. Determining the probiotic potential of cholesterol-reducing *Lactobacillus* and *Weissella* strains isolated from gherkins (fermented cucumber) and south Indian fermented koozh. *Res. Microbiol.* 166, 428–439.
- Antharam, V.C., McEwen, D.C., Garrett, T.J., Dossey, A.T., Li, E.C., Kozlov, A.N., Mesbah, Z., Wang, G.P., 2016. An integrated metabolomics and microbiome analysis identified specific gut microbiota associated with fecal cholesterol and coprostanol in *Clostridium difficile* infection. *PLoS One* 11 (2), e014882. <http://dx.doi.org/10.1371/journal.pone.0148824>.
- Argyri, A.A., Zoumpopoulou, G., Karatzas, K.-A.G., Tsakalidou, E., Nychas, G.-J.E., Panagou, E.Z., Tassou, C.C., 2013. Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol.* 33, 282–291.
- Aslim, B., Onal, D., Beyatli, Y., 2007. Factors influencing autoaggregation and aggregation of *Lactobacillus delbrueckii* subsp. *bulgaricus* isolated from handmade yogurt. *J. Food Prot.* 70, 223–227.
- Backes, J.M., Ruisinger, J.F., Gibson, C.A., Moriarty, P.M., 2017. Statin-associated muscle symptoms – managing the highly intolerant. *J. Clin. Lipidol.* 11, 24–33.
- Begley, M., Hill, C., Gahan, C.G.M., 2006. Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* 72, 1729–1738.
- Bernstein, C., Holubec, H., Bhattacharyya, A.K., Nguyen, H., Payne, C.M., Zaitlin, B., Bernstein, H., 2011. Carcinogenicity of deoxycholate, a secondary bile acid. *Arch. Toxicol.* 85, 863–871.
- Bi, J., Fang, F., Lu, S., Du, G., Chen, J., 2013. New insight into the catalytic properties of bile salt hydrolase. *J. Mol. Catal. B Enzym.* 96, 46–51.
- Boente, R.F., Pauer, H., Silva, D., Filho, J.S., Sandim, V., Antunes, L., Ferreira, R., Zngali, R.B., Domingues, R., Lobo, L.A., 2016. Differential proteomic analysis of outer membrane enriched extracts of *Bacteroides fragilis* grown under bile salts stress. *Anaerobe* 39, 84–90.
- Boke, H., Aslim, B., Alp, G., 2010. The role of resistance to bile salts and acid tolerance of exopolysaccharides (EPSS) produced by yogurt starter bacteria. *Arch. Biol. Sci.* 62, 323–328.
- Bron, P.A., Marco, M., Hoffer, S.M., Van Mulleken, E., de Vos, W.M., Kleerebezem, M., 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. *J. Bacteriol.* 186, 7829–7835.
- Bustos, A.Y., Raya, R., Font de Valdez, G., Taranto, M.P., 2011. Efflux of bile acids in *Lactobacillus reuteri* is mediated by ATP. *Biotechnol. Lett.* 33, 2265–2269.
- Bustos, A.Y., Saavedra, L., Font de Valdez, G., Raya, R.R., Taranto, M.P., 2012. Relationship between bile salt hydrolase activity, changes in the internal pH and tolerance to bile acids in lactic acid bacteria. *Biotechnol. Lett.* 34, 1511–1518.
- Bustos, A.Y., de Valdez, G.F., Raya, R., Almeida, A.M., Fadda, S., Taranto, M.P., 2015. Proteomic analysis of the probiotic *Lactobacillus reuteri* CRL1098 reveals novel tolerance biomarkers to bile acid-induced stress. *Food Res. Int.* 77, 599–607.
- Caggia, C., De Angelis, M., Pitino, I., Pino, A., Randazzo, C.L., 2015. Probiotic features of *Lactobacillus* strains isolated from Ragusano and Pecorino Siciliano cheeses. *Food Microbiol.* 50, 109–117.
- Campaniello, D., Bevilacqua, A., Sinigaglia, M., 2015. Screening of *Propionibacterium* spp. for potential probiotic properties. *Anaerobe* 34, 169–173.
- Chae, J.P., Valeriano, V.D., Kim, G.-B., Kang, D.-K., 2012. Molecular cloning, characterization and comparison of bile salt hydrolases from *Lactobacillus johnsonii* PF01. *J. Appl. Microbiol.* 114, 121–133.
- Chand, D., Ramasamy, S., Suresh, C.G., 2016. A highly active bile salt hydrolase from

- Enterococcus faecalis* shows positive cooperative kinetics. *Process Biochem.* 51, 263–269.
- Cháver-Talavera, O., Tailleur, A., Lefebvre, P., Staels, B., 2017. Bile acid control of metabolism and inflammation in obesity, type 2 diabetes, dyslipidemia, and nonalcoholic fatty liver disease. *Gastroenterology* 152, 1679–1694.
- Chiang, J.Y.L., 2013. Bile acid metabolism and signaling. *Compr. Physiol.* 3, 1191–1212.
- Chien, Y.L., Wu, L.Y., Lee, T.C., Hwang, L.S., 2010. Cholesterol-lowering effect of phytosterol-containing lactic-fermented milk powder in hamsters. *Food Chem.* 119, 1121–1126.
- Collado, M.C., Meriluoto, J., Salminen, S., 2008. Adhesion and aggregation properties of probiotic and pathogen strains. *Eur. Food Res. Technol.* 226, 1065–1073.
- Conterno, L., Fava, F., Viola, R., Tuohy, K.M., 2011. Obesity and the gut microbiota: does up-regulating colonic fermentation protect against obesity and metabolic disease? *Genes Nutr.* 6, 241–260.
- Dambekodi, P.C., Gilliland, S.E., 1998. Incorporation of cholesterol into the cellular membrane of *Bifidobacterium longum*. *J. Dairy Sci.* 81, 1818–1824.
- Darilimaz, D.O., Beyatli, Y., 2012. Acid-bile, antibiotic resistance and inhibitory properties of propionibacteria isolated from Turkish traditional home-made cheeses. *Anaerobe* 18, 122–127.
- Davis, A.P., 2007. Bile acid scaffolds in supramolecular chemistry: the interplay of design and synthesis. *Molecules* 12, 2106–2122.
- Davis, H.R., Zhu, L., Hoos, L.M., Tetzloff, G., Maguire, M., Liu, J., Yao, X., Iyer, S.P.N., Lam, M.-H., Lund, E.G., Detmers, P.A., Graziano, M.P., Altmann, S.W., 2004. Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J. Biol. Chem.* 279, 33586–33592.
- De Smet, I., Van Hoorde, L., Woestyne, V., Christiaens, H., Verstraete, W., 1995. Significance of bile salt hydrolytic activities of lactobacilli. *J. Appl. Bacteriol.* 79, 292–301.
- Dora, I., Pereira, A., Glenn, R.G., 2002. Cholesterol assimilation by lactic acid bacteria and *Bifidobacteria* isolated from the human gut. *Appl. Environ. Microbiol.* 68, 4689–4693.
- Dunn-Ekke, S., Weidner, G., Ornish, D., 2001. Benefits of a low-fat plant-based diet. *Obes. Res.* 9, 731–736.
- Dussurget, O., Cabanes, D., Dehoux, P., Lecuit, M., Buchrieser, C., Glaser, P., Cossart, P., 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. *Mol. Microbiol.* 45, 1095–1106.
- Dziedziol, M., Wagner, M., Hein, I., 2011. CmeR-dependent gene Cj0561c is induced more effectively by bile salts than the CmeABC efflux pump in both human and poultry *Campylobacter jejuni* strains. *Res. Microbiol.* 162, 991–998.
- Eggert, T., Bakonyi, D., Hummel, W., 2014. Enzymatic routes for the synthesis of ursodeoxycholic acid. *J. Biotechnol.* 191, 11–21.
- Elkins, C.A., Moser, S.A., Savage, D.C., 2001. Genes encoding bile salt hydrolases and conjugated bile salt transporters in *Lactobacillus johnsonii* 100-100 and other *Lactobacillus* species. *Microbiology* 147, 3403–3412.
- FAO/WHO, 2002. Guidelines for the evaluation of probiotics in food. In: Report of a joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London, ON, Canada.
- Favier, M.L., Moundras, C., Dénigé, C., Rémy, C., 1995. Fermentable carbohydrates exert a more potent cholesterol-lowering effect than cholestyramine. *Biochim. Biophys. Acta* 1258, 115–121.
- Fukui, S., Arata, M., Kawashima, Y., Yoshida, D., Kaneko, M., Minamida, K., Watanabe, J., Ogura, Y., Uchida, K., Itoh, K., Wada, M., Ito, S., Yokota, A., 2009. Conversion of cholic acid and chenodeoxycholic acid into their 7-oxo derivatives by *Bacteroides intestinalis* AM-1 isolated from human feces. *FEMS Microbiol. Lett.* 293, 263–270.
- García-Ruiz, A., González de Llano, D., Esteban-Fernández, A., Requena, T., Barolomé, B., Moreno-Arriba, M.V., 2014. Assessment of probiotic properties in lactic acid bacteria isolated from wine. *Food Microbiol.* 44, 220–225.
- Gérard, P., 2014. Metabolism of cholesterol and bile acids by the gut microbiota. *Pathogens* 3, 14–24.
- Gérard, P., Lepercq, P., Leclerc, M., Gavini, F., Raibaud, P., Juste, C., 2007. *Bacteroides* sp. strain D8, the first cholesterol-reducing bacterium isolated from human feces. *Appl. Environ. Microbiol.* 73, 5742–5749.
- Gopal-Srivastava, R., Hylemon, P.B., 1988. Purification and characterization of bile salt hydrolase from *Clostridium perfringens*. *J. Lipid Res.* 29, 1079–1085.
- Gorenjak, M., Gradišnik, L., Trapežar, M., Pistello, M., Kozmus, C.P., Škorjanc, D., Skok, P., Langerholc, T., Cencić, A., 2014. Improvement of lipid profile by probiotic/protective cultures: study in a non-carcinogenic small intestinal cell model. *New Microbiol.* 37, 51–64.
- Gueimonde, M., Garrigues, C., van Sinderen, D., de los Reyes-Gavilán, C.G., Margolles, A., 2009. Bile-inducible efflux transporter from *Bifidobacterium longum* NCC2705, conferring bile resistance. *Appl. Environ. Microbiol.* 75, 3153–3160.
- Hemaraajata, P., Versalovic, J., 2013. Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. *Therap. Adv. Gastroenterol.* 6, 39–51.
- Hernando-Amado, S., Blanco, P., Alcalde-Rico, M., Corona, F., Reales-Calderón, A.R., Sánchez, B., Martínez, J.L., 2016. Multidrug efflux pumps as main players in intrinsic and acquired resistance to antimicrobials. *Drug Resist. Updat.* 28, 13–27.
- Hofmann, A.F., Eckmann, L., 2006. How bile acids confer gut mucosal protection against bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 103, 4333–4334.
- Hofmann, A.F., Sjövall, J., Kurz, G., Radominska, A., Schteingart, C.D., Tint, G.S., Vlahcevic, Z.R., Setchell, K.D.R., 1992. A proposed nomenclature for bile acids. *J. Lipid Res.* 33, 599–604.
- Horáčková, Š., Žaludová, K., Plocková, M., 2011. Stability of selected lactobacilli in the conditions simulating those in the gastrointestinal tract. *Czech J. Food Sci.* 29, 30–35.
- Horáčková, Š., Mühlhansová, A., Plocková, M., 2017. Interakce žlučových kyselin a probiotických mikroorganismů. *Chem. Listy* 111, 246–250.
- Huang, Y., Zheng, Y., 2010. The probiotic *Lactobacillus acidophilus* reduces cholesterol absorption through the down-regulation of Niemann-Pick C1-like 1 in Caco-2 cells. *Br. J. Nutr.* 103, 473–478.
- Huang, L., Sun, Y., Zhu, H., Zhang, Y., Xu, J., Shen, Y.-M., 2009. Synthesis and antimicrobial evaluation of bile acid tridentate conjugates. *Steroids* 74, 701–706.
- Huang, Y., Wang, J., Cheng, Y., Zheng, Y., 2010. The hypocholesterolaemic effects of *Lactobacillus acidophilus* ATCC 4356 in rats are mediated by the down-regulation of Niemann-Pick C1-Like 1. *Br. J. Nutr.* 104, 807–812.
- Huang, Y., Wu, F., Wang, X., Sui, Y., Yang, L., Wang, J., 2013. Characterization of *Lactobacillus plantarum* Lp27 isolated from Tibetan kefir grains: a potential probiotic bacterium with cholesterol-lowering effects. *J. Dairy Sci.* 96, 2816–2825.
- Ishimwe, N., Daliri, E.B., Lee, B.H., Fang, F., Du, G., 2015. The perspective on cholesterol-lowering mechanisms of probiotics. *Mol. Nutr. Food Res.* 59, 94–105.
- Islam, K.B., Fukui, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., 2011. Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 141, 1773–1781.
- Ismail, W., Chiang, I.R., 2011. Oxidative and anoxic metabolism of steroids by bacteria. *J. Biomed. Biotechnol.* S1, 001. <http://dx.doi.org/10.4172/2155-6199.S1-001>.
- Ivey, K.L., Hodgson, J.M., Kerr, D.A., Thompson, P.L., Stojceski, B., Prince, R.L., 2015. The effect of yoghurt and its probiotics on blood pressure and serum lipid profile; a randomised controlled trial. *Nutr. Metab. Cardiovasc. Dis.* 25, 46–51.
- Jarocki, P., Podleśny, M., Glibowski, P., Targoński, Z., 2014. A new insight into the physiological role of bile salt hydrolase among intestinal bacteria from the genus *Bifidobacterium*. *PLoS One* 9 (12), e114379. <http://dx.doi.org/10.1371/journal.pone.0114379>.
- Jeun, J., Kim, S., Cho, S.J., Jun, H., Park, H.J., Seo, J.G., Chung, M.J., Lee, S.J., 2010. Hypocholesterolemic effects of *Lactobacillus plantarum* KCTC3928 by increased bile acid excretion in C57BL/6 mice. *Nutrition* 26, 321–330.
- Jia, L., Betters, J.L., Yu, L., 2011. Niemann-Pick C1-Like 1 (NPC1L1) protein in intestinal and hepatic cholesterol transport. *Annu. Rev. Physiol.* 73, 239–259.
- Jiang, J., Hang, X., Zhang, M., Liu, X., Li, D., Yang, H., 2010. Diversity of bile salt hydrolase activities in different lactobacilli toward human bile salts. *Ann. Microbiol.* 60, 81–88.
- Jones, B.V., Begley, M., Hill, C., Gahan, C.G.M., Marchesi, J.R., 2008. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proc. Natl. Acad. Sci. U. S. A.* 105, 13580–13585.
- Jones, M.L., Martoni, C.J., Parent, M., Prakash, S., 2012. Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active *Lactobacillus reuteri* NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. *Br. J. Nutr.* 107, 1505–1513.
- Jones, M.L., Tomaro-Duchesneau, C., Martoni, C.J., Prakash, S., 2013. Cholesterol lowering with bile salt hydrolase-active probiotic bacteria, mechanism of action, clinical evidence, and future direction for heart health applications. *Expert. Opin. Biol. Ther.* 13, 631–642.
- Khaleghi, M., Kermanshahi, R.K., Yaghoobi, M.M., Zarkesh-Esfahani, S.H., Baghizadeh, A., 2010. Assessment of bile salt effects on s-layer production, *slp* gene expression and some physicochemical properties of *Lactobacillus acidophilus* ATCC 4356. *J. Microbiol. Biotechnol.* 20, 749–756.
- Kim, G.-B., Lee, B.H., 2008. Genetic analysis of a bile salt hydrolase in *Bifidobacterium animalis* subsp. *lactis* KL612. *J. Appl. Microbiol.* 105, 778–790.
- Kim, G., Miyamoto, C.M., Meighen, E., Lee, B.H., 2004. Cloning and characterization of the bile salt hydrolase genes (*bsh*) from *Bifidobacterium bifidum* strains. *Appl. Environ. Microbiol.* 70, 5603–5612.
- Kimoto, H., Ohmomo, S., Okamoto, T., 2002. Cholesterol removal from media by *Lactococci*. *J. Dairy Sci.* 85, 3182–3188.
- Kociubinski, G., Zavaglia, A.G., Pérez, P.F., Disalvo, E.A., De Antoni, G.L., 2002. Effect of bile components on the surface properties of bifidobacteria. *J. Dairy Res.* 69, 293–302.
- Koppel, N., Redkal, V.M., Balskus, E.P., 2017. Chemical transformation of xenobiotics by the human gut microbiota. *Science* 356, 1246–1257.
- Kotal, P., Demnerová, K., Pazlarová, J., Plocková, M., Šmidrkal, J., 2014. Inhibition of exoproteins using bacteria of the *Lactobacillus* genus. Republic of South Africa, P54330ZA00.
- Kotzamanidis, C., Kourelis, A., Litopoulou-Tzanetaki, E., Tzanetakis, N., Yiangou, M., 2010. Evaluation of adhesion capacity, cell surface traits and immunomodulatory activity of presumptive probiotic *Lactobacillus* strains. *Int. J. Food Microbiol.* 140, 154–163.
- Kumar, A., Kumar, D., 2015. Characterization of *Lactobacillus* isolated from dairy samples for probiotic properties. *Anaerobe* 33, 117–123.
- Kumar, R., Rajkumar, H., Kumar, M., Varikuti, S.R., Athimamula, R., Shujaiddin, M., Ramagoni, R., Kondapalli, N., 2013. Molecular cloning, characterization and heterologous expression of bile salt hydrolase (Bsh) from *Lactobacillus fermentum* NCD0394. *Mol. Biol. Rep.* 40, 5057–5066.
- Kurdi, P., Kawanishi, K., Mizutani, K., Yokota, A., 2006. Mechanisms of growth inhibition by free bile acids in lactobacilli and bifidobacteria. *J. Bacteriol.* 188, 1979–1986.
- Lambert, J.M., Bongers, R.S., de Vos, W.M., Kleerebezem, M., 2008. Functional analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus plantarum* WCF51. *Appl. Environ. Microbiol.* 74, 4719–4726.
- Lee, J.Y., Pajarillo, E.A.B., Kim, M.J., Chae, J.P., Kang, D.-K., 2013. Proteomic and transcriptional analysis of *Lactobacillus johnsonii* PF01 during bile salt exposure by iTRAQ shotgun proteomics and quantitative RT-PCR. *J. Proteome Res.* 12, 432–443.
- Lee, N.-K., Son, S.-H., Jeon, E.B., Jung, G.H., Lee, J.-Y., Paik, H.-D., 2015. The prophylactic effect of probiotic *Bacillus polyfermenticus* KU3 against cancer cells. *J. Funct. Foods* 14, 513–518.
- Lee, S., Lee, J., Jin, Y.-I., Jeong, J.-C., Chang, Y.-H., Lee, Y., Jeong, Y., Kim, M., 2017.

- Probiotic characteristics of *Bacillus* strains isolated from Korean traditional soy sauce. *LWT-Food Sci. Technol.* 79, 518–524.
- Lepercq, P., Gérard, P., Béquet, F., Raibaud, P., Grill, J.-P., 2004. Epimerization of chenodeoxycholic acid to ursodeoxycholic acid by *Clostridium baratii* isolated from human feces. *FEMS Microbiol. Lett.* 235, 65–72.
- Li, G., 2012. Intestinal probiotics: interactions with bile salts and reduction of cholesterol. *Procedia Environ Sci* 12, 1180–1186.
- Li, T., Chiang, J.Y.L., 2015. Bile acids as metabolic regulators. *Curr. Opin. Gastroenterol.* 31, 159–165.
- Lilly, D.M., Stillwell, R.H., 1965. Probiotics: growth-promoting factors produced by microorganisms. *Science* 147, 747–748.
- Lin, W.-H., Jang, S.-H., Tsen, H.-Y., 2007. Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. *Anaerobe* 13, 107–113.
- Lin, J., Negga, R., Zeng, X., Smith, K., 2014. Effect of bile salt hydrolase inhibitors on a bile salt hydrolase from *Lactobacillus acidophilus*. *Pathogens* 3, 947–956.
- Liong, M.-T., Shah, N.P., 2005a. Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. *Int. Dairy J.* 15, 391–398.
- Liong, M.-T., Shah, N.P., 2005b. Bile salt deconjugation and BSH activity of five bifidobacterial strains and their cholesterol co-precipitating properties. *Food Res. Int.* 38, 135–142.
- López-Garrido, J., Cheng, N., García-Quintanilla, F., García-del Portillo, F., Casades, J., 2010. Identification of the *Salmonella enterica* *damX* gene product, an inner membrane protein involved in bile resistance. *J. Bacteriol.* 192, 893–895.
- Lubanda, H., Vecka, M., 2009. Cholesterol – přítel či nepřítel? *Chem. Listy* 103, 40–51.
- Lv, L.X., Yan, R., Shi, H.Y., Shi, D., Fang, D.Q., Jiang, H.Y., Wu, W.R., Guo, F.F., Jiang, X.W., Gu, S.L., Chen, Y.B., Yao, J., Li, L.J., 2017. Integrated transcriptomic and proteomic analysis of the bile stress response in probiotic *Lactobacillus salivarius* LI01. *J. Proteomics* 150, 216–229.
- Lye, H.-S., Rahmat-Ali, G.R., Liong, M.-T., 2010a. Mechanisms of cholesterol removal by lactobacilli under conditions that mimic the human gastrointestinal tract. *Int. Dairy J.* 20, 169–175.
- Lye, H.-S., Rusul, G., Liong, M.-T., 2010b. Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol. *J. Dairy Sci.* 93, 1383–1392.
- Lye, H.-S., Alias, K.A., Rusul, G., Liong, M.-T., 2012. Ultrasound treatment enhances cholesterol removal ability of lactobacilli. *Ultrason. Sonochem.* 19, 632–641.
- Madiedo, P.R., Gueimonde, M., Arigoni, F., Reyes-Gavila, C.G., Margolles, A., 2009. Bile affects the synthesis of exopolysaccharides by *Bifidobacterium animalis*. *Appl. Environ. Microbiol.* 75, 1204–1207.
- Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., Mackie, A., 2011. The role of bile salts in digestion. *Adv. Colloid Interf. Sci.* 165, 36–46.
- Masuda, N., 1981. Deconjugation of bile salts by *Bacteroides* and *Clostridium*. *Microbiol. Immunol.* 25, 1–11.
- McAuliffe, O., Cano, R.J., Klaenhammer, T.R., 2005. Genetic analysis of two bile salt hydrolase activities in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 71, 4925–4929.
- Melo, J., Schrama, D., Hussey, S., Andrew, P.W., Faleiro, M.L., 2013. *Listeria monocytogenes* dairy isolates show a different proteome response to sequential exposure to gastric and intestinal fluids. *Int. J. Food Microbiol.* 163, 51–63.
- Merritt, M.E., Donaldson, J.R., 2009. Review-effect of bile salts on the DNA and membrane integrity of enteric bacteria. *J. Med. Microbiol.* 58, 1533–1541.
- Michael, D.R., Moss, J.W.E., Calvente, D.L., Garaiova, I., Plummer, S.F., Ramji, D.P., 2016. *Lactobacillus plantarum* CUL66 can impact cholesterol homeostasis in Caco-2 enterocytes. *Benefic. Microbes* 7, 443–451.
- Michael, D.R., Davies, T.S., Moss, J.W.E., Calvente, D.L., Ramji, D.P., Marchesi, J.R., Pechlivanis, A., Plummer, S.F., Hughes, T.S., 2017. The anti-cholesterolaemic effect of a consortium of probiotics: an acute study in C57BL/6J mice. *Sci. Rep.* 7, 2883. <http://dx.doi.org/10.1038/s41598-017-02889-5>.
- Miremadi, F., Ayyasch, M., Sherkat, F., Stojanovska, L., 2014. Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic Lactobacilli and Bifidobacteria. *J. Funct. Foods* 9, 295–305.
- Miremadi, F., Sherkat, F., Stojanovska, L., 2016. Hypocholesterolaemic effect and anti-hypertensive properties of probiotics and prebiotics. *J. Funct. Foods* 25, 497–510.
- Moser, S.A., Savage, D.C., 2001. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in Lactobacilli. *Appl. Environ. Microbiol.* 67, 3476–3480.
- Mukhopadhyay, S., Maitra, U., 2004. Chemistry and biology of bile acids. *Curr. Sci.* 87, 1666–1683.
- Nascimento, P.G.G., Lemos, T.L.G., Almeida, M.C.S., de Souza, J.M.O., Bizerra, A.M.C., Santiago, G.M.P., da Costa, J.G.M., Coutinho, H.D.M., 2015. Lithocholic acid and derivatives: antibacterial activity. *Steroids* 104, 8–15.
- Nguyen, T.D.T., Kang, J.H., Lee, M.S., 2007. Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *Int. J. Food Microbiol.* 113, 358–361.
- Nickerson, K.P., Chanin, R.B., Sistrunk, J.R., Rasko, D.A., Fink, P.J., Barry, E.M., Nataro, J.P., Faherty, C.S., 2017. Analysis of *Shigella flexneri* resistance, biofilm formation, and transcriptional profile in response to bile salts. *Infect. Immun.* 85, e01067-16. <http://dx.doi.org/10.1128/IAI.01067-16>.
- Noh, D.O., Kim, S.H., Gilliland, S.E., 1997. Incorporation of cholesterol into the cellular membrane of *Lactobacillus acidophilus* ATCC 43121. *J. Dairy Sci.* 80, 3107–3113.
- Noriega, L., Gueimonde, M., Sánchez, B., Margolles, A., de los Reyes-Gavilán, C.G., 2004. Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in *Bifidobacterium*. *Int. J. Food Microbiol.* 94, 79–86.
- Noriega, L., Cuevas, I., Margolles, A., de los Reyes-Gavilán, C.G., 2006. Deconjugation and bile salt hydrolase activity by *Bifidobacterium* strains with resistance to bile. *Int. Dairy J.* 16, 850–855.
- Norin, E., 2008. Intestinal cholesterol conversion in adults and elderly from four different European countries. *Ann. Nutr. Metab.* 52, 12–14.
- Norlin, M., Wikvall, K., 2007. Enzymes in the conversion of cholesterol into bile acids. *Curr. Mol. Med.* 7, 199–218.
- Oelschlaeger, T.A., 2010. Mechanisms of probiotic actions – a review. *Int. J. Med. Microbiol.* 300, 57–62.
- Olejniková, P., Kaszonyi, A., Šimkovič, M., Lakatoš, B., Kaliňák, M., Valachovičová, M., Bírošová, L., 2017. Differences in gut microbiota activity (antimicrobials, potential mutagens, and sterols) according to diet. *Acta Aliment* 46, 61–68.
- Oliveira, T., Ramalhosa, E., Nunes, L., Pereira, J.A., Colla, E., Pereira, E.L., 2017. Probiotic potential of indigenous yeasts isolated during the fermentation of table olives from northeast of Portugal. *Innov. Food Sci. Emerg. Technol.* 44, 167–172.
- Öner, Ö., Aslim, B., Aydaş, S.B., 2014. Mechanisms of cholesterol-lowering effects of lactobacilli and bifidobacteria strains as potential probiotics with their bsh gene analysis. *J. Mol. Microbiol. Biotechnol.* 24, 12–18.
- Ooi, L.-G., Liong, M.-T., 2010. Cholesterol-lowering effects of probiotics and prebiotics: a review of *in vivo* and *in vitro* findings. *Int. J. Mol. Sci.* 11, 2499–2522.
- Park, M.Y., Kim, S.J., Ko, E.K., Ahn, S.H., Seo, H., Sung, M.K., 2016. Gut microbiota-associated bile acid deconjugation accelerates hepatic steatosis in ob/ob mice. *J. Appl. Microbiol.* 121, 800–810.
- Patel, A.K., Singhania, R.R., Pandey, A., Chincholkar, S.B., 2010. Probiotic bile salt hydrolase: current developments and perspectives. *Appl. Biochem. Biotechnol.* 162, 166–180.
- Payne, C.M., Holubec, H., Crowley-Skillicorn, C., Nguyen, H., Bernstein, H., Wilcox, G., Bernstein, C., 2011. Maspin is a deoxycholate-inducible, anti-apoptotic stress-response protein differentially expressed during colon carcinogenesis. *Clin. Exp. Gastroenterol.* 4, 239–253.
- Pfeiler, E.A., Azcarate-Peril, M.A., Klaenhammer, T.R., 2007. Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. *J. Bacteriol.* 189, 4624–4634.
- Philipp, B., 2011. Bacterial degradation of bile salts. *Appl. Microbiol. Biotechnol.* 89, 903–915.
- Pieniz, S., Andreazza, R., Anghinoni, R., Camargo, F., Brandelli, A., 2014. Probiotic potential, antimicrobial and antioxidant activities of *Enterococcus durans* strain LAB18s. *Food Control* 37, 251–256.
- Pos, K.M., 2009. Drug transport mechanism of the AcrB efflux pump. *Biochim. Biophys. Acta* 1794, 782–793.
- Price, C.E., Reid, S.J., Driessen, A.J.M., Abratt, V.R., 2006. The *Bifidobacterium longum* NCIMB 70225⁹ *ctr* gene codes for a novel cholate transporter. *Appl. Environ. Microbiol.* 72, 923–926.
- Prieto, A.I., Ramos-Morales, F., Casades, J., 2004. Bile-induced DNA damage in *Salmonella enterica*. *Genetics* 168, 1787–1794.
- Pumbwe, L., Skilbeck, C.A., Nakano, V., Avila-Campos, M.J., Piazza, R.M.F., Wexler, H.M., 2007. Bile salts enhance bacterial co-aggregation, bacterial-intestinal epithelial cell adhesion, biofilm formation and antimicrobial resistance of *Bacteroides fragilis*. *Microb. Pathog.* 43, 78–87.
- Quillin, S.J., Schwartz, K.T., Leber, J.H., 2011. The novel *Listeria monocytogenes* bile sensor BrtA controls expression of the cholic acid efflux pump MdrT. *Mol. Microbiol.* 81, 129–142.
- Ramasamay, K., Abdullah, N., Wong, M.C., Karuthan, C., Ho, Y.W., 2010. Bile salt deconjugation and cholesterol removal from media by *Lactobacillus* strains used as probiotics in chickens. *J. Sci. Food Agric.* 90, 65–69.
- Rani, R.P., Anandharaj, J., Ravindran, A.D., 2017. Characterization of bile salt hydrolase from *Lactobacillus gasseri* FR4 and demonstration of its substrate specificity and inhibitory mechanism using molecular docking analysis. *Front. Microbiol.* 8, 1004. <http://dx.doi.org/10.3389/fmicb.2017.01004>.
- Rasras, A.J.M., Al-Tel, T.H., Al-Aboudi, A.F., Al-Qawasmeh, R.A., 2010. Synthesis and antimicrobial activity of cholic acid hydrazone analogues. *Eur. J. Med. Chem.* 45, 2307–2313.
- Razin, S., 1975. Cholesterol incorporation into bacterial membranes. *J. Bacteriol.* 124, 570–572.
- Ren, D., Li, L., Schwabacher, A.W., Young, J.W., Beitz, D.C., 1996. Mechanism of cholesterol reduction to coprostanol by *Eubacterium coprostanoligenes* ATCC 51222. *Steroids* 61, 33–40.
- Ren, J., Sun, K., Wu, Z., Yao, J., Guo, B., 2011. All 4 bile salt hydrolase proteins are responsible for the hydrolysis activity in *Lactobacillus plantarum* ST-III. *J. Food Sci.* 76, 622–628.
- Reyes-Nava, L.A., Rivera-Espinoza, Y., 2014. Isolation sources of bile salt hydrolase-microorganisms. *Herald J. Agric. Food Sci. Res.* 3, 49–54.
- Ridlon, J.M., Hylemon, P.B., 2012. Identification and characterization of two bile acid coenzyme A transferases from *Clostridium scindens*, a bile acid 7 α -dehydroxylating intestinal bacterium. *J. Lipid Res.* 53, 66–76.
- Ridlon, J.M., Kang, D.J., Hylemon, P., 2006. Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* 47, 241–259.
- Ridlon, J.M., Alves, J.M., Hylemon, P.B., Bajaj, J.S., 2013. Cirrhosis, bile acids and gut microbiota. *Gut Microbes* 4, 382–387.
- Ridlon, J.M., Harris, S.C., Bhowmik, S., Kang, D.-J., Hylemon, P.B., 2016. Consequences of bile salt biotransformations by intestinal bacteria. *Gut Microbes* 7, 22–39.
- Rodríguez-Betrán, J., Rodríguez-Rojas, A., Guelfo, J.R., Couce, A., Blazquez, J., 2012. The *Escherichia coli* SOS gene *dinF* protects against oxidative stress and bile salts. *PLoS One* 7, e34971.
- Rossocha, M., Schultz-Heienbrok, R., von Moeller, H., Coleman, J.P., Saenger, W., 2005. Conjugated bile acid hydrolase is a tetrameric N-terminal thiol hydrolase with specific recognition of its chylol but not of its tauryl product. *Biochemistry* 19, 5739–5748.

- Ruiz, L., Sanchez, B., Ruas-Madiedo, P., de los Reyes-Bavil, C.G., Margolles, A., 2007. Cell envelope changes in *Bifidobacterium animalis* ssp. *lactis* as a response to bile. *FEMS Microbiol. Lett.* 274, 316–322.
- Ruiz, L., Zomer, A., O'Connell-Motherway, M., van Sinderen, D., Margolles, A., 2012. Discovering novel bile protection systems in *Bifidobacterium breve* UCC2003 through functional genomics. *Appl. Environ. Microbiol.* 78, 1123–1131.
- Sagdic, O., Ozturk, I., Yapar, N., Yetim, H., 2014. Diversity and probiotic potentials of lactic acid bacteria isolated from gilaburu, a traditional Turkish fermented European cranberrybush (*Viburnum opulus* L.) fruit drink. *Food Res. Int.* 64, 537–545.
- Sedláčková, P., Horáková, Š., Shi, T., Kosová, M., Plocková, M., 2015. Two different methods for screening of bile salt hydrolase activity in *Lactobacillus* strains. *Czech J. Food Sci.* 33, 13–18.
- Shao, C., Zhang, Q., Sun, Y., Liu, Z., Zeng, J., Zhou, Y., Yu, X., Jia, J., 2008. *Helicobacter pylori* protein response to human bile stress. *J. Med. Microbiol.* 57, 151–158.
- Solheim, M., Aakra, A., Vebø, H., Snipen, L., Nes, I.F., 2007. Transcriptional responses of *Enterococcus faecalis* V583 to bovine bile and sodium dodecyl sulfate. *Appl. Environ. Microbiol.* 73, 5767–5774.
- Sridevi, N., Vishwe, P., Prabhune, A., 2009. Hypocholesteremic effect of bile salt hydrolase from *Lactobacillus buchneri* ATCC 4005. *Food Res. Int.* 42, 516–520.
- Studer, N., Desharnais, L., Beutler, M., Brugiroux, S., Terrazos, M.A., Menin, L., Schürch, C.M., McCoy, K.D., Kuehne, S.A., Minton, N.P., Stecher, B., Bernier-Latmani, R., Hapfelmeier, S., 2016. Functional Intestinal Bile Acid 7 α -dehydroxylation by *Clostridium scindens* associated with protection from *Clostridium difficile* infection in gnotobiotic mouse model. *Front. Cell. Infect. Microbiol.* 6, 191.
- Sun, J., Deng, Z., Yan, A., 2014. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochem. Biophys. Res. Commun.* 453, 254–267.
- Suzuki, S., Yakabe, T., Suganuma, H., Fukao, M., Saito, T., Yajima, N., 2013. Cell-bound exopolysaccharides of *Lactobacillus brevis* KB290: protective role and monosaccharide composition. *Can. J. Microbiol.* 59, 549–555.
- Suzuki, S., Kimoto-Nira, H., Suganuma, H., Suzuki, C., Saito, T., Yajima, N., 2014. Cellular fatty acid composition and exopolysaccharide contribute to bile tolerance in *Lactobacillus brevis* strains isolated from fermented Japanese pickles. *Can. J. Microbiol.* 60, 183–191.
- Taheur, F.B., Kouidhi, B., Fdhila, K., Elabed, H., Slama, R.B., Mahdouani, K., Bakhrouf, A., Chaieb, K., 2016. Anti-bacterial and anti-biofilm activity of probiotic bacteria against oral pathogens. *Microb. Pathog.* 97, 213–220.
- Tanaka, H., Hashib, H., Kok, J., Mireau, I., 2000. Bile salt hydrolase of *Bifidobacterium longum* – biochemical and genetic characterization. *Appl. Environ. Microbiol.* 66, 2502–2512.
- Taranto, M.P., Murga, M.L.F., Lorca, G., De Valdez, G.F., 2003. Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reuteri*. *J. Appl. Microbiol.* 95, 86–91.
- Tareb, R., Bernardeau, M., Gueguen, M., Vernoux, J.-P., 2013. *In vitro* characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic *Lactobacillus* strains and interaction with foodborne zoonotic bacteria, especially *Campylobacter jejuni*. *J. Med. Microbiol.* 62, 637–649.
- Tomaro-Duchesneau, C., Jones, M.L., Shah, D., Jain, P., Saha, S., Prakash, S., 2014. Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an *in vitro* investigation. *Biomed. Res. Int.*, ID380316. <http://dx.doi.org/10.1155/2014/380316>.
- Tyagi, P., Edwards, D.R., Coyne, M.S., 2008. Use of sterol and bile acid biomarkers to identify domesticated animal sources of fecal pollution. *Water Air Soil Pollut.* 187, 263–274.
- Valkonen, A., Lahtinen, M., Tamminen, J., Kolehmainen, E., 2008. Solid state structural studies of five bile acid derivatives. *J. Mol. Struct.* 886, 197–206.
- Vasiljevic, T., Shah, N.P., 2008. Probiotics—from Metchnikoff to bioactives. *Int. Dairy J.* 18, 714–728.
- Vinderola, C.G., Reinheimer, J.A., 2003. Lactic acid starter and probiotic bacteria: a comparative “in vitro” study of probiotic characteristics and biological barrier resistance. *Food Res. Int.* 36, 895–904.
- Virtanen, E., Kolehmainen, E., 2004. Use of bile acids in pharmacological and supra-molecular applications. *Eur. J. Org. Chem.* 3385–3399. <http://dx.doi.org/10.1002/ejoc.200300699>.
- Wacher-Rodarte, M.C., Trejo-Munúuri, T.P., Montiel-Aguirre, J.F., Drago-Serrano, M.E., Gutiérrez-Lucas, R.L., Castaneda-Sánchez, J.I., Sainz-Espunes, T., 2016. Antibiotic resistance and multidrug-resistant efflux pumps expression in lactic acid bacteria isolated from pozol, a nonalcoholic Mayan maize fermented beverage. *Food Sci. Nutr.* 4, 423–430.
- Wexler, H.M., 2012. Pump it up: occurrence and regulation of multidrug efflux pumps in *Bacterioides fragilis*. *Anaerobe* 18, 200–208.
- Whitehead, K., Versalovic, J., Roos, S., Britton, R.A., 2008. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. *Appl. Environ. Microbiol.* 74, 1812–1819.
- Ye, W., Li, Y., Zhou, Z., Wang, X., Yao, J., Liu, J., Wang, C., 2013. Synthesis and antibacterial activity of new long-chain-alkyl bile acid-based amphiphiles. *Bioorg. Chem.* 51, 1–7.
- Yokota, A., Fukiya, S., Saiful Islam, K.B.M., Ooka, T., Ogura, Y., Hayashi, T., Hagio, M., Ishizuka, S., 2012. Is bile acid a determinant of the gut microbiota on a high-fat diet? *Gut Microbes* 3, 455–459.
- Yoon, H.-S., Ju, J.-H., Kim, H.-N., Park, H.-J., Ji, Y., Lee, J.-E., Shin, H.-K., Do, M.-S., Holzapfel, W., 2013. Reduction in cholesterol absorption in Caco-2 cells through the down-regulation of Niemann-Pick C1-like 1 by the putative probiotic strains *Lactobacillus rhamnosus* BFE5264 and *Lactobacillus plantarum* NR74 from fermented foods. *Int. J. Food Sci. Nutr.* 64, 44–52.
- Yoon, S., Yu, J., McDowell, A., Kim, S.H., You, H.J., Ko, G.P., 2017. Bile salt hydrolase-mediated inhibitory effect of *Bacteroides ovatus* on growth of *Clostridium difficile*. *J. Microbiol.* 55, 892–899.
- Yoshimoto, S., Loo, T.M., Atarashi, K., Kanda, H., Sato, S., Oyadomari, S., Iwakura, Y., Oshima, K., Morita, H., Hattori, M., Honda, K., Ishikawa, Y., Hara, E., Ohtani, N., 2013. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 499, 97–101.
- Zhang, W.Y., Wu, R.N., Sun, Z.H., Sun, T.S., Meng, H., Zhang, H.P., 2009. Molecular cloning and characterization of bile salt hydrolase in *Lactobacillus casei* Zhang. *Ann. Microbiol.* 59, 721–726.



Bile salt hydrolase activity, growth characteristics and surface properties in *Lactobacillus acidophilus*

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Abstract

Thirteen strains of *Lactobacillus acidophilus* of various origins were tested for bile salt hydrolase (BSH) activity, growth characteristics and surface properties during cultivation in the presence of ox bile. Seven strains were BSH positive, three strains were found to hydrolyse both sodium glycocholate and taurocholate and four hydrolysed only glycocholate. The addition of ox bile (3; 5 or 10 g/l) to MRS growth medium affected the growth rate of both BSH-positive and BSH-negative strains. The lag phases were increased when 5 or 10 g/l of ox bile were added. Growth rates and lag phases were significantly different between BSH-positive and BSH-negative strains in MRS medium with bile. BSH-positive strains were better able to adapt to increasing bile concentrations. The concentration of 2 g/l sodium cholate completely inhibited growth of all *lactobacilli* tested. Its toxicity was influenced by pH of medium. The addition of 3 g/l ox bile affected the properties related to ability of *lactobacilli* to adhere to surfaces—autoaggregation increased in all strains and zeta potential only in BSH-negative strains. In all strains tested, *bshA* and *bshB* genes were identified with the exception of *L. acidophilus* Lafti-L10. Repeated cultivation in MRS with bile did not cause the expression of genes in BSH-negative strains.

Keywords Bile · *Lactobacilli* · Autoaggregation · Zeta potential

Introduction

Lactobacillus acidophilus is one of the well-characterized species of the genus *Lactobacillus*, immobile, non-sporulating rods with homofermentative metabolism. *L. acidophilus* is often used as a microbial culture during food processing. It occurs naturally in the oral, intestinal and vaginal cavities of mammals. Several strains of *L. acidophilus* have been confirmed to have positive effects on human physiological status and therefore are used as probiotic bacteria [1]. One of the important criteria for these bacteria is their ability to survive conditions in the gastrointestinal tract (GIT), i.e. low pH and toxicity of bile salts. This property is strain specific and may occur in *lactobacilli* either as genetic resistance or acquired by adaptation to surrounding conditions [2]. Several mechanisms have been described to explain the ability to tolerate and grow in the presence of bile. These mechanisms include alterations of the cell wall or differences in

gene transcription and protein expression. Only few studies have been dedicated to efflux pumps in *lactobacilli*. These pumps are responsible for active transport of antibiotics out of the cell, i.e. antibiotic resistance [3]. The ATP-dependent active efflux pump could also serve as a transporter of bile salts [4]. The third option is the activity of bile salt hydrolase (BSH)—choloylglycine hydrolase (EC 3.5.1.24). Nevertheless, some findings do not support the hypothesis that BSH protects cells against bile salt toxicity [5], and generally the exact function of BSH has not been fully explained.

BSH is an intracellular enzyme that catalyses the hydrolysis of amide bonds between glycine or taurine and the steroid nucleus of bile salts. This hydrolysis can be followed by subsequent microbial transformation of bile acids [6, 7]. The activity of BSH was confirmed in several bacterial genera originating from the GIT [8, 9] among them, *lactobacilli* [10]. The number of BSH-encoding genes differs for each species of *lactobacilli*. The largest number of genes (four) was detected in *L. plantarum* [11], whereas only one gene was found in *L. fermentum* [12] or *L. casei* [13]. In *L. acidophilus* NCFM, two genes (*bshA* and *bshB*) were confirmed by McAuliffe et al. [14].

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The aim of this study was to determine the presence of *bshA* and *bshB* genes and the activity of BSH enzyme in selected *L. acidophilus* strains obtained from various sources, and to compare the growth characteristics of BSH-positive and BSH-negative strains in the presence of ox bile and its effect on the surface properties of cells.

Materials and methods

Lactobacillus acidophilus strains for testing were obtained from the Czech Collection of Dairy Microorganisms (CCDM) Laktoflora® Milcom (Prague, Czech Republic), ATCC and as commercial probiotic cultures. *Lactobacillus plantarum* CCM 1904 (Czech Collection of Microorganisms, Brno, Czech Rep.) and *E. coli* CCM 4517 were used as control strains for PCR reactions. All microorganisms used are summarized in Table 1.

All strains were routinely cultivated in MRS broth (Merck, Darmstadt, Germany) (pH 6.5) at 37 °C and 5% v/v CO₂ atmosphere.

Screening for BSH enzyme activity

Screening was performed by the TLC method according to Guo et al. [15]. Strains were inoculated (2% v/v) into 2 ml of MRS broth (pH 6.5) with the addition of 3 g/l sodium glycocholate or sodium taurocholate (both Sigma-Aldrich, St. Louis, USA) and cultivated at 37 °C in an atmosphere of 5% v/v CO₂. After 8 and 24 h, 1 ml of sample was collected and centrifuged (9000 rpm, 15 min, 4 °C). The supernatant (0.5 ml) was evaporated in a vacuum evaporator (R-114, Büchi, Switzerland) and then dissolved in 0.5 ml

of methanol. This sample (3 µl) was loaded onto a silica gel plate (TLC Silica gel 60 F 254, Merck), and isoamylacetate: propionic acid: 1-propanol: water (40: 30: 20: 10) was used as the mobile phase. After separation, the plate was dried and detection of liberated cholic acid was performed using 10% w/w phosphomolybdic acid. Solutions of cholic acid (Sigma-Aldrich), sodium glycocholate and sodium taurocholate (all 3 g/l in methanol) were used as standards.

Cultivation measurements

Growth curves in all media tested, i.e. MRS broth, MRS broth with 3 g/l, 5 g/l and 10 g/l ox bile (all at pH 6.5), with sodium cholate (VWR Amresco Life Science, Solon, USA) (0.1–3 g/l, pH 6.5) or with 0.5 g/l sodium cholate at different pHs (5.0; 5.5; 6.0; 6.5; 7.2), were measured spectrophotometrically at A = 600 nm in microtiter plates on a Powerwave XS instrument (BioTek Instruments, Winooski, USA) at 37 °C. Growth curves were used to determine the lag phase and calculate the specific growth rate. Growth curves were constructed using the mean absorbance values from two independent replicates, and the measurements were carried out in triplicate (*n* = 6). Growth rate was expressed as a slope of a linear part of the growth curve, i.e. in the period of exponential growth of the bacteria.

Identification of BSH genes

DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used to isolate DNA from overnight cultures. Amplification reactions were performed in a total volume of 20 µl containing 0.5 µl (10 µmol/l) of each primer, 4 µl of 5xFIRE-Pol Master Mix (12.5 mmol/l MgCl₂; Solis BioDyne, Tartu,

Table 1 List of microorganisms used and their origin

<i>L. acidophilus</i>	origin	
CCDM 149	milk	Laktoflora® Milcom, Czech Republic
CCDM 151	food supplement	Laktoflora® Milcom, Czech Republic
CCDM 152	fermented milk	Laktoflora® Milcom, Czech Republic
CCDM 193	fermented milk	Laktoflora® Milcom, Czech Republic
CCDM 197	fermented milk	Laktoflora® Milcom, Czech Republic
CCDM 217	fermented milk	Laktoflora® Milcom, Czech Republic
CCDM 406	dry milk powder	Laktoflora® Milcom, Czech Republic
CCDM 476	fermented milk	Laktoflora® Milcom, Czech Republic
ATCC® 314™	human gut	Oxoid, UK
ATCC® 4356™	human gut	Oxoid, UK
LA5	probiotic culture	Chr. Hansen, Denmark
Howaru LA14	probiotic culture	Danisco, Denmark
Lafti L10	probiotic culture	DSM Food Specialties, Nietherland
Control culture for PCR:		
<i>E.coli</i> CCM 4517		Czech Collection of Microorganisms, CR
<i>L. plantarum</i> CCM 1904		Czech Collection of Microorganisms, CR

Estonia), 14 µl of nuclease-free water (ThermoFisher Scientific, Pardubice, Czech Rep.) and 1 µl of DNA (10 ng) on a PCR Thermo labcycler gradient SensoQuest (Schoeller Instruments, Prague, Czech Rep.) according to the following cycling program: an initial denaturation step at 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension cycle of 72 °C for 10 min.

Two different primer pairs were used to identify *bshA* and *bshB* genes in *L. acidophilus* (Table 2). Electrophoresis was performed for one hour at 100 V, on 1% agarose gels in 0.5×TBE buffer (Sigma Aldrich). A fixed volume of sample (5 µl) with Gel Loading Buffer was loaded in each lane. PCR products were visualized using Red Safe staining.

Autoaggregation assay

Autoaggregation was measured according to Kos et al. [16]. *Lactobacilli* were cultivated in MRS broth or in MRS broth with 3 g/l ox bile (Hi-Media, Mumbai, India) at 37 °C, 18 h, and pH 6.5 in an atmosphere of 5% v/v CO₂. Cells were centrifuged (9000 rpm, 4 °C, 10 min), washed twice with PBS buffer (pH 7.0±0.1) and resuspended in 20 ml of PBS buffer to reach an absorbance $A_{600}=0.5\pm0.05$. At time 0 and 24 h (maintained at 37 °C), 1 ml of sample was carefully removed from the top of the vessel and the absorbance was measured at 600 nm. Two independent measurements were carried out in duplicate ($n=4$). The resulting autoaggregation was counted as follows:

$$\text{Autoaggregation} = \left(1 - \frac{A_t}{A_0}\right) \cdot 100\%,$$

where A_0 is the absorbance at time 0; A_t is the absorbance at time 24 h.

Measurement of zeta potential

Fresh cultures (18 h in MRS broth or MRS broth with 3 g/l ox bile) were used to determine the zeta potential. Cells were centrifuged (9000 rpm, 10 min, 4 °C), washed twice

with PBS buffer and resuspended to an absorbance (A_{600}) of 0.5 ± 0.05 . The electrophoretic mobility was measured on the Zetasizer Nano ZS-ZEN 3601 (Malvern Instruments, Malvern, UK) at 25 °C and at a direct current of 40 V. Each sample was analysed three times.

Standard statistical methods (F test, t test) were used to evaluate the results. Differences were considered significant at $p\leq0.05$.

Results and discussion

Influence of bile on growth characteristics of *lactobacilli*

The ability of microorganisms to detoxify bile salts by hydrolysis (activity of BSH enzyme) has often been included among the required properties for probiotic strain selection [17]. In addition to detoxification, the possibility to lower serum cholesterol levels is often mentioned in this context. Bile salts are secreted from the gallbladder into the small intestine where they can be hydrolysed by the action of microorganisms. Hydrolysed bile acids are more readily secreted from the body, leading to the synthesis of new bile salts from serum cholesterol and thereby to its depletion [18]. The strains tested in this work were different origin, and the aim was to include strains isolated from the GIT as well as commercial probiotic strains and strains from fermented products. The activity of BSH was detected in 7 strains of *L. acidophilus* from a total of 13 tested. All strains that were BSH positive after 24 h (Table 3) showed BSH activity even after 8 h of cultivation, except for strains CCDM 193 and Howaru LA14. An example of the TLC plate is shown in Fig. 1. Only three strains showed positive reactions with both sodium taurocholate (Na-TCA) and sodium glycocholate (Na-GCA). This confirmed the view that glycine-conjugated bile salts are more easily and rapidly hydrolysed than those containing taurine [19]. Strain ATCC 4356 has also been shown to be BSH positive by other authors [15].

Table 2 Primers used in this study

Primer	Sequence (5'→3')	Product length (bp)	Literature
<i>bsh-laAf</i>	Fwd: TACAACATATTCATTTAGACGCAATATCC	1246	Jiang et al [43]
<i>bsh-laAr</i>	Re: CACTCTGCCAACACTCCATAACG		
<i>bsh-laBf</i>	Fwd: CAAAAGCCATTTATTCGACTGA	1243	McAuliffe et al. [14]
<i>bsh-laBr</i>	Re: CATAATTTATTACTTCCTTTGTTAGACAGC		
<i>bshA</i>	Fwd: AAAGTCGACGAAAAGGGGCTTGTA Re: AAGAATTCCTATCAGGTTGTTCTAC	588	
<i>bshB</i>	Fwd: AGGATCCAGTTAGTTCCATCAGAATA Re: TATAAGCTTGGTATGGCCGGACTCAAC	618	

Table 3 Screening of bile salt hydrolase activity by TLC method after 24 h cultivation at 37 °C

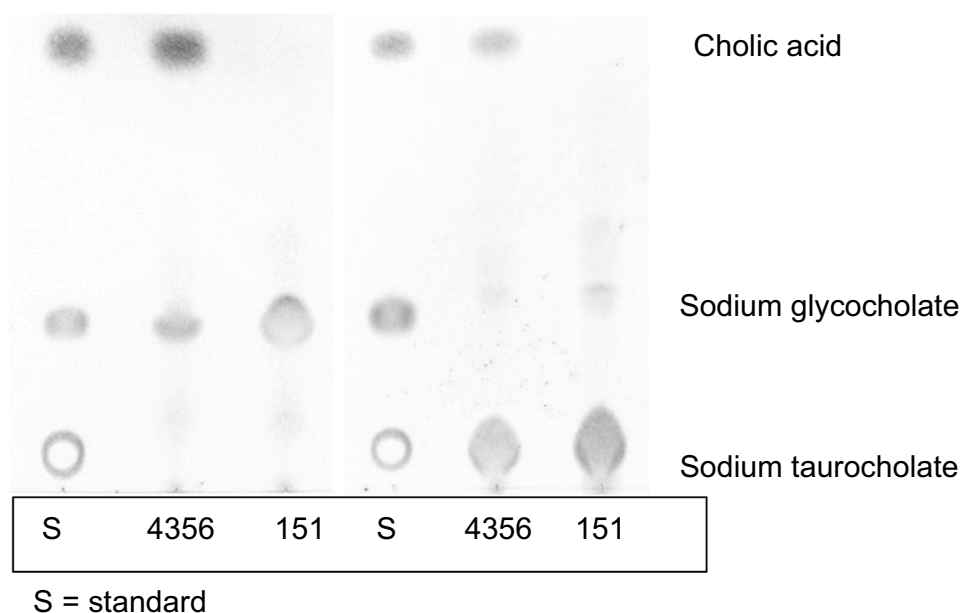
Strain	Na-TCA	Na-GCA
ATCC 4356	P	P
CCDM 149	N	P
CCDM 151	N	N
CCDM 152	N	N
CCDM 217	N	P
CCDM 197	N	N
CCDM 193	P	P
CCDM 406	P	P
CCDM 476	N	N
ATCC 314	N	N
LA 5	N	N
Howaru LA 14	N	P
Lafti L-10	N	P

Na-TCA sodium taurocholate, *Na-GCA* sodium glycocholate, *P* positive reaction (liberated cholic acid detected), *N* negative

Tolerance to bile salts is considered to be a very important functional property of bacteria, through which they can survive and reproduce in the GIT. This property is strain specific [2] and is tested in all studies that deal with the selection of probiotic bacteria [20, 21]. The growth of *L. acidophilus* at various bile concentrations has been investigated. For example, in the study of Soomro and Masud [22], two of five tested strains were not able to survive 3 h in 0.3% ox bile. On the other hand, in the study of Pan et al. [23], *L. acidophilus* NIT showed good growth in 1% bile.

In this study, the growth of *L. acidophilus* strains in MRS broth with different concentrations of ox bile (3, 5 and

10 g/l) was evaluated and compared to growth in pure MRS broth. These concentrations were chosen since under normal physiological conditions, the concentration of bile salts in the human small intestine ranges from 2 to 0.05% depending on the concentration of fat in the diet [24]. Some authors have reported values between 2 and 1.5% in the first hour of digestion, followed by a decrease to 0.3% [25]. Examples of the growth curves of the BSH-positive strain (CCDM 406) and the BSH-negative strain (CCDM 152) are shown in Fig. 2. From the growth curves, the lag phase length and growth rates were calculated (Tables 4 and 5). The results show that the addition of ox bile significantly affected the growth rate and the length of lag phase of BSH-negative strains. In the case of BSH-positive strains, growth rate was significantly more affected by 10 g/l bile compared to growth in pure MRS medium, but no statistically significant differences were found between 3 g/l and 5 g/l bile. In studies reported by other authors, prolongation of the lag phase in BSH-negative strains was confirmed. Pan et al. [23] found that in *L. acidophilus* NIT cultivated in the presence of 1% bile, the lag phase was prolonged by 2 h and in the presence of 2% bile, by 4 h. Similar results were achieved by Ahn et al. [26], who tested the effect of bile on the growth of four *L. acidophilus* strains. They reported a smaller prolongation of the lag phase in the presence of 1 mol/l with the increasing activity of the BSH enzyme. There were no apparent differences in growth rate between strains that showed activity against both salts (sodium glycocholate and sodium taurocholate) and strains that were only positive for glycocholate. This can be explained by the fact that bile affinity for glycocholate may be more important in reducing bile toxicity, as the ratio of glyco-conjugated and tauro-conjugated acids is about 3:1 in bile [27]. A comparison of mean growth rates

Fig. 1 Example of thin layer chromatography for BSH detection in *L. acidophilus* ATCC 4356 and *L. acidophilus* CCDM 151

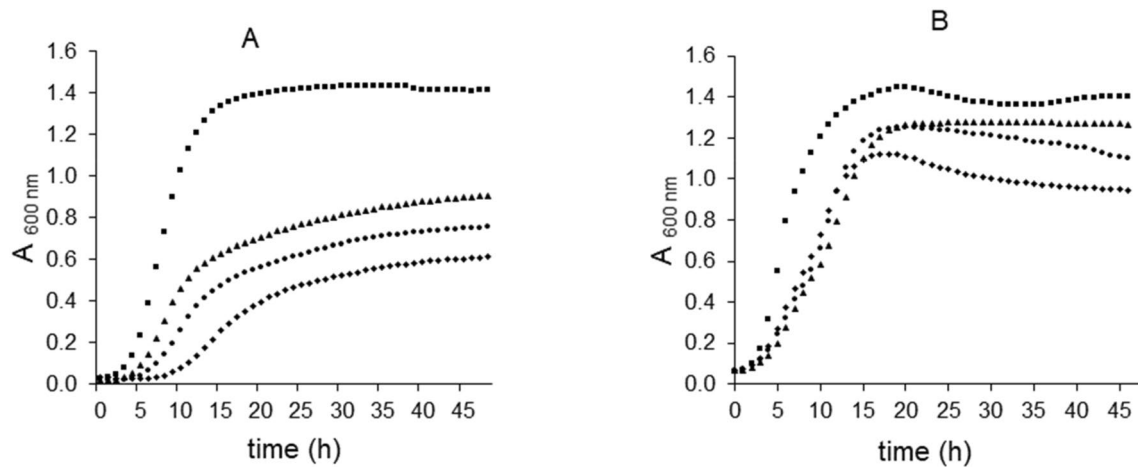


Fig. 2 Growth curves of BSH negative *L. acidophilus* CCDM 152 (a) and BSH positive, *L. acidophilus* CCDM 406 (b) in MRS broth (■) and MRS broth with 3 g/l (▲); 5 g/l (●) and 10 g/l (◆) w/v ox bile at 37 °C

Table 4 The values of growth rate (h^{-1}) during cultivation of *L. acidophilus* strains in MRS broth and in MRS broth MRS broth with 3; 5 and 10 g/l of ox-bile at 37 °C

Strain	MRS	Ox-bile		
		3 g/l	5 g/l	10 g/l
BSH negative				
LA5	0.138 ± 0.002	0.074 ± 0.002	0.041 ± 0.006	0.024 ± 0.005
151	0.200 ± 0.006	0.105 ± 0.005	0.034 ± 0.001	0.032 ± 0.004
152	0.133 ± 0.002	0.065 ± 0.003	0.042 ± 0.006	0.028 ± 0.005
197	0.137 ± 0.007	0.075 ± 0.004	0.050 ± 0.012	0.036 ± 0.013
476	0.142 ± 0.012	0.071 ± 0.004	0.048 ± 0.008	0.039 ± 0.013
314	0.148 ± 0.005	0.076 ± 0.005	0.049 ± 0.007	0.029 ± 0.006
Mean ± SD	0.150 ± 0.023 ^{a,A}	0.078 ± 0.013 ^{b,B}	0.044 ± 0.006 ^{c,C}	0.031 ± 0.005 ^{d,E}
BSH positive				
L10	0.151 ± 0.005	0.095 ± 0.013	0.073 ± 0.007	0.065 ± 0.007
149	0.137 ± 0.004	0.070 ± 0.005	0.065 ± 0.006	0.049 ± 0.009
217	0.140 ± 0.004	0.067 ± 0.001	0.044 ± 0.003	0.032 ± 0.000
LA14	0.136 ± 0.003	0.097 ± 0.006	0.061 ± 0.005	0.048 ± 0.007
193	0.136 ± 0.000	0.052 ± 0.001	0.041 ± 0.004	0.039 ± 0.008
406	0.140 ± 0.000	0.097 ± 0.003	0.089 ± 0.005	0.075 ± 0.012
4356	0.141 ± 0.001	0.139 ± 0.001	0.109 ± 0.006	0.078 ± 0.004
Mean ± SD	0.140 ± 0.005 ^{a,A}	0.088 ± 0.026 ^{b,B}	0.069 ± 0.022 ^{b,c,D}	0.055 ± 0.016 ^{c,F}

Values in the same row followed by different lowercase letters are significantly different ($p < 0.05$)

Values in the same column followed by different capital letters are significantly different ($p < 0.05$)

and lag phase lengths between BSH-negative and BSH-positive strains shows statistically significant differences in growth in medium with 5 g/l and 10 g/l bile. A concentration of 3 g/l bile did not affect these values. This result confirms the view that BSH activity helps microorganisms to avoid bile toxicity [28] since conjugated bile acids reduce growth of microorganisms in the proximal small intestine [29]. On the other hand, the released cholic acid, which is liberated by BSH, can also be toxic to bacteria, as shown in Fig. 3 for *L. acidophilus* CCDM 406. A concentration of 2 g/l of

sodium cholate inhibited bacterial growth completely. The minimal inhibitory concentration in all strains tested was 1 or 2 g/l of sodium cholate at pH 6.5 (data not shown), which is lower than that reported by Kurdi et al. [30]. These authors tested nine species of *Lactobacilli* including *L. acidophilus* and MICs ranged from 0.2 to 0.5% w/w sodium cholate. *L. acidophilus* JCM 1034 strain isolated from the human intestine had an MIC of 0.35% w/w. Cholic acid (or sodium cholate) itself is more hydrophobic compared to its conjugated derivatives and can enter the cells freely.

Table 5 The length of lag phase (h) during cultivation of *L. acidophilus* strains in MRS broth and in MRS broth and in MRS broth with 3; 5 and 10 g/l of ox-bile at 37 °C

Strain	MRS	Ox bile		
		3 g/l	5 g/l	10 g/l
BSH negative				
LA5	2.5	3	4.5	5
151	2	2.2	4	8.5
152	2.5	2	3	7
197	2	3	4.5	7
476	2	3	5	6.5
314	1.5	2	3.5	10
Mean±SD	2.1±0.3 ^{a,A}	2.6±0.4 ^{a,B}	4.1±0.7 ^{b,C}	7.3±1.6 ^{c,E}
BSH positive				
L10	2	3.5	4	5
149	2.5	3.5	4	6
217	1.5	2	2.5	5
LA14	2	2	3	4
193	2.5	3.5	5	6.5
406	2	2	2.5	3.5
4356	1.5	2	2	3
Mean±SD	2.0±0.4 ^{a,A}	2.6±0.7 ^{a,b,B}	3.1±1.0 ^{b,D}	4.4±1.3 ^{c,F}

Values in the same row followed by different lowercase letters are significantly different ($p < 0.05$)

Values in the same column followed by different capital letters are significantly different ($p < 0.05$)

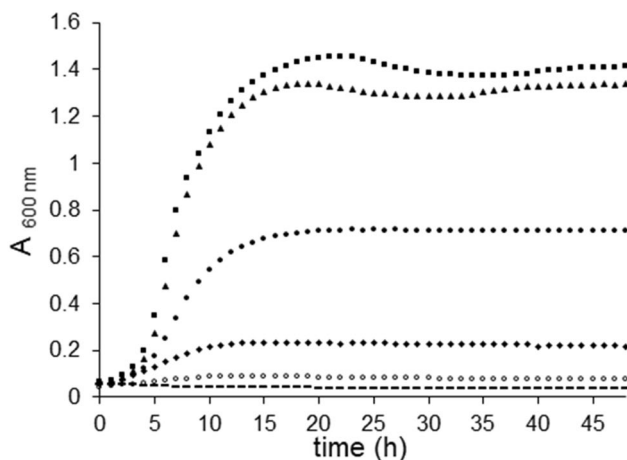


Fig. 3 Growth curves of BSH positive *L. acidophilus* CCDM 406 (a) in MRS broth (■) and MRS broth with 0.1 (▲); 0.5 (●); 1.0 (◆); 2.0 (○) and 3.0 (–) g/l sodium cholate at 37 °C, pH 6.5

The pH can also have a significant effect on the survival of bacteria in the small intestine and on the toxic effect of cholic acid. Figure 4 shows the growth curve of *L. acidophilus* CCDM 406 in MRS broth with 0.5 g/l sodium cholate at different pH values. At increasing pH of the medium, a decrease in sodium cholate toxicity was observed. At higher

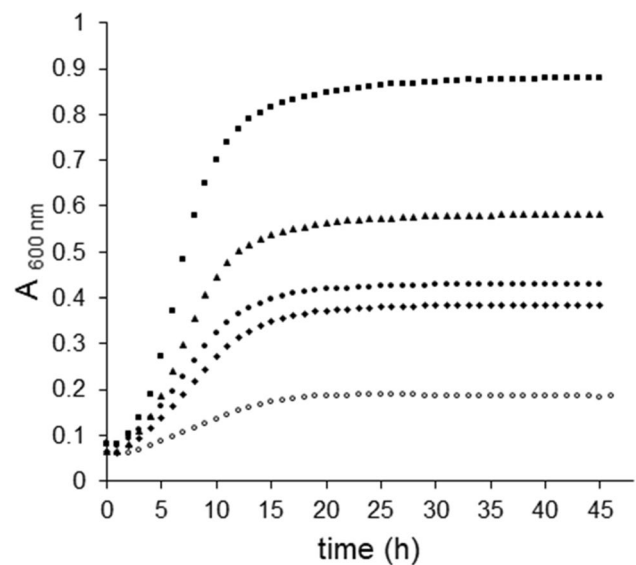


Fig. 4 Growth curves of BSH positive *L. acidophilus* CCDM 406 in MRS broth with 0.5 g/l sodium cholate at pH 7.2 (■); pH 6.5 (▲); pH 6.0 (●); pH 5.5 (◆) and pH 5.0 (○) at 37 °C

pH, the ratio of dissociated and non-dissociated molecules changes. Cholates are more dissociated (cholic acid $pK_a = 6.4$ [31]) and therefore less toxic. In an earlier study, De Boever and Verstraete [32] also described a reduction in the toxic effect of cholates using a higher pH. Growth studies with the addition of bile are usually carried out at neutral pH, but due to the acidic content of the stomach, the intestinal pH may initially be reduced to about pH 5 [33]. Thus, in vivo, this effect may be significant. In addition, BSH activity is the highest at pH 5.0–6.0 [34].

Influence of cultivation in the presence of bile on cell surface properties

In the following part of this study, it was tested how cultivation of cells in the presence of bile affects autoaggregation and zeta potential. These methods are used to evaluate cell adhesion properties in vitro [35]. Autoaggregation is the interconnection of cells through chemical–physical interactions and surface structures. Due to this ability, lactic acid bacteria can prevent the adhesion of pathogenic bacteria to the intestinal mucosa by creating a barrier or directly co-aggregating with pathogens [36]. Autoaggregation values are summarized in Fig. 5. Of 13 strains tested, 12 showed a significant increase in autoaggregation, which can be assumed to increase adhesion ability. Autoaggregation is a strain-specific property. Its values vary widely among authors [16, 37]. The influence of bile on autoaggregation is not widely discussed in the literature. Kociubinski et al. [38] described an increase in autoaggregation of *Bifidobacterium bifidum* NCC 189 after cultivation in the presence of sodium

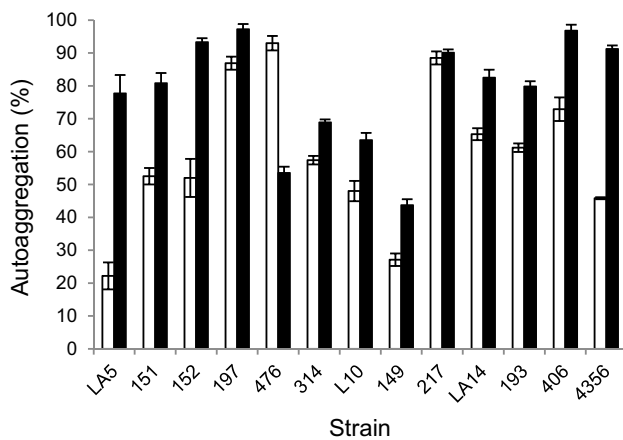


Fig. 5 The values of autoaggregation (%) after cultivation in MRS broth (□) and in MRS broth with 3 g/l ox-bile (■) at 37 °C, pH 6.5

taurocholate, deoxycholic or cholic acid. Conversely, Aslim et al. [39] found a reduction in autoaggregation in three *L. delbrueckii* subsp. *bulgaricus* strains in the presence of bile.

Bacterial cell surfaces are characterized by their electrical charge [35]. The cell surface charge and the hydrophobicity of the cell, and material surfaces, affect adhesion. The cell surface has a net negative charge at neutral pH, allowing easier adhesion to hydrophobic surfaces [40]. One way how to evaluate surface charge is to measure the zeta potential which characterizes electrophoretic mobility of the bacteria. Zeta potential was measured in cells cultivated in MRS broth and in cells after cultivation in MRS broth with 3 g/l bile (Fig. 6). There was a significant increase in zeta potential in BSH-negative strains when cultured in bile medium. BSH-positive strains showed only insignificant increases, and also a decrease in strains Lafti L-10 and ATCC 4356.

These data show that cultivation in ox bile medium influenced cell surface properties. To study adhesive ability in vitro, conditions simulating those in the GIT should be taken into account to obtain more accurate results.

BSH genes detection

The last part of this study was devoted to the detection of BSH genes. Two genes, *bshA* and *bshB*, have been described in *L. acidophilus* [14]. Figure 7 shows the results of electrophoresis after PCR reaction. Both genes were detected in all *L. acidophilus* strains tested, including those that did not directly show enzyme activity, with the exception of *L. acidophilus* Lafti-L10. The strains used in this study are both probiotic strains and strains isolated from fermented food. It is clear that BSH activity is not connected with strains isolated from the GIT. Some authors suggest that the tolerance of microorganisms to bile salts is probably due to the expression of bile salt hydrolase as an inducible enzyme, along

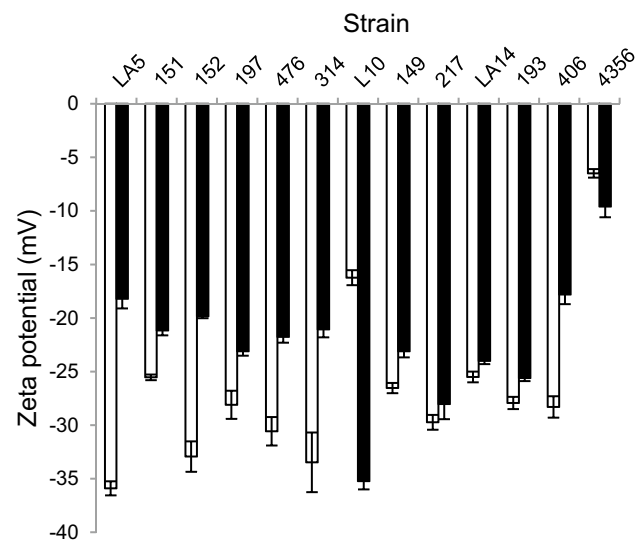


Fig. 6 The values of zeta-potential (mV) after cultivation in MRS broth (□) and in MRS broth with 3 g/l ox-bile (■) at 37 °C, pH 6.5

with some transport proteins [41]. Duany et al. [42] tested *bsh* gene expression in *L. plantarum* Lp9 and Lp91 strains. They found that after cultivation in MRS broth with 2% w/w ox bile, their BSH activity and bile tolerance were increased. In this study, after detection of the genes in BSH-negative strains, these strains were re-inoculated and cultivated three times in MRS broth supplemented with 3 g/l ox bile. Subsequently, BSH activity for sodium glycocholate and sodium taurocholate was checked again by TLC. However, cholic acid was not repeatedly detected in any of these strains (data not shown). This would suggest that the presence of an additional inducer may be required for *bsh* genes expression.

Conclusion

Knowledge of intestinal microbiota interactions with bile salts is important not only for basic research but also for the selection of proper probiotic LAB strains for food applications, dietary supplements or drugs. This research has demonstrated the effect of bile salts and pH, corresponding to conditions in the small intestine, on growth and surface properties of BSH-positive and BSH-negative strains *Lactobacillus acidophilus*. It also proved that the exposure of BSH-negative strains of *L. acidophilus*, but possessing *bshA* and *bshB* genes, to bile was not sufficient to express these genes. Although this work did not fully answer the question of the importance of BSH activity for *L. acidophilus*, it indicated a different behaviour of BSH-positive and BSH-negative strains in the presence of bile salts. Clearly, this issue needs further research focused on the behaviour of LAB strains in vitro, using systems modelling conditions of

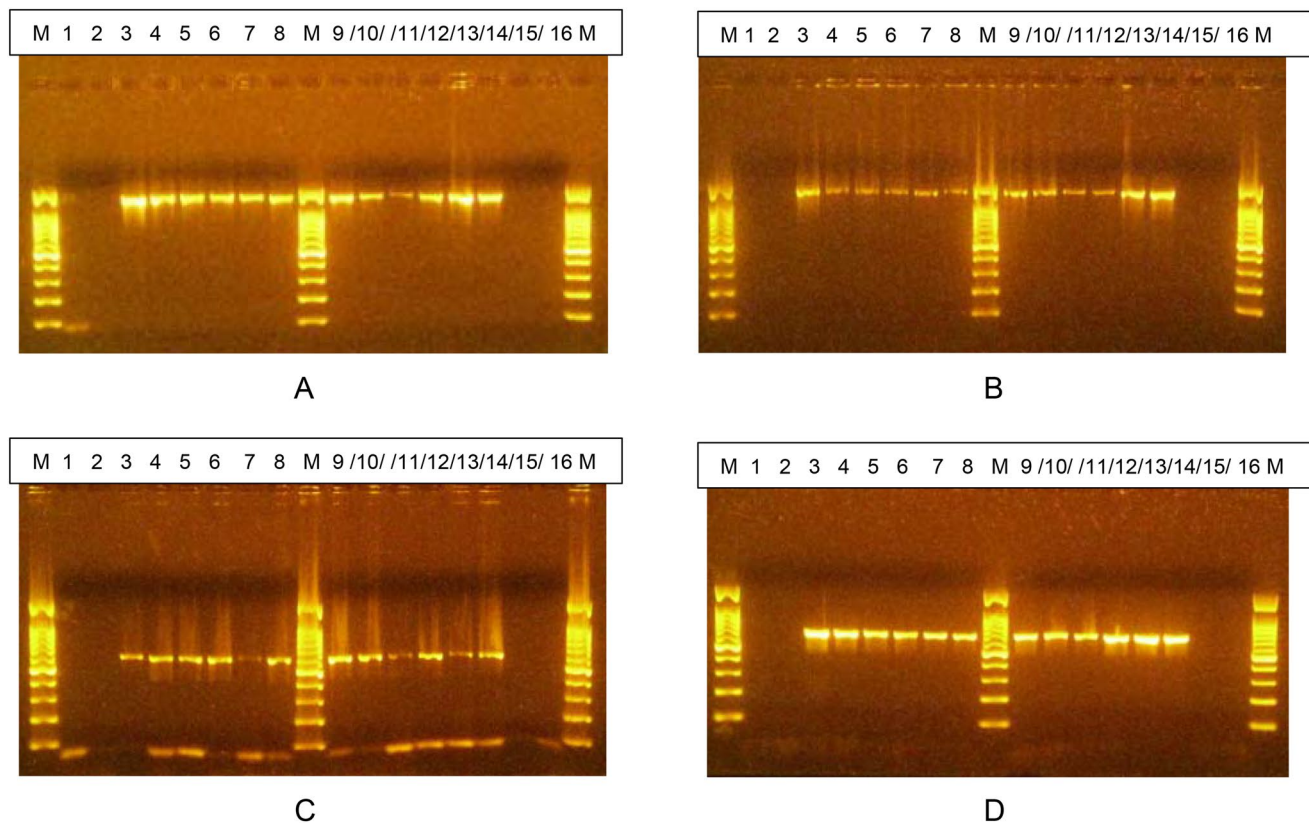


Fig. 7 Electrophoresis after PCR reaction with primers by Jiang et al. [43] (A=*bshA*; B=*bshB*) and by McAuliffe et al. [14] (C=*bshA*; D=*bshB*). M marker, 1=negative control H₂O, 2=negative control *L. plantarum* CCM 1904, 3=ATCC 4356, 4=CCDM 149,

5=CCDM 151, 6=CCDM 152, 7=CCDM 217, 8=CCDM 197, 9=CCDM 193, 10=CCDM 406, 11=CCDM 476, 12=ATCC 314, 13=LA5, 14=LA14, 15=Lafti L10, 16=negative control *E. coli* CCM 4517

the human GIT. Furthermore, bile salts (as emulsifiers) have been shown to affect the surface properties of lactobacilli. These properties can significantly influence the adhesion of microorganisms in the GIT. Other emulsifiers that are widely used in the food industry can affect these important properties in the same way.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

References

1. Bull M, Plumme S, Marchesi J, Mahenthiralingam E (2013) The life history of *Lactobacillus acidophilus* as a probiotic: a tale of revisionary taxonomy, misidentification and commercial success. FEMS Microbiol Lett 349:77–87. <https://doi.org/10.1111/1574-6968.12293>
2. Sumeri I, Arike L, Stekolššikova J, Uusna R, Adamberg S, Adamberg K, Paalme T (2010) Effect of stress pretreatment on survival of probiotic bacteria in gastrointestinal tract simulator. Appl Microbiol Biotechnol 86:1925–1931. <https://doi.org/10.1007/s00253-009-2429-2>
3. Dec M, Urban-Chmiel R, Stepien-Pysniak D, Wernicki A (2017) Assessment of antibiotic susceptibility in *Lactobacillus* isolates from chickens. Gut Pathog. <https://doi.org/10.1186/s13099-017-0203-z>
4. Bustos AY, Raya R, Font de Valdez G, Taranto MP (2011) Efflux of bile acids in *Lactobacillus reuteri* is mediated by ATP. Biotechnol Lett 33:2265–2269. <https://doi.org/10.1007/s10529-011-0696-3>
5. Moser SA, Savage DC (2001) Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in Lactobacilli. Appl Environ Microbiol 67:3476–3480. <https://doi.org/10.1128/AEM.67.8.3476-3480.2001>
6. Studer N, Desharnais L, Beutler M, Brugiroux S, Terrazos MA, Menin L, Schürch CM, McCoy KD, Kuehne SA, Minton NP, Stecher B, Bernier-Latmani R, Hapfelmeier S (2016) Functional intestinal bile acid 7 α -dehydroxylation by *Clostridium scindens* associated with protection from *Clostridium difficile* infection in gnotobiotic mouse model. Front Cell Infect Microbiol. <https://doi.org/10.3389/fcimb.2016.00191> (Article 191)

7. Eggert T, Bakonyi D, Hummel W (2014) Enzymatic routes for the synthesis of ursodeoxycholic acid. *J Biotechnol* 191:11–21. <https://doi.org/10.1016/j.jbiotec.2014.08.006>
8. Yoon S, Yu J, McDowell A, Kim SH, You HJ, Ko GP (2017) Bile salt hydrolase-mediated inhibitory effect of *Bacteroides ovatus* on growth of *Clostridium difficile*. *J Microbiol* 55:892–899. <https://doi.org/10.1007/s12275-017-7340-4>
9. Jones BV, Begley M, Hill C, Gahan CGM, Marchesi JR (2008) Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proc Natl Acad Sci USA* 105:13580–13585. <https://doi.org/10.1073/pnas.0804437105>
10. O'Flaherty S, Briner Crawley A, Theriot CM, Barrangou R (2018) The *Lactobacillus* bile salt hydrolase repertoire reveals niche-specific adaptation. *mSphere* 3:e00140–e218. <https://doi.org/10.1128/mSphere.00140-18>
11. Ren J, Sun K, Wu Z, Yao J, Guo B (2011) All 4 bile salt hydrolase proteins are responsible for the hydrolysis activity in *Lactobacillus plantarum* ST-III. *J Food Sci* 76:622–628. <https://doi.org/10.1111/j.1750-3841.2011.02431.x>
12. Kumar R, Rajkumar H, Kumar M, Varikuti SR, Athimamula R, Shujaiddin M, Ramagoni R, Kondapalli N (2013) Molecular cloning, characterization and heterologous expression of bile salt hydrolase (Bsh) from *Lactobacillus fermentum* NCDO394. *Mol Biol Rep* 40:5057–5066. <https://doi.org/10.1007/s11033-013-2607-2>
13. Zhang WY, Wu RN, Sun ZH, Sun TS, Meng H, Zhang HP (2009) Molecular cloning and characterization of bile salt hydrolase in *Lactobacillus casei* Zhang. *Ann Microbiol* 59:721–726. <https://link.springer.com/content/pdf/10.1007%2F978-3-642-14214-4.pdf>. Accessed 17 July 2019.
14. McAuliffe O, Cano RJ, Klaenhammer TR (2005) Genetic analysis of two bile salt hydrolase activities in *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* 71:4925–4929. <https://doi.org/10.1128/AEM.71.8.4925-4929.2005>
15. Guo CF, Zhang LW, Han X, Li JY, Du M, Yi HX, Feng Z, Zhang YC, Xu RX (2011) Short communication: a sensitive method for qualitative screening of bile salt hydrolase-active lactobacilli based on thin-layer chromatography. *J Dairy Sci* 94:1732–1737. <https://doi.org/10.3168/jds.2010-3801>
16. Kos B, Šušković J, Vuković S, Šimpraga M, Frece J, Matošić S (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J Appl Microbiol* 94:981–987. <https://doi.org/10.1046/j.1365-2672.2003.01915.x>
17. Allain T, Chaouch S, Thomas M, Vallée I, Buret AG, Langella P, Grellier P, Polack B, Bermúdez-Humarán LG, Florent I (2018) Bile-salt-hydrolases from the probiotic strain *Lactobacillus johnsonii* La1 mediate anti-giardial activity *in vitro* and *in vivo*. *Front Microbiol*. <https://doi.org/10.3389/fmicb.2017.02707> (Article 2707)
18. Tsai C-C, Lin P-P, Hsieh Y-M, Zhang Z, Wu H-C, Huang C-C (2014) Cholesterol-lowering potentials of lactic acid bacteria based on bile-salt hydrolase activity and effect of potent strains on cholesterol metabolism *in vitro* and *in vivo*. *Sci World J*. <https://doi.org/10.1155/2014/690752> (Article ID 690752)
19. Corzo G, Gilliland SE (1999) Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. *J Dairy Sci* 82:472–480. [https://doi.org/10.3168/jds.S0022-0302\(99\)75256-2](https://doi.org/10.3168/jds.S0022-0302(99)75256-2)
20. Pereira GVM, Coelho BO, Magalhães Júnior AI, Soccol VT, Soccol CR (2018) How to select a probiotic? A review and update of methods and criteria. *Biotechnol Adv* 36:2060–2076. <https://doi.org/10.1016/j.biotechadv.2018.09.003>
21. Shewale RN, Sawale PD, Khedkar CD, Singh A (2014) Selection criteria for probiotics: a review. *Int J Probiotics Prebiotics* 9:17–22
22. Soomro AH, Masud T (2012) Probiotic characteristics of *Lactobacillus* spp. isolated from fermented milk product dahi. *Food Sci Technol Res* 18:91–98. https://www.jstage.jst.go.jp/article/fstr/18/1/18_91/_pdf. Accessed 17 July 2019
23. Pan X, Chen F, Wu T, Tang H, Zhao Z (2009) The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. *Food Control* 20:598–602. <https://doi.org/10.1016/j.foodcont.2008.08.019>
24. Islam KB, Fukiya S, Hagio M, Fujii N, Ishizuka S, Ooka T (2011) Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 141:1773–1781. <https://doi.org/10.1053/j.gastro.2011.07.046>
25. Noriega L, Gueimonde M, Sánchez B, Margolles A, de los Reyes-Gavilán CG (2004) Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in *Bifidobacterium*. *Int J Food Microbiol* 94:79–86. <https://doi.org/10.1016/j.ijfoodmicro.2004.01.003>
26. Ahn YT, Kim GB, Lim KS, Baek YJ, Kim HU (2003) Deconjugation of bile salts by *Lactobacillus acidophilus* isolates. *Int Dairy J* 13:303–311. [https://doi.org/10.1016/S0958-6946\(02\)00174-7](https://doi.org/10.1016/S0958-6946(02)00174-7)
27. De Aguiar Vallim TQ, Tarling EJ (2013) Pleiotropic roles bile acids in metabolism. *Cell Metab* 17:657–669. <https://doi.org/10.1016/j.cmet.2013.03.013>
28. Gérard P (2014) Metabolism of cholesterol and bile acids by the gut microbiota. *Pathogens* 3:14–24. <https://doi.org/10.3390/pathogens3010014>
29. Hofmann AF, Eckmann L (2006) How bile acids confer gut mucosal protection against bacteria. *Proc Natl Acad Sci USA* 103:4333–4334. <https://doi.org/10.1073/pnas.0600780103>
30. Kurdi P, Kawanishi K, Mizutani K, Yokota A (2006) Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. *J Bacteriol* 188:1979–1986. <https://doi.org/10.1128/JB.188.5.1979-1986.2006>
31. Philipp B (2011) Bacterial degradation of bile salts. *Appl Microbiol Biotechnol* 89:903–915. <https://doi.org/10.1007/s00253-010-2998-0>
32. De Boever P, Verstraete W (1999) Bile salt deconjugation by *Lactobacillus plantarum* 80 and its implication for bacterial toxicity. *J Appl Microbiol* 87:345–352
33. Liong MT, Shah NP (2005) Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. *Int Dairy J* 15:391–398. <https://doi.org/10.1016/j.idairyj.2004.08.007>
34. Li G (2012) Intestinal probiotics: Interactions with bile salts and reduction of cholesterol. *Procedia Environ Sci* 12:1180–1186. <https://doi.org/10.1016/j.proenv.2012.01.405>
35. Kurinčić M, Jeršek B, Klančnik A, Možina SS, Fin R, Dražić G, Raspor P, Bohinc K (2016) Effects of natural antimicrobials on bacterial cell hydrophobicity, adhesion, and zeta potential. *Arh Hig Rada Toksikol* 67:39–45. <https://doi.org/10.1515/aht-2016-67-2720>
36. Ershadian M, Soleimani NA, Ajoudan Far H, Kakhki MRV (2015) Antimicrobial and co-aggregation effects of probiotic lactobacilli against some pathogenic bacteria. *Iran J Med Microbiol* 9:14–22
37. Grigoryan S, Bazukyan I, Trchounian A (2018) Aggregation and adhesion activity of lactobacilli isolated from fermented products *in vitro* and *in vivo*: a potential probiotic strain. *Probiotics Antimicrob Proteins* 10:269–276. <https://doi.org/10.1007/s12602-017-9283-9>
38. Kociubinski G, Zavaglia AG, Pérez PF, Disalvo EA, De Antoni GL (2002) Effect of bile components on the surface properties of bifidobacteria. *J Dairy Res* 69:293–302
39. Aslim B, Onal D, Beyatli Y (2007) Factors influencing autoaggregation and aggregation of *Lactobacillus delbrueckii* subsp. *bulgaricus* isolated from handmade yogurt. *J Food Prot* 70:223–227. <https://doi.org/10.4315/0362-028X-70.1.223>
40. Tassell MLV, Miller MJ (2011) *Lactobacillus adhesion to mucus*. *Nutrients* 3:613–636. <https://doi.org/10.3390/nu3050613>

41. Sue D, Boor KJ, Wiedmann M (2003) Sigma(B)-dependent expression patterns of compatible solute transporter genes *opuCA* and *lmo1421* and the conjugated bile salt hydrolase gene *bsh* in *Listeria monocytogenes*. Microbiology 149:3247–3256. <https://doi.org/10.1099/mic.0.26526-0>
42. Duany RK, Batish VK, Grover S (2012) Relative gene expression of bile salt hydrolase and surface proteins in two putative indigenous *Lactobacillus plantarum* strains under *in vitro* gut conditions. Mol Biol Rep 39:2541–2552. <https://doi.org/10.1007/s11033-011-1006-9>
43. Jiang J, Hang X, Zhang M, Liu X, Li D, Yang H (2010) Diversity of bile salt hydrolase activities in different lactobacilli toward human bile salts. Ann Microbiol 60:81–88. <https://doi.org/10.1007/s13213-009-0004-9>

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Two Different Methods for Screening of Bile Salt Hydrolase Activity in *Lactobacillus* Strains

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Abstract

SEDLÁČKOVÁ P., HORÁČKOVÁ Š., SHI T., KOSOVÁ M., PLOCKOVÁ M. (2015): **Two different methods for screening of bile salt hydrolase activity in *Lactobacillus* strains.** Czech J. Food Sci., 33: 13–18.

Bile salt hydrolase (BSH) activity of intestinal bacteria (including lactobacilli) is one of the indirect ways of decreasing a cholesterol level in human body. Tested *Lactobacillus* strains were isolated from various sources (faeces of fully breast-fed infants, cow's colostrum, cow's raw milk cheeses, and cow's raw milk) and identified by genotypic and phenotypic methods. All strains, including three commercial probiotic strains and six culture collection strains, were subsequently tested for their BSH activity via two methods – thin layer chromatography (TLC) and plate assay. Among all the 59 *Lactobacillus* strains, 15 strains were shown to be BSH positive by TLC method and only 8 of them by plate assay. Most of the BSH positive strains (9 strains) were isolated from faeces. Differences between BSH activities for sodium salts of cholytaurine and cholyglycine were demonstrated. The TLC method was shown to be more sensitive compared to the plate assay for BSH activity determination.

Keywords: *Lactobacillus*; bile salt hydrolase activity

Historically, lactobacilli have been used for a long time in food technology, mainly in the production of dairy products and are considered to be Generally Recognised as Safe (GRAS) by the World Health Organization (FAO/WHO 2002). Lactobacilli can be safely added into foods as food additives. Lactobacilli have multiple beneficial properties, including antimicrobial activity (LEE *et al.* 2011; ZHANG *et al.* 2011), immunostimulation (CROSS 2002), antimutagenicity (CALDINI *et al.* 2005), anticarcinogenicity (BURNS & ROWLAND 2000) or bile salt hydrolysing (DASHKEVICZ & FEIGHNER 1989; AHN *et al.* 2003; LIONG & SHAH 2005a; SRIDEVI *et al.* 2009), which makes lactobacilli valuable as probiotic microorganisms.

Cholyglycine hydrolase (EC 3.5.1.24), also known as bile salt hydrolase (BSH), hydrolyses bile salts to form glycine or taurine, as well as steroid core (LIONG & SHAH 2005a; BEGLEY *et al.* 2006). Bile salts recirculate within human bodies. Hydrolysed bile salts compared to non-hydrolysed ones are less absorbed

in the human intestine, leaving more free bile acids to be excreted out of human bodies via faeces (BEGLEY *et al.* 2006). Increased excretions of bile salts, therefore, decrease the total amount of bile salts available in human bodies. The lost bile salts can be replenished via *de novo* synthesis from cholesterol, which could subsequently reduce the level of serum cholesterol in human body (BEGLEY *et al.* 2006). BSH activity has been found in several bacterial genera, i.e. *Lactobacillus* (DASHKEVICZ & FEIGHNER 1989; AHN *et al.* 2003; LIONG & SHAH 2005a; SRIDEVI *et al.* 2009; LEE *et al.* 2011), *Bifidobacterium* (LIONG & SHAH 2005b; NORIEGA *et al.* 2006), *Enterococcus* (FRANZ *et al.* 2001), *Clostridium* (KISHINAKA *et al.* 1994), *Bacteroides* (STELLWAG & HYLEMON 1976), etc. However, based on TANAKA *et al.* (1999), not all strains of lactobacilli isolated from gastrointestinal tracts possess BSH activity, and some lactobacilli and bifidobacteria isolated from environments other than gastrointestinal tracts may also have BSH activity.

The aim of this study was to isolate and identify lactobacilli obtained from different sources and screen them for BSH activity using two methods: thin layer chromatography (TLC) and plate assay.

MATERIAL AND METHODS

Bacterial strains. In total 59 lactobacilli strains were used in this study. 27 strains of lactobacilli were isolated from faeces of fully breast-fed infants at Czech University of Life Science, Prague, Czech Republic and kindly provided for this study. Their identification as well as the isolation and identification of strains from colostrum (4), cow's raw milk (7), and cheeses made of cow's raw milk (12) were done at the University of Chemistry and Technology, Prague, Czech Republic. Six culture collection strains (*L. acidophilus* CCDM 151, *L. rhamnosus* CCDM 150, *L. casei* CCDM 198, *L. acidophilus* CCDM 406 Laktoflora®; Milcom, Prague, Czech Republic; *L. acidophilus* ATCC® 4356™, *L. acidophilus* ATCC® 314™; Oxoid, Basingstoke, Hampshire, UK), three commercial probiotic strains (*L. acidophilus* LAFTI® L10, *L. casei* LAFTI® L26; DSM Food Specialties, JH Heerlen, the Netherlands, *L. acidophilus* LA-5; Ch. Hansen, Hørsholm, Denmark) and *Escherichia coli* CNCTC 6859 (Czech National Collection of Type Cultures, Prague, Czech Republic) were also included in this study.

Isolation of bacteria. The strains were isolated from Rogosa agar (pH 5.5; Merck, Darmstadt, Germany) at 37°C under an atmosphere of 5% v/v CO₂. Selected colonies were streaked onto MRS agar (pH 5.6; Oxoid, Basingstoke, Hampshire, UK). Subsequent cultivations were carried out in the same conditions and individual selected colonies were transferred into MRS broth (pH 5.6; Oxoid).

Identification of bacteria. Isolated bacteria were identified thereafter, using phenotypic methods which included Gram staining, bacteria morphology, and catalase activity. The Gram-positive, catalase negative, rod-shaped bacteria with proved BSH activity were further identified by genotypic methods. The first method was PCR for identification of the genus *Lactobacillus* (VENTURA *et al.* 2000) and the second one was 16S rRNA gene sequence analysis for determination of *Lactobacillus* species (GODON *et al.* 1997). The identification of isolated strains was achieved by comparing the results with the bacterial genome database of NCBI.

Cultivation of bacteria. Cultivations of *Lactobacillus* strains were carried out at 37°C for 18 h under an atmosphere of 5% v/v CO₂ in MRS broth (pH 5.6; Oxoid). *Escherichia coli* CNCTC 6859 was cultivated in Brain Heart Infusion (BHI) broth (pH 7; Himedia, Mumbai, India) in the same conditions as *Lactobacillus* strains.

BSH activity – Plate assay. The protocol for bile salt hydrolase activity measurement was based upon DASHKEVICZ and FEIGNER (1989) and AHN *et al.* (2003) with modifications. Soft MRS agar (pH 5.6; Oxoid), which contained MRS broth (52.5 g/l; Oxoid), bacteriological agar (7.5 g/l; Oxoid), bile salts (0.3% w/v; Ox Bile, Himedia, India), and CaCl₂ (0.375 g/l; Lach-Ner, Neratovice, Czech Republic), was used. Petri dishes with agar were incubated under an anaerobic atmosphere at 37°C for 48 hours. Various lactobacilli strains (10 µl), which had been cultivated for 18 h under an atmosphere of 5% v/v CO₂, were inoculated on MRS agar by puncturing into the agar. Subsequent cultivation of the agar media containing bacterial strains was carried out at 37°C for 72 h under an anaerobic atmosphere. Visible halos around the punctures indicate the positive BSH activity of the strains. Results were then assessed by measuring the diameters of halos. Lactobacilli strains grown on MRS agar without bile salts were used as the negative control. Measurements were repeated three times.

BSH activity – thin layer chromatography (TLC). According to GUO *et al.* (2011), lactobacilli strains were cultivated in MRS broth (5 ml; Oxoid) at 37°C for 18 h, followed by centrifugation at 9000 g at 4°C for 10 minutes. The centrifuged samples were then washed with 2 ml of physiological solution. After washing, 5 ml of physiological solution was added to the cell pellets of lactobacilli. Bacterial suspension (1 ml) was mixed with reaction mix (1 ml). The reaction mix contained MRS broth (Oxoid), with either sodium salt of cholyglycine (Na-GCA, 0.3% w/v) or sodium salt of cholytaurine (Na-TCA, 0.3% w/v; both St. Louis, USA) in phosphate buffer (0.1 mol/l), which gave a final pH of 6.5. The strains were then cultivated at 37°C for 8 h under an atmosphere of 5% v/v CO₂. After the cultivation, the samples were vacuum evaporated and the residuals were subsequently dissolved in 1 ml of methanol (Lach-Ner, Neratovice, Czech Republic), followed by centrifugation at 14 000 g at 4°C for 1 minute. The supernatants were spotted onto the baselines on silica gel plates (10 × 10 cm, TLC silica gel 60 F₂₅₄; Merck), along with spotting of standard solutions,

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which were cholic acid (CA) in methanol (5 mmol/l), Na-TCA in methanol (5 mmol/l) and Na-GCA in methanol (5 mmol/l; all Sigma-Aldrich, St. Louis, USA). The mobile phase contained isoamyl acetate (Merck, Darmstadt, Germany), propionic acid (Fluka, Buchs, Switzerland), *n*-propanol (Penta, Prague, Czech Republic) and water at a ratio of 40:30:20:10. The mobile phase was allowed to migrate along TLC plates for 30 minutes. The plates were then dried and sprayed with 10% w/v solution of phosphomolybdic acid (Lachema, Brno, Czech Republic) in ethanol, followed by drying with hot air. Cholic acid was liberated from bile salts by BSH positive strains. Results of TLC were evaluated by comparing with standards. Measurements were repeated three times.

RESULTS AND DISCUSSION

Survival of probiotics while passing through the gastrointestinal tract is of great importance for maintaining the health benefits of probiotics (ELLI *et al.* 2006). Many factors can affect the survival of bacteria in the gastrointestinal tract, i.e. pH of the environment, time of exposure to certain pH, presence and concentration of bile salts, etc. In the environment of small intestine, presence of bile salts is the most undesirable factor for the survival of probiotics (BEZKOROVAINY 2001). Therefore, probiotic lactobacilli with BSH enzymes, which hydrolyse bile salts, were found to have a higher possibility of survival in the gastrointestinal tract (DU TOIT *et al.* 1998). Furthermore, BSH activity is one of the indirect possibilities how to decrease a cholesterol level in human body (BEGLEY *et al.* 2006).

In this study, 59 Gram-positive, rod-shaped, and catalase negative strains were isolated, of which 27 strains were isolated from faeces of fully breast-fed infants (from 5 to 207 days old), 4 strains were

isolated from cow's colostrum, 12 strains from cheeses made from cow's raw milk, and 7 strains from cow's raw milk. All the isolates were confirmed by PCR identification as lactobacilli.

All of 50 newly isolated strains, 6 collection strains and 3 commercial probiotic strains were screened for their BSH activity by use of plate assay and TLC. *L. acidophilus* ATCC 4356 was used as a comparative BSH positive strain (DASHKEVICZ & FEIGNER 1989; LIONG & SHAH 2005a), and *Escherichia coli* CNCTC 6859 was used as the BSH negative strain (BEGLEY *et al.* 2006; GUO *et al.* 2011). The results are summarised in Table 1. It was found that 15 out of the 59 strains were demonstrated to have BSH activity using TLC method.

In Table 2 the results from TLC method and plate assay are compared for BSH positive strains. The strain specification is also included. Only 8 of these 15 positive strains were confirmed to be BSH positive using the plate assay. All strains which were found BSH negative by TLC method were also confirmed as negative by the plate assay. TLC method was shown to be more sensitive compared to the plate assay for BSH activity determination. Figure 1 shows examples of TLC plates, where hydrolysis of Na-GCA and Na-TCA is visible. Figure 2 demonstrates precipitated zones around BSH positive lactobacilli strains on the Petri dish which varied among the strains from 15 mm to 32 mm.

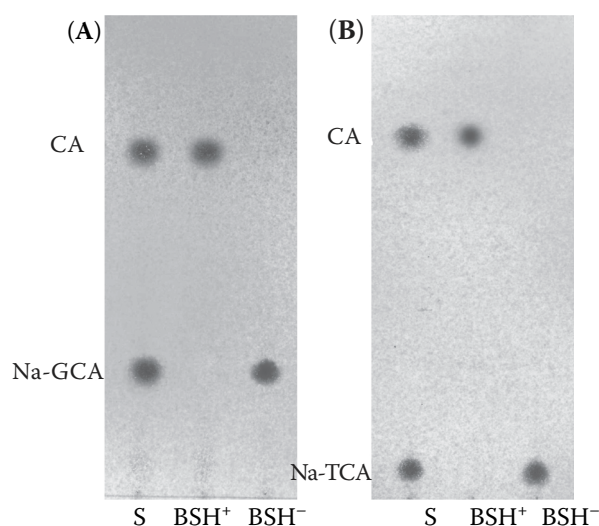


Figure 1. Example of TLC method for bile salt hydrolase (BSH) activity determination: (A) BSH activity for sodium salt of cholyglycine (Na-GCA), (B) BSH activity for sodium salt of cholytaurine (Na-TCA)

S – standard of Na-TCA, Na-GCA, and cholic acid (CA) in 0.1 mol/l phosphate buffer (0.3% w/v); BSH⁺ – BSH positive strain, BSH[–] – BSH negative strain

Table 1. Origin of strains used in this study with the ratio between bile salt hydrolase (BSH) positive and negative strains (TLC method)

Strain origin	Total	BSH	
		positive	negative
Faeces of fully breast-fed infants	27	9	18
Raw milk	7	1	6
Colostrum	4	0	4
Cheeses from raw milk	12	0	12
Culture collection strains	6	4	2
Commercial probiotic strains	3	1	2

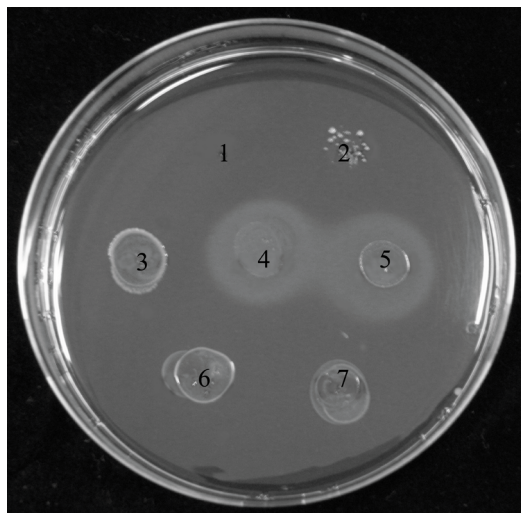


Figure 2. Plate assay for bile salt hydrolase (BSH) activity determination in soft MRS agar with Ox Bile (0.3% w/v) and CaCl_2 (0.37 g/l)

1 – *E. coli* CNCTC 6859, 2 – *L. casei* JN, 3 – *L. brevis* S1, 4 – *L. gasseri* VI, 5 – *L. acidophilus* ATCC 4356, 6 – *L. gasseri* Z3, 7 – *L. rhamnosus* AN

It was demonstrated in this study that most of the BSH positive strains (9 out of 15) were isolated from faeces. It is in accordance with the statement of TANAKA *et al.* (1999) that lactobacilli strains isolated

from the gastrointestinal tract are more likely to be BSH positive, as compared to those without exposures to bile salts. Other BSH positive lactobacilli were the culture collection of strains (*L. acidophilus* ATCC 314, *L. acidophilus* ATCC 4356, *L. acidophilus* CCDM 151, and *L. acidophilus* CCDM 406), commercial probiotic strain *L. acidophilus* LA-5 and one strain from cow's raw milk (*L. brevis* S1). The rest of the strains from cow's raw milk, cheeses, and cow's colostrum did not show any BSH activity, nor did the commercial probiotic strains of *L. acidophilus* LAFTI L10 and *L. casei* LAFTI L26, and collection strains of *L. rhamnosus* CCDM 150 and *L. casei* CCDM 198.

Moreover, as demonstrated in the study by RIDLON *et al.* (2006), *Lactobacillus* spp. may perform different BSH activities in the presence of different bile salts. Specifically, bile salts formed from glycine are more readily to be hydrolysed than bile salts formed from taurine (SUVARNA & BOBY 2005; BEGLEY *et al.* 2006). CORZO and GILLILAND (1999) showed in their study that three strains of *L. acidophilus* performed a higher hydrolysis rate on the sodium salt of cholylglycine than on cholyltaurine. In this study, *L. casei* JN from faeces and *L. brevis* S1 from cow's raw milk did not show any BSH activity on Na-TCA whereas *L. rhamnosus* AN from faeces had no BSH activity

Table 2. Strain specification and the results of plate assay and TLC method of bile salt hydrolase (BSH) positive lactobacilli

Species	Origin	Plate assay (mm) ²	TLC	
			Na-GCA	Na-TCA
<i>L. casei</i> JN	faeces (6 days) ¹	0	P	N
<i>L. gasseri</i> VI	faeces (6 days) ¹	24	P	P
<i>L. gasseri</i> Z2	faeces (131 days) ¹	20	P	P
<i>L. gasseri</i> LBA	faeces (139 days) ¹	24	P	P
<i>L. gasseri</i> Z3	faeces (62 days) ¹	0	P	P
<i>L. gasseri</i> R	faeces (19 days) ¹	15	P	P
<i>L. gasseri</i> ZU 11	faeces (5 days) ¹	0	P	P
<i>L. gasseri</i> Z1	faeces (42 days) ¹	0	P	P
<i>L. rhamnosus</i> AN	faeces (48 days) ¹	0	N	P
<i>L. brevis</i> S1	cow's raw milk	0	P	N
<i>L. acidophilus</i> ATCC 4356	Oxoid	20	P	P
<i>L. acidophilus</i> ATCC 314	Oxoid	30	P	P
<i>L. acidophilus</i> CCDM 151	Laktoflora [®] , Milcom	0	P	P
<i>L. acidophilus</i> CCDM 406	Laktoflora [®] , Milcom	25	P	P
<i>L. acidophilus</i> LA-5	Ch. Hansen	32	P	P

¹faeces of fully breast-fed infants with the infants' age; ²diameter of the zone formed; Na-GCA – sodium salt of cholylglycine; Na-TCA – sodium salt of cholyltaurine; P – positive reaction; N – negative reaction

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on Na-GCA. The rest of the strains were shown to be BSH positive on both salts.

CONCLUSION

Bile salt hydrolase activity of probiotic lactobacilli is more often found in strains isolated from the gastrointestinal tract, where bile salts are present, rather than in strains from the non-bile salt environment. However, some strains isolated from the non-bile salt environment may also possess BSH activity. Also, each lactobacillus strain has its distinct specificity towards bile salts. Moreover, salts of cholyglycine are more readily to be hydrolysed by lactobacilli than salts of cholytaurine. In terms of the methods of BSH activity assessment, we found that the TLC method is easier to evaluate and more sensitive compared to the plate assay.

References

- Ahn Y.T., Kim G.B., Lim K.S., Baek Y.J., Kim H.U. (2003): Deconjugation of bile salts by *Lactobacillus acidophilus* isolates. *International Dairy Journal*, 13: 303–311.
- Begley M., Hill C., Gahan C.G.M. (2006): Bile salt hydrolase activity in probiotics. *Applied and Environmental Microbiology*, 72: 1729–1738.
- Bezkorovainy A. (2001): Probiotics: determinants of survival and growth in the gut. *American Journal of Clinical Nutrition*, 73: 399S–405S.
- Burns A.J., Rowland I.R. (2000): Anti-carcinogenicity of probiotics and prebiotics. *Current Issues in Intestinal Microbiology*, 1: 13–24.
- Caldini G., Trotta F., Villarini M., Moretti M., Pasquini R., Scassellati-Sforzolini G., Cenci G. (2005): Screening of potential lactobacilli antigenotoxicity by microbial and mammalian cell-based tests. *International Journal of Food Microbiology*, 102: 37–47.
- Corzo G., Gilliland S.E. (1999): Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. *Journal of Dairy Science*, 82: 472–480.
- Cross M.L. (2002): Immunoregulation by probiotic lactobacilli: Pro-Th1 signals and their relevance to human health. *Clinical and Applied Immunology Reviews*, 3: 115–125.
- Dashkevich M.P., Feighner S.D. (1989): Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. *Applied and Environmental Microbiology*, 55: 11–16.
- Du Toit M., Franz C.M.A.P., Dicks L.M.T., Schillinger U., Haberer P., Warlies B., Ahrens F., Holzapfel W.H. (1998): Characterisation and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. *International Journal of Food Microbiology*, 40: 93–104.
- Elli M., Callegari M.L., Ferrari S., Bessi E., Cattivelli D., Soldi S., Morelli L., Feuillerat N.G., Antoine J.M. (2006): Survival of yogurt bacteria in the human gut. *Applied and Environmental Microbiology*, 72: 5113–5117.
- FAO/WHO (2002): Guidelines for the Evaluation of Probiotics in Food 2002. Available at http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf (accessed Feb 20, 2013).
- Franz C.M.A.P., Specht I., Haberer P., Holzapfel W.H. (2001): Bile salt hydrolase activity of enterococci isolated from food: screening and quantitative determination. *Journal of Food Protection*, 64: 725–729.
- Godon J.-J., Zumstein E., Dabert P., Habouzit F., Moletta R. (1997): Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis. *Applied and Environmental Microbiology*, 63: 2802–2813.
- Guo C.F., Zhang L.W., Han X., Li J.Y., Du M., Yi H.X., Feng Z., Zhang Y.C., Xu X.R. (2011): Short communication: a sensitive method for qualitative screening of bile salt hydrolase-active lactobacilli based on thin-layer chromatography. *Journal of Dairy Science*, 94: 1732–1737.
- Kishinaka M., Umeda A., Kuroki S. (1994): High concentrations of conjugated bile acids inhibit bacterial growth of *Clostridium perfringens* and induce its extracellular cholyglycine hydrolase. *Steroids*, 59: 485–489.
- Lee H., Yoon H., Ji Y., Kim H., Park H., Lee J., Shin H., Holzapfel W. (2011): Functional properties of *Lactobacillus* strains isolated from kimchi. *International Journal of Food Microbiology*, 145: 155–161.
- Liong M.T., Shah N.P. (2005a): Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. *International Dairy Journal*, 15: 391–398.
- Liong M.T., Shah N.P. (2005b): Bile salt deconjugation and BSH activity of five bifidobacterial strains and their cholesterol co-precipitating properties. *Food Research International*, 38: 135–142.
- Noriega L., Cuevas I., Margolles A., de los Reyes-Gavilán C.G. (2006): Deconjugation and bile salts hydrolase activity by *Bifidobacterium* strains with acquired resistance to bile. *International Dairy Journal*, 16: 850–855.
- Ridlon J.M., Kang D.J., Hylemon P.B. (2006): Bile salt biotransformations by human intestinal bacteria. *Journal of Lipid Research*, 47: 241–259.
- Sridevi N., Vishwe P., Prabhune A. (2009): Hypocholesteremic effect of bile salt hydrolase from *Lactobacillus bu-*

- chneri* ATCC 4005. Food Research International, 42: 516–520.
- Stellwag E.J., Hylemon P.B. (1976): Purification and characterization of bile salt hydrolase from *Bacteroides fragilis* subsp. *fragilis*. BBA – Enzymology, 452: 165–176.
- Suvarna V.C., Bobby V.U. (2005): Probiotics in human health: A current assessment. Current Science, 88: 1744–1748.
- Tanaka H., Doesburg K., Iwasaki T., Mierau I. (1999): Screening of lactic acid bacteria for bile salt hydrolase activity. Journal of Dairy Science, 82: 2530–2535.
- Ventura M., Callegari M.L., Morelli L. (2000): S-layer gene as a molecular marker for identification of *Lactobacillus helveticus*. FEMS Microbiology Letters, 189: 275–279.
- Zhang Y., Zhang L., Du M., Yi H., Guo C., Tuo Y., Han X., Li J., Zhang L., Yang L. (2011): Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild lactobacilli from fermented food. Microbiological Research, 167: 27–31.

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Emulsion Encapsulation of *Bifidobacterium animalis* subsp. *lactis* BB12 with the Addition of Lecithin

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Abstract

LISOVÁ I., HORÁČKOVÁ Š., KOVÁČOVÁ R., RADA V., PLOCKOVÁ M. (2013): **Emulsion encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 with the addition of lecithin.** Czech J. Food Sci., **31**: 270–274.

The commercial probiotic strain *Bifidobacterium animalis* subsp. *lactis* Bb12 was encapsulated using emulsion encapsulation into milk protein matrix without and with the addition of 0.5% w/w lecithin into the oil. Different agitation speeds were used during the encapsulation process. The examination of microcapsules was carried out by optical microscope and fluorescence *in situ* hybridisation. The particle size distribution as volume based median $d_{0.5}$ was evaluated by the laser diffraction method. In the case of no lecithin addition, the agitation speed did not influence significantly the size of the microcapsules. The addition of 0.5% (w/w) of lecithin into the oil caused a decrease of $d_{0.5}$ value from $196 \pm 37 \mu\text{m}$ to $79 \pm 3 \mu\text{m}$ at an agitation speed of 500 rpm, and from $193 \pm 24 \mu\text{m}$ to $39 \pm 3 \mu\text{m}$ at 1200 rpm. It can improve the sensory properties of the products with the added microcapsules.

Keywords: microencapsulation, bifidobacteria, emulsifier, particle size distribution

The research on probiotic microorganisms and their application to different types of products have become one of the trends of recent years. Previously, probiotic microorganisms were only included in dairy products and food supplements. The possibilities of probiotics application in a larger number of products such as fruit juices, cereals and non-food products for preventive and also therapeutic purposes are now fully developed (HELLER 2001; CRITTENDEN 2009). The application of probiotics in sufficient quantities is important for positive probiotic effects in the gastrointestinal tract of humans. Products containing probiotic microorganisms should include 10^6 probiotic viable

cells per 1 g at the time of expiration in fermented dairy products (PICOT & LACROIX 2004). The viability of probiotic cells is reduced by a number of factors during the production and storage of the products, and also in the gastrointestinal tract of the consumer. These factors include low pH, high temperature, aerobic environment, concentrated bile salts, digestive enzymes, and others. The encapsulation of probiotic microorganisms is one way to protect the cells from these stress factors (ADHIKARI *et al.* 2000; GBASSI *et al.* 2009). Nevertheless, some authors did not find any protective effect of encapsulation against gastric juice for encapsulated cells (BRINQUES & AYUB 2011).

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The encapsulation was developed from the immobilisation technology of cell cultures used by the biotechnology industry (HEIDEBACH *et al.* 2009). The process of encapsulation is a physico-chemical or mechanical process that leads to the production of particles ranging in size from several nanometres to several millimetres (BURGAIN *et al.* 2011). Materials such as alginate, κ -carrageenan, chitosan, starch, gelatine, or milk proteins are primarily used to encapsulate probiotic cells (PICOT & LACROIX 2004). Calcium alginate is most often used for the encapsulation of probiotics as it is nontoxic, biocompatible, and inexpensive (BURGAIN *et al.* 2011). However, its disadvantage is its low resistance to acidic pH and high porosity which can be compensated by combining with other polymers such as starch (HANSEN *et al.* 2002; VIDHYALAKSHMI *et al.* 2009). Natural κ -carrageenan is another commonly used polymer in the food industry. The main disadvantage of carrageenan microcapsules is their fragility (BURGAIN *et al.* 2011). Resistant starch, which is not split by pancreatic enzymes in the small intestine and which allows the release of probiotic cells in the large intestine, is also used for encapsulation (CHARALAMPOPOULOS *et al.* 2002). Milk proteins possess good structural and physico-chemical properties (low viscosity, nondescript flavour, ability to form gels) which promote them as ideal encapsulation matrices (DOHERTY *et al.* 2011). Emulsion, extrusion, and spray drying are the most commonly used techniques for the microencapsulation of probiotics (HEIDEBACH *et al.* 2009; BURGAIN *et al.* 2011).

Emulsion encapsulation of probiotic cells into a milk protein matrix was successfully carried out in the past by HEIDEBACH *et al.* (2009). It is based on the principle of cold renneting of milk containing the probiotic cells, followed by emulsification with oil. The method was later modified by replacing rennet with transglutaminase which allows cross-linking between milk proteins (HEIDEBACH *et al.* 2010).

The aim of this study was to modify the above mentioned method by the addition of lecithin and to evaluate the effect of the lecithin added as well

as the influence of the speed of agitation during the procedure on the capsule size and size distribution.

MATERIAL AND METHODS

Microorganisms. Freeze-dried commercial probiotic strain *Bifidobacterium animalis* subsp. *lactis* Bb12 from Christian Hansen (Hoersholm, Denmark) was used in this study.

Encapsulation procedure. The strain was encapsulated using the method of HEIDEBACH *et al.* (2009) with some modification. Briefly, 30 g of 35% (w/w) reconstituted skimmed milk (skimmed-milk powder Laktino (PROMIL, Nový Bydžov, Czech Republic) was mixed with 1 g of lyophilised probiotic culture. After 15 min of agitation, 400 μ l of rennet Naturen™ PREMIM 145 (Christian Hansen, Nienburg, Germany), diluted with distilled water in ratio 1:4 (w/w), was added. The mixture was stirred at a speed of 500 rpm at 5°C for 1 h and subsequently 180 μ l of 10% (w/w) CaCl_2 was added. Then, the whole mixture was transferred to 150 ml of sunflower oil (Vegetol; Setuza, Ústí nad Labem, Czech Republic) at 5°C without the addition of soya lecithin or with the addition of 0.5, 1.0, 5.0% (w/w) of soya lecithin SOLEC F-10 (Solae, Le Grand-Saconnex, Switzerland) into the resulting mixture while the agitation speed was adjusted to 500 rpm or 1200 rpm. After 5 min agitation, the mixture was heated to 40°C and the aggregation and formation of a gel matrix were observed. The encapsulated cells were separated from the oil by gentle centrifugation (500 g, 2 min, 4°C), washed by distilled water, and stored in refrigerator at 4°C for further analysis. A scheme of the encapsulation procedure is shown in Figure 1.

Optical examination. Both native and fixed preparations were used to check the encapsulated cells by optical microscope (Leica MD LF; Leica Camera AG, Solms, Germany). Furthermore, the fluorescence hybridisation *in situ* was used to visualise the encapsulated cells by an epifluorescence microscope Nikon E800 (Nikon, Tokyo, Japan)

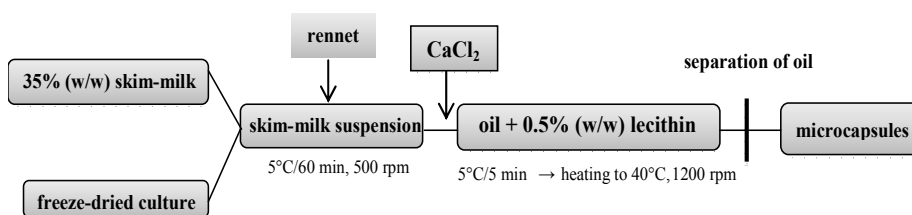


Figure 1. Scheme of emulsion encapsulation of probiotic microorganism with the addition of lecithin

with the filter 495/520 nm excitation/emission. To bind the fluorescent probe, FISH-kitTM for *Bifidobacterium* spp. stained with FITC (Ribo-Technologies, Groningen, Netherlands) was used.

Particle size determination. Laser diffraction, based on the optical properties of dispersions, was used to determine the size of the microcapsules. The capsules were re-suspended in demineralised water with the refractive index 1.33 and the mean size distribution was measured using Mastersizer 2000 with the dispersion unit Hydro G (both Malvern, Worcestershire, UK). A refractive index of 1.45 and absorption coefficient of 0.001 were selected for a sample of microcapsules. The measurement was performed at 25°C. The results are the means from two independent encapsulation procedures, both parallel encapsulated cell samples were analysed three times. The mean of volume based median ($d_{0.5}$) (i.e. 50% of total volume is composed of microcapsules with diameters equal or lower than $d_{0.5}$), and 90% fractiles ($d_{0.9}$) were calculated.

RESULTS AND DISCUSSION

First, the capsules with *B. animalis* subsp. *lactis* Bb12 cells were prepared without the lecithin addition and at a constant agitation speed of 500 rpm. An optical microscope and fluorescence hybridisation *in situ* (FISH) were used for the visual characterisation of the capsules (Figure 2). It was concluded that the microcapsules were mostly of a globular shape of different sizes and were not fully covered by a compact film of oil on their surface

Table 1. Volume based fractiles ($d_{0.5}$; $d_{0.9}$) of microcapsules prepared at different agitation speed and with or without the addition of lecithin

Encapsulation process		$d_{0.5}$	$d_{0.9}$
Agitation speed (rpm)	lecithin addition* % (w/w)	(µm)	
500	0.0	196 ± 37 ^a	519 ± 85 ^a
1200	0.0	193 ± 24 ^a	450 ± 39 ^b
500	0.5	79 ± 3 ^b	245 ± 12 ^c
1200	0.5	39 ± 3 ^c	85 ± 6 ^d

*lecithin addition into the oil; different letters in the same column indicate statistically significant differences ($P < 0.05$)

(Figure 2A). FISH method confirmed the presence of cells inside the microcapsules (Figure 2B).

In contrast to the result of HEIDEBACH *et al.* (2009) who found the volume based median diameter $d_{0.5}$ of only 68 ± 5 µm and $d_{0.9}$ below 93 µm, the results of this study showed much higher values. As shown in Table 1, $d_{0.5}$ value for the microcapsules of *B. animalis* subsp. *lactis* Bb12 was 196 µm. The size of microcapsules is one of the most important parameters for their further use in food because it can significantly influence the sensory characteristics of the product. The emulsion technique usually creates smaller beads (25 µm to 2 mm) compared to the extrusion method (2–5 mm) as reported by BURGAIN *et al.* (2011). The applications of microcapsules prepared by different methods of encapsulation in dairy products have been described by many authors (HANSEN *et al.* 2002; PICOT & LACROIX 2002; KOSIN & RAKSHIT 2006). The size of microcapsules reported to have no potential

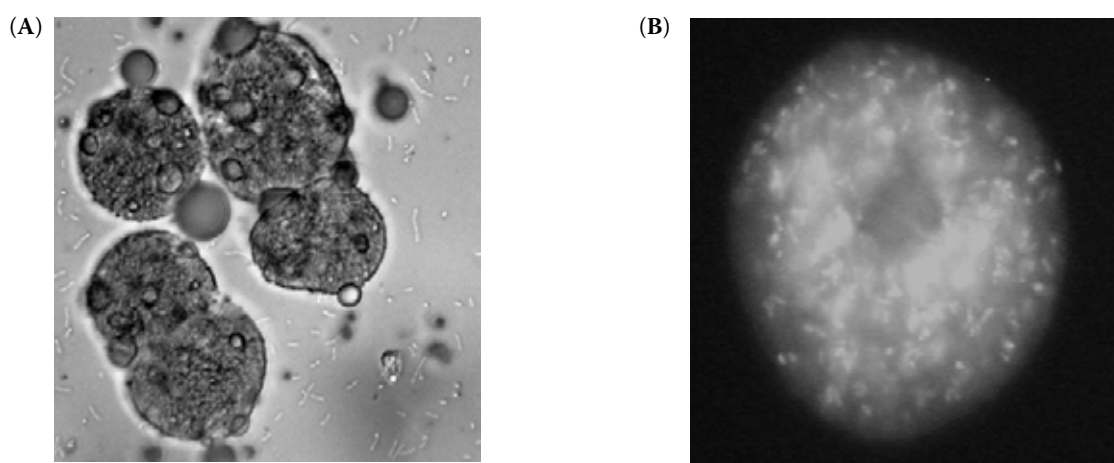


Figure 2. Optical visualisation of microcapsules with *B. animalis* subsp. *lactis* Bb12: (A) native preparation (80×); (B) FISH method preparation (1000×)

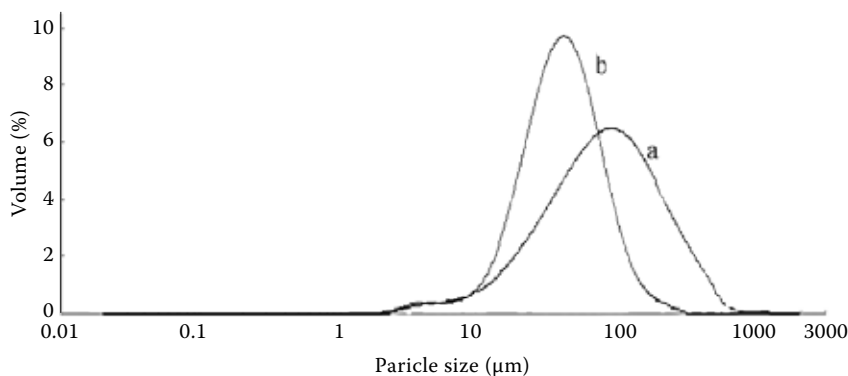


Figure 3. Particle size distribution curves of microcapsules prepared without (line a) and with (line b) the addition of 0.5% (w/w) lecithin into the oil at 1200 rpm agitation speed

sensory defect was approximately 250 μm (HEIDEBACH *et al.* 2009). Therefore, in further experiments the agitation speed was adjusted to 1200 rpm and the particle size was measured again to check the influence of this parameter on the particle size distribution. As shown in Table 1, the increase of the agitation speed did not significantly affect $d_{0.5}$ or $d_{0.9}$ values.

Hence the addition of 0.5, 1.0, and 5.0% (w/w) of lecithin into the oil was applied in order to help the preparation of an emulsion with smaller particles. The addition of 1.0 and 5.0% (w/w) lecithin into the oil caused a significant colour change of the microcapsules. The microcapsules of light brown colour were evaluated as undesirable for the application in dairy products and therefore only 0.5% (w/w) addition of lecithin into the oil was chosen for further experiments. Lecithin addition led to a decrease of $d_{0.5}$ value by a factor of 5 in the case of 1200 rpm agitation. However, when the agitation speed of 500 rpm was used, both $d_{0.5}$ and $d_{0.9}$ values decreased by only a factor of 2 (Table. 1). Also the microcapsules' surface was fully covered by oil layer (picture not shown). Figure 3 shows an example of the particle size distribution curves for the microcapsules of *B. animalis* subsp. *lactis* Bb12 prepared without (line a) or with (line b) lecithin at 1200 rpm. A number of other authors have observed a decrease in the bead size when an emulsifier such as sodium lauryl sulphate and Tween 80 was used during the emulsification process of calcium alginate beads (ROKKA & RANTAMÄKI 2010). Above that, the application of lecithin brings several other positive effects as it is non-toxic, well tolerated by humans, and many scientific studies have confirmed its contribution to lowering the cholesterol level (JIMENEZ *et al.* 1990; WILSON *et al.* 1998).

To conclude, the lecithin application for the preparation of microcapsules with probiotics offers the

possibility to decrease the particle size and to enrich food in which the microcapsules will be used by a nutritionally important substance.

References

- ADHIKARI K., MUSTAPHA A., GRÜN I.U., FERNANDO L. (2000): Viability of microencapsulated bifidobacteria in set yogurt during refrigerated storage. *Journal of Dairy Science*, **83**: 1946–1951.
- BURGAIN J., GAIANI C., LINDER M., SCHER J. (2011): Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of Food Engineering*, **104**: 467–483.
- BRINQUES G.B., AYUB M.A.Z. (2011): Effect of microencapsulation on survival of *Lactobacillus plantarum* in simulated gastrointestinal conditions, refrigeration and yogurt. *Journal of Food Engineering*, **103**: 123–128.
- CHARALAMPOPOULOS D., WANG R., PANDIELLA S. (2002): Application of cereals and cereal components in functional foods: a review. *International Journal of Food Microbiology*, **79**: 131–141.
- CRITTENDEN R. (2009): Incorporating probiotics into foods. In: LEE Y.L., SALMINEM S. (eds): *Handbook of Probiotics and Prebiotics*. 2nd Ed. Wiley, New Jersey: 58–75.
- DOHERTY S.B., GEE V.L., ROSS R.P., STANTON C., FITZGERALD G.F., BRODKORB A. (2011): Development and characterisation of whey protein micro-beads as potential matrices for probiotic protection. *Food Hydrocolloids*, **25**: 1604–1617.
- GBASSI G.K., VANDAMME T., ENNAHAR S., MARCHIONI E. (2009): Microencapsulation of *Lactobacillus plantarum* spp. in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology*, **129**: 103–105.
- HANSEN L.T., IAN-WOJTAS P.M., JIN Y.T., PAULSON A.T. (2002): Survival of Ca-alginate microencapsulated *Bifidobacterium* spp. in milk and simulated gastrointestinal conditions. *Food Microbiology*, **19**: 35–45.

- HELLER K.J. (2001): Probiotic bacteria in fermented foods: product characteristics and starter organisms. *American Journal of Clinical Nutrition*, **73**: 374–379.
- HEIDEBACH T., FORST P., KULOZIK U. (2009): Microencapsulation of probiotic cells by means of rennet-gelation of milk proteins. *Food Hydrocolloids*, **23**: 1670–1677.
- HEIDEBACH T., FORST P., KULOZIK U. (2010): Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. *Journal of Food Engineering*, **98**: 309–316.
- JIMENEZ M.A., SCARINO M.L., VIGNOLINI F., MENGHERI E. (1990): Evidence that polyunsaturated lecithin induces a reduction in plasma cholesterol level and favorable changes in lipoprotein composition in hypercholesterolemic rats. *Journal of Nutrition*, **120**: 659–667.
- KOSIN B., RAKSHIT S.K. (2006): Microbial and processing criteria for production of probiotics: a review. *Food Technology and Biotechnology*, **44**: 371–379.
- PICOT A., LACROIX C. (2004): Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. *International Dairy Journal*, **14**: 505–515.
- ROCCA S., RANTAMÄKI P. (2010): Protecting probiotic bacteria by microencapsulation: challenges for industrial applications. *European Food Research and Technology*, **231**: 1–12.
- VIDHYALAKSHMI R., BHAKYARAJ R., SUBHASREE R.S. (2009): Encapsulation “The future of probiotics” – a review. *Advances in Biological Research*, **3**: 96–103.
- WILSON T.A., MESERVEY C. M., NICOLosi R.J. (1998): Soy lecithin reduces plasma lipoprotein cholesterol and early atherogenesis in hypercholesterolemic monkeys and hamsters: beyond linoleate. *Atherosclerosis*, **140**: 147–153.

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Influence of co-encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 with inulin and ascorbic acid on its viability

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Abstract: Eight types of capsules containing *Bifidobacterium animalis* subsp. *lactis* Bb12 with addition of inulin and/or ascorbic acid were prepared by emulsion method with milk protein matrix or by extrusion method with alginate matrix. The size of protein and alginate capsules containing only Bb12 was $204 \pm 18 \mu\text{m}$ and $1.7 \pm 0.1 \text{ mm}$, respectively. Addition of both inulin (1% w/w) and ascorbic acid (0.5% w/w) increased the size of alginate capsules. Both methods of encapsulation prevented efficiently the manifestation of Bb12 cell metabolic activity. All types of encapsulation provided higher resistance of Bb12 cells to the conditions of a model gastrointestinal tract (GIT) compared to free cells. The influence of co-encapsulation with inulin (1% w/w) and ascorbic acid (0.5% w/w) on viability in model GIT was not demonstrable in alginate capsules but it was significant in protein capsules. The most efficient was co-encapsulation in a protein matrix with 1% w/w inulin and 0.5% w/w ascorbic acid.

Keywords: alginate; emulsion encapsulation; extrusion encapsulation; gastrointestinal tract; milk protein

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer health benefits on the host” (FAO/WHO 2002). The role of *Bifidobacterium* spp., which represents one of the widely used probiotics, in the human microbiota was summarized by O’callaghan & Van Sinderen (2016). *Bifidobacterium* spp. are Gram-positive, strictly anaerobic, non-sporeforming and non-motile rod-shaped bacteria that belong to the phylum *Actinobacteria*. They mainly occur in the gastrointestinal tract of mammals, birds and insects, and they are also present in sewage or human breast milk. Bifidobacteria are saccharolytic microorganisms, the fermentation via the typical fructose-6-phosphate shunt results in the production of acetic and lactic acid (Lee & O’Sullivan 2010). *Bifidobacterium animalis* subsp. *lactis* has been isolated from different sources (Bunesova et al. 2017) and is commonly found in the gut of healthy humans (Turrone et al. 2017). The specific

strain Bb12, selected by Chr. Hansen, is the world’s most documented probiotic with widespread commercial use (Jungersen et al. 2014).

When probiotic bifidobacteria are applied into food, it is necessary to ensure their stability during processing and storage of the final product, and protection when passing through GIT. Encapsulation of cells may be an effective protection option. It is a physicochemical and mechanical process by which bacteria are coated with or entrapped within different materials through the use of emulsions, extrusions or through drying technologies. Commonly used materials for the encapsulation of bacteria include polysaccharides (starch, carrageenan), polysaccharide derivatives (gum Arabic, xanthan gum, alginate) and some proteins (gelatine, milk proteins) (Martín et al. 2015).

Co-encapsulation is an encapsulation process which uses a combination of two or more bioactive substances which can positively influence each other (Cham-

pagne & Fustier 2007). Co-encapsulation of probiotics with prebiotics has been tested in order to protect their viability during the passage through GIT (Atia et al. 2017). As the viability of probiotics is negatively influenced by a detrimental effect of oxygen, which damages the membrane lipids, antioxidants (ascorbic acid, l-cysteine and tocopherol) were also tested to prevent oxidative stress when added into an encapsulation matrix with probiotics (Chen et al. 2017).

The aim of this study was to prepare microcapsules containing *Bifidobacterium animalis* subsp. *lactis* Bb12 (Bb12) with the addition of inulin and/or ascorbic acid both by emulsion method using milk proteins and by extrusion method using an alginate matrix, and further to compare the viability of the free and encapsulated bifidobacteria during storage and in conditions simulating those in GIT.

MATERIALS AND METHODS

Cultivation and determination of bifidobacteria count. The freeze-dried commercial probiotic strain of *Bifidobacterium animalis* subsp. *lactis* Bb12 was obtained from Christian Hansen (Hørsholm, Denmark). The strain was cultivated in 200 mL of MRS broth (Oxoid Ltd., Hampshire, UK; pH 6.5) with 0.05% (w/v) L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany) anaerobically at 37 °C for 18 hours. The cells were harvested by centrifugation at 9 000 rpm for 10 min at 4 °C (Universal 32R; Hettich, Tuttlingen, Germany), washed three times with physiological saline solution (pH 7) and immediately used for encapsulation.

For determination of their counts, after appropriate dilution bifidobacteria were cultivated in an anaerobic jar (Oxoid) at 37 °C for 72 h on L-cysteine-MRS agar, pH 6.5, and counted as colony-forming units (CFU mL⁻¹). Sterile sodium citrate solution [2% (w/v), pH 7.5] instead of saline solution was used for the dilution of samples containing protein or alginate capsules to allow their solubilisation and a release of cells from the matrix.

Encapsulation process. Emulsion encapsulation was done according to Heidebach et al. (2009) and Lisová et al. (2013) with some modifications. In brief, 120 g of 35% (w/w) reconstituted skimmed milk (Laktino skimmed milk powder, Promil, Nový Bydžov, Czech Republic) was mixed with fresh culture from 200 mL cultivation in MRS (de Man, Rogosa and Sharpe medium). After agitation (800 rpm, 5 min), 1.6 mL of Naturen™ Premim 145 rennet (Chr. Hansen, Nienburg, Germany), diluted with distilled water at the ratio of 1 : 4 (w/w), was added. After that, the stirring speed

was reduced to 500 rpm for 30 min and subsequently 720 µL of 10% (w/w) sterile CaCl₂ was added. Then, the whole mixture was transferred to 600 g of pre-tempered (5 °C) sunflower oil (Vegetol; Setuza, Ústí nad Labem, Czech Republic) with 0.5% (w/w) soya lecithin (Epikuron 100 PIP; Cargill GmbH, Krefeld, Germany) and stirred at 500 rpm. After 5-min agitation the mixture was heated to 40 °C with continued stirring for 15 minutes. The encapsulated cells were separated from the oil by gentle centrifugation (6 000 rpm, 4 °C, 2 min), washed three times in physiological solution and stored at 6 ± 1 °C before the following use.

For extrusion encapsulation, alginate was used as a coating material (Krasaekoopt et al. 2004). Fresh culture (1 g) was added to 10 mL of sterile 1% (w/v) sodium alginate solution (Sigma-Aldrich, St. Louis, USA). The mixture was injected through a sterile disposable 0.5-mm needle into 100 mL of sterile 0.1 mol L⁻¹ CaCl₂ solution. The alginate beads were kept in the solution for gelification for 30 min; then they were washed twice with saline solution and stored at 6 ± 1 °C.

The addition of 1 % (w/w) inulin (Orafti® HPX, Be-neo-Orafti, Oreye, Belgium) and/or 0.5% (w/w) ascorbic acid (Penta, Prague, Czech Rep.) was performed directly into reconstituted skimmed milk or into sodium alginate solution.

Survival of free and encapsulated cells in simulated gastrointestinal conditions. Freeze-dried culture (0.1 g) or microcapsules (1 g) were added into 50 mL of a solution simulating the stomach gastric juice containing distilled water, 0.5% w/v NaCl and 0.3% w/v pepsin (Sigma-Aldrich, St. Louis, USA). The final pH was adjusted to pH 2 with 10% w/v HCl. The samples were incubated at 37 °C in anaerobic atmosphere. After 2 h, pH was adjusted to 6.8 (with sterile 10% w/v NaOH) and ox bile (0.3% w/v; Merck) and pancreatin (0.1% w/v; Sigma-Aldrich, St. Louis, USA) were added to simulate the conditions in the ileum for another 4 hours. At the time of 0, 2, 4 and 6 h the number of viable cells was determined. The results are expressed as the means from three independent measurements (*n* = 3).

Viability of free and encapsulated bacteria during storage. The storage stability (UHT skim milk, Madeta, Planá, Czech Republic) of free and encapsulated cells in milk was tested for 6 weeks at 6 ± 1 °C. Every 14 days the number of viable cells and pH value (Jenway pH meter; Jenway, Staffordshire, UK) were measured. The results are the means from two independent replications, both parallel samples were analysed twice (*n* = 4).

Capsule size determination. Laser diffraction, based on the optical properties of dispersion, was used

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to determine the size of microcapsules prepared by the emulsion method. The capsules were re-suspended in demineralised water with refractive index 1.33 and the mean size distribution was measured using a Mastersizer 2 000 particle size analyzer (Malvern, Worcestershire, UK) with the Hydro G dispersion unit (Malvern, Worcestershire, UK) at 25 °C. The refractive index 1.45 and absorption coefficient 0.001 were selected for samples. Ninety percent fractiles ($d_{0.9}$) were calculated (i.e. 90% of the total volume is composed of microcapsules with diameters equal or lower than $d_{0.9}$). The results are the means from two independent encapsulation procedures, both parallel encapsulated cell samples were analysed three times.

The size of alginate capsules was measured with a digital calliper. The results are the means of measuring at least 30 randomly selected capsules, each from two independent encapsulation processes.

RESULTS AND DISCUSSION

Eight types of capsules with inulin and/or ascorbic acid were prepared by emulsion method with protein matrix or by extrusion method with alginate (Table 1). Ascorbic acid can affect the growth of bifidobacteria (Shu et al. 2013) and it can also have a physiological effect (Garaiova et al. 2015). Inulin is a well-known prebiotic, its effect on bifidobacterial growth and human microbiota has been confirmed in many studies (Meyer & Stasse-Wolthuis 2009). It can be concluded that the addition of ascorbic acid reduced the size of protein microcapsules prepared by the emulsion method. On the contrary, in the case of alginate capsules (extrusion method), the addition of inulin and/or ascorbic acid increased the size of capsules. In the study of Heidebach et al. (2009) protein microcapsules containing probiotics with a volume-based median $d_{0.5} = 68 \pm 5 \mu\text{m}$ were prepared. The authors concluded that there was no difference in the size of microcapsules containing bacterial cells or without them but they reported the influence of encapsulation conditions on $d_{0.5}$. Other authors (Vivek 2013) also proved the influence of various factors on the capsule size such as stirring speed, water/oil ratio, emulsifier concentration. In accordance with results of Valero-Cases & Frutos (2015), we also found that the addition of inulin increases the size of capsules. When applying encapsulated material into different types of food, the size of capsules must be taken into account. The application of larger particles obtained by the extrusion method is not suitable for liquid and

Table 1. Type and size of capsules prepared by different methods containing *Bifidobacterium animalis* subsp. *lactis* Bb12 with the addition of inuline and/or ascorbic acid

Encapsulation method	Matrix	Inulin 1% (w/w)	Ascorbic acid 0.5 % (w/w)	
				v.b. fractiles (d _{0.9}) (μm)
Emulsion	milk protein	0	0	204 ± 18 ^a
		+	0	192 ± 27 ^a
		0	+	87 ± 10 ^b
		+	+	227 ± 11 ^a
				size (mm)
Extrusion	alginate	0	0	1.7 ± 0.1 ^a
		+	0	2.3 ± 0.1 ^b
		0	+	2.2 ± 0.1 ^b
		+	+	2.3 ± 0.1 ^b

Values in the same column followed by different lowercase letters are significantly different ($P < 0.05$); v.b. – volume based

semi-liquid foods, but they can be used in solid foods (Chen et al. 2017).

In the next part of this work, the influence of encapsulation and co-encapsulation with inulin and/or ascorbic acid on the stability of Bb12 compared to the stability of free Bb12 cells during 6-week storage at 6 ± 1 °C in sterile milk was tested. The results are summarized in Table 2. Milk is known for its protective effects on the viability of microorganisms (Sagheddu et al. 2018). Free Bb12 cells were found quite stable; nevertheless the number of cells at the end of storage was significantly different. It decreased by about 1.5 log cycles while the number of encapsulated cells remained statistically unchanged. The encapsulation had a positive effect on the viability of cells but the addition of inulin and ascorbic acid had no demonstrable effect either in protein or alginate capsules (at $P < 0.05$). This result is different from the conclusion of Valero-Cases & Frutos (2015), who found a positive effect of inulin on the viability of *L. plantarum* in alginate capsules.

Changes in the pH value of milk (considered as metabolic activity) were also monitored. Table 3 shows that free cells were able to produce organic acids and decreased pH from 6.33 ± 0.03 to 5.10 ± 0.02 . In contrast, the pH of milk with encapsulated cells remained the same as at the beginning of storage. Both encapsulation methods prevented efficiently the manifestation of Bb12 fermentation activity.

The main objective of encapsulation is to protect cells against adverse conditions. The viability of bacte-

Table 2. The viability (log CFU mL⁻¹) of free, encapsulated and co-encapsulated (with 1% (w/w) inulin and/or 0.5% (w/w) ascorbic acid) cells of *Bifidobacterium animalis* subsp. *lactis* Bb12 during 6-week storage in sterile skimmed milk at 6 ± 1 °C

	Week				Week			
	0	2	4	6	0	2	4	6
Free Bb12	8.1 ± 0.2 ^{a,A}	7.5 ± 0.1	6.8 ± 0.1	6.5 ± 0.1 ^{b,A}	10.4 ± 0.1 ^{a,A}	8.8 ± 0.1	8.5 ± 0.1	8.2 ± 0.1 ^{b,A}
Encapsulated Bb12	8.8 ± 0.1 ^{a,A}	8.7 ± 0.1	8.3 ± 0.1	8.2 ± 0.3 ^{a,B}	10.4 ± 0.1 ^{a,A}	10.2 ± 0.1	10.2 ± 0.1	9.9 ± 0.3 ^{a,B}
Co-encapsulated Bb12 + inulin	8.3 ± 0.3 ^{a,A}	8.2 ± 0.2	8.2 ± 0.1	8.0 ± 0.3 ^{a,B}	10.3 ± 0.2 ^{a,A}	10.2 ± 0.1	9.9 ± 0.1	9.8 ± 0.1 ^{a,B}
Co-encapsulated Bb12 + ascorbic acid	8.2 ± 0.2 ^{a,A}	8.3 ± 0.1	8.4 ± 0.1	8.1 ± 0.1 ^{a,B}	10.3 ± 0.1 ^{a,A}	10.1 ± 0.1	10.2 ± 0.1	9.9 ± 0.2 ^{a,B}
Co-encapsulated Bb12 + inulin + ascorbic acid	8.2 ± 0.3 ^{a,A}	8.1 ± 0.2	8.3 ± 0.1	8.1 ± 0.1 ^{a,B}	10.3 ± 0.1 ^{a,A}	10.0 ± 0.1	9.9 ± 0.1	9.9 ± 0.1 ^{a,B}

Values are in mean ± SD; values in the same row followed by different lowercase letters are significantly different ($P < 0.05$); values in the same column followed by different capital letters are significantly different ($P < 0.05$).

ria in the stomach environment and in the presence of bile is one of the main requirements for probiotics (Karimi et al. 2011). Therefore, the stability of free and encapsulated Bb12 cells was checked in conditions simulating those when passing through GIT. The results are presented in Figure 1. All types of encapsulated cells showed a greater resistance to the conditions of the model digestive tract compared to free cells of Bb12. Differences in stability were particularly evident in conditions simulating the ileum. During the experiment a decrease in the number of encapsulated cells was only by 0.9–2.8 log cycles compared to 8 log cycles for free cells. No significant differences in cell survival were found when using protein or alginate capsules in the case of encapsulation into the matrix itself (decrease in cell number by 2.7–2.8 orders). In alginate capsules, the positive effect of inulin and ascorbic acid was not demonstrable, in all cases there was a total cell loss of 2.5–2.8 orders. On the other hand, the enriched protein capsules showed a lower cell loss; only by 0.9–1.8 orders. Our results are in good agreement with previously published data concerning the effect of encapsulation on the stability of bifidobacteria in human GIT. Comparable results were achieved by Madureira et al. (2011) with whey protein encapsulation of Bb12. The positive effect of encapsulation into an alginate matrix on resistance of *B. infantis* against gastric juices was also published by Cook et al. (2012). Fritzen-Freire et al. (2013) proved a positive effect of co-encapsulation of Bb12 with inulin into a protein matrix on the cell resistance to gastric juice and bile.

Table 3. The change in pH of milk with free, encapsulated and co-encapsulated [with 1% (w/w) inulin and/or 0.5% (w/w) ascorbic acid] cells of *Bifidobacterium animalis* subsp. *lactis* Bb12 after 6-week storage at 6 ± 1 °C

	Week		
	0	6	
Free Bb12	6.33 ± 0.03	5.10 ± 0.02	
Encapsulated Bb12	6.36 ± 0.03	6.15 ± 0.03	6.25 ± 0.04
Co-encapsulated Bb12 + inulin	6.36 ± 0.03	6.30 ± 0.03	6.45 ± 0.03
Co-encapsulated Bb12 + ascorbic acid	6.35 ± 0.04	6.32 ± 0.02	6.14 ± 0.04
Co-encapsulated Bb12 + inulin + ascorbic acid	6.32 ± 0.03	6.40 ± 0.02	6.08 ± 0.04

Values are in mean ± SD

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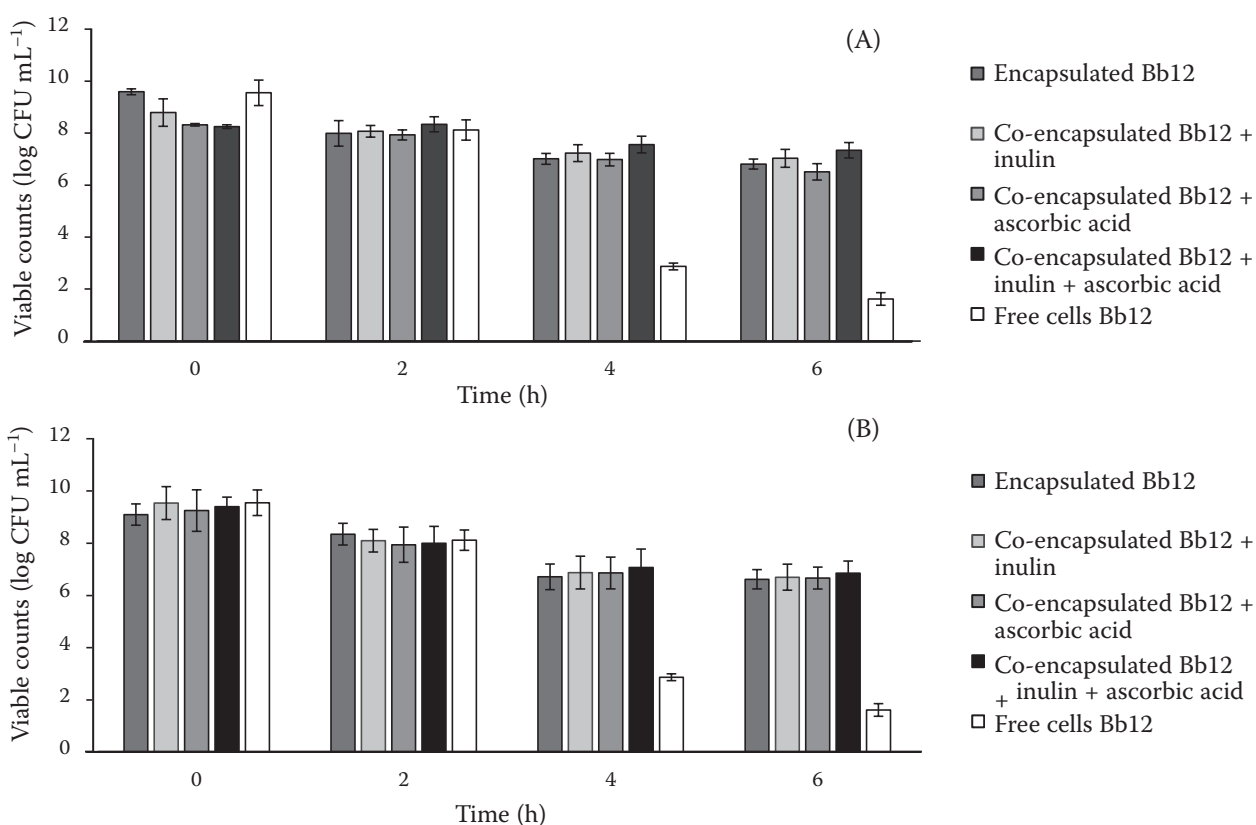


Figure 1. The viability (log CFU mL⁻¹) of free, encapsulated and co-encapsulated [with 1% (w/w) inulin and/or 0.5% (w/w) ascorbic acid] cells of *Bifidobacterium animalis* subsp. *lactis* Bb12 in conditions simulating those in stomach (0–2 h) and in ileum (2–6 h); (A) – emulsion encapsulation method with milk protein matrix; (B) – extrusion encapsulation method with alginate matrix

CONCLUSION

In this study, protein microcapsules of $204 \pm 18 \mu\text{m}$ in size and alginate capsules of $1.7 \pm 0.1 \text{ mm}$ in size were obtained by various methods of encapsulation of *B. animalis* subsp. *lactis* Bb12 bacteria. Co-encapsulation with inulin (1% w/w) and/or ascorbic acid (0.5% w/w) affected the size of alginate capsules. Both encapsulation techniques resulted in the protection of Bb12 cells during 6-week storage in milk at a refrigerator temperature. Protein and alginate capsules as well as those with added inulin and/or ascorbic acid greatly enhanced the viability of Bb12 in conditions simulating the gastrointestinal tract. Capsules with probiotics, prebiotic inulin and antioxidant ascorbic acid can be considered as a suitable way of enriching food with biologically active ingredients with a high level of protection of sensitive bacteria. However, for application, the particle size must also be taken into account not to negatively affect the sensory properties of a product.

REFERENCES

- Atia A., Gomma A.I., Fliss I., Beyssac E., Garrait G., Subirade M. (2017): Molecular and biopharmaceutical investigation of alginate-inulin symbiotic to target the colon. *Journal of Microencapsulation*, 34: 171–184.
- Bunesova V., Killer J., Javurkova B., Vlkova E., Tejnecky V., Musilova S., Rada V. (2017): Diversity of the subspecies *Bifidobacterium animalis* subsp. *lactis*. *Anaerobe*, 44: 40–47.
- Champagne C.P., Fustier P. (2007): Microencapsulation for the improved delivery of bioactive compounds into foods. *Current Opinion in Biotechnology*, 18: 184–190.
- Chen J., Wang Q., Liu C.-M., Gong J. (2017): Issues deserve attention in encapsulating probiotics: Critical review of existing literature. *Critical Reviews in Food Science and Nutrition*, 57: 1228–1238.
- Cook M.T., Tzortzis G., Charalampopoulos D., Khutoryanskiy V.V. (2011): Production and evaluation of dry alginate-chitosan microcapsules as an enteric delivery vehicle for probiotic bacteria. *Biomacromolecules*, 12: 2834–2840.

<https://doi.org/10.17221/292/2019-CJFS>

- FAO/WHO (2002): Guidelines for the Evaluation of Probiotics in Food. Available at https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf (accessed Sept 5, 2019).
- Fritzen-Freire C.B., Prudêncio E.S., Pinto S.S., Muñoz I.B., Amboni R.D.M.C. (2013): Effect of microencapsulation on survival of *Bifidobacterium* BB-12 exposed to simulated gastrointestinal conditions and heat treatments. *LWT – Food Science and Technology*, 50: 39–44.
- Garaiova I., Muchová J., Nagyová Z., Wang D., Li J.V., Országhová Z., Michael D.R., Plummer S.F., Ďuračková Z. (2015): Probiotics and vitamin C for the prevention of respiratory tract infections in children attending preschool: a randomised controlled pilot study. *European Journal of Clinical Nutrition*, 69: 373–379.
- Heidebach T., Först P., Kulozik U. (2009): Microencapsulation of probiotic cells by means of rennet-gelation of milk proteins. *Food Hydrocolloids*, 23: 1670–1677.
- Jungersen M., Wind A., Johansen E., Christensen J.E., Stuer-Lauridsen B., Eskesen D. (2014): The science behind the probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12. *Microorganisms*, 2: 92–110.
- Karimi R., Mortazavian A.M., Da Cruz A.G. (2011): Viability of probiotic microorganisms in cheese during production and storage. *Dairy Science & Technology*, 91: 283–308.
- Krasaekoopt W., Bhandari B., Deeth H. (2004): The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. *International Dairy Journal*, 14: 737–743.
- Lee J., O'Sullivan D.J. (2010): Genomic insight into bifidobacteria. *Microbiology and Molecular Biology Reviews*, 74: 378–416.
- Lisová I., Horáčková Š., Kováčová R., Rada V., Plocková M. (2013): Emulsion encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 with the addition of lecithin. *Czech Journal of Food Sciences*, 31: 270–274.
- Madureira A.R., Amorim M., Gomes A.M., Pintado M.E., Malcata F.X. (2011): Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. *Food Research International*, 44: 465–470.
- Martín M.J., Lara-Villoslada F., Ruiz M.A., Morales M.E. (2015): Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. *Inovative Food Science & Emerging Technologies*, 27: 15–25.
- Meyer D., Stasse-Wolthuis M. (2009): The bifidogenic effect of inulin and oligofructose and its consequences for gut health. *European Journal of Clinical Nutrition*, 63: 1277–1289.
- O'Callaghan A., van Sinderen D. (2016): Bifidobacteria and their role as members of the human gut microbiota. *Frontiers in Microbiology*, 7: 925.
- Sagheddu V., Elli M., Biolchi C., Lucido J., Morelli L. (2018): Impact of mode of assumption and food matrix on probiotic viability. *Journal of Food Microbiology*, 2: 1–6.
- Shu G., Yang H., Tao Q., He Ch. (2013): Effect of Ascorbic Acid and Cysteine Hydrochloride on Growth of *Bifidobacterium bifidum*. *Advance Journal of Food Science and Technology*, 5: 678–681.
- Turroni F., Berry D., Ventura M. (2017): Editorial: *Bifidobacteria* and their role in the human gut microbiota. *Frontiers in Microbiology*, 7: 2148.
- Valero-Cases E., Frutos M.J. (2015): Effect of different types of encapsulation on the survival of *Lactobacillus plantarum* during storage with inuline and *in vitro* digestion. *LWT – Food Science and Technology*, 64: 824–828.
- Vivek K.B. (2013): Use of encapsulated probiotics in dairy based foods. *International Journal of Food, Agriculture and Veterinary Sciences*, 3: 188–199.

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Fermentation of Soymilk by Yoghurt and Bifidobacteria Strains

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Abstract

HORÁČKOVÁ Š., MÜHLHANSOVÁ A., SLUKOVÁ M., SCHULZOVÁ V., PLOCKOVÁ M. (2015): **Fermentation of soymilk by yoghurt and bifidobacteria strains.** Czech J. Food Sci., 33: 313–319.

Soy and milk based products fermented by yoghurt culture YC-381 alone or in combination with two probiotic cultures (*Bifidobacterium animalis* subsp. *lactis* BB 12 and *Bifidobacterium bifidum* CCDM 94) were prepared. Bacteria growth, the amounts of lactic acid, acetic acid, and acetaldehyde were compared after 16 h of fermentation at 37°C. The changes of isoflavones concentrations were also monitored in soy products. The growth of *Streptococcus thermophilus* and bifidobacteria was similar in both media, but *Lactobacillus delbrueckii* subsp. *bulgaricus* showed a better growth in milk. Titratable acidity and the concentrations of acids were consistently higher in cow milk than in soymilk at the end of fermentation. Bifidobacteria, compared to the yoghurt culture, were only able to acidify the media to the half values. Comparing the bifidobacteria strains, *Bifidobacterium animalis* subsp. *lactis* BB 12 exhibited a better ability to acidify milk. The strain *Bifidobacterium bifidum* CCDM 94 was able to release 6.90 mg/100 ml of isoflavone aglycones in soymilk.

Keywords: acetaldehyde; acetic acid; isoflavones; lactic acid

The fermented soy products represent an interesting alternative to the fermented milk products. The metabolism of lactic acid bacteria (LAB) shows some differences between soymilk and cow milk as a result of their different media compositions. The higher concentration of proteins in cow milk increases the buffering capacity of media which is beneficial for the growth of bacteria. Soymilk – lactose free medium – contains galactooligosaccharides which are considered as prebiotics, i.e. compounds supporting probiotic bacteria growth (FARNWORTH 2007). The metabolism of saccharides and proteins caused by LAB can influence both the nutritional value and final sensory quality of the fermented products. On the other hand, Europeans are not familiar with the organoleptic quality of fermented soy products (ANG *et al.* 2003).

The yoghurt culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) exhibits symbiotic character of growth in cow milk but the support of the growth was not observed in soymilk

(CHUMCHUERE & ROBINSON 1999). *Bifidobacterium* spp. normally inhabits the human gastrointestinal tract and is one of the main probiotic cultures (SHAH 2007). As an example of the positive health effects of bifidobacteria can be their ability to bio-activate some substances which can increase their impact on human body.

Isoflavones belong to a group phytoestrogens with the highest level of estrogenic activity. In plants, they occur as free aglycones or are very commonly bonded with glycosides or their malonyl- and acetyl-conjugates. Isoflavone glycosides derived from soymilk require bacterial-induced hydrolysis in order to be transformed into a bioavailable aglycone form (TSANGALIS *et al.* 2003). IZUMI *et al.* (2000) and SETCHELL *et al.* (2001) discovered that isoflavone aglycones were absorbed faster and in higher amounts than their respective β -glucoside forms.

The reduction of the beany flavour of soymilk, which improves the sensory quality of the final product,

could be also connected with fermentation. Bifidobacteria strains, specifically, are able to metabolise *n*-hexanal and pentanal in soymilk – the former is responsible for the undesirable flavour (DESAI *et al.* 2002). Furthermore, fermentation also reduces the content of soy galactooligosaccharides: indigestible carbohydrates, which can in higher amounts cause undesirable flatulence (SCALABRINI *et al.* 1998).

The aims of the present study were to compare the growths of the yoghurt culture and two different strains of bifidobacteria in cow milk and soymilk, to evaluate the production of short fatty acids and acetaldehyde, and to confirm the biotransformation of isoflavone glycosides to aglycons during the fermentation process.

MATERIAL AND METHODS

Microorganisms. *Bifidobacterium animalis* subsp. *lactis* BB 12 – commercial probiotic strain (Ch. Hansen, Hørsholm, Denmark); *Bifidobacterium bifidum* CCDM 94 (Culture Collection of Dairy Microorganisms; Laktoflora®, Prague, Czech Republic); Yoghurt culture YC-381 – commercial culture (Ch. Hansen, Hørsholm, Denmark).

Preparation of model fermented products. As the cultivation media, whole-fat UHT cow milk (Madeta, Planá nad Lužnicí, Czech Republic) and soymilk Sunfood klasik (Sunfood, Dobruška, Czech Republic) were used after sterilisation at 100°C for 20 minutes. The appropriate amounts (0.5 g) of lyophilised cultures (*B. animalis* subsp. *lactis* BB 12 and yoghurt culture YC-381) were dissolved in 100 ml of soymilk or cow milk which were then stored at 4°C for 12 h to activate the cultures with the aim to shorten their lag phase. The activated microorganisms or the liquid culture (*B. bifidum* CCDM 94) were transferred in the amount of 1% (v/v) to sterile soymilk or cow milk. The inoculated media were cultivated at 37°C for 16 h with the aim to enhance the growth of bifidobacteria. The number of cells at the beginning of fermentation was as follows: *S. thermophilus* 10⁶ CFU/ml, *L. delbrueckii* subsp. *bulgaricus* 10⁴ CFU/ml, and *Bifidobacterium* spp. 10⁶ CFU/ml. All experiments were replicated twice and all results are the mean from three independent measurements (*n* = 6).

Determination of cell count. The number of *L. delbrueckii* subsp. *bulgaricus* in the samples was determined after 72 h of incubation at 37°C in 4%

(v/v) CO₂ atmosphere on MRS agar (Oxoid, Hampshire, UK), pH 5.2; the number of *S. thermophilus* after 48 h of incubation at 37°C on M17 agar (Oxoid, Hampshire, UK), pH 6.8, and the number of *Bifidobacterium* spp. after 4 days at 37°C in an anaerobic jar with an anaerobic gas generating kit AnaeroGen on MRS agar (both Oxoid, Hampshire, UK) with 0.5 g/l L-cysteine (Merck, Darmstadt, Germany) and 2 mg/l dicloxacilin (Sigma, St. Louis, USA), pH 6.8.

Physico-chemical analyses. Total solids were determined by Halogen Moisture Analyzer HR 73 (Mettler Toledo, Greifensee, Switzerland). The fat content was measured according to EN ISO 1211:2010 (Milk – Determination of fat content – Gravimetric method (Reference method). The total nitrogen content was evaluated using a Kjelttec analyzer (Kjelttec 8400 Analyzer; FOSS Analytical AB, Höganäs, Sweden); the protein content was calculated as total nitrogen multiplied by 6.38 for cow milk and 5.80 for soymilk. Ash content was determined by samples incineration in the furnace Ht40P (Lac, Rajhrad, Czech Republic) at 525°C.

pH and titratable acidity. The pH values were determined with a pH meter (Jenway, Staffordshire, UK) provided with a combined electrode. The titratable acidity was evaluated by titration of 25 g of product with 0.1 mol/l NaOH solution to pH 8.3 with phenolphthalein indicator and expressed as mmol H⁺/kg. All experiments were replicated twice.

Measurement of metabolic products. Enzymatic kits (Megazyme, Bray, Co., Wicklow, Ireland) were used for the determination of the content of lactic acid isomers (D,L-lactic acid assay), acetic acid and acetaldehyde according to the manufacturer's instruction. All experiments were replicated twice.

Extraction of isoflavones from soymilk. The soymilk or the fermented soy product (1 ml) with chrysin 10 ng/ml (Sigma-Aldrich, Seelze, Germany) as an inner standard was extracted two times with 6 ml of ethylacetate. The solution was centrifuged at 11 000 rcf (Rotina 35 R; Hettich Zentrifugen, Tuttlingen, Germany) for 5 min and the supernatant was dried in a rotary evaporator (BUCHI Rotavapor R-114; Büchi, Flawil, Switzerland) under nitrogen. The dried pellet was resuspended in 10 ml of 50% (v/v) methanol and then diluted 10 times with 50% (v/v) methanol. The extract was filtered through microfilter and transferred to vials for UPLC-MS/MS analysis.

HPLC analysis of isoflavones. The separation was performed using an Acquity Ultra-Performance LC system (UPLC) (Waters, Milford, USA) with an

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Acquity UPLC BEH C18 column (50 mm × 2.1 mm i.d., 1.8 µm particle size; Waters, Milford, USA) connected to a 5500 QTRAP tandem mass spectrometer (AB SCIEX, Toronto, Canada), equipped with an TurboIonTM electrospray (ESI) ion source operated in both positive and negative modes. Aqueous solutions of acetic acid and methanol (0.1% v/v) were used as mobile phase with gradient elution; the column temperature was 35°C. Daidzein, glycitein, and genistein (Sigma-Aldrich, Seelze, Germany) standard solutions in methanol were used to identify the products in Buchi (Flawil, Switzerland) samples tested. The isoflavone concentration was calculated by interpolation of the calibration curve prepared by the function of the ratio of the standard area and inner standard area (chrysin) and the ratio of isoflavone and inner standard area in the sample. All experiments were replicated twice.

RESULTS AND DISCUSSION

The compositions of soymilk and cow milk were found to be different with respect to the compounds which could be utilised by the starter and probiotic cultures tested. The parameters of soymilk were as follows: total solids $13.57 \pm 0.04\%$ w/w, fat $3.75 \pm 0.35\%$ w/w, proteins $2.17 \pm 0.02\%$ w/w, and ash $0.61 \pm 0.00\%$ w/w, while with cow milk: total solids $12.20 \pm 0.05\%$ w/w, fat $3.33 \pm 0.29\%$ w/w, proteins $3.59 \pm 0.01\%$ w/w, and ash $0.80 \pm 0.00\%$ w/w. The pH of soymilk and cow milk were 6.9 and 6.6, respectively. The titratable acidity of soymilk was lower (11.18 ± 0.39 mmol H⁺/kg) compared to cow milk (15.85 ± 0.30 mmol H⁺/kg).

The variety and quantity of live microorganisms is an important characteristic of the product quality. Cell counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *Bifidobacterium* spp. in cow milk and soymilk after 16 h of fermentation at 37°C are shown in Table 1. At the end of fermentation, no difference in the counts of bifidobacteria fermented alone or in a mixture with the yoghurt culture was observed in either media. The counts of *S. thermophilus* predominated in both media fermented with the yoghurt culture. *L. delbrueckii* subsp. *bulgaricus* achieved counts of 10^7 CFU/ml in milk and of 10^6 CFU/ml in soymilk. The difference could be explained by the inability of *L. delbrueckii* subsp. *bulgaricus* to utilise sucrose, the main sugar in soymilk (PINTHONG *et al.* 1980). The same results were found by DONKOR *et al.* (2007) who confirmed a better growth of *S. thermophilus* St1342 than that of *L. delbrueckii* subsp. *bulgaricus* Lb1466 in soymilk. A sufficient amount of bifidobacteria in the final product is important in terms of ensuring probiotic properties. In both media, bifidobacteria counts reached more than 10^7 CFU/ml with no difference between the strains used. Several factors influence the growth and viability of *Bifidobacterium* spp. co-cultivated with the yoghurt culture. The acidity, pH and hydrogen peroxide concentration have been identified as having an effects during the manufacture and storage of yoghurt (LANKAPUTHRA *et al.* 1996). On the other hand FARNWORTH (2007) found that the presence of the probiotic bacteria did not affect the growth of the yoghurt strains in soymilk and cow milk.

The acidification ability has been defined as the difference between the active and titratable acidity measured immediately after inoculation and at the

Table 1. Number of cells (log CFU/ml) in cow milk and soymilk fermented by yoghurt culture YC-381 (YC-381), *B. animalis* subsp. *lactis* BB 12 (BB 12), *B. bifidum* CCDM 94 (CCDM 94), and mixed cultures after 16 h of fermentation at 37°C

Culture	Species	Cow milk	Soy milk
YC-381	<i>S. thermophilus</i>	8.20 ± 0.12	8.08 ± 0.11
	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	7.45 ± 0.18	6.46 ± 0.57
YC-381 + BB 12	<i>S. thermophilus</i>	8.40 ± 0.45	8.15 ± 0.40
	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	6.97 ± 0.52	6.38 ± 0.36
	<i>B. animalis</i> subsp. <i>lactis</i>	8.36 ± 0.42	8.11 ± 0.18
YC-381 + CCDM 94	<i>S. thermophilus</i>	7.28 ± 0.11	8.15 ± 0.12
	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	7.54 ± 0.19	6.73 ± 0.29
	<i>B. bifidum</i>	8.15 ± 0.21	7.00 ± 0.32
BB 12	<i>B. animalis</i> subsp. <i>lactis</i>	8.34 ± 0.43	7.08 ± 0.19
CCDM 94	<i>B. bifidum</i>	8.15 ± 0.41	7.08 ± 0.64

Table 2. Changes in pH and titratable acidity (mmol H⁺/kg) of cow milk and soymilk fermented by yoghurt culture YC-381 (YC-381), *B. animalis* subsp. *lactis* BB 12 (BB 12), *B. bifidum* CCDM 94 (CCDM 94), and mixed cultures after 16 h of fermentation at 37°C

Culture	Cow milk		Soymilk	
	$\Delta\text{pH}16^*$	$\Delta\text{TA}16^*$	$\Delta\text{pH}16^*$	$\Delta\text{TA}16^*$
YC-381	2.25 ± 0.06	74.0 ± 0.8	2.20 ± 0.14	39.0 ± 0.9
YC-381 + BB 12	2.14 ± 0.15	66.1 ± 0.6	2.25 ± 0.17	46.8 ± 0.7
YC-381 + CCDM 94	2.25 ± 0.10	71.8 ± 0.9	2.27 ± 0.16	46.3 ± 0.7
BB 12	1.60 ± 0.02	38.7 ± 0.4	1.52 ± 0.09	22.8 ± 0.6
CCDM 94	1.05 ± 0.10	17.0 ± 1.2	1.21 ± 0.17	17.5 ± 1.0

*difference in pH or titratable acidity (TA) between 0 and 16 h of fermentation at 37°C

end of fermentation (16 h) at 37°C. The results of all measurements are summarised in Table 2. The addition of bifidobacteria to the yoghurt culture did not influence the pH of either medium but slightly increased the titratable acidity of the soymilk fermented product. The differences in pH between the fermented cow milk and soymilk were not significant (2.2–2.3 in both media). It was assumed that cow milk is a more suitable medium for the acids production. This could be influenced by the fact that starter cultures are adapted to cow milk. Bifidobacteria were only able to acidify the media by half the value compared to the yoghurt culture.

Acetaldehyde and acetic and lactic acids significantly contribute to the products organoleptic quality.

The contents of these substances in cow milk and soymilk fermented by all combinations of cultures tested are shown in Table 3. D(–)-Lactic acid is metabolised to a smaller extent than L(+)-lactic acid in the human body and therefore it causes acidosis. Bifidobacteria did not produce D(–)-lactic acid in either medium. Also, TSANGALIS and SHAH (2004) attempted to quantify D(–) lactic acid in soymilk, they found, however, that none of the strains investigated (*Bifidobacterium pseudolongum* CSCC 1944, *B. longum* CSCC 5550 and CSCC 1941) produced this isomer during the fermentation. ISHIBASHI and SHIMAMURA (1993) stated that the unique aspect of bifidobacteria is their production of the L(+) form only. Acetic acid is responsible for the undesirable

Table 3. Concentration of lactic acid isomers, acetic acid, and acetaldehyde in cow's milk and soymilk fermented by yoghurt culture YC-381 (YC-381), *B. animalis* subsp. *lactis* BB 12 (BB 12), *B. bifidum* CCDM 94 (CCDM 94), and mixed cultures after 16 h of fermentation at 37°C

Culture	L(+)-Lactic acid	D(–)-Lactic acid	Acetic acid	Acetaldehyde
		(g/100 g)		(mg/kg)
Cow milk				
YC-381	0.43 ± 0.00	0.25 ± 0.00	N	5.63 ± 0.36
YC-381 + BB 12	0.41 ± 0.03	0.18 ± 0.01	0.15 ± 0.03	5.25 ± 0.44
YC-381 + CCDM 94	0.39 ± 0.01	0.21 ± 0.01	0.22 ± 0.00	5.96 ± 0.20
BB 12	0.12 ± 0.01	N	0.11 ± 0.00	0.44 ± 0.13
CCDM 94	0.08 ± 0.02	N	0.12 ± 0.01	1.94 ± 0.26
Soymilk				
YC-381	0.29 ± 0.01	0.11 ± 0.01	traces	3.84 ± 0.30
YC-381 + BB 12	0.21 ± 0.01	0.15 ± 0.01	0.03 ± 0.01	4.36 ± 0.39
YC-381 + CCDM 94	0.25 ± 0.01	0.01 ± 0.00	0.12 ± 0.00	6.25 ± 0.33
BB 12	0.08 ± 0.01	N	0.05 ± 0.00	0.37 ± 0.11
CCDM 94	0.12 ± 0.01	N	0.09 ± 0.02	3.59 ± 0.04

N – not detected

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vinegar taste in the products. Both bifidobacteria produced a certain amount of acetic acid. In general, the production of organic acids (sum of lactic and acetic acids) by all organisms in cow milk was higher than in soymilk. DONKOR *et al.* (2007) observed that the selected organisms (*Bifidobacterium lactis* B94 and *B. longum* B1 536, *L. delbrueckii* subsp. *bulgaricus* Lb 1466 and *S. thermophilus* St 1342) produced lower amounts of organic acids in soymilk than in MRS broth, even if they grew well.

Acetaldehyde is an important volatile flavour compound of yoghurt and is produced by LAB, most frequently from glucose via pyruvate or acetyl phosphate, from amino acids (threonine, methionine, and valine) and from the nucleic acid thymidine. The most important ingredients of cow milk required by the yoghurt starter cultures to produce acetaldehyde are lactose (mainly the glucose fraction) and the amino acids threonine and methionine. The content of acetaldehyde in yoghurt from cow milk usually ranged from 1.3–3.5 mg/kg (XANTHOPOULOS *et al.* 2001) to 40 mg/kg (OZER & ATASOY 2002). In this study, milk fermented only by the yoghurt culture contained 5.63 ± 0.36 mg/kg of acetaldehyde while in soymilk yoghurt 3.84 ± 0.30 mg/kg of acetaldehyde was found. This is consistent with the results of BLAGDEN *et al.* (2005) who detected acetaldehyde content of 1.4–3.5 mg/kg in a soy product fermented by *S. thermophilus* after 12 h incubation at 37°C. The production of acetaldehyde by bifidobacteria is also possible. NOSOVA *et al.* (2000) discovered bifidobacteria strains possessing alcohol dehydrogenase activity, which were capable to oxidise ethanol to acetaldehyde. MARGOLLES and SANCHES (2012) found a strain of *Bifidobacterium animalis* subsp. *lactis* CECT 7953 producing acetate, ethanol and yoghurt related volatile compounds (including acetaldehyde) in skim milk. MURTI *et al.* (1992) fermented soy extract and

cow milk with a yeast extract with *Bifidobacterium* spp. CNRZ 1494, which resulted in the amount of acetaldehyde being higher in the supplemented cow milk (12 ppm) as compared with soy extract (5 ppm). A noteworthy result was obtained with the strain of *B. bifidum* CCDM 94, which produced a significant amount of acetaldehyde in both cow milk and soymilk. In soymilk, the amount of acetaldehyde was almost the same as in the case of the yoghurt culture (3.59 and 3.84 mg/kg, respectively)

Isoflavones can be found in plants as free aglycones, glycosides and malonyl- and acetyl-glycosides. Glycosides belong to the main group of isoflavones which are considered less bioactive. During fermentation, isoflavone glycosides are transformed to aglycons with a higher phytoestrogen activity. Table 4 shows the concentrations of isoflavonoid aglycones in non-fermented soymilk and fermented products. In non-fermented soymilk the bioactive aglycone structure contributes only 8–10% to the total isoflavone content (TSANGALIS *et al.* 2003). For example, DING and SHAH (2010) found 0.402 mg/100 ml of total aglycones in soymilk made from soy protein isolate while CHEN *et al.* (2010) and REKHA and VIJAYALAKSHMI (2010) who prepared soymilk from soybeans, detected 4.85 mg/100 ml and 2.91 mg/100 ml aglycone forms, respectively. In our samples the total amount of isoflavone aglycones was 1.59 ± 0.06 mg/100 ml in non-fermented soymilk. Only the *B. bifidum* CCDM 94 strain was able to release free aglycons – daidzein and genistein, and therefore to increase their concentrations in the media roughly by a factor of 4. Hydrolysis of β -glycosides by *B. animalis* subsp. *lactis* BB 12 was insignificant and also no aglycons release either was observed after fermentation with the yoghurt culture. The conversion of glycosides to aglycones differed in published data from 0.9–13.9 mg/100 ml in soymilk fermented by probiotic cultures including

Table 4. Concentration of isoflavone aglycones (mg/100 ml) in soymilk and soymilk fermented by yoghurt culture YC-381 (YC-381), *B. animalis* subsp. *lactis* BB 12 (BB 12), *B. bifidum* CCDM 94 (CCDM 94), and mixed cultures after 16 h of fermentation at 37°C

	Daidzein	Genistein	Glycitein	Sum
Soymilk	0.61 ± 0.02	0.95 ± 0.04	0.03 ± 0.00	1.59 ± 0.06
YC-381	0.50 ± 0.01	0.80 ± 0.02	0.03 ± 0.01	1.32 ± 0.04
YC-381 + BB 12	0.63 ± 0.14	1.08 ± 0.25	0.04 ± 0.01	1.74 ± 0.40
YC-381 + CCDM 94	2.23 ± 0.17	4.02 ± 0.23	0.09 ± 0.01	6.33 ± 0.41
BB 12	0.69 ± 0.04	1.09 ± 0.15	0.02 ± 0.01	1.80 ± 0.21
CCDM 94	2.56 ± 0.74	4.25 ± 1.73	0.10 ± 0.01	6.90 ± 2.47

bifidobacteria (PYO *et al.* 2005; CHEN *et al.* 2010), however some cultures, for example *Bifidobacterium longum* R0175, were unable to hydrolyse isoflavone glycoside at all (CHAMPAGNE *et al.* 2010).

CONCLUSION

All three cultures, followed, i.e. the yoghurt culture YC-381 and probiotic cultures (*Bifidobacterium animalis* subsp. *lactis* BB 12, *Bifidobacterium bifidum* CCDM 94) were able to grow in both tested media. *B. animalis* subsp. *lactis* BB 12 is suitable for the production of soy and cow milks fermented products due to a lower level of acetic acid. In soymilk, *B. bifidum* CCDM 94 showed an extensive isoflavone hydrolysis and a higher production of acetaldehyde. The initial counts of LAB and conditions of fermentation were desirable for preparing soy and cow milks fermented products with probiotic microflora.

References

- Ang E.C., Boatright W.L. (2003): Olfactory perception of major odorants found in the headspace of aqueous soy protein isolate slurries. *Journal of Food Science*, 68: 388–393.
- Blagden T.D., Gilliland S.E. (2005): Reduction of levels of volatile components associated with the beany flavor in soymilk by Lactobacilli and Streptococci. *Food Microbiology and Safety*, 70: 186–189.
- Champagne C.P., Tompkins T.A., Buckley N.D., Green-Johnson J.N. (2010): Effect of fermentation by pure and mixed cultures of *Streptococcus thermophiles* and *Lactobacillus helveticus* on isoflavone and B-vitamin content of a fermented soy beverage. *Food Microbiology*, 27: 968–972.
- Chen T.R., Su R.Q., Wei Q.K. (2010): Hydrolysis of isoflavone phytoestrogens in soymilk fermented by *Lactobacillus* and *Bifidobacterium* cocultures. *Journal of Food Biochemistry*, 34: 1–12.
- Chumchuere S., Robinson R.K. (1999): Selection of starter cultures for the fermentation of soya milk. *Food Microbiology*, 16: 129–137.
- Desai A., Small D., McGill A.E.J., Shah N.P. (2002): Metabolism of raffinose and stachyose in reconstituted skim milk and of *n*-hexanal and pentanal in soymilk by bifidobacteria. *Bioscience and Microflora*, 21: 245–250.
- Ding W.K., Shah N.P. (2010): Enhancing the biotransformation of isoflavones in soymilk supplemented with lactose using probiotic bacteria during extended fermentation. *Journal of Food Science*, 75: 140–149.
- Donkor O.N., Henriksson A., Vasiljevic T., Shah N.P. (2007): α -Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk. *Food Chemistry*, 104: 10–20.
- Farnworth E.R., Mainville I., Desjardins M.P., Gardner N., Fliss I., Champagne C. (2007): Growth of probiotic bacteria and bifidobacteria in soy yoghurt formulation. *International Journal of Food Microbiology*, 116: 174–181.
- Ishibashi N., Shimamura S. (1993): Bifidobacteria: research and development in Japan. *Food Technology*, 6: 126–136.
- Izumi T., Piskula M.K., Osawa S., Obata A., Tobe K., Saito M., Kataoka S., Kubota Y., Kikuchi M. (2000): Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *Journal of Nutrition*, 130: 1695–1699.
- Lankaputhra W.E.V., Shah N.P., Britz M.L. (1996): Survival of bifidobacteria during refrigerated storage in the presence of acid and hydrogen peroxide. *Milchwissenschaft*, 51: 65–70.
- Margolles A., Sánchez B. (2012): Selection of a *Bifidobacterium animalis* subsp. *lactis* strain with a decreased ability to produce acetic acid. *Applied and Environmental Microbiology*, 78: 3338–3342.
- Murti T.W., Roger S., Bouillianne C., Landon M., Desmazeaud M. (1992): Growth of *Bifidobacterium* sp. CNRZ 1494 in soybean milk and cow milk: effects on aroma compounds. *Sciences des Aliments*, 12: 429–439.
- Nosova T., Jousimies-Somer H., Jokelainen K., Heine R., Salaspuro M. (2000): Acetaldehyde production and metabolism by human indigenous and probiotic *Lactobacillus* and *Bifidobacterium* strains. *Alcohol & Alcoholism*, 35: 561–568.
- Ozer B., Atasoy F. (2002): Effect of addition of aminoacids, treatment with β -galactosidase and use of heat-shocked cultures on the acetaldehyde level in yoghurt. *International Journal of Dairy Technology*, 55: 166–170.
- Pinthong R., Macrae R., Rothwell J. (1980): The development of a soya-based yoghurt. I. Acid production by lactic acid bacteria. *Journal of Food Technology* 15: 647–652.
- Pyo Y.H., Lee T.C., Lee Y.C. (2005): Enrichment of bioactive isoflavones in soymilk fermented with β -glucosidase-producing lactic acid bacteria. *Food Research International*, 38: 551–559.
- Rekha C.R., Vijayalakshmi G. (2010): Bioconversion of isoflavone glycosides to aglycones, mineral bioavailability and vitamin B complex in fermented soymilk by probiotic bacteria and yeast. *Journal of Applied Microbiology*, 109: 1198–1208.
- Scalabrini P., Rossi M., Spettoli P., Matteuzzi D. (1998): Characterization of *Bifidobacterium* strains for use in soymilk fermentation. *International Journal of Food Microbiology*, 39: 213–219.

doi: 10.17221/115/2015-CJFS

Setchell K.D.R., Brown N.M., Desai P., Zimmer-Nechemias L., Wolfe B.E., Brashear W.T., Kirschner A.S., Cassidy A., Heubi J.E. (2001): Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *Journal of Nutrition*, 131: 1362–1375.

Shah P. (2007): Functional cultures and health benefits. *International Dairy Journal*, 17: 1262–1277.

Tsangalis D., Ashton J.F., McGill A.E.J., Shah N.P. (2003): Biotransformation of isoflavones by bifidobacteria in fermented soymilk supplemented with D-glucose and L-cysteine. *Journal of Food Science*, 68: 623–631.

Tsangalis D., Shah N.P. (2004): Metabolism of oligosaccharides and aldehydes and production of organic acids in soymilk by probiotic bifidobacteria. *International Journal of Food Science and Technology*, 39: 541–554.

Xanthopoulos V., Petridis D., Tyanetakis N. (2001): Characterization and classification of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains isolated from traditional Greek yoghurt. *Food Microbiology and Safety*, 66: 747–752.

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Fruit Juices With Probiotics – New Type of Functional Foods

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Abstract

Horáčková Š., Rokytová K., Bialasová K., Klojdová I., Sluková M. (2018): Fruit juices with probiotics – new type of functional foods. Czech J. Food Sci., 36.

The cells of commercial strain *Bifidobacterium animalis* subsp. *lactis* Bb12 were encapsulated using emulsion encapsulation in a milk protein matrix. The volume based median of the microcapsules was $52.1 \pm 6.2 \mu\text{m}$. The stability of free end encapsulated cells was compared during 28 day-storage in pineapple juice and in strawberry-apple juice at $8 \pm 1^\circ\text{C}$ and $22 \pm 1^\circ\text{C}$. Encapsulation ensured a higher number of cells compared to the free cells only at $8 \pm 1^\circ\text{C}$. Strawberry-apple juice was found to be not suitable as probiotic vehicle. Both free and encapsulated cells lost their viability after 14 days at $22 \pm 1^\circ\text{C}$. The number of bifidobacteria cells, pH and lactic and acetic acid content did not change in pineapple and strawberry-apple juice after 24 h cultivation at 37°C .

Keywords: bifidobacteria; encapsulation; pineapple juice; strawberry juice

Currently, consumers are increasingly interested in so-called 'functional foods' containing bioactive compounds such as fibre, oligosaccharides or probiotic microorganisms whose consumption can lead to the prevention or reduction of the risk of food-borne illness. Probiotics together with prebiotics (non-digestible food ingredients that positively and selectively stimulate the growth and/or activity of beneficial bacteria in the colon (GIBSON & ROBERFROID 1995), phytonutrients and unsaturated fatty acids complement the traditional functional food ingredients such as vitamins, minerals and micronutrients (JANKOVIC *et al.* 2010).

Many foodstuffs containing probiotics or prebiotics are of milk origin but the interest of both manufacturers and consumers is extended also to non-dairy products. Greater attention is now given to fruit juices that are considered to be healthy due to their high content of bioactive compounds and are consumed

by all age groups of the population. Fruit juices with added probiotics can be a choice for people who cannot consume dairy products for health (lactose intolerance) or other reasons (not available in the market, lifestyle habits) (ANTUNES *et al.* 2013; MARTINS *et al.* 2013). Fruit juices containing probiotics are commercially available only in limited variants, mainly supplemented with *L. plantarum* 299v or *L. rhamnosus* GG (PERRICONE *et al.* 2015). They are not always suitable for a probiotics application because of their composition (minimum proteins and aminoacids content, presence of phenolic compounds inhibiting bacteria, flavonoids and organic acids lowering pH) (NUALKAEKUL *et al.* 2013). Paradoxically, fruit juices can contain ingredients which promote the viability of probiotics such as ascorbic acid, which reduces redox potential (ANTUNES *et al.* 2013), saccharides or organic acids usable as a carbon source (NUALKAEKUL & CHARALAMPOPOULOS 2011)

or cellulose, which may help to protect probiotics during manufacturing and storage (MARTINS *et al.* 2013). Factors influencing the stability of probiotics in fruit matrices include the microorganism strain and its inoculum, the juice's composition, which determines pH, oxygen concentration, the presence of antimicrobial compound, artificial dyes and flavours, and last but not least the production processes and subsequent handling (pasteurization, storage temperature and packaging material used) (PERRICONE *et al.* 2015). Many studies demonstrating how to increase the stability of probiotics in fruit juices have been published. The most successful methods are fortification by prebiotics, cellulose, β -glucans (SAARELA *et al.* 2006), storage at low temperature in atmosphere enriched by carbon dioxide (CORBO *et al.* 2014), antioxidants addition (NAG & DAS 2013) or the probiotics encapsulation (TSEN *et al.* 2004; DING & SHAH 2008). The most commonly used process is an extrusion method using alginate, chitosan, gelatine or cellulose derivatives as an encapsulation material (NUALKAEKUL *et al.* 2013). Emulsion encapsulation in a protein matrix (HEIDEBACH *et al.* 2009) has not been mentioned in literature regarding the application of probiotics in fruit juices so far.

The aim of this study was to verify the viability of a commercial strain *Bifidobacterium animalis* subsp. *lactis* Bb12 (Bb12) in the form of free and encapsulated cells in pineapple and strawberry-apple juices during long-term storage at $8 \pm 1^\circ\text{C}$ and $22 \pm 1^\circ\text{C}$, as well as to test the effect of encapsulation on maintaining the original sensory properties of juices.

MATERIAL AND METHODS

Used microorganisms. Commercial probiotic lyophilized DVS culture *Bifidobacterium animalis* subsp. *lactis* Bb12 (Ch. Hansen, Denmark) was cultured in MRS broth (Merck, Germany) at pH 6.5 with the addition of L-cysteine hydrochloride monohydrate (0.5 g/l) (Sigma, USA) anaerobically for 24 h at 37°C .

Fruit juices. Relax® 100% pineapple juice made from pineapple concentrate (pineapple juice from concentrate, pineapple pulp, pasteurized, proteins 0 g/100 ml, carbohydrates 12 g/100 ml, of which sugar 12 g/100 ml) and Relax® Strawberry-apple juice, partly made from concentrates and fruit puree (water, juice from concentrates and puree (33%), of which strawberries 25%, apples 8%, citric acid, concentrate

of black carrots, flavour, proteins 0 g/100 ml, carbohydrates 9.3 g/100 ml, of which sugars 9.3 g/100 ml, fats 0 g/100 ml) were purchased on the market.

Encapsulation process. Encapsulation of *B. animalis* subsp. *lactis* Bb12 was performed according to HEIDEBACH *et al.* (2009) in a modification according to LISOVÁ *et al.* (2013). Briefly, 30 g of 35% (w/w) reconstituted skimmed milk (skimmed-milk powder Laktino; PROMIL, Czech Republic) was kept for 2 h at 5°C with agitation (500 rpm) in a closed 50 ml vessel. Afterwards, about 2 g of fresh culture and 400 μl of diluted rennet (1 : 4) Naturen™ PREMIM 145 (Christian Hansen, Germany) were added. The mixture was stirred (500 rpm) at 5°C for 1 h and subsequently 180 μl of 10% (w/w) CaCl_2 was added. Fifteen grams of this mixture was immediately transferred to 150 ml of canola oil with 0.5% (w/w) soya lecithin (Solae Europe S.A., Switzerland; purity of 96%) and stirred for 5 min to emulsify the mixture into the oil. Then, still stirring, the mixture was heated up to 40°C with 15 min duration. The capsules were separated by gentle centrifugation (500 rpm, 2 min and 4°C), washed 3 times with distilled water and stored in wet form at 4°C before further use.

Particle size determination and capsules optical control. The mean size distribution of microcapsules was measured using Mastersizer 2000 (Malvern, UK) with dispersion unit Hydro G (Malvern, UK) at 25°C . A refractive index 1.45 and absorption coefficient 0.001 were selected for the measurement. The results are the means from two independent encapsulation processes; the sample analyses were repeated seven times. The mean of volume based median ($d_{0.5}$) (i.e. 50% of total volume is composed of microcapsules with diameters equal or lower than $d_{0.5}$) and 90% fractiles ($d_{0.9}$) were calculated. Microscopic observations of the microcapsules were performed on a Leica DM LF (Leica Microsystems, Germany) after methylene blue staining.

Analytical determination. To determine the number of cells in the microcapsules, 9 ml of citrate buffer supplemented with Tween 80 (0.5 g/l), pH 7.5 was added to 1 g of wet microcapsules, followed by dilution with physiological solution and using the cultivation plate method at MRS agar (Merck, Germany), pH 6.8 with the addition of L-cysteine hydrochloride monohydrate (0.5 g/l) (anaerobic cultivation, 72 h and 37°C). The same plate method was used to count the number of free cells in samples. The active acidity was measured with a pH meter (Jenway, UK) provided with a combined electrode.

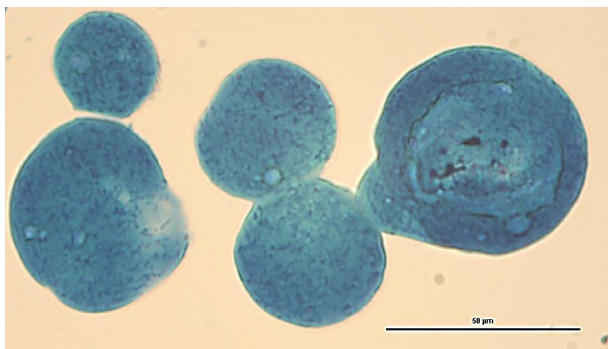


Figure 1. Microcapsules with *B. animalis* subsp. *lactis* Bb12 in milk protein matrix after methylene-blue staining

The metabolic activity of free and encapsulated cells in both juices was tested based on cell count, pH, lactic and acetic acids contents determined by capillary isotachopheresis (ITP analyser IONOSEP 2003; Recman, Czech Republic) after 24 h cultivation at 37°C. The anionic lead electrolyte was composed of 10 mmol/l HCl, 1% hydroxypropyl methylcellulose and 22 mmol/l ϵ -aminocaproic acid (pH 4.5). The end electrolyte contained 5 mmol/l of capric acid. Lithium lactate and ammonium acetate were used as standard for calibration curves measurements. The results are the means from two parallel assays, both were analysed twice ($n = 4$).

Storage. The stability of free (cultivated in MRS broth and washed in physiological solution) and encapsulated cells were monitored during storage in pineapple and strawberry juice for 28 days at $8 \pm 1^\circ\text{C}$ and $20 \pm 1^\circ\text{C}$. Initial cells concentration was approx. 10^8 CFU/ml of juice. The number of viable cells was determined at weekly intervals.

RESULTS AND DISCUSSION

The Figure 1 illustrates the example of microcapsules obtained by the encapsulation process. The mean of volume based median $d_{0.5}$ was $52.1 \pm 8.2 \mu\text{m}$ and $d_{0.9}$ value was $193.6 \pm 24.7 \mu\text{m}$. The size of microcapsules can significantly influence the sensory characteristics. The size reported to have no potential sensory defect was approx. $250 \mu\text{m}$ (HEIDEBACH *et al.* 2009) and is usually achieved by emulsion technique rather than by the extrusion method of encapsulation (BURGAIN *et al.* 2011). The changes in the shape and size of the microcapsules were monitored microscopically during the storage period. Microcapsules were compact even after 28

days storage at both temperatures in pineapple juice and at $8 \pm 1^\circ\text{C}$ in strawberry-apple juice, while total disruption in strawberry-apple juice stored for 21 days at room temperature was observed.

The main goal of this experiment was to assess bacteria stability when applied in different fruit juices. Figure 2 shows changes in the number of viable cells (free and encapsulated) during storage in both media tested at $8 \pm 1^\circ\text{C}$ (Figure 2A) and $22 \pm 1^\circ\text{C}$ (Figure 2B). During the first 3 weeks of storage, no effect of encapsulation on cell viability in pineapple juice at $8 \pm 1^\circ\text{C}$ was observed. But a more significant difference in a decrease of cells number of free cells (1.7 log cycles) compared to encapsulated cells (only 0.7 log cycle) was detected after 28 days storage. The same trend was noted at room temperature storage. Acidity of pineapple juice (pH 3.8 ± 0.1) did not change during the whole period at either temperature. Some previously published studies also confirmed pineapple juice as a suitable carrier of probiotics due to its high fibre content, low content of antimicrobial phenolic compounds and higher pH (ESPINOSA & NAVARRO 2010, NUAL- KAEKUL & CHARALAMPOPOULOS 2011).

The level of cell count reached when applying free and encapsulated *B. animalis* subsp. *lactis* Bb12 in

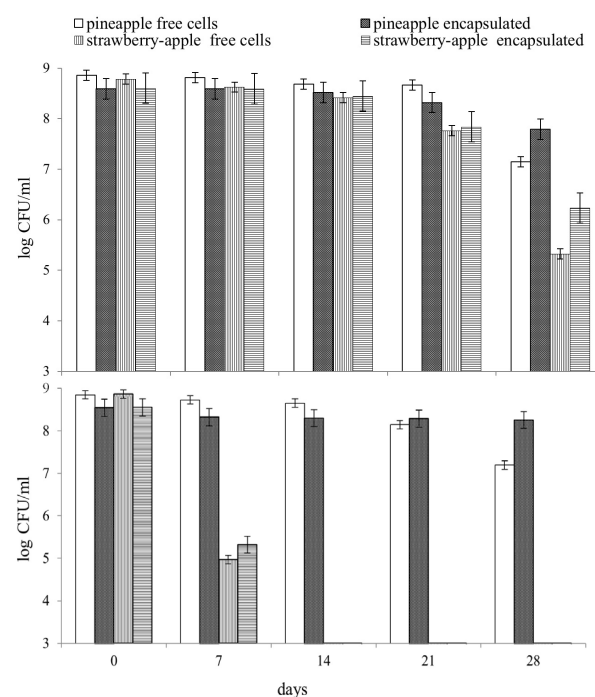


Figure 2. The number of free and encapsulated cells in milk protein matrix during storage in pineapple and strawberry-apple juices at $8 \pm 1^\circ\text{C}$ (A) and $22 \pm 1^\circ\text{C}$ (B)

Table 1. Viable count and metabolic activity of free and encapsulated cells in juices after 24 h cultivation at 37°C in aerobic condition

Cells	Pineapple juice				Strawberry-apple juice			
	free		encapsulated		free		encapsulated	
Time (h)	0	24	0	24	0	24	0	24
Number (log CFU/ml)	8.72 ± 0.05	7.89 ± 0.08	8.56 ± 0.12	7.99 ± 0.16	8.57 ± 0.08	6.91 ± 0.10	9.20 ± 0.17	9.23 ± 0.12
pH	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1
Lactic acid (g/l)	0.12 ± 0.03	0.13 ± 0.01	0.21 ± 0.06	0.23 ± 0.05	0.19 ± 0.02	0.15 ± 0.02	0.23 ± 0.03	0.18 ± 0.04
Acetic acid (g/l)	nd	0.01 ± 0.0	nd	0.02 ± 0.0	nd	nd	nd	nd

nd – not detected

strawberry-apple juice showed that bifidobacteria were significantly less stable in this environment. The acidity of strawberry-apple juice was stable during storage at both temperatures and reached pH 3.2 ± 0.1 . The cells stability was influenced both by storage temperature and encapsulation. The difference in the cell count decrease between free and encapsulated cells was 1.1 log cycle (3.5 and 2.4, respectively) at $8 \pm 1^\circ\text{C}$ after 28 days. Juice with encapsulated cells still contained more than 10^6 CFU/ml, which corresponds with the requirements for functional foods (BOYLSTON *et al.* 2004; ANAL & SINGH 2007). At the same time the encapsulation did not affect cells viability when strawberry-apple juice was stored at $22 \pm 1^\circ\text{C}$. Already during the first week there was a decrease by 3.9 (free cells) and 3.2 (encapsulated) log cycles. After 14 day-storage no live cells were detected in either of the samples. Juices obtained from small-berry fruits contain a high amount of phenolic compounds and have lower pH and are therefore not suitable for probiotics application (ESPINOZA & NAVARRO 2010). This was observed in the study by PERRICONE *et al.* (2015) and the positive effect on the probiotic lactobacilli viability was demonstrated only for peach, orange and tomato juices and for carrot juice in case of bifidobacteria.

Further, the metabolic activity of free and encapsulated cells in both tested juices was compared after 24 h cultivation at optimal temperature 37°C in order to determine whether bifidobacteria can cause any undesirable changes in sensory properties because acetic acid production. The results are summarized in Table 1. It is evident that both type of cells were neither able to grow nor increase the concentration of lactic and acetic acids in the samples. The tested parameters did not significantly change. Some authors have demonstrated that pro-

biotics do not affect the acceptability of juices for consumers (PERRICONE *et al.* 2014 for *Lactobacillus reuteri* in pineapple juice, RODRÍGUEZ *et al.* 2009 for *L. casei* in apple juice), but in other studies the addition of probiotics degraded their sensory properties (KRASAEKOOP & KITSAWAD 2010, LUCKOW & DELAHUNTY 2004). In this case tentative sensory valuation confirmed that encapsulated cells did not influence either the taste of pineapple juice by acid off-flavour or by the negative effect of capsules on consistency. Juice with free bifidobacteria cells were not positively accepted by evaluators.

CONCLUSIONS

It was confirmed that juice type selection is a key factor for the stability of added probiotics. In this case, pineapple juice was proved to be suitable for *B. animalis* subsp. *lactis* Bb12 cells viability. Encapsulation increased the cells stability after 28 days of storage. Microcapsules were not stable in strawberry-apple juice at $22 \pm 1^\circ\text{C}$ and both free and encapsulated cells died after 14 days. Encapsulation was important for maintaining the sensory properties of pineapple juice. Based on these results, pineapple juice with a commercial probiotic Bb12 strain encapsulated in a milk protein matrix seems to be a promising area for probiotic fruit juice development.

References

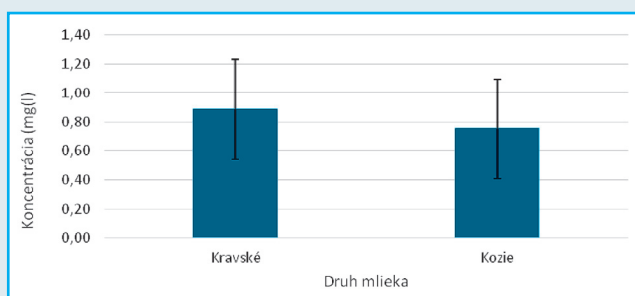
- Anal A.K., Singh H. (2007): Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends in Food Science and Technology, 18: 240–251.

- Antunes A.E.C., Liserre A.M., Coelho A.L.A., Menezes C.R., Moreno I., Yotsuyanagi K., Azambuja N.C. (2013): Acerola nectar with added microencapsulated probiotic. *Food Science and Technology*, 54: 125–131.
- Boylston T.D., Vinderola C.G., Ghoddusi H.B., Reinheimer J.A. (2004): Incorporation of bifidobacteria into cheeses: challenges and rewards. *International Dairy Journal*, 14: 375–387.
- Corbo M.R., Bevilacqua A., Petrucci L., Casanova F.P., Sinigaglia M. (2014): Functional beverages: The emerging side of functional foods: commercial trends, research and health implications. *Comprehensive Review in Food Science and Food Safety*, 13: 1192–1206.
- Ding W.K., Shah N.P. (2008): Survival of free and microencapsulated probiotic bacteria in orange and apple juices. *International Food Research Journal*, 15: 219–232.
- Espinoza Y.R., Navarro Y.G. (2010): Non-dairy probiotic products. *Food Microbiology*, 27: 1–11.
- Gibson G.R., Roberfroid M.B. (1995): Dietary modulation of the human colonic microbiota – Introducing the concept of prebiotics. *Journal of Nutrition*, 125: 1401–1412.
- Heidebach T., Först P., Kulozik U. (2009): Microencapsulation of probiotic cells by means of rennet-gelation of milk proteins. *Food Hydrocolloids*, 23: 1670–1677.
- Jankovic I., Sybesma W., Phothirath P., Ananta E., Mercenier A. (2010): Application of probiotics in food products-challenges and new approaches. *Current Opinion in Biotechnology*, 21: 175–181.
- Krasaekoopt W., Kitsawad K. (2010): Sensory characteristics and consumer acceptance of fruit juice containing probiotics beads in Thailand. *AU Journal of Technology*, 14: 33–38.
- Lisová I., Horáčková Š., Kováčová R., Rada V., Plocková M. (2013): Emulsion encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb 12 with the addition of lecithin. *Czech Journal of Food Science*, 31: 270–274.
- Luckow T., Delahunty C. (2004): Which juice is ‘healthier’? A consumer study of probiotic non-dairy juice drinks. *Food Quality and Preferences*, 15: 751–759.
- Martins E.M.F., Ramos A.M., Vanzela E.S.L., Stringheta P.C., de Oliveira Pinto C.L., Martins J.M. (2013): Products of vegetable origin: A new alternative for the consumption of probiotic bacteria. *Food Research International*, 51: 764–770.
- Nag A., Das S. (2013): Improving ambient temperature stability of probiotics with stress adaptation and fluidized bed drying. *Journal of Functional Foods*, 5: 170–177.
- Nuallkaekul S., Charalampopoulos D. (2011): Survival of *Lactobacillus plantarum* in model solutions and fruit juices. *International Journal of Food Microbiology*, 146: 111–117.
- Nuallkaekul S., Cook M.T., Khutoryanskiy V.V., Charalampopoulos D. (2013): Influence of encapsulation and coating materials on the survival of *Lactobacillus plantarum* and *Bifidobacterium longum* in fruit juices. *Food Research International*, 53: 304–311.
- Perricone M., Bevilacqua A., Altieri C., Sinigaglia M., Corbo M.R. (2015): Challenges for the production of probiotic fruit juices. *Beverages*, 1: 95–103.
- Perricone M., Corbo M.R., Sinigaglia M., Speranza B., Bevilacqua A. (2014): Viability of *Lactobacillus reuteri* in fruit juices. *Journal of Functional Foods*, 10: 421–426.
- Rodríguez H., Curiel J.A., Landete J.M., de las Rivas B., de Felipe F.L., Gómez-Cordovés C., Mancheño J.M., Muñoz R. (2009): Food phenolics and lactic acid bacteria. *International Journal of Food Microbiology*, 132: 79–90.
- Saarela M., Virkajarvi I., Nohynek L., Vaari A., Matto J. (2006): Fibres as carriers for *Lactobacillus rhamnosus* during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals. *International Journal of Food Microbiology*, 112: 171–178.
- Tsen J.H., Lin Y.P., King V.A.E. (2004): Fermentation of banana media by using κ -carrageenan immobilized *Lactobacillus acidophilus*. *International Journal of Food Microbiology*, 91: 215–220.

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Graf 1 Koncentrácia vitamínu A (mg/l ± SD) v kravskom a kozom mlieku

U mlieka kozieho bola pozorovaná variabilita vitamínu A v priebehu celej laktácie. Na grafe č. 2 je vidno zmeny koncentrácií v jednotlivých mesiacoch. Najvyššia koncentrácia bola zaznamenaná v júni a auguste a následne klesala. Tento jav okrem zmien v kŕmení (pastva vs kŕmenie koncentrované) môže súvisieť i so zasušovaním a prípravou organizmu na ďalšiu graviditu a laktáciu. Štatistickým porovnaním koncentrácie vitamínu A u fariem konvenčných a EKO neboli pozorované žiadne rozdiely.

Záver

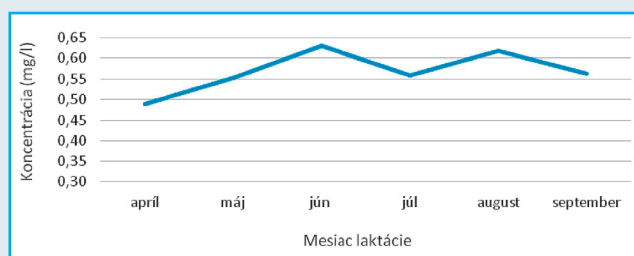
Vitamín A patrí k významným nutričným parametrom mlieka. Z pohľadu potravinových zdrojov tohoto vitamínu, patrí mlieko ako základná potravina k jeho najvýznamnejším zdrojom. Na základe stanovenia obsahu retinolu v mlieku kravskom a kozom je možno konštatovať, že 1 liter mlieka kravského, nakupovaného mliekárňami, resp. mlieka z priameho predaja obsahuje $0,89 \pm 0,34$ mg/l retinolu (resp. u mlieka kozieho $0,75 \pm 0,34$ mg/l).

Podakovanie

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Použitá literatúra

- EFSA (2013): Vitamin A and normal development and function of the immune system. *EFSA Journal*, 11, s. 1-11.
- HULSHOF, P. J. M., ROEKEL-JANSEN, T., BOVENKAMP, P., WEST, C. E. (2006): Variation in retinol and carotenoid content of milk and milk products in The Netherlands. *J. Food Compos. Anal.*, 19, s. 67-75.
- LIDÉN, M., ERISSON, U. (2006): Understanding retinol metabolism: structure and function of retinol dehydrogenases. *J. Biol. Chem.*, s.13001-13004.
- PARK Y.W. ET AL. (2007): Physico-chemical characteristics of goat and sheep milk. *Small Rum Res*; 68, s. 88-113.
- PARK Y.W., HAENLEIN H.F.W. (2013): *Milk and Dairy Products in Human Nutrition: Production, Composition and Health*. West Sussex, Wiley- Blackwell & Sons, 728 s. ISBN: 978-0-470-67418-5
- RAMALHO, H. M. M., SANTOS, J., CASAL S., ALVES, M. R., OLIVEIRA M. B. P. P. (2012): Fat soluble vitamin (A, D, E and β -carotene) contents from a Portuguese autochthonous cow breed-Minhota. *J. of Dairy Sci.*, 95, s. 5476-5484.
- Regulation (EC) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers. OJ L 304/18, 22.11.2011, s. 1-46.
- SAUVANT, P., GROLIER, P., AZAIS-BRAESCO V. (2002): Vitamin A, Nutritional significance. In Roginski, H., Foquay, J., W., Fox, P. F. *Encyclopedia of dairy sciences*. 2nd ed. London: Academic Press, s. 2657-2664. ISBN: 978-0-12-227235-6.



Graf 2 Variabilita koncentrácie vitamínu A v priebehu celého laktáčného obdobia kôz

WEBER, P., BENDICH, A., MACHLIN, L. J. (1997): Vitamin E and human health: Rationale for determining recommended intake levels. *Nutrition*, 13, s. 450-460.

WHO, (2009): *Global prevalence of vitamin A deficiency in populations at risk 1995-2005 (online)*. Staženo 15.01.2016. Dostupné z: http://apps.who.int/iris/bitstream/10665/44110/1/9789241598019_eng.pdf

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STANOVENÍ ŽIVOTASCHOPNÝCH LAKTOBACILŮ S VYUŽITÍM PROPIDIUM MONOAZIDU POMOCÍ QPCR

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Determination of viable lactobacilli using propidium monoazide and qPCR

Abstrakt

Byla optimalizována a testována kvantitativní polymerázová řetězová reakce s interkalačními barvivými propidium monoazidem (PMA) a ethidium monoazidem (EMA) pro stanovení pouze životaschopných laktobacilů. Byly hodnoceny podmínky metody jako je výběr barviva, koncentrace barviva, přídavek chlótu sodného a doba fotoindukce. Daná metoda PMA-qPCR byla porovnána s plotnovými metodami a qPCR bez použití propidium monoazidu. Pro *Lactobacillus casei* a *Lactobacillus acidophilus* byl jako vhodné barvivo vybrán propidium monoazid o konečné koncentraci 50 mmol l^{-1} s přídavkem 0,5 % hm. chlótu sodného a dobou fotoindukce 15 minut pomocí LED lampy. Výsledky získané touto metodou byly ve větší shodě s výsledky z kultivačních metod než samotná qPCR.

Klíčová slova: propidium monoazid, qPCR, laktobacily

Abstrakt

The quantitative polymerase chain reaction technique with the use of intercalating dyes (propidium monoazide and ethidium monoazide) was tested for determination of viable lactobacilli. Conditions of the methods such as selecting dyes, dye concentration, the addition of sodium cholate and time of photoinduction were evaluated. The given method PMA-qPCR was compared with plate methods and qPCR methods without the use of propidium monoazide. Propidium monoazide with the final concentration of 50 mmol l⁻¹ with addition of 0,5 % wt. sodium cholate and 15 min of the photoinduction period with LED lamp was the most suitable for the determination of *Lactobacillus acidophilus* and *Lactobacillus casei*. Results obtained by this method were more comparable to those obtained by cultivation media than qPCR alone.

Key words: propidium monoazide, qPCR, lactobacilli

Úvod

V dnešní době roste zájem veřejnosti o informace týkající se potravin, ať už jsou to údaje o krajině původu potravin, jejím zpracovateli či vlivu konzumace určitých potravin na lidské zdraví. Zároveň čím dál více spotřebitelů od potravin očekává i její pozitivní vliv na zdraví. Výrobci se snaží na tuto poptávku reagovat a například přidávají do svých potravin probiotika.

Probiotika jsou živé mikroorganismy, které při podání v adekvátním množství přinášejí hostiteli zdravotní výhody (FAO/WHO, 2002). Dle mnohých autorů je třeba, aby výrobek obsahoval alespoň 10⁶ až 10⁹ KTJ g⁻¹ životaschopných probiotických mikroorganismů (Shah, 2000). Z tohoto důvodu je důležité mít ověřené metody selektivního stanovení probiotických bakterií (Shah, 2000, Lima a kol., 2009). Někteří autoři upozorňují na problém stanovení selektivně probiotických mikroorganismů ve směsi s ostatními bakteriemi mléčného kvašení pomocí plotnových metod a nabízejí jako možné řešení stanovení pomocí kvantitativní polymerázové řetězové reakce, které by navíc umožňovalo určit, zda se jedná o deklarovaný kmen (Furet a kol., 2004; Huang, Lee, 2009).

Klasické stanovení počtu kolonií pomocí ploten se selektivní půdou je metodou, kterou se zachycují pouze živé buňky, což je její velkou výhodou, na druhou stranu jedná se o časově náročnou metodu, která může vykazovat odchylku až 30 % (García-Cayuela a kol., 2009). Z tohoto důvodu by bylo vhodné použití qPCR, která je rychlejší a přesnější (Furet a kol., 2004; García-Cayuela a kol., 2009). Výhodou qPCR je její vysoká citlivost, přesnost, dobrá opakovatelnost, minimální riziko kontaminace a nejsou třeba další kroky (po qPCR). Mezi její nevýhody patří, že data získaná pomocí qPCR značně závisí na přesnosti odběru a přípravě vzorku, kvalitě standardu a správném výběru primerů. Další její nevýhodou je, že se stanovují nejen živé buňky, ale též mrtvé (García-Cayuela a kol., 2009). V současnosti jsou již vyvinuty techniky pro

stanovení pouze živých buněk pomocí propidium monoazidu (García-Cayuela a kol., 2009; Nocker a kol., 2006) či ethidium monoazidu (Nocker a kol., 2006).

Tato práce se zabývala kvantitativním stanovením *Lactobacillus casei* (*L. casei*) a *Lactobacillus acidophilus* (*L. acidophilus*) a optimalizací metody s využitím propidium monoazidu pro stanovení pouze živých buněk *L. casei* a *L. acidophilus* ve srovnání s plotnovými metodami a s výsledky získanými pomocí kvantitativní polymerázové řetězové reakce (qPCR).

Materiál a metody

Použité kmeny

Byly použity kmeny: *Lactobacillus acidophilus* LA 5[®] - Christian Hansen (Dánsko) a CCDM 151 - Laktoflora[®] Milcom (ČR) dále *Lactobacillus casei* Lafti[®] L26 - DSM (Nizozemsko) a CCDM 198 - Laktoflora[®] Milcom (ČR).

Kultivace kmenů rodu *Lactobacillus*

Zkoumané kmeny rodu *Lactobacillus* byly kultivovány v bujónu MRS, pH 5,6 (po sterilaci), bylo zaočkováno 1 obj. % inokula. Laktobacily byly kultivovány v upravené atmosféře s obsahem 5 obj. % CO₂ při 37 °C.

Plotnové metody

V práci byly vyzkoušeny a porovnávány plotnové metody pro *L. casei* MRS agar s vankomycinem (MRS-V) při podmínkách anaerobní inkubace za teploty 37 °C po dobu 72 hodin. pH bylo upraveno na hodnotu 5,2-4,6 a přidán vankomycin; výsledná koncentrace 1 mg l⁻¹. A LC agar, jehož složení je následující: na jeden litr 10 g bakteriologického peptonu, 1 g kvasničného extraktu, 4 g Lab Lemco, 2 g KH₂PO₄, 3 g trihydrátu acetátu sodného, 1 g citrátu triamonného, 0,2 g heptahydrátu síranu hořečnatého, 0,05 g tetrahydrátu síranu hořečnatého, 1 g hydrolyzátu kyselého kaseinu, 1 g tweenu 80. pH bylo upraveno na 5,1 ± 0,1, bylo přidáno 6 ml bromkresolové zeleně a 12 g bakteriologického agaru. Těsně před očkovaním byla k médiu přidána D-ribosa; konečná koncentrace 1 hm. %. Misky byly inkubovány anaerobně při 27 °C po dobu 72 až 96 hodin (Shah, 2000). Pro stanovení presumptivního počtu *L. acidophilus* na selektivní živné půdě byl použit postup dle ČSN ISO 20 128. Dle této normy byla připravena MRS půda s clindamycinem hydrochloridem a ciprofloxacinem hydrochloridem (MRS/CL/CP); pH 6,2 ± 0,2. Výsledná koncentrace clindamycinu hydrochloridu byla 0,1 mg l⁻¹, ciprofloxacinu hydrochloridu 10 mg l⁻¹. Misky byly inkubovány anaerobně při 37 °C po dobu 72 ± 3 hodiny (ČSN ISO 20128). Dále byl použit agar MRS pH 5,6 používaný obecně pro stanovení laktobacilů.

Izolace DNA s obarvením propidium monoazidem nebo ethidium monoazidem

Byl odstředěn 1 ml čerstvě narostlé kultury (10 000 g, 10 min, 4 °C) a odsán supernatant. Buňky byly promyty

v 1 ml 1,5 % (hm.) roztoku NaCl a odstředěny 10 000 g, 10 min, 4 °C). Po odsátí supernatantu bylo přidáno 500 µl sterilní vody. K suspenzi bylo přidáno 1,25 µl roztoku PMA (roztok 20 % obj.) sterilního dimethylsulfoxidu s PMA; finální koncentrace 50 mmol l⁻¹ PMA) a proběhla inkubace 5 minut ve tmě. Následovala fotoindukce azidové skupiny použitím LED lampy (Phastblue, GenIUL, Španělsko) (15 minut). Suspenze byla odstředěna (10 000 g, 10 min, 4 °C), supernatant byl odsán, buňky byly promyty nejprve v 1 ml 1,5 % (hm.) roztoku NaCl, odstředěny (10 000 g, 10 min, 4 °C), a poté v 1 ml sterilní vody a opět odstředěny (10 000 g, 10 min, 4 °C). Dále bylo přidáno 400 µl lytického pufru a proběhla inkubace 1 hodinu při 37 °C. Poté bylo přidáno 25 µl proteinasy K (20 mg ml⁻¹) a 200 µl pufru AL a následovala inkubace 30 min při 70 °C. Suspenze byla převedena do 2 ml mikrozkumavek s 0,3 g zirkoniových kuliček (diameter 0,5 mm). Mikrozkumavky byly protřepány 3krát po dobu 30 s. Suspenze byla převedena zpět do Eppendorfových zkumavek a odstředěna (10 000 g, 10 min, 25 °C). Po převedení do kolonky (DNeasy Mini spin z kitu DNeasy Blood and Tissue kit, Qiagen, Německo) bylo přidáno 200 µl ethanolu (98 %, -20 °C) a inkubováno 5 minut při 25 °C. Poté byl vzorek odstředěn (5 750 g, 2 min, 4 °C). Po odstředění byl vylit obsah sběrné zkumavky, přidáno 500 µl pufru AW1 a následovalo odstředění (6 750 g, 2 min, 4 °C). Opět byl vylit obsah sběrné zkumavky, bylo přidáno 500 µl pufru AW2 a odstředěno (13 900 g, 3 min, 4 °C). Nakonec byl ještě jednou vylit obsah sběrné zkumavky a následovalo další odstředění (13 900 g, 3 min, 4 °C). Před elucí DNA byla vyměněna sběrná zkumavka. Na střed membrány v kolonce bylo napipetováno 50 µl elučního pufru AE, proběhla inkubace 2 minuty při 25 °C a poslední odstředění (5 750 g, 1 min, 4 °C). V případě obarvení ethidium monoazidem byl PMA pouze nahrazen EMA.

Izolace DNA s použitím cholátu sodného před obarvením PMA a EMA upravenou metodou dle Yanga a kol. (2011)

Byl odstředěn 1 ml čerstvě narostlé kultury (10 000 g, 10 min, 4 °C) a odsán supernatant. K peletě byl přidán cholát sodný (konečná koncentrace 0,5 hm. %) a poté proběhla inkubace 30 minut při 37 °C. Dále se v práci pokračovalo dle předchozího odstavce.

Kvantitativní polymerázová řetězová reakce

Nejprve byl připraven mix dle tabulky I. Následně bylo do každé jamky napipetováno 9 µl mixu. A poté byl do každé jamky napipetován 1 µl stanovované DNA (duplikát-

Tab. I PCR mix

PCR mix	výrobce	objem (µl)
iQ SYBR Green Supermix	Biorad	5
Primer Fwd (10 mol l ⁻¹)	GeneriBiotech	0,5
Primer Re (10 mol l ⁻¹)	GeneriBiotech	0,5
Demineralizovaná sterilní voda	-	3
Celkový objem		9

Tab. II Průběh qPCR reakce

Počet cyklů	Teplota (°C)	čas (s)
1	95	180
39	95	30
	65/62	30
	72	30
Křivka tání	55-95	0,05 s / 0,5 °C

ně byla napipetována kalibrační řada i neznámé vzorky). Vlastní reakce probíhala dle tabulky II.

Výsledky a diskuse

Porovnání kvantitativního stanovení počtu buněk *L. acidophilus* a *L. casei* pomocí propidium monoazidu a ethidium monoazidu

Bylo provedeno porovnání stanovení počtu kolonií tvořících jednotek dvou kmenů *L. casei* a dvou kmenů *L. acidophilus*, jak je uvedeno v tabulkách III-VI, pomocí kultivačních metod, qPCR, PMA-qPCR a pomocí qPCR s obarvením EMA (EMA-qPCR). Počet buněk stanovený pomocí qPCR byl o 1,0-1,3 řádu vyšší než plotnovými metodami. Lepší korelace s kultivačními metodami byla dosažena u qPCR s interkalačními barvivky.

Tab. III. Porovnání stanovení počtu buněk čisté kultury *L. casei* Lafti L26 pomocí plotnových metod, qPCR a qPCR s obarvením PMA a EMA (log KTJ ml⁻¹)

MRS 5,6	MRS-V	LC	qPCR	PMA-qPCR	EMA-qPCR
8,49	8,45	8,46	9,78	9,77	9,62

Tab. IV. Porovnání stanovení počtu buněk čisté kultury *L. casei* CCDM 198 pomocí plotnových metod, qPCR a qPCR s obarvením PMA a EMA (log KTJ ml⁻¹)

MRS 5,6	MRS-V	LC	qPCR	PMA-qPCR	EMA-qPCR
8,75	8,74	8,74	9,73	9,70	9,49

Tab. V. Porovnání stanovení počtu buněk čisté kultury *L. acidophilus* LA 5 pomocí plotnových metod, qPCR a qPCR s obarvením PMA a EMA (log KTJ ml⁻¹)

MRS 5,6	MRS/CL/CP	qPCR	PMA-qPCR	EMA-qPCR
8,00	8,11	11,46	10,21	9,33

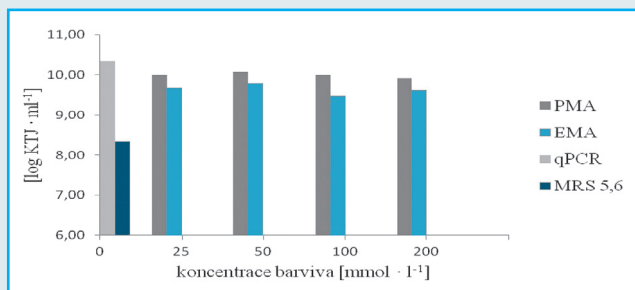
Tab. VI. Porovnání stanovení počtu buněk čisté kultury *L. acidophilus* CCDM 151 pomocí plotnových metod, qPCR a qPCR s obarvením PMA a EMA (log KTJ ml⁻¹)

MRS 5,6	MRS/CL/CP	qPCR	PMA-qPCR	EMA-qPCR
8,08	8,04	8,31	7,91	5,02

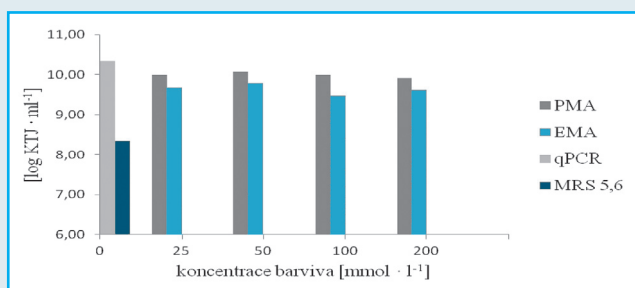
Nocker a kol. uvádí, že v jejich práci došlo k poklesu počtu buněk stanovených pomocí EMA-qPCR o 1-2 řádu oproti stanovení PMA-qPCR. Tento jev je dán tím, že EMA může proniknout také buněčnou stěnou živých buněk a zkreslovat tak výsledky, zatímco PMA buněčnou stěnou živých buněk neproniká (Nocker a kol., 2006; Yáñez a kol., 2011). Množství živých buněk, do kterých EMA pronikne

Tab. VII. Porovnání stanovení počtu buněk pomocí qPCR, qPCR-PMA, qPCR-PMA-CHS, qPCR-EMA, qPCR-EMA-CHS a plotnovými metodami *L. casei* Lafti L26 (log KTJ ml⁻¹)

MRS 5,6	qPCR	PMA-qPCR	PMA-qPCR s CHS	EMA-qPCR	EMA-qPCR s CH
9,00	11,55	9,74	8,94	9,31	9,10



Obr. 1 Stanovení počtu buněk *L. casei* Lafti L26 při různé koncentraci interkalačních barviv



Obr. 2 Stanovení počtu buněk *L. acidophilus* CCDM 151 při různé koncentraci interkalačních barviv

je dáno druhem buněk (Nocker a kol., 2006), použitou koncentrací barviva (Kobayashi a kol., 2009; Wang a kol., 2009) či hustotou bakteriální suspenze (Flekna a kol., 2007; Kobayashi a kol., 2009). Existují názory, že EMA není vhodný k odlišení živých a mrtvých buněk (Flekna a kol., 2007; Kobayashi a kol., 2009; Nam a kol., 2011). PMA byl navržen jako vhodná alternativa EMA, neboť je specifitější. Důvodem by mohlo být to, že PMA má vyšší náboj molekuly (PMA²⁺, EMA¹⁺) (Nocker a kol., 2006). Nevýhodou PMA je generace falešně pozitivních výsledků z důvodu potlačení neúplného signálu. Na druhou stranu, EMA-qPCR způsobuje vyšší snížení CT hodnoty než PMA-qPCR při srovnání s qPCR (Fittipaldi a kol., 2012). Ke stejnému výsledku se došlo i v této práci u *L. casei* Lafti L26, *L. casei* CCDM 198, *L. acidophilus* LA 5 a *L. acidophilus* CCDM 151.

Vliv změny podmínek na kvantitativní stanovení počtu živých buněk *L. acidophilus* a *L. casei* pomocí PMA-qPCR a EMA-qPCR

Byla popsána nutnost stanovení efektivní koncentrace barviva a doby ozáření pro stanovovaný mikroorganismus (Yáñez a kol., 2011). Byla provedena stanovení pro optimalizaci metody pro použití PMA-qPCR (EMA-qPCR) pro *L. casei* a *L. acidophilus* se zaměřením na koncentraci použitého barviva a dobu fotoindukce barviva. Čisté kmeny *L. casei* Lafti L26 a *L. acidophilus* CCDM 151 byly kultivovány v bujónu a dále byla provedena izolace DNA se

změnou koncentrace barviva (konečná koncentrace barviva 25 mmol l⁻¹, 50 mmol l⁻¹, 100 mmol l⁻¹ a 200 mmol l⁻¹) v prvním případě (obr. 1 a 2), ve druhém případě (obr. 3 a 4 byla použita koncentrace barviva 50 mmol l⁻¹, ale byla pozměněna doba fotoindukce (7,5 min, 15 min a 30 min).

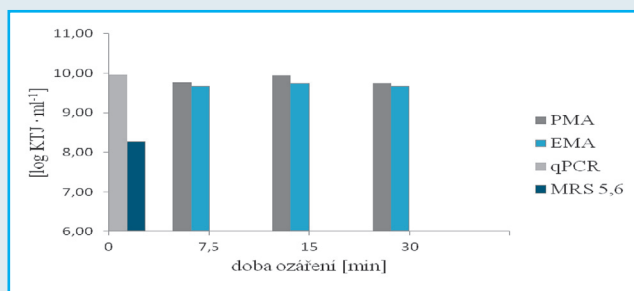
Počet buněk stanovený plotnovou metodou byl u *L. casei* Lafti L 26 i *L. acidophilus* CCDM 151 o 2 řády nižší než qPCR, oproti PMA-qPCR o cca 1,5 řádu. V případě EMA-qPCR se od sebe *L. casei* a *L. acidophilus* lišili více. U *L. casei* došlo k poklesu od PMA-qPCR o 0,3 řádu. U stanovení počtu buněk *L. acidophilus* s rostoucí koncentrací EMA klesal stanovený počet buněk. EMA se nejspíše navázala na DNA i ze živých buněk *L. acidophilus*.

Z obrázků 3 a 4 je patrné, že doba fotoindukce neměla v těchto případech příliš velký vliv na stanovení počtu buněk *L. casei* a *L. acidophilus* (pouze v případě doby fotoindukce 7,5 minuty u EMA-qPCR byl zaznamenán pokles počtu stanovených buněk o 2,5 řádu).

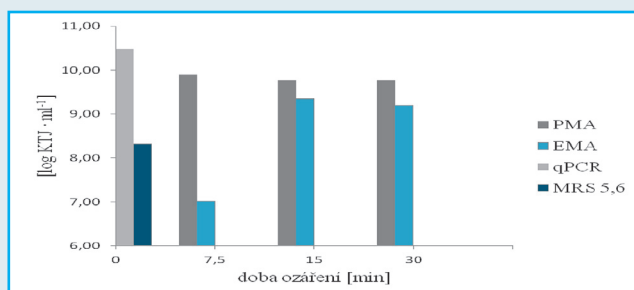
Fotoindukci po dobu 15 minut pro obarvení PMA používají ve své práci například Guarcía-Caguella a kol. (2009).

Použití cholátu sodného před obarvením buněk PMA a EMA u qPCR upravenou metodou dle Yanga a kol. (2011)

Dle Yanga a kol. (2011) je vhodné před obarvením buněk PMA použít deoxycholát sodný. Účinek cholátu sodného byl testován v této práci u *L. casei* Lafti L26, který byl kultivován v bujónu. Byla vyizolována DNA s cholátem sodným (CHS). Bylo také provedeno stanovení počtu buněk plotnovou metodou na MRS agaru pH 5,6; Porovnání výsledků je patrné v tabulce VII.



Obr. 3 Stanovení počtu buněk *L. casei* Lafti L26 při různé době fotoindukce interkalačních barviv



Obr. 4 Stanovení počtu buněk u *L. acidophilus* CCDM 151 při různé době fotoindukce Interkalačních barviv

Počet buněk obarvených PMA (EMA) bez cholátu sodného byl vyšší než počet buněk obarvených PMA (EMA) s cholátem sodným. Počtu buněk stanovených plotnovými metodami bylo nejbližší stanovení PMA-qPCR s cholátem sodným. Ke stejnému zjištění došli Yang a kol. (2011) v práci zaměřené na kvantitativní stanovení *E. coli*. V naší práci pro *L. casei* Lafti L26 jsou výsledky získané EMA-qPCR s použitím cholátu sodného bližší plotnové metodě nežli pouze EMA-qPCR.

Závěr

Byla provedena optimalizace qPCR s použitím PMA a EMA. Pro stanovení počtu buněk *L. casei* a *L. acidophilus* byla vybrána PMA-qPCR s konečnou koncentrací PMA 50 mmol l⁻¹ a optimální doba fotoindukce byla stanovena na 15 minut při použití LED lampy Phastblue, GenIUL (Španělsko). Při použití ošetření buněk cholátem sodným (konečná koncentrace 0,5 hm. %) před samotnou reakcí PMA-qPCR bylo dosaženo nejvyšší shody s hodnotami počtu buněk stanovenými kultivačními metodami. Pomocí této metody by mělo být možné stanovit kmeny *L. casei* a *L. acidophilus* selektivně ve směsi s ostatními probiotickými bakteriemi či kvasinami.

Poděkování

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Literatura

- FITTIPALDI M., NOCKER A., CODONY F. (2012): Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of Microbiological Methods*, 91, s. 276-289.
- FLEKNA G., ŠTEFANIČ P., WAGNER M., SMULDERS F. J. M., MOŽINA S. S., HEIN I. (2007): Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Research in Microbiology*, 158, s. 405-412.
- FURET J.P., QUÉNÉE P., TAILLIEZ P. (2004): Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *International Journal of Food Microbiology*, 97, s. 197-207.
- GARCÍA-CAYUELA T., TABASCO R., PELÁEZ C., REQUENA T. (2009): Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR. *International Dairy Journal*, 19, s. 405-409.
- HUANG CH. H., LEE F. L. (2009): Development of novel species-specific primers for species identification of the *Lactobacillus casei* group based on RAPD fingerprints. *Journal of the Science of Food Agriculture*, 89, s. 1831-1837.
- KLEIN D. (2002): Quantification using real-time PCR technology: Applications and limitations. *Trends in Molecular Medicine*, 8, s. 257-260.
- KOBAYASHI H., OETHINGER M., TUOHY M. J., HALL G. S., BAUER T. W. (2009): Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermidis* by ethidium bromide monoazide. *Letters in Applied Microbiology*, 48, s. 633-638.
- LIMA K. G. DE C., KRUGER M. F., BEHRENS J., DESTRO M. T., LANDGRAF M., MELO FRANCO B. G. (2009): Evaluation of culture media for enumeration of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium animalis* in the presence of *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*. *Food and Technology*, 42, s. 491-495.

- NAM S., KWON S., KIM M.-J., CHAE J.-CH., MAENG P. J., PARK J.-G., LEE G.-CH. (2011): Selective detection of viable *Helicobacter pylori* using ethidium monoazide or propidium monoazide in combination with real-time polymerase chain reaction. *Microbiology and Immunology*, 55, s. 841-846.
- NOCKER A., CHEUNG CH.-Y., CAMPER A. K. (2006): Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods*, 67, s. 310 - 320.
- YÁÑEZ M. A., NOCKER A., SORIA-SORIA E., MÚRTULA R., MARTÍNEZ L., CATALÁN V. (2011): Quantification of viable *Legionella pneumophila* cells using propidium monoazide. *Journal of Microbiological Methods*, 85, s. 124-130.
- SHAH N. P. (2000): Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods. *Journal of Dairy Science*, 83, s. 894-907.
- TALWALKAR A., KAILASAPATHY K. (2004): Comparison of selective and differential media for the accurate enumeration of strains of *Lactobacillus acidophilus*, *Bifidobacterium* ssp. and *Lactobacillus casei* complex from commercial yogurts. *International dairy journal*, 14, s. 143-149.
- YANG X., BADONI M., GILL C. O. (2011): Use of propidium monoazide and quantitative PCR for differentiation of viable *Escherichia coli* from *E. coli* killed by mild or pasteurizing heat treatments. *Food Microbiology*, 28, s. 1478-1482.

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VLIV ZVÝŠENÉHO POČTU SOMATICKÝCH BUNĚK NA KVALITU MLÉKA

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The influence of somatic cells count on milk quality

Souhrn

Práce rozebírá příčiny vzniku mastitidy a vliv zvýšeného počtu somatických buněk na technologické zpracování mléka. Pojednává o změnách ve složení a kvalitě mléka v důsledku mastitidy, rizicích zvýšeného počtu somatických buněk v mléce pro spotřebitele i zpracovatele mléka. Popisuje diagnostiku mastitidy pomocí sledování počtu somatických buněk a enzymatické aktivity v mléce.

Klíčová slova: složení mléka, obsah laktózy, somatické buňky, inhibiční látky, enzymy

Summary

This work analyzes the possible causes of mastitis and the impact of increased somatic cell count on the techno-

QUANTIFICATION OF *LACTOBACILLUS HELVETICUS* IN A MIXTURE OF LACTIC ACID BACTERIA USING qPCR IN CHEESE

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Conventional quantification of *L. helveticus*, in presence of other lactobacilli species, using classical plate method employing low selective media is very inaccurate. Determination of *L. helveticus* using quantitative PCR (qPCR) was performed in six artisanal Kazakh soft cheeses made from cow's milk or from a mixture of cow's and goat's milk. *L. helveticus* was quantified by species-specific qPCR, monitoring the presence of genes encoding for peptidoglycan hydrolases. Quantification of *L. helveticus* based on qPCR ranged from 2.6×10^6 to 4.1×10^8 CFU·g⁻¹ according to the type of the cheese. The microflora of cheese consisted of a mixture of starter and non-starter lactic acid bacteria.

Keywords: *L. helveticus*, cheese, qPCR, peptidoglycan hydrolases

National Kazakh cheese is an acid-rennet curd cheese ripened over a short period of time containing *L. helveticus*, a thermophilic lactic acid bacterium used traditionally for production of Gruyère, Emmental, Grana, and Parmesan cheeses (BERESFORD et al., 2001). This variety was first produced with a mixture of goat's milk, and currently is made from cow's milk. *L. helveticus* can be determined using culture-dependent or culture-independent methods. Culture-dependant methods are generally labour intensive, time consuming, and more or less specific of a microbial group (ACHIELLOS & BERTHIERE, 2013). Only strains able to grow under the defined environmental conditions can be monitored, apart from viable but non-cultivable cells. Moreover, *L. helveticus* occurs in cheese in the presence of other lactobacilli; therefore, its quantification using classical plating method is difficult (FORTINA et al., 2001). In a food matrix, such as cheese, culture-independent methods have rapidly been recognized as valuable alternatives to culture-dependant methods. These methods are based on the direct analysis of DNA extracted from the cheese matrix with no enrichment steps. Among culture-independent methods, qPCR represents a powerful tool for the quantification of microbial populations through the determination of targeted gene copies (ACHIELLOS & BERTHIERE, 2013). Both dead and viable cells can be detected by qPCR (GARCIA-CAYULE et al., 2009). The design and use of specific primers has been proven to be a valuable tool for quantifying bacteria in cheeses like Emmental (FALENTIN et al., 2010, 2012), fresh cheese (FURET et al., 2004), and other cheese-types (LADERO et al., 2008, 2010). The qPCR has been used for quantification of technologically important bacteria such as *Lactococcus lactis*, *Lactobacillus rhamnosus*, *Brevibacterium linens*, *Lactobacillus fermentum*, *Streptococcus thermophilus*,

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Lactobacillus delbrueckii, *Lactobacillus casei*, *Lactobacillus paracasei*, or *Lactobacillus acidophilus* (ACHIelloS & BERTHIERE, 2013). Many studies showed that *L. helveticus* has technological importance, especially regarding probiotic activity and bioactive peptides production by certain strains (SADAT-MEKMENE et al., 2011). To our knowledge, a simple PCR method to quantify *L. helveticus* in a mixture of lactic acid bacteria in cheese has not been reported yet. Therefore, the aim of this work was to quantify *L. helveticus* in soft cheeses produced in Kazakhstan by a species specific qPCR method in a mixture of starter and non-starter cultures.

1. Materials and methods

1.1. Cheese manufacture

Six cheese samples were manufactured in a dairy pilot plant in Kazakhstan. Three cheeses were produced from cow's milk and three cheeses were the new samples of Kazakh national soft cheese (irimshik). They were made from a mixture of goat's and cow's milk with the addition of skimmed cow's milk up to 15% mass fraction of dry matter in the milk. Milk for cheese production was treated with a low pasteurization (72 ± 2 °C, 15–20 s). Christian Hansen FD-DVS DCC-260 was used as starter culture and contained these lactic acid bacteria: *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Lactobacillus casei*, *Lactobacillus helveticus*, and *Streptococcus thermophilus*.

Two different cheese types were made following the traditional technology in three different moments to obtain the 7, 14, and 21 day-old cheeses: CCM (cheese made from cow's milk), and CGCM (cheese made from goat's milk and cow's milk, 1:1 ratio). The 7, 14, and 21 day-old cheeses were analysed at the same time. The pH was determined by using a pH-meter (expert-001, Russia).

1.2. Determination of bacterial composition

Serial dilutions of cheese were prepared in sterile 1% (w/v) peptone solution and plated on MRS agar medium (pH 5.4) for the determination of *Lactobacillus* spp., M17 agar medium for the determination of *Lactococcus* spp. and *Streptococcus thermophilus*, agar medium according to NICKELS and LEESMENT (1964) for the determination of citrate-fermenting bacteria (ISO, 2006), and agar medium according to NICKELS and LEESMENT (1964) with vancomycin supplementation for the determination of *Leuconostoc* spp. (ISO, 2006). Plates with MRS agar medium (pH 5.4) were incubated anaerobically at 37 °C for 72 h. Plates with M17 agar medium were incubated aerobically at 37 °C for 48 h. Plates with medium according to NICKELS and LEESMENT (1964) with and without vancomycin were incubated aerobically at 25 °C for 5 days. Pure cultures of *L. helveticus* DPC 4571 were determined on MRS agar (pH 5.4) and incubated at 37 °C for 48 h.

1.3. PCR amplification

DNA was isolated from pure cultures and cheeses as published by PARAYRE and co-workers (2007). This method uses combination of mechanical and enzymatic lysis. DNA concentration was determined by using Nanodrop ND-1000 (NanoDrop Technologies, USA). *L. helveticus* DPC 4571, *L. acidophilus* LA5, *L. casei* L26, and *L. rhamnosus* CCM1825 were cultured in

culture tubes with MRS broth (pH 5.4). Total DNA from cell culture was extracted by using DNeasy Blood & Tissue kit (Qiagen, Germany) as described in the manufacturer's instructions. All DNA samples were frozen before analyses.

Real-time PCR amplification and analysis were performed using Real-time PCR cycler CFX-96 (Bio-Rad, USA) with species-specific primers for *L. helveticus* for Lhv_0190 gene (Fwd: CAGTTGTGTTGACTTCCACAAT, Re: CAAATTGTGGCTGGTGATTCT) and Lhv_0191 gene (Fwd: GGGCTGATTACAGTGGCTAAT, Re: CTTGCCCTTTTCGGTGTAAG). The selected primers are highly selective and are able to discriminate accurately *L. helveticus* from other closely related homofermentative lactobacilli, especially *L. gallinarum*, *L. acidophilus*, and *L. delbrueckii* (JEBAVA et al., 2014). The initial composition of the PCR reaction mix was as follows: 2 µl genomic DNA, 10 µl iQSybr Green Supermix (BioRad), 7 µl demineralised sterile water, 1 µl of each primer (50 µM) in a final volume of 20 µl. The PCR conditions were: initial denaturation step at 95 °C for 5 min, followed by 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 45 s).

The PCR conditions and composition of the PCR reaction were the same for pure culture of *L. helveticus* DPC 4571 (described above) as well as for negative control strains (*L. acidophilus* LA5, *L. casei* L26, *L. rhamnosus* CCM1825). Non-template control was included in all PCR assays. Purification of the PCR product was carried out using commercially available QIAquick PCR Purification Kit (Qiagen, Germany) as described in the manufacturer's instructions. The concentration of the purified PCR product was measured by spectrophotometry using Nanodrop ND-1000 and the corresponding copy number was calculated using the following equation (LEE et al., 2006).

$$\text{DNA(copy)} = \frac{6.02 \times 10^{23} (\text{copy/mol}) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 (\text{g/mol/bp})}$$

Decimal dilutions for the calibration curve were prepared according to a calculation of the copy number of DNA. Two calibration curves were prepared using two sets of primers (Lhv_0190, Lhv_0191). The same method for absolute quantification using qPCR as describe above was used to quantify counts of *L. helveticus* in pure cultures and in cheeses.

2. Results and discussion

2.1. Cheese manufacture and determination of bacterial composition

Characteristics of six Kazakh cheeses and results for culture-dependent plating methods used for the identification and quantification of microorganisms in cheeses are presented in Table 1. No significant differences in counts of bacteria were detected, and they ranged from 10^8 to 10^9 CFU g⁻¹. Only cheese N°6 showed lower numbers of citrate fermenting bacteria (10^6 CFU g⁻¹). The most important mesophilic lactic acid bacteria that ferment citrate belong to *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. Diagnostic characteristics of lactococci and leuconostoc were distinguishable by this method (ISO, 2006). *Leuconostoc* colonies are blue; *Lactococcus* colonies are white with a zone of clearing. Present knowledge of microbial diversity and dynamics in cheese is mainly based on culture-dependent methods, involving traditional numeration followed by

identification of dominant microorganisms using phenotypic and molecular methods (FALENTIN et al., 2012). Cheese contains a complex combination of microorganisms that changes with time; initially containing large numbers of starter lactic acid bacteria and then with maturation, an increasing number of non-starter lactic acid bacteria (KARIMI et al., 2012).

Table 1. Cheese characterization, bacterial counts by plating method and quantification of *L. helveticus* using qPCR method with two primer sets

Sample	Used milk	Period of ripening (day)	pH	
Cheese 1	CGCM ^a	7	5.06	
Cheese 2	CGCM ^a	14	4.91	
Cheese 3	CGCM ^a	21	4.95	
Cheese 4	CCM ^b	7	4.86	
Cheese 5	CCM ^b	14	4.67	
Cheese 6	CCM ^b	21	4.70	
Bacterial counts [CFU·g ⁻¹]				
	<i>Lactococcus</i> spp., <i>St. thermophilus</i>	citrate-fermenting bacteria	<i>Leuconostoc</i> spp.	<i>Lactobacillus</i> spp.
Cheese 1	2.4·10 ⁹	3.4·10 ⁸	2.5·10 ⁸	1.0·10 ⁹
Cheese 2	2.8·10 ⁹	2.0·10 ⁸	1.5·10 ⁸	1.9·10 ⁹
Cheese 3	1.2·10 ⁹	1.8·10 ⁹	1.7·10 ⁸	9.8·10 ⁸
Cheese 4	1.8·10 ⁹	2.5·10 ⁸	1.7·10 ⁸	9.4·10 ⁹
Cheese 5	2.1·10 ⁹	1.7·10 ⁸	1.7·10 ⁸	7.6·10 ⁸
Cheese 6	1.4·10 ⁹	5.0·10 ⁶	3.6·10 ⁸	6.3·10 ⁸
Number of <i>L. helveticus</i> cells [CFU·g ⁻¹]				
	Lhv_0190	Lhv_0191		
Cheese 1	3.7·10 ⁷ ±6.9·10 ³	8.9·10 ⁷ ±4.1·10 ³		
Cheese 2	4.3·10 ⁷ ±4.7·10 ³	1.0·10 ⁸ ±7.1·10 ³		
Cheese 3	2.5·10 ⁸ ±4.2·10 ⁴	4.1·10 ⁸ ±4.6·10 ³		
Cheese 4	3.9·10 ⁶ ±5.9·10 ²	1.0·10 ⁷ ±3.3·10 ⁴		
Cheese 5	4.8·10 ⁷ ±6.3·10 ³	1.2·10 ⁸ ±3.4·10 ³		
Cheese 6	2.6·10 ⁶ ±1.1·10 ²	6.5·10 ⁶ ±3.6·10 ²		

^aCGCM (cheese made from goat's milk and cow's milk, 1:1 ratio), ^bCCM (cheese made from cow's milk)

Because of the presence of other lactobacilli closely related to *L. helveticus* in the culture, and the deficiency in a reliable species selective agar medium, it was impossible to determine selectively *L. helveticus* using plating method based on MRS agar. Thus, total counts of lactobacilli were determined. Most plating methods are based on pure cultures of these organisms and fail to work in products because of the presence of multiple and closely related species making the differential or selective enumeration of probiotic and starter bacteria difficult due to similarity in growth requirements and overlapping biochemical characteristics of the species (ASHRAF & SHAH, 2011).

Acta Alimentaria 45, 2016

2.2. qPCR determination

First the absolute quantification method was verified in pure cultures of *L. helveticus* DPC 4571 in comparison with the plate count method. The results were as follows: plate count on MRS (pH 5.4) agar $8.5 \cdot 10^7 \pm 2.1 \cdot 10^1$ CFU·ml⁻¹, qPCR with Lhv_0190 $9.2 \cdot 10^7 \pm 1.2 \cdot 10^2$ CFU·ml⁻¹, qPCR with Lhv_0191 $1.1 \cdot 10^8 \pm 3.1 \cdot 10^2$ CFU·ml⁻¹. These results are averages of 10 assessments and do not differ substantially, and thus the suitability of chosen qPCR method is confirmed.

The soft cheese irimshik was selected for the first trial, because DNA isolation from this soft cheese was unambitious and defined starter culture was used for its production. The DNA concentration (quantified by Nanodrop ND-1000) extracted from 6 different cheeses varied from 4.7 to 56.7 µg·g⁻¹ cheese. The DNA concentration was in agreement with previously published data quantifying DNA extracted from cheese matrix (JACQUIN et al., 2001; LEE et al., 2006), reporting concentrations of DNA extracted from 10 different dairy products between 0.7 to 29.2 µg·g⁻¹ using picogreen spectrofluorimetry, respectively, 4 to 19 µg DNA·g⁻¹ of Emmental cheese using spectrophotometric method.

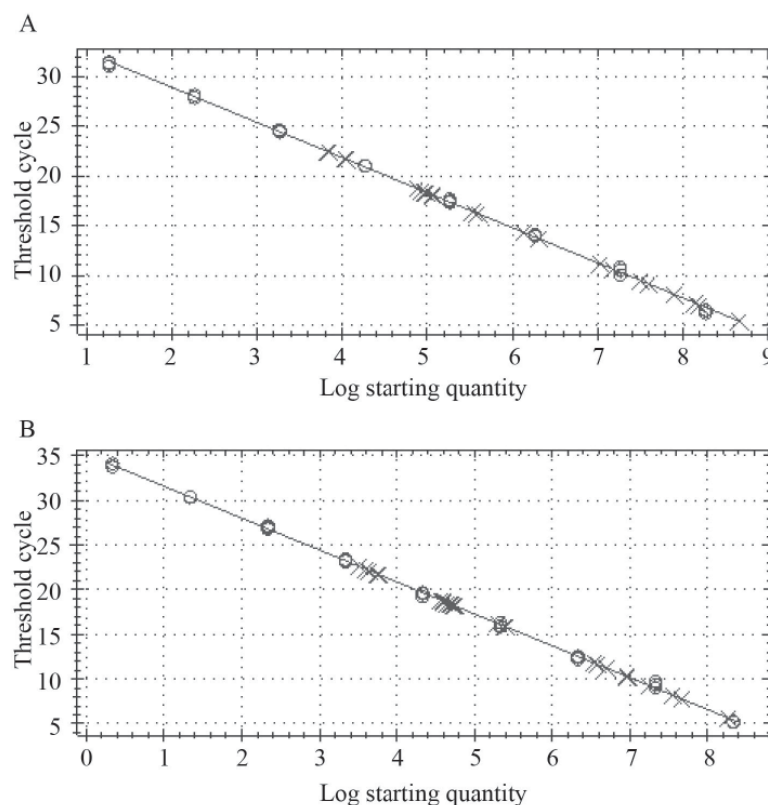


Fig. 1. Calibration curves for A: Lhv_0190 and B: Lhv_0191 from qPCR assay
 o: standard – *L. helveticus* DPC 4571; X: unknown samples (cheeses)
 –: SYBR (A: efficiency 91.9%, slope –3.53; B: efficiency 90.6%, slope –3.57)

Two qPCR assays targeting peptidoglycan hydrolases were developed for the absolute quantification of *L. helveticus* in food matrices. One pair of primers Lhv_0190 encoding *N*-acetylmuramidase and the other one Lhv_0191 encoding amidase. Two pairs of primers were used to observe possible differences. Ascertained differences between obtained results for these two pairs of primers were minimal and error is within the standard deviation. Quantification of *L. helveticus* using qPCR ranged from $2.6 \cdot 10^6$ to $4.1 \cdot 10^8$ CFU·g⁻¹, and particular results are shown in Table 1. The standard curves for Lhv_0190 and Lhv_0191 were linear in the range tested ($R^2 > 0.999$) by the triplicate reactions (Fig. 1).

Amplification was linear over the range of $1.8 \cdot 10^1$ to $1.8 \cdot 10^8$ for Lhv_0190 and $2.1 \cdot 10^0$ to $2.1 \cdot 10^8$ for Lhv_0191, respectively, of DNA copies. The slope of the standard curves for Lhv_0190 and Lhv_0191 were -3.53 and -3.57, respectively. Quantitation cycle (Cq) for positive control was 16.2 for Lhv_0190 and 15.1 for Lhv_0191, respectively, Cq for all three negative controls were above 34 for both primer sets, Cq for NTC was >35. Efficiencies of qPCR were 91.9% for Lhv_0190 and 90.6% for Lhv_0191, respectively. Intra-assay CV was 2.3% for Lhv_0190 and 0.9% for Lhv_0191.

Because both *L. helveticus* and *L. casei* were present in the used starter culture and cheese contains probably other lactobacilli of non-starter origin as well, the determination of *L. helveticus* was difficult using non-selective MRS (pH 5.4) agar. It was not possible to compare the results of plating methods to qPCR. Direct qPCR quantification has proven to be useful, providing additional information to plate counts. In addition, the use of species-specific primer pairs at the given PCR conditions proved to be a very rapid and effective method for quantification of the species. Our results confirmed the necessity of using the species-specific qPCR for the determination and quantification of lactobacilli in mixtures.

When we critically assess both the pros and cons of qPCR, we have to say that usage of qPCR for quantification of *L. helveticus* is an alternative to plating methods. More particularly, qPCR is considered as the first choice method, and its major advantage is that it is less-time consuming than conventional culture based methods. It is also highly sensitive, specific, enables simultaneous detection of different microorganisms, and requires no post-processing (MARTÍNEZ et al., 2011; POSTOLLEC et al., 2011). Method of qPCR has also some limitations, for example the precision of molecular quantification by PCR depends on the effectiveness of bacterial lysis of DNA extraction, and on the presence of PCR inhibitors in the DNA solution (FURET et al., 2004). Reliable quantification depends on optimized and carefully performed qPCR reactions. The accuracy of qPCR is influenced by primer design, the quality of the template DNA, and the handling and storage of samples, primers, and enzymes. With food samples, special attention must be paid to the possible presence of inhibitors and to the efficiency of DNA extraction. Provided appropriate control (e.g. positive PCR control, non-template control, control for environmental contamination, etc.) are included in the analyses, proposed qPCR appears to be highly accurate and reliable for quantification of targeted genes (POSTOLLEC et al., 2011).

3. Conclusions

In conclusion, our results suggest that application of simple, fast, and accurate qPCR method is eligible for species specific quantification of *L. helveticus* in cheese matrix containing other lactic acid bacteria including lactobacilli. In this work soft cheese was used as matrix. Other experiments with different cheeses and other dairy products will follow in the very near future to verify appropriateness of this method and its independence of food matrix and composition of microflora.

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References

- ACHIELLOS, C. & BERTHIERE, F. (2013): Quantitative PCR for the specific quantification of *Lactococcus lactis* and *Lactobacillus paracasei* and its interest for *Lactococcus lactis* in cheese samples. *Food Microbiol.*, 36, 286–295.
- ASHRAF, R. & SHAH, N. (2011): Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt – A review. *Int. J. Food Microbiol.*, 149, 194–208.
- BERESFORD, T., FITZMOL, N., BRENNAN, N. & COGAN, T. (2001): Recent advances in cheese microbiology. *Int. Dairy J.*, 11, 259–274.
- FALENTIN, H., POSTOLLEC, F., PARAYRE, S., HENAFF, N., LE BIVIC, P., RICHOUX, R., THIERRY, A. & SOHIER, D. (2010): Specific metabolic activity of ripening bacteria quantified by real-time reverse transcription PCR throughout Emmental cheese manufacture. *Int. J. Food Microbiol.*, 144, 10–19.
- FALENTIN, F., HENAFF, N., LE BIVIC, P., DEUTSCH, S.M., PARAYRE, S., RICHOUX, R., SOHIER, D., THIERRY, A., LORTAL, S. & POSTOLLEC, F. (2012): Reverse transcription quantitative PCR revealed persistency of thermophilic lactic acid bacteria metabolic activity until the end of the ripening of Emmental cheese. *Food Microbiol.*, 29, 132–140.
- FORTINA, M.G., RICCI, G., MORA, D., PARINI, C. & MANACHINI, P.L. (2001): Specific identification of *Lactobacillus helveticus* by PCR with *pepC*, *pepN* and *htrA* target primers. *FEMS Microbiol. Lett.*, 198, 85–89.
- FURET, J.P., QUÉNÉE, P. & TAILLIEZ, P. (2004): Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *Int. J. Food Microbiol.*, 97, 197–207.
- GARCIA-CAYULE, T., TABASCO, R., PELAEY, C. & REQUENA, T. (2009): Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR. *Int. Dairy J.*, 19, 405–409.
- ISO (2006): *Milk, milk products and mesophilic starter cultures — Enumeration of citrate-fermenting lactic acid bacteria — Colony-count technique at 25 degrees C*. ISO 17792, IDF, 2006, pp. 1–12.
- JACQUIN, J., ARAGNO, M. & ROSSI, P. (2001): Application of the bead beater technique to the extraction of bacterial DNA from cheese samples. *Milchwissenschaft*, 56, 563–565.
- JEBAVA, I., CHUAT, V., LORTAL, S. & VALENCE, F. (2014): Peptidoglycan hydrolases as species-specific markers to differentiate *Lactobacillus helveticus* from *Lactobacillus gallinarum* and other closely related homofermentative lactobacilli. *Curr. Microbiol.*, 68, 551–557.
- KARIMI, R., MORTAYAVIAN, A. & AMIRI-RIGI, A. (2012): Selective enumeration of probiotic microorganisms in cheese. *Food Microbiol.*, 29, 1–9.
- LADERO, V., LINAREZ, D., FERNÁNDEZ, M. & ALVAREZ, M. (2008): Real time quantitative PCR detection of histamine-producing lactic acid bacteria in cheese: Relation with histamine content. *Food Res. Int.*, 41, 1015–1019.
- LADERO, V., FERNÁNDEZ, M., CUESTA, I. & ALVAREZ, M. (2010): Quantitative detection and identification of tyramine-producing enterococci and lactobacilli in cheese by multiplex qPCR. *Food Microbiol.*, 27, 933–939.
- LEE, C., KIM, J., SHIN, S. & HWANG, S. (2006): Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. *J. Biotechnol.*, 123, 273–280.
- MARTÍNEZ, N., MARTÍN, M.C., HERRERO, A., FERNÁNDEZ, M., ALVAREZ, M.A. & LADERO, V. (2011): qPCR as a powerful tool for microbial food spoilage quantification: Significance for food quality. *Trends Food Sci. Tech.*, 22, 367–376.
- NICKELS, C. & LEESMENT, H. (1964): Methode zur Differenzierung und quantitative Bestimmung von Säureweckerbakterien. *Milchwissenschaft*, 19, 374–378.
- PARAYRE, S., FALENTIN, H., MADEC, M.N., SIVIERI, K., LE DIZES, A.S., SOHIER, D. & LORTAL, S. (2007): Easy DNA extraction method and optimization of PCR-Temporal Temperature Gel Electrophoresis to identify the predominant high and low GC-content bacteria from dairy products. *J. Microbiol. Meth.*, 69, 431–441.
- POSTOLLEC, F., FALENTIN, H., PAVAN, S., COMBRISSE, J. & SOHIER, D. (2011): Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food Microbiol.*, 28, 848–861.
- SADAT-MEKMENE, L., GENAY, M., ATLAND, D., LORTAL, S. & GAGNAIRE, V. (2011): Original features of cell-envelope proteinases of *Lactobacillus helveticus*. A review. *Int. J. Food Microbiol.*, 146, 1–13.



Selective culture medium for the enumeration of *Lactobacillus plantarum* in the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*

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ABSTRACT

Selective enumeration of *Lactobacillus plantarum* in a mixed culture with yogurt bacteria is difficult, due to their similar growth conditions. We evaluated several culture media to determine their suitability for selective determination. The following media were tested to enumerate the strains of *Lactobacillus plantarum*, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*: M17, MRS pH 5.2; 5.6 and 6.2, and modified MRS where glucose was replaced by maltose, sorbitol, mannitol, sucrose or galactose. Further, the addition of vancomycin (20 mg/l) and the use of different cultivation conditions – aerobic or anaerobic, and different cultivation temperatures 30 °C, 37 °C and 45 °C, were chosen to improve selectivity. MRS medium with vancomycin (pH 5.6), and cultivation under aerobic conditions at 30 °C was successfully used for selective enumeration of *L. plantarum* in a mixed culture with yogurt bacteria after cultivation both in broth and in milk. To distinguish between the remaining bacteria, M17 medium under aerobic conditions at 45 °C and MRS pH 5.2 under anaerobic conditions under 45 °C were recommended for counting of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, respectively. No statistical differences were found between pour plate and surface plate methods of inoculation ($P > 0.05$).

1. Introduction

Lactobacillus plantarum belongs to a group of technologically useful lactic acid bacteria (LAB) that are mainly used in the production of fermented dairy products (de Vries, Vaughan, Kleerebezem, & de Vos, 2006). Moreover, some of these bacteria are used as probiotics associated with beneficial health effects in human and animals (Butel, 2014; Kerry et al., 2018; Seddik et al., 2017). Probiotic LAB strains have been used in functional foods, fermented dairy products or dietary supplements, but to ensure the quality of the products, it is necessary to monitor the viability of probiotic bacteria during storage. Thus, it is very important to have reliable methods for the quantification of particular species of probiotic bacteria (Ashraf & Shah, 2011; Tripathi & Giri, 2014).

The addition of probiotics in a mixture with other LAB to different types of foods requires high resolution methods to quantify the number of individual bacteria in complex matrices of dairy, cereal, fruit or vegetable products. In general, lactobacilli are usually cultivated and

determined on MRS agar (de Man, Rogosa and Sharpe agar), but this medium only enables the determination of total lactobacilli. Most products available on the market include a combination of probiotic microorganisms and non-probiotic starter cultures (Ashraf & Shah, 2011). Different species of lactobacilli are closely related in terms of their nutritional demands and therefore selective cultivation is difficult to achieve (Boyer & Combrisson, 2013; Galat et al., 2016).

Usually, different cultivation temperatures, times, atmospheres and modified culture media (pH, carbon source etc.) are used for selective enumeration of LAB (Oberg, Moyes, Domek, Brotherson, & McMahon, 2011), and several culture media have been designed to distinguish between probiotic and starter cultures. MRS agar modified with various selective agents, for example bile salts (de Carvalho Lima et al., 2009; Mortazavian, Ehsani, Sohrabvandi, & Reinheimer, 2007), lithium chloride (Tabasco, Paarup, Janer, Peláez, & Requena, 2007), antibiotics or their combinations (de Carvalho Lima et al., 2009; di Lena et al., 2015; Sakai et al., 2010) are frequently used. Many authors (de Carvalho Lima et al., 2009; Lankaputhra & Shah, 1996; Ravula & Shah,

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1998; Tabasco et al., 2007) successfully tested media where glucose was replaced with other carbon sources (maltose, fructose, trehalose or ribose). The main focus was on selective enumeration of *L. acidophilus* and *L. casei* in combination with a yoghurt culture comprising *L. rhamnosus*, *L. reuteri* or bifidobacteria (de Carvalho Lima et al., 2009; Karimi, Mortazavian, & Amiri-Rigi, 2012). Only two international standards have been validated. A selective MRS agar with clindamycin hydrochloride and ciprofloxacin hydrochloride (MRS/CL/CP) has been used for estimation of the presumptive number of *L. acidophilus* according to international standard ISO 20128:2006 (ISO, 2006), acidic MRS agar has been used for *L. delbrueckii* subsp. *bulgaricus*, and M17 agar has been used for enumeration of *S. thermophilus* in a yoghurt culture according to international standard ISO 7889:2003 (ISO, 2003).

Recently, attention has been paid to *L. plantarum*, which has been included in many products such as fermented dairy foods, fruit juices, cereal products and dietary supplements (Hashemi et al., 2017; Li et al., 2017; Russo et al., 2016). With increasing knowledge of the functional and probiotic properties of *L. plantarum* (Bengtsson et al., 2016; Liu et al., 2017; Jabbari et al., 2017; Garcia-Gonzalez, Prete, Battista, & Corsetti, 2018), new applications in different probiotic foods are expected. One possibility is its inclusion in a yoghurt that is often used as a vehicle for delivery of probiotic bacteria to the host intestinal tract (Granato, Branco, Cruz, Faria, & Shah, 2010).

However, despite the importance of accurate bacterial enumeration, no selective medium for quantification of *L. plantarum* has yet been proposed. The aim of this study was to design a selective medium and appropriate cultivation conditions for selective enumeration of *L. plantarum* in the presence of a yoghurt culture.

We tested ten culture media, three different temperatures (30 °C, 37 °C and 45 °C) and 2 atm (aerobic and anaerobic). The influence of pour and surface plate methods on growth were also examined.

2. Materials and methods

2.1. Microorganisms

Strains of *L. plantarum*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were routinely cultivated in broths under conditions summarized in Table 1. Strains were also cultivated in 10% (w/v) reconstituted powdered skimmed milk (Moravia Lacto, Czech Republic, sterilized at 100 °C for 10 min) and incubated aerobically at 37 °C for 18 h.

2.2. Culture media

The following ten media were tested to verify their selectivity for

enumeration of both lactobacilli and *S. thermophilus*: M17 agar (Oxoid Ltd., Hampshire, United Kingdom), MRS agar (Merck, Darmstadt, Germany) adjusted to pH 5.6; 5.2 and 6.2, modified MRS agars containing different sugars (Penta, Prague, Czech Republic) at concentration of 20 g/l instead of glucose: MRS agar with maltose (M-MRS), with sorbitol (S-MRS), with mannitol (MA-MRS), with sucrose (SU-MRS) and with galactose (GA-MRS) and MRS agar with vancomycin (Sigma Chemical Co., St. Louis, USA) (20 mg/l, VA-MRS), all at pH 5.6. All media were sterilized at 121 °C for 15 min, 0.15 MPa. A vancomycin solution was sterilized by filtration (0.22 µm) and added to the medium after heat treatment. Lactose, peptone, yeast extract, Lab-Lemco powder and bacteriological agar were obtained from Oxoid Ltd. (Hampshire, United Kingdom), manganese sulphate tetrahydrate from Merck (Darmstadt, Germany); di-potassium hydrogen phosphate, sodium acetate trihydrate from Penta Ltd. (Prague, Czech Republic); triammonium citrate and magnesium sulphate heptahydrate from Carl Roth GmbH (Karlsruhe, Germany); sorbitan mono-oleate and vancomycin from Sigma Chemical Co. (St. Louis, USA).

2.3. Selectivity of media tested

Pure cultures of four different strains of each LAB species from MRS (*L. plantarum*, *L. delbrueckii* subsp. *bulgaricus*) or M17 (*S. thermophilus*) broths were streaked using a 1 µl loop onto each of ten tested media. Each strain was cultivated on two plates each under anaerobic (AnaeroGen system™, Oxoid, Hampshire, United Kingdom) and aerobic conditions at 30 °C, 37 °C and 45 °C for 72 h. After incubation, the growth of microorganisms were evaluated as + (visible growth), +/- (weak growth) and - (no growth). All experiments were carried out in triplicate (n = 6).

2.4. Quantitative microbiological analysis of pure cultures

For the quantification of pure cultures after cultivation in broth, five tested media (MRS 5.6, M17, MRS 5.2, GA-MRS and VA-MRS) were selected. After cultivation in milk, only four media (MRS 5.6, M17, MRS 5.2 and VA-MRS) were chosen based on results from testing in broth. After cultivation in broth, both pour plates (1 ml inoculum) and surface plates (0.1 ml inoculum) of serially diluted samples was used, whereas after cultivation in milk only pour plates were used. Cultivation on M17, GA-MRS and VA-MRS agar was carried out under aerobic conditions. The cultivation conditions on MRS agar (pH 5.6 or 5.2) are specified at Tables 3 and 5. Cultivation temperatures of 30 °C, 37 °C and 45 °C for 48 h were used for all samples. Plates with 25–300 colonies were enumerated and results were expressed as log CFU/ml.

Table 1
Microorganisms used in this study and culture conditions.

Microorganism ^a	Origin	Culture media ^b	Incubation
<i>L. plantarum</i> 299v	Human	MRS broth, pH 5.6	37 °C, 5% CO ₂ , 18 h
<i>L. plantarum</i> ATCC 8014	Unknown		
<i>L. plantarum</i> CCDM 183	Fermented milk		
<i>L. plantarum</i> CCDM 375	Human faeces		
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	Unknown	MRS broth, pH 5.6	37 °C, anaerobic, 18 h
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> YC-381	Yoghurt culture Ch. Hansen		
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> CCDM 31	Yoghurt culture CCDM 31		
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> CCDM 664	Yoghurt		
<i>S. thermophilus</i> ATCC 19258	Pasteurized milk	M17 broth, pH 6.5	37 °C, aerobic, 18 h
<i>S. thermophilus</i> YC-381	Yoghurt culture Ch. Hansen		
<i>S. thermophilus</i> CCDM 31	Yoghurt culture CCDM 31		
<i>S. thermophilus</i> CCDM 130	Milk		

^a 299v and YC-381 – commercial culture, ATCC – American type culture collection, CCDM – Czech Collection of Dairy Microorganisms.

^b MRS broth – de Man, Rogosa and Sharpe broth (Merck, Germany), M17 broth – M17 broth with lactose (Oxoid, United Kingdom).

Table 2
Growth of tested bacteria on selected media at 30 °C, 37 °C and 45 °C, under aerobiosis and anaerobiosis (72 h).

Culture media ^a	<i>L. plantarum</i> (n = 4)				<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (n = 4)				<i>S. thermophilus</i> (n = 4)			
	Anaerobiosis		Aerobiosis		Anaerobiosis		Aerobiosis		Anaerobiosis		Aerobiosis	
	30 °C	37 °C	45 °C	30 °C	37 °C	45 °C	30 °C	37 °C	30 °C	37 °C	45 °C	30 °C
MRS 5.6	+	(4) ^b	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
M17	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
MRS 5.2	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
MRS 6.2	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
M-MRS	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
S-MRS	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
MA-MRS	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
SU-MRS	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
GA-MRS	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)
VA-MRS	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)	+	(4)

^a MRS 5.6 - de Man, Rogosa and Sharpe agar, pH adjusted to 5.6; M17 agar - M17 agar with lactose; MRS 5.2 - pH adjusted to 5.2; MRS 6.2 - pH adjusted to 6.2; M - mannitol; S - sorbitol; MA - maltose; SU - sucrose; GA - galactose; VA - vancomycin.

^b + visible growth, + / - weak growth, - no growth.

2.5. Quantitative microbiological analysis of strain mixtures

The following four mixtures of strains, both in broth and in milk, were prepared in a ratio of 1:1:1: mixture A - *L. plantarum* 299v, *L. delbrueckii* subsp. *bulgaricus* YC-381 and *S. thermophilus* YC-381; mixture B - *L. plantarum* ATCC 8014, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and *S. thermophilus* ATCC 19258; mixture C - *L. plantarum* ATCC 8014, *L. delbrueckii* subsp. *bulgaricus* YC-381 and *S. thermophilus* YC-381; and mixture D - *L. plantarum* 299v, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and *S. thermophilus* ATCC 19258.

The pour plate technique was used for quantitative analysis of mixtures. The schema of incubation and enumeration were the same as above. The purity of cultures on a selective medium was monitored by colony morphology, Gram staining and microscopy (Olympus CX23; Olympus, Tokyo, Japan).

All experiments were carried out in triplicate. The counts of microorganisms tested on different media were compared by ANOVA to determine the significance of differences.

3. Results and discussion

For the purpose of this study, extended pre-screening of growth on 15 saccharides at 37 °C, growth at 7 different temperatures (in the range of 20–48 °C), resistance to 25 antibiotics and their minimal inhibitory concentrations for 33 LAB strains of different origins (*L. plantarum* – 13 strains, *L. delbrueckii* subsp. *bulgaricus* – 10 strains and *S. thermophilus* – 10 strains) were carried out to choose the optimal selective agents for further testing (data not shown).

Table 2 shows the results of a growth test of 12 chosen strains on ten media. Strains of *L. plantarum* were able to grow on all media tested at 30 °C and 37 °C regardless of an aerobic or anaerobic atmosphere. An increase in temperature to 45 °C inhibited the growth of *L. plantarum*, although some weak growth still occurred under aerobic conditions. *L. delbrueckii* subsp. *bulgaricus* preferred higher cultivation temperatures (37 °C or 45 °C) and anaerobic conditions for satisfactory growth on certain media. *L. delbrueckii* subsp. *bulgaricus* did not grow or grew very weakly at 30 °C. This bacterium did not grow on MRS with galactose (GA-MRS) or with vancomycin (VA-MRS), but showed weak growth on MRS with other sugars (maltose, sucrose, mannitol and sorbitol). Changing pH in MRS agar had no effect on either lactobacilli. On the other hand, the pH of MRS agar influenced the growth of streptococci. On MRS agar at pH 6.2, streptococci grew well, but at a lower pH (5.2 or 5.6), growth was suppressed. Therefore, MRS agars at pH 5.2 or 5.6 were selected for further experiments. In the study of Van de Castele et al. (2006), a change in pH of MRS agar did not act as a sufficient selective factor for discrimination between different lactobacilli. From Table 2 it is evident that *L. plantarum* and *L. delbrueckii* subsp. *bulgaricus* both grew on M17 agar, which is intended for enumeration of streptococci by IDF (International Dairy Federation) and ISO 7889:2003 (ISO, 2003). MRS with mannitol, sorbitol, maltose and sucrose could not be used to distinguish between *L. plantarum* and *L. delbrueckii* subsp. *bulgaricus*. High selectivity was proven only for MRS agar with galactose because only *L. plantarum* grew on it. It is well known that yoghurt culture microorganisms are able to effectively metabolize only the glucose portion of lactose and secrete galactose back into the medium. The reason for this metabolic shortcoming is the lack of galactokinase (Anbukkarasi et al., 2014). Nevertheless, some strains are galactose positive, especially when grown under conditions of lactose limitation (Hou, Hannon, McSweeney, Beresford, & Guinee, 2017). In the case of using GA-MRS as a selective agar for *L. plantarum*, it would first be necessary to verify whether the strains contained in the yoghurt culture could use galactose. *S. thermophilus* was able to grow well only on M17 agar at a higher cultivation temperature (37 °C, 45 °C) in either atmospheres, and was rarely detected on MRS 6.2 or SU-MRS. Streptococci did not grow on MRS agar when glucose was replaced with mannitol, sorbitol, maltose or galactose. Vancomycin (20 mg/l) inhibited the

Table 3
Microbial analysis (log₁₀ CFU/ml) of pure cultures from broth using different culture media and surface-plate and pour-plate technique.

culture media ^a		30 °C			37 °C			45 °C			30 °C			37 °C			45 °C		
		pp ^b			SP			PP			SP			PP			SP		
<i>L. plantarium</i> 299v																			
MRS 5.6		9.33 ± 0.01 ^c	9.33 ± 0.03	9.32 ± 0.01	9.36 ± 0.01	0 ^d		9.26 ± 0.01	9.26 ± 0.04	9.27 ± 0.03	9.26 ± 0.02	0 ^d		9.26 ± 0.02	0 ^d		0 ^d		
M17		nd	nd	9.34 ± 0.01	9.38 ± 0.01	0		nd	nd	9.20 ± 0.05	9.21 ± 0.02	0		9.21 ± 0.02	0		0		
MRS 5.2		nd	nd	9.35 ± 0.03 ^d	9.35 ± 0.01 ^d	0 ^d		nd	nd	9.25 ± 0.03 ^d	9.21 ± 0.02 ^d	0 ^d		9.21 ± 0.02 ^d	0 ^d		0 ^d		
GA-MRS		9.31 ± 0.03	9.34 ± 0.03	9.34 ± 0.03	9.34 ± 0.01	nd		9.26 ± 0.02	9.23 ± 0.03	9.25 ± 0.02	9.26 ± 0.01	nd		9.26 ± 0.01	nd		nd		
VA-MRS		9.33 ± 0.04	9.35 ± 0.02	9.32 ± 0.02	9.35 ± 0.03	nd		9.21 ± 0.02	9.22 ± 0.03	9.24 ± 0.03	9.28 ± 0.02	nd		9.28 ± 0.02	nd		nd		
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842																			
MRS 5.6		nd	nd	8.07 ± 0.02	8.07 ± 0.03	8.06 ± 0.01 ^d	8.08 ± 0.02 ^d	nd	nd	7.95 ± 0.05	7.92 ± 0.03	8.01 ± 0.01 ^d		7.96 ± 0.03 ^d			7.96 ± 0.03 ^d		
M17		nd	nd	0	0	0	0	nd	nd	0	0	0		0			0		
MRS 5.2		nd	nd	8.06 ± 0.03 ^d	8.00 ± 0.01 ^d	8.06 ± 0.03 ^d	8.08 ± 0.04 ^d	nd	nd	7.98 ± 0.03 ^d	7.96 ± 0.06 ^d	7.95 ± 0.03 ^d		7.96 ± 0.04 ^d			7.96 ± 0.04 ^d		
GA-MRS		0	0	0	0	nd	nd	0	0	0	0	nd		nd			nd		
VA-MRS		0	0	0	0	nd	nd	0	0	0	0	nd		nd			nd		
<i>S. thermophilus</i> ATCC 19258																			
MRS 5.6		nd	nd	0	0	0 ^d	0 ^d	nd	nd	0	0	8.27 ± 0.01		8.27 ± 0.01			8.27 ± 0.01		
M17		nd	nd	8.16 ± 0.02	8.13 ± 0.01	8.06 ± 0.02	8.06 ± 0.02	nd	nd	8.31 ± 0.02	8.29 ± 0.01	8.27 ± 0.01		8.27 ± 0.01			8.27 ± 0.01		
MRS 5.2		nd	nd	0 ^d	0 ^d	0 ^d	0 ^d	nd	nd	0 ^d	0 ^d	0 ^d		0 ^d			0 ^d		
GA-MRS		0	0	0	0	nd	nd	0	0	0	0	nd		nd			nd		
VA-MRS		0	0	0	0	nd	nd	0	0	0	0	nd		nd			nd		

^a MRS - de Man, Rogosa and Sharpe agar; M17 agar - M17 agar with lactose; MRS 5.2 - pH adjusted to 5.2; GA - galactose; VA - vancomycin.

^b SP - surface plate, PP - pour plate method.

^c log₁₀ CFU/ml ± standard deviation of three replicates, nd - not done, 0 - no growth.

^d anaerobic conditions.

Table 4Counts (log₁₀ CFU/ml) of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* in mixture after broth cultivation, using different culture media.

Microorganism	Medium ^a					
	M17, 45 °C, aerobiosis	MRS 5.2 45 °C, anaerobiosis	GA-MRS, 30 °C, aerobiosis	GA-MRS, 37 °C, aerobiosis	VA-MRS, 30 °C, aerobiosis	VA-MRS, 37 °C, aerobiosis
Mixture A						
<i>S. thermophilus</i> YC-381	7.84 ± 0.04 ^b	0 ^c	0	0	0	0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> YC-381	0	7.49 ± 0.06	< 1	< 1	0	0
<i>L. plantarum</i> 299v	0	0	8.87 ± 0.02	8.87 ± 0.01	8.89 ± 0.02	8.87 ± 0.03
Mixture B						
<i>S. thermophilus</i> ATCC 19258	7.49 ± 0.03	0	0	0	0	0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	0	7.57 ± 0.11	< 1	< 1	0	0
<i>L. plantarum</i> ATCC 8014	0	0	8.82 ± 0.06	8.84 ± 0.03	8.86 ± 0.03	8.81 ± 0.04
Mixture C						
<i>S. thermophilus</i> YC-381	7.85 ± 0.07	0	0	0	0	0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> YC-381	0	7.41 ± 0.02	< 1	< 1	0	0
<i>L. plantarum</i> ATCC 8014	0	0	8.77 ± 0.03	8.84 ± 0.03	8.87 ± 0.04	8.79 ± 0.07
Mixture D						
<i>S. thermophilus</i> ATCC 19258	7.42 ± 0.04	0	0	0	0	0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	0	7.53 ± 0.03	< 1	< 1	0	0
<i>L. plantarum</i> 299v	0	0	8.84 ± 0.05	8.85 ± 0.02	8.92 ± 0.01	8.86 ± 0.04

^a M17 agar - M17 agar with lactose; MRS 5.2 – de Man, Rogosa and Sharpe agar, pH adjusted to 5.2; GA – galactose; VA – vancomycin.^b log₁₀ CFU/ml ± standard deviation of three replicates.^c 0 - no growth on this medium.

growth of both yoghurt culture microorganisms. The addition of an antibiotic or a combination of them is often a factor in the design of a selective medium. Ong and Shah (2009) used MRS with vancomycin to enumerate *L. casei* in cheese products. Also, Tharmaraj and Shah (2003) used vancomycin for determination of *L. rhamnosus* and *L. casei* in a mixture. In the study of Colombo, de Oliveira, de Carvalho, and Nero (2014), the influence of vancomycin (10 mg/l) on growth of *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus* and *L. casei* was tested. Based on the results summarized in Table 2, two MRS agars at pH 5.6 (control) and pH 5.2 (cultivation of acid-tolerant *L. delbrueckii* subsp. *bulgaricus*), M17 agar for streptococci, MRS with galactose and MRS with vancomycin for enumeration of *L. plantarum* were chosen for further work.

Bacterial counts of pure strains on these media after broth cultivation are shown in Table 3. The mean counts of *L. plantarum* on all media tested at 30 °C and 37 °C, inoculated either by pour plate or surface plate methods, were similar with no significant differences ($P > 0.05$) in comparison with the control (MRS, 37 °C).

At a temperature of 45 °C, *L. plantarum* did not grow on M17 (aerobically) or on MRS 5.2 agar (anaerobically). *L. delbrueckii* subsp. *bulgaricus* grew only on MRS 5.6 or MRS 5.2 agar and at temperatures of 37 °C and 45 °C under anaerobic conditions. No difference ($P > 0.05$) in *S. thermophilus* growth was observed on M17 agar at 37 °C and 45 °C (aerobically).

Compared with the control growth on MRS 5.6, the same counts

Table 5Microbial analysis (log₁₀ CFU/ml) of pure culture *L. plantarum*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* after milk cultivation using different culture media.

culture media ^a	30 °C	37 °C	45 °C	30 °C	37 °C	45 °C
	<i>L. plantarum</i> 299v			<i>L. plantarum</i> ATCC 8014		
MRS 5.6	8.46 ± 0.01 ^b	8.40 ± 0.01	0 ^c	8.50 ± 0.02	8.46 ± 0.01	0 ^c
M17	nd ^d	nd	0	nd	nd	0
MRS 5.2	nd	nd	0 ^c	nd	nd	0 ^c
VA-MRS	8.41 ± 0.03	nd	nd	8.44 ± 0.01	nd	nd
	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842			<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> YC-381		
MRS 5.6	nd	7.78 ± 0.06 ^c	7.75 ± 0.09 ^c	nd	8.21 ± 0.04 ^c	8.24 ± 0.03 ^c
M17	nd	nd	0	nd	nd	0
MRS 5.2	nd	nd	7.65 ± 0.02 ^c	nd	nd	8.24 ± 0.02 ^c
VA-MRS	0	nd	nd	0	nd	nd
	<i>S. thermophilus</i> ATCC 19258			<i>S. thermophilus</i> YC-381		
MRS 5.6	nd	nd	0 ^c	nd	nd	0 ^c
M17	nd	8.82 ± 0.04	8.82 ± 0.03	nd	8.62 ± 0.04	8.69 ± 0.03
MRS 5.2	nd	nd	0 ^c	nd	nd	0 ^c
VA-MRS	0	nd	nd	0	nd	nd

^a MRS 5.6 - de Man, Rogosa and Sharpe agar, pH adjusted to 5.6; M17 agar - M17 agar with lactose; MRS 5.2 – pH adjusted to 5.2, VA - vancomycin.^b log₁₀ CFU/ml ± standard deviation of three replicates.^c anaerobic cultivations.^d not done.

were obtained for both strains of *L. plantarum* when cultivated on GA-MRS and VA-MRS. Also, the counts were not affected ($P > 0.05$) by inoculation techniques and therefore only the pour plate method was used in further analyses. Naghili et al. (2013) compared drop plate and spread plate methods for enumeration of bacteria and also found no significant difference between these two methods.

The counts of strains tested in mixtures after broth cultivation are summarized in Table 4.

Only *L. delbrueckii* subsp. *bulgaricus* strains were able to grow on MRS 5.2 at 45 °C, anaerobically. Tharmaraj and Shah (2003) found that MRS at pH 5.2 and incubation at 45 °C under anaerobic conditions were suitable for the selective assay of *L. delbrueckii* subsp. *bulgaricus* in the presence of *L. acidophilus*, *L. casei* and *L. rhamnosus*. As shown in Table 3, *L. plantarum* strains were able to grow on M17 agar at 37 °C (forming small colonies ~0.7 mm) but an increase in cultivation temperature to 45 °C stopped their growth. Under these conditions (45 °C, aerobically) on M17, *S. thermophilus* strains were able to be quantified selectively. On M17 agar at 37 °C and aerobic conditions, both *S. thermophilus* and *L. plantarum* were supported. In the study of Van de Castele et al. (2006), M17 agar was used for enumeration of *S. thermophilus* at 45 °C under aerobic conditions in combination with several lactobacilli and bifidobacteria species. Cardarelli, Buriti, Castrom, and Saad (2008) used a lower cultivation temperature (37 °C) for *S. thermophilus*, but for our mixture, this temperature was non-selective. VA-MRS agar was selective for *L. plantarum* at both temperatures (30 °C and 37 °C), where the other LAB species tested were suppressed by vancomycin. Furthermore, 30 °C was chosen in order to limit the growth of thermophilic yoghurt microorganisms. *L. delbrueckii* subsp. *bulgaricus* showed occasional and weak growth on GA-MRS, so this medium was omitted from further analysis.

Where a combination of *L. plantarum* and yoghurt culture was used for the production of fermented dairy products, selectivity and efficiency of the proposed media after cultivation of individual strains in milk were tested compared to the control medium (MRS 5.6).

Table 5 shows that the same counts were achieved on MRS 5.6 and VA-MRS (30 °C, aerobically) for *L. plantarum* and MRS 5.2 (45 °C, anaerobically) for *L. delbrueckii* subsp. *bulgaricus*. *S. thermophilus* did not grow on MRS 5.6 or MRS 5.2 at 45 °C, anaerobically. The selectivity of M17 at 45 °C aerobically was again confirmed for this microorganism.

The enumeration of each bacteria species in mixed cultures after

cultivation in milk is shown in Table 6. The results confirmed the previous findings concerning medium selectivity. The selectivity of each medium tested was checked by colony morphology of microorganism on plates and also by microscopy. No differences were observed in size, appearance or colour of the colonies of given species cultivated on the appropriate media. To confirm our findings, at least six colonies from each plate were checked by Gram staining and microscopy. Only cocci were formed on M17 agar, short rods (approx. length 3 µm) of *L. plantarum* on MRS agar with vancomycin and only long rods (approx. length 8 µm) of *L. delbrueckii* subsp. *bulgaricus* on MRS agar pH 5.2.

The selective enumeration of different *Lactobacillus* species in food matrices is difficult due to their similar growth characteristics. Several methods for selective enumeration have been already designed for *L. acidophilus*, *L. casei*, *L. rhamnosus* and *Bifidobacterium* in a mixture (de Carvalho Lima et al., 2009; Colombo et al., 2014; di Lena et al., 2015; Tabasco et al., 2007; Sakai et al., 2010). To our knowledge, this is the first report of a selective medium for quantification of *L. plantarum* in the presence of a yogurt culture. Previously, only one study dealing with a selective medium for *L. plantarum* in faecal samples had been published (Bujalance, Jiménez-Valera, Moreno, & Ruiz-Bravo, 2006).

4. Conclusions

Based on the results of our study, the pour plate method using MRS agar with vancomycin (20 mg/l, pH 5.6) and cultivation at 30 °C aerobically for 48 h can be recommended for selective enumeration of *L. plantarum* in a mixed yoghurt culture. In contrast to the standard method for enumeration of bacteria in a yoghurt culture, the cultivation temperature should be increased to 45 °C to preclude *L. plantarum* growth and to enumerate *L. delbrueckii* subsp. *bulgaricus* on MRS agar, pH 5.2 and *S. thermophilus* on M17 agar.

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Table 6

Counts ($\log_{10}$ CFU/ml) of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* in mixtures after milk cultivation using different culture media.

Microorganism	Medium ^a		
	M17, 45 °C aerobiosis	MRS 5.2, 45 °C anaerobiosis	VA-MRS, 30 °C aerobiosis
Mixture A			
<i>S. thermophilus</i> YC-381	8.14 ± 0.04 ^b	0 ^c	0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> YC-381	0	7.81 ± 0.05	0
<i>L. plantarum</i> 299v	0	0	8.10 ± 0.05
Mixture B			
<i>S. thermophilus</i> ATCC 19258	8.29 ± 0.01	0	0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	0	6.73 ± 0.04	0
<i>L. plantarum</i> ATCC 8014	0	0	7.99 ± 0.02
Mixture C			
<i>S. thermophilus</i> YC-381	8.03 ± 0.01	0	0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> YC-381	0	7.84 ± 0.03	0
<i>L. plantarum</i> ATCC 8014	0	0	7.94 ± 0.05
Mixture D			
<i>S. thermophilus</i> ATCC 19258	8.29 ± 0.02	0	0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	0	6.85 ± 0.03	0
<i>L. plantarum</i> 299v	0	0	8.04 ± 0.04

^a M17 agar - M17 agar with lactose; MRS 5.2 - de Man, Rogosa and Sharpe agar, pH adjusted to 5.2; VA - vancomycin.

^b $\log_{10}$ CFU/ml ± standard deviation of three replicates.

^c no growth on this medium.

References

- Anbukkarasi, K., UmaMaheswari, T., Hemelatha, T., Nanda, D. K., Singh, P., & Singh, R. (2014). Preparation of low galactose yogurt using cultures of *Gal+ Streptococcus thermophilus* in combination with *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Journal of Food Science and Technology*, 51, 2183–2189. <https://doi.org/10.1007/s13197-014-1262-5>.
- Ashraf, R., & Shah, N. P. (2011). Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt - a review. *International Journal of Food Microbiology*, 149, 194–208. <https://doi.org/10.1016/j.ijfoodmicro.2011.07.008>.
- Bengtsson, J., Adlerbert, I., Östblom, A., Sakseña, P., Öresland, T., & Börjesson, L. (2016). Effect of probiotics (*Lactobacillus plantarum* 299 plus *Bifidobacterium* Cure21) in patients with poor ileal pouch function: A randomised controlled trial. *Scandinavian Journal of Gastroenterology*, 51, 1087–1092. <https://doi.org/10.3109/00365521.2016.1161067>.
- Boyer, M., & Combrisson, J. (2013). Analytical opportunities of quantitative polymerase chain reaction in dairy microbiology. *International Dairy Journal*, 30, 45–52. <https://doi.org/10.1016/j.idairyj.2012.11.008>.
- Bujalance, C., Jiménez-Valera, M., Moreno, E., & Ruiz-Bravo, A. (2006). A selective differential medium for *Lactobacillus plantarum*. *Journal of Microbiological Methods*, 66, 572–575. <https://doi.org/10.1016/j.mimet.2006.02.005>.
- Butel, M. J. (2014). Probiotics, gut microbiota and health. *Medecine et Maladies Infectieuses*, 1–14. <https://doi.org/10.1016/j.medmal.2013.10.002>.
- Cardarelli, H. R., Buriti, F. C. A., Castrom, I. A., & Saad, M. I. (2008). Inulin and oligofructose improve sensory quality and increase the probiotic viable count in potentially synbiotic *petit-suisse* cheese. *LWT-Food Science and Technology*, 41, 1037–1046. <https://doi.org/10.1016/j.lwt.2007.07.001>.
- Colombo, M., de Oliveira, A. E. Z., de Carvalho, A. F., & Nero, L. A. (2014). Development of an alternative culture medium for the selective enumeration of *Lactobacillus casei* in fermented milk. *Food Microbiology*, 39, 89–95. <https://doi.org/10.1016/j.fm.2013.11.008>.
- de Carvalho Lima, K. G., Kruger, M. F., Behrens, J., Destro, M. T., Landgraf, M., & Franko, B. D. G. M. (2009). Evaluation of culture media for enumeration of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium animalis* in the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. *LWT- Food Science and Technology*, 42, 491–495. <https://doi.org/10.1016/j.lwt.2008.08.011>.
- Galat, A., Dufresne, J., Combrisson, J., Thépaut, J., Boumghar-Bourthai, L., Boyer, M., et al. (2016). Novel method based on chromogenic media for discrimination and selective enumeration of lactic acid bacteria in fermented milk products. *Food Microbiology*, 55, 86–94. <https://doi.org/10.1016/j.fm.2015.11.005>.
- Garcia-Gonzalez, N., Prete, R., Battista, N., & Corsetti, A. (2018). Adhesion properties of food-associated *Lactobacillus plantarum* strains on human intestinal epithelial cells and modulation of IL-8 release. *Frontiers in Microbiology*, 9, 2392, 1–11. <https://doi.org/10.3389/fmicb.2018.02392>.
- Granato, D., Branco, G. F., Cruz, A. G., Faria, J. de A. F., & Shah, N. P. (2010). Probiotic dairy products as functional foods. *Comprehensive Reviews in Food Science and Food Safety*, 9, 455–470. <https://doi.org/10.1111/j.1541-4337.2010.00120.x>.
- Hashemi, S. M. B., Khaneghah, A. M., Barba, F. J., Nemati, Z., Shokofiti, S. S., & Alizadeh, F. (2017). Fermented sweet lemon juice (*Citrus limetta*) using *Lactobacillus plantarum* LS5: Chemical composition, antioxidant and antibacterial activities. *Journal of Functional Foods*, 38, 409–414. <https://doi.org/10.1016/j.jff.2017.09.040>.
- Hou, J., Hannon, J. A., McSweeney, P. L. H., Beresford, T. P., & Guinee, T. P. (2017). Effect of galactose metabolizing and non-metabolising strains of *Streptococcus thermophilus* as a starter culture adjunct on the properties of Cheddar cheese made with low or high pH at whey drainage. *International Dairy Journal*, 65, 44–55. <https://doi.org/10.1016/j.idairyj.2016.10.003>.
- ISO (2003). *Yogurt - enumeration of characteristic microorganisms - colony-count technique at 37 degrees C*.
- ISO (2006). *Milk products - enumeration of presumptive Lactobacillus acidophilus on a selective medium - colony-count technique at 37 degrees C*.
- Jabbari, V., Khiabani, M. S., Mokarram, R. R., Hassanzadeh, A. M., Ahmadi, E., Gharenaghadeh, S., et al. (2017). *Lactobacillus plantarum* as a probiotic potential from Kouzeh cheese (Traditional Iranian Cheese) and its antimicrobial activity. *Probiotics and Antimicrobial Proteins*, 9, 189–193. <https://doi.org/10.1007/s12602-017-9255-0>.
- Karimi, R., Mortazavian, A. M., & Amiri-Rigi, A. (2012). Selective enumeration of probiotic microorganisms in cheese. *Food Microbiology*, 29, 1–9. <https://doi.org/10.1016/j.fm.2011.08.008>.
- Kerry, R. G., Patra, J. K., Gouda, S., Park, Y., Shin, H.-S., & Das, G. (2018). Benefaction of probiotics for human health: A review. *Journal of Food and Drug Analysis*, 26, 927–939. <https://doi.org/10.1016/j.jfda.2018.01.002>.
- Lankaputhra, W. E. V., & Shah, N. P. (1996). A simple method for selective enumeration of *Lactobacillus acidophilus* in yogurt supplemented with *L. acidophilus* and *Bifidobacterium* spp. *Milchwissenschaft*, 51, 446–450.
- di Lena, M., Quero, G. M., Santovito, E., Verran, J., De Angelis, M., & Fusco, V. (2015). A selective medium for isolation and accurate enumeration of *Lactobacillus casei*-group members in probiotic milks and dairy products. *International Dairy Journal*, 47, 27–36. <https://doi.org/10.1016/j.idairyj.2015.01.018>.
- Li, C., Song, J., Kwok, L., Wang, J., Dong, Y., Yu, H., et al. (2017). Influence of *Lactobacillus plantarum* on yogurt fermentation properties and subsequent changes during postfermentation storage. *Journal of Dairy Science*, 100, 2512–2525. <https://doi.org/10.3168/jds.2016-11864>.
- Liu, D.-M., Guo, J., Zeng, X.-A., Sun, D.-W., Brennan, C. S., Zhou, Q.-X., et al. (2017). The probiotic role of *Lactobacillus plantarum* in reducing risks associated with cardiovascular disease. *International Journal of Food Science and Technology*, 52, 127–136. <https://doi.org/10.1111/ijfs.13234>.
- Mortazavian, A. M., Ehsani, M. R., Sohrabvandi, S., & Reinheimer, J. A. (2007). MRS-bile agar: Its suitability for the enumeration of mixed probiotic cultures in cultured dairy products. *Milchwissenschaft*, 62, 270–272.
- Naghili, H., Tajik, H., Mardani, K., Rouhani, S., M.R., Ehsani, A., et al. (2013). Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests. *Veterinary Research Forum*, 4, 179–183.
- Oberg, C. J., Moyes, L. V., Domek, M. J., Brotherson, C., & McMahon, D. J. (2011). Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media. *Journal of Dairy Science*, 94, 2220–2230. <https://doi.org/10.3168/jds.2010-3934>.
- Ong, L., & Shah, N. P. (2009). Probiotic cheddar cheese: Influence of ripening temperatures on proteolysis and sensory characteristics of cheddar cheeses. *Journal of Food Science*, 74, S182–S191. <https://doi.org/10.1111/j.1750-3841.2009.01164.x>.
- Ravula, R. R., & Shah, N. P. (1998). Selective enumeration of *Lactobacillus casei* from yogurts and fermented milk drinks. *Biotechnology Techniques*, 12, 819–822.
- Russo, P., de Chiara, M. L. V., Capozzi, V., Arena, M. P., Amodio, M. L., Rascón, A., et al. (2016). *Lactobacillus plantarum* strains for multifunctional oat-based foods. *LWT-Food Science and Technology*, 68, 288–294. <https://doi.org/10.1016/j.lwt.2015.12.040>.
- Sakai, T., Oishi, K., Asahara, T., Takada, T., Yuki, N., Matsumoto, K., et al. (2010). M-RTLV agar, a novel selective medium to distinguish *Lactobacillus casei* and *Lactobacillus paracasei* from *Lactobacillus rhamnosus*. *International Journal of Food Microbiology*, 139, 154–160. <https://doi.org/10.1016/j.ijfoodmicro.2010.03.019>.
- Seddik, H. A., Bendali, F., Gancel, F., Fliss, I., Spano, G., & Drider, D. (2017). *Lactobacillus plantarum* and its probiotic and food potentialities. *Probiotics and Antimicrobial Proteins*, 9, 111–112. <https://doi.org/10.1007/s12602-017-9264-z>.
- Tabasco, R., Paarup, T., Janer, C., Peláez, C., & Requena, T. (2007). Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk. *International Dairy Journal*, 17, 1107–1114. <https://doi.org/10.1016/j.idairyj.2007.01.010>.
- Tharmaraj, N., & Shah, N. P. (2003). Selective enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, bifidobacteria, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and propionibacteria. *Journal of Dairy Science*, 86, 2288–2296. [https://doi.org/10.3168/jds.S0022-0302\(03\)73821-1](https://doi.org/10.3168/jds.S0022-0302(03)73821-1).
- Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, 9, 225–241. <https://doi.org/10.1016/j.jff.2014.04.030>.
- Van de Castele, S., Vanheuverzwijn, T., Ruysen, T., Assche, P. V., Swings, J., & Huys, G. (2006). Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. *International Dairy Journal*, 16, 1470–1476. <https://doi.org/10.1016/j.idairyj.2005.12.002>.
- de Vries, M. C., Vaughan, E. E., Kleerebezem, M., & de Vos, W. M. (2006). *Lactobacillus plantarum*—survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal*, 16, 1018–1028. <https://doi.org/10.1016/j.idairyj.2005.09.003>.