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Vliv probiotik a prebiotik na střevní mikrobiotu

Dizertační práce

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Obsah

1 Úvod

Vztahem člověka a jeho střevní mikrobioty se výzkumníci zabývali již v 19. století. S nástupem nových mikrobiologických a biochemických metod v první polovině století dvacátého tento zájem výrazně vzrostl, výzkumníci pozorovali bakterie ve výkalech i jinde, izolovali je, kultivovali a popisovali jejich biochemické vlastnosti. Již tehdy si, na základě učení Mečnikova a dalších, všímali vlivu výživy na střevní mikroby a popisovali jejich diverzitu.

Svůj prozatímní vrchol zažívá mikrobiologie posledních cca 30 let, kdy se do popředí dostaly metody molekulárně-genetické. V posledních letech se do popředí zájmu dostalo dělení střevní mikrobioty podle enterotypů, které není nepodobné dělení starému bezmála sto let, kdy měli vědci k dispozici bezesporu primitivnější metody. Dnes ovšem dovedeme charakterizovat i obtížně kultivovatelné bakterie, což podstatně prohlubuje naše znalosti střevní mikrobioty vzhledem k tomu, že v ní sídlí více než 1000 různých druhů bakterií. Vše tak nasvědčuje tomu, že se podstatně blížíme poznání všech mikrobů v našem střevě.

Teoretická část této dizertační práce je zaměřena na popis střevní mikrobioty v rámci nejnovějších poznatků, dále je zde popsán princip fungování probiotik a prebiotik, jednotlivá prebiotika jsou pak krátce charakterizována.

Experimentální část je složena ze souboru publikovaných prací, skládá se ze dvou prvoautorských článků, z toho jeden je přijat k publikaci v časopise s IF, druhý byl publikován v časopise v databázi SJR. Dále jsou přiloženy čtyři články z časopisů s IF, na kterých se autor dizertační práce podílel jako spoluautor a jeden spoluautorský článek z časopisu v databázi SJR.

Všechny přiložené články se zabývají probiotiky nebo prebiotiky, přičemž větší část je zaměřena na netradiční prebiotické či potenciálně prebiotické látky, jako jsou oligosacharidy mateřského mléka a oligosacharidy rafinosové řady. Hlavní článek je zaměřen na netradiční probiotickou bakterii rodu *Clostridium*.

Dizertační práce si klade za cíl přispět svým dílem k aktuálním znalostem vztahu probiotik, prebiotik a střevní mikrobioty tím, že se věnuje především méně známým zdrojům probiotik a prebiotik.

2 Současný stav problematiky

Střevní mikrobiota je komplexní ekosystém, který je tvořen řadou rozličných organismů. Tyto organismy, především bakterie, přeměňují nestravitelné zbytky potravy, tvoří některé vitaminy, metabolizují cizorodé látky, stimulují imunitu a rovněž tvoří přirozenou bariéru pro patogenní mikroorganismy, čímž ovlivňují zdravotní stav hostitele (Cummings and Macfarlane 1997; Guarner and Malagelada 2003; Nicholson et al. 2012). Mikroorganismy se nacházejí jak v lumenu střeva, tak ve slizké vrstvě přilehlé střevní stěně, která je nazývána mukosou. Mikrobiální populace těchto dvou nik jsou do určité míry odlišné a částečně na sobě nezávislé (Donaldson et al. 2016). Výživa má bezesporu velký vliv na jejich výskyt, avšak některé druhy bakterií jsou schopny přežívat nezávisle na přijaté potravě za pomoci živin, které jim poskytuje mukosní vrstva, jako třeba glykanů (Schluter and Foster 2012). Tímto představuje střevní mukosa, podobně jako třeba apendix, jistý rezervoár mikroorganismů (Donaldson et al. 2016). Význam střevní mikrobioty je dokonce tak velký, že ji někteří výzkumníci označují za samostatný orgán (O'Hara and Shanahan 2006).

2.1 Lidská střevní mikrobiota

Lidské střevo obsahuje přes 1000 různých druhů bakterií (Qin et al. 2010; Lozupone et al. 2012), které dohromady dosahují počtu až 10^{11} v jednom gramu stolice (Sender et al. 2016). Dále byly ve střevě nalezeny také archea[1], eukaryotní organismy, jako kvasinky a prvoci a rovněž nebuněční viry (fágy), jež často parazitují na přítomných bakteriích (Lagier et al. 2016; Bakhshinejad and Ghiasvand 2017; Reyes et al. 2010). Mikrobiom[2] v lidském střevě je dokonce tak rozmanitý, že jeho genom je 150krát větší než genom lidský (Lepage et al. 2013).

Sekvenační metody studia lidské mikrobioty ukazují, že většina bakterií v lidském střevě patří do dvou druhově rozsáhlých kmenů – Bacteroidetes a Firmicutes, přičemž kmen Bacteroidetes zahrnuje gramnegativní a Firmicutes grampozitivní rody bakterií. Dále se ve střevě pravidelně, ale v nižších počtech nacházejí zástupci kmenů Actinobacteria, Proteobacteria a Verrucomicrobia (Lozupone et al. 2012).

Je známo, že lidé se dají rozdělit na dvě skupiny podle tzv. enterotypů., což jsou vzájemně odlišitelné, nikoliv však zcela diskrétní klastry, které sdružují mikrobioty podobné druhové skladby (Arumugam et al. 2011; Knights et al. 2014). Podle Wu et al. (2011a) jsou enterotypy odvislé od dlouhodobých stravovacích návyků. Zatímco typ Bacteroides je spojený se zvýšenou konzumací proteinů a živočišného tuku, tak typ Prevotella zahrnuje převážně sacharolytické a mucinolytické bakterie, které jsou spojené s vysokým zastoupením sacharidů v dietě.

[1] Archea jsou organismy podobné bakteriím, dnes již víme, že jde o samostatnou doménu.

[2] Pojem mikrobiom se používá pro souhrn všech genů přítomných mikroorganismů. Některé práce tak nazývají i mikroby samotné, v této diertační práci ale bude pro skupinu mikrobů používán výhradně pojem „mikrobiota".

2.1.1 Ontogeneze lidské střevní mikrobioty

Střevní mikrobiota člověka se s věkem prokazatelně vyvíjí. Jsou známy studie, které tvrdí, že plod je kolonizován již v těle matky přes placentu, názory na tento jev jsou ovšem protichůdné (Milani et al. 2017), není ale sporu, že dítě je kolonizováno během porodu. Způsob porodu je prvním faktorem, který má zásadní vliv na vývoj střevní mikrobioty novorozence (Munyaka et al. 2014; Biasucci et al. 2010). Děti, porozené přirozeně jsou zpravidla osidlovány fekálními a vaginálními bakteriemi od matky, především rody *Bifidobacterium*, *Lactobacillus* a *Bacteroides* (Musilova et al. 2015b). Děti, přivedené na svět císařským řezem jsou oproti tomu nejprve kolonizovány bakteriemi z prostředí nemocnice nebo kůže matky či zdravotnických pracovníků a vykazují nižší počty rodů *Bifidobacterium* a *Bacteroides*, jejichž místo zaujímají klostridie, stafylokoky, nebo bakterie rodu *Acinetobacter* (Dominguez-Bello et al. 2010). Není zcela jasné, jak dlouho trvá normalizace tohoto stavu, studie se liší v odhadech od 6 měsíců (Gronlund et al. 1999), až do 7 let (Salminen et al. 2004). Předpokládá se, že narušení střevní mikrobioty při porodu císařským řezem má za následek nárůst výskytu jak autoimunních chorob, jako jsou diabetes 1. typu, Crohnova choroba nebo roztroušená skleróza, tak i alergických onemocnění, jako astma, alergická rýma nebo atopický ekzém (Okada et al. 2010; Bach 2002). Střevo novorozence obsahuje těsně po porodu kyslík, z toho důvodu dochází nejprve k pomnožení fakultativně anaerobních bakterií z čeledi Enterobacteriaceae či některých koků. Tyto bakterie rychle vytvoří redukované prostředí příznivé pro růst striktně anaerobních bifidobakterií, *Bacteroides* spp. i klostridií (Del Chierico et al. 2015).

Je nasnadě, že již od raného věku bude mít na složení střevní mikrobioty velký vliv výživa. Je dobře zdokumentováno, že kojení má pozitivní vliv nejen na samotný organismus kojence, ale i na jeho střevní mikroby a je známo, že děti, které jsou krmeny náhražkou mateřského mléka, mají odlišnou střevní mikrobiotu (O'Sullivan et al. 2015). Mateřské mléko je unikátní směsí živin a antimikrobiálních látek, která zabraňuje růstu některých potenciálně patogenních mikrobů a zároveň podporuje růst bifidobakterií. Hlavním faktorem podporujícím růst bifidobakterií jsou oligosacharidy mateřského mléka. Tyto unikátní látky jsou popsány v samostatné kapitole. Bylo vysledováno, že stolice kojených dětí obsahuje více laktobacilů a bifidobakterií a méně potenciálně patogenních bakterií než stolice dětí na umělé výživě (Guaraldi and Salvatori 2012).

Ustálení střevní mikrobioty se děje ve věku 2 až 3 let (Yatsunenko et al. 2012). Po odstavu a přechodu na konvenční stravu dochází ve střevě batolete ke kodominanci kmenů Firmicutes a Bacteroidetes a jeho mikrobiota se tak začíná podobat mikrobiotě dospělého člověka, začíná se zvyšovat množství butyrátu ve střevním obsahu a v mikrobiomu se objevují geny zodpovědné za rozklad složitých sacharidů, škrobu a cizorodých látek a výrobu vitaminů (Koenig et al. 2011). Mikrobiota dospělého člověka je tudíž více přizpůsobena pro rozklad nestravitelných rostlinných polysacharidů (Bergstrom et al. 2014). Příjem nestravitelných sacharidů má dozajista příznivý vliv na střevní mikrobiotu (Flint et al. 2012; Valdes et al. 2018).

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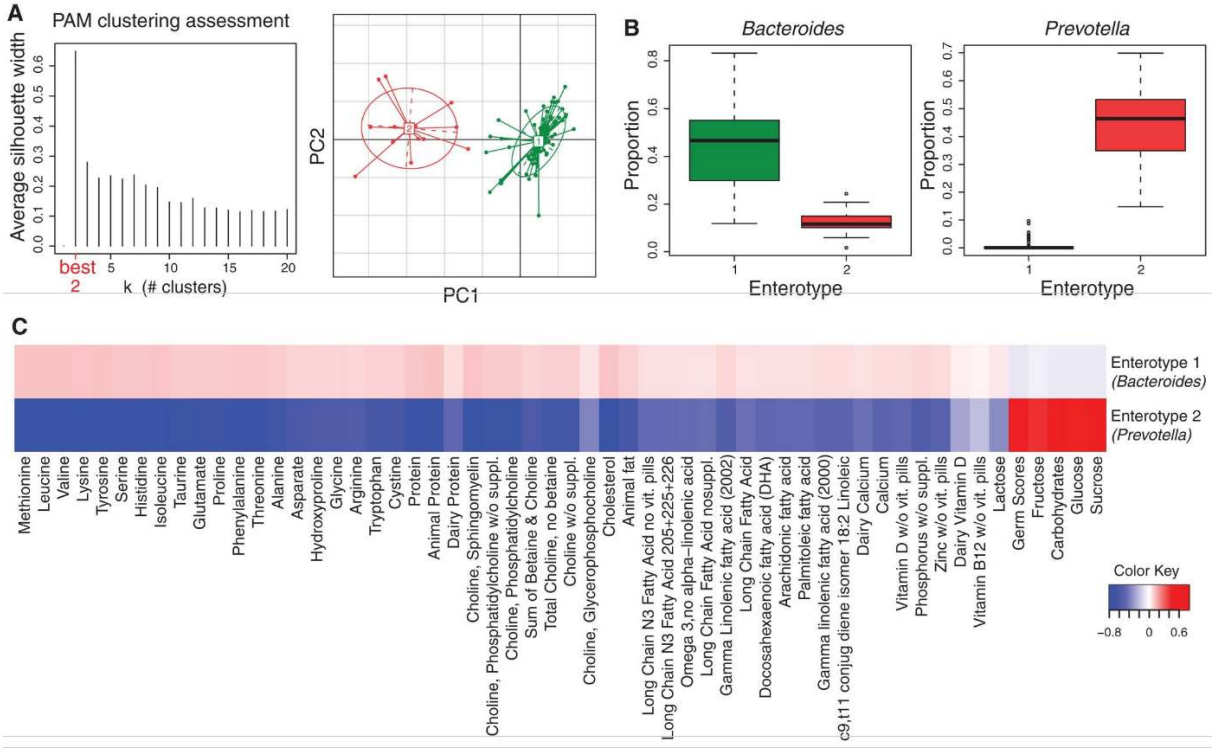
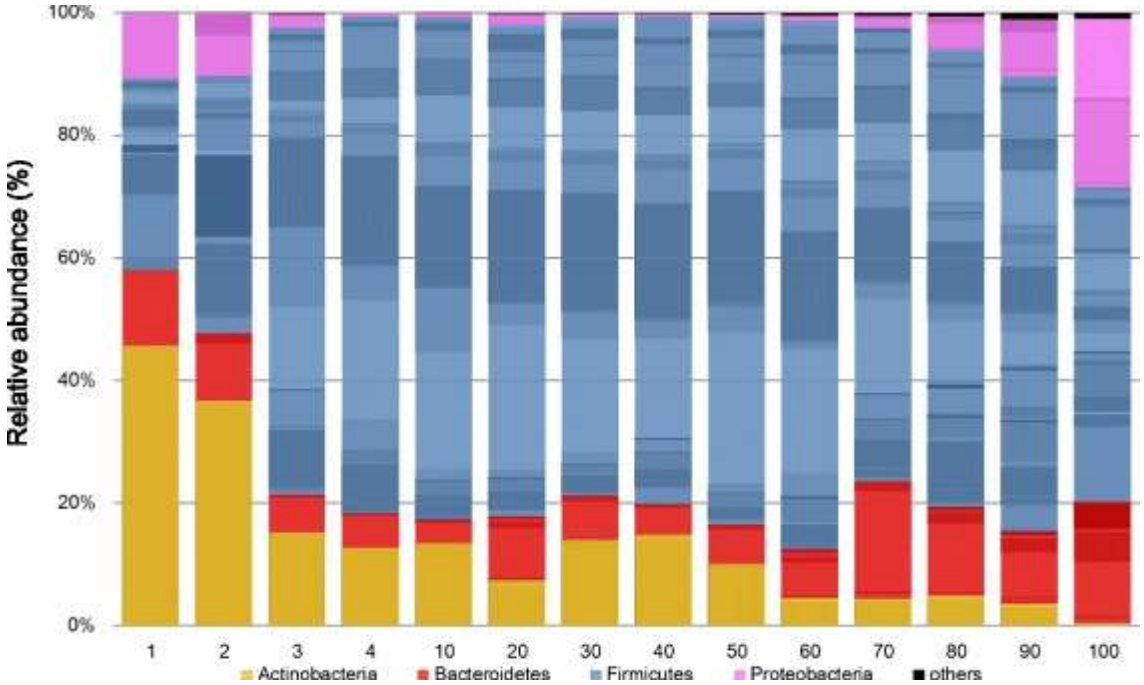
Enterotypy, které byly zmíněny v předchozí kapitole, se zdají být přímo návazné na dlouhodobé stravovací návyky. Zatímco konzumace živočišného tuku a bílkovin, obsahujících širokou škálu aminokyselin typická pro tzv. západní dietu, je spojena s enterotypem Bacteroides, tak enterotyp Prevotella se nachází u jedinců, kteří dlouhodobě přijímají hlavně rostlinnou stravu s vysokým obsahem různých poly-, oligo- i monosacharidů (Obr. 1). Tento enterotyp je tedy zastoupen mezi vegetariány, vegany a u obyvatel rozvojových zemí, kde není tak vysoká spotřeba živočišných produktů, jako ve vyspělých státech západní Evropy a Severní Ameriky (Wu et al. 2011a). Myšlenka enterotypů nicméně není úplně nová. Již v první polovině 20. století Dudgeon (1926) popsal tři typy mikrobioty – sacharolytickou, proteolytickou a normální a komentoval i vliv stravy na toto dělení.



Obr. 1: Shluková analýza bakteriálních taxonů rozdělených do enterotypů Bacteroides a Prevotella ve vztahu k dietě (Wu et al. 2011a).

Typ Bacteroides je spojován s příjmem živočišného proteinu, aminokyselin a živočišného tuku, zatímco typ Prevotella má silnou pozitivní korelaci s rostlinnými sacharidy.

Stárnutí člověka se, jako na všech orgánech, projevuje i na střevní mikrobiotě. Odamaki et al. (2016) tvrdí, že stárnutí mikrobioty se začíná projevovat po dosažení 70 let života, kdy dochází k poklesu počtu bifidobakterií, a naopak k nárůstu klostridií a bakterií z kmene Proteobacteria. Podle Kim and Jazwinski (2018) dochází během stárnutí k přirozenému úbytku diverzity mikrobiální populace ve střevě. Následkem toho dochází k narušení rovnováhy, což může snadno vyústit v dysbiosu, která může pro změnu iniciovat zánětlivou odpověď ve střevě nebo narušit komunikaci střevní mikrobioty s hostitelem. Celkovým výsledkem pak může být zhoršení zdravotního stavu.



Obr. 2: Změny relativní četnosti základních skupin mikroorganismů vyskytujících se v tlustém střevě v průběhu stárnutí (Odamaki et al. 2016)

2.1.2 Lidská střevní mikrobiota ve zdraví a nemoci

Člověk koexistuje se střevní mikrobiotou v mutualistickém svazku, někdy je ale toto spojení narušeno a přechází v negativní vztah, jako je to mu při obezitě, diabetu, ateroskleróze nebo zánětlivých střevních onemocněních (Hooper and Gordon 2001).

V předchozí kapitole bylo pojednáno o vývoji střevní mikrobioty a vlivu vnějších faktorů na její složení. Jaké složení je ale správné? Na tuto otázku nelze jednoznačně odpovědět, střevní mikrobiota je velice komplexní a zároveň variabilní v závislosti na vnějších podmínkách. I přesto známe jisté druhy bakterií, které jsou brány jako ukazatelé zdravé střevní mikrobioty. Mezi ně dozajista patří bakterie rodu *Bifidobacterium*, které mají prokazatelně příznivé účinky na střevní rovnováhu zejména u malých dětí (Fijan 2014). Naproti tomu u rodu *Clostridium* není zařazení jednoznačné. Existují klostridie patogenní, oportunně patogenní, komensální, ale i probiotické (Lopetuso et al. 2013).

Kromě výživy, která byla diskutována v předchozí kapitole, mají na střevní mikrobiotu vliv i další individuální faktory, jako třeba Body Mass Index (BMI). Jakkoli je BMI relativně nedostatečným ukazatelem tělesné kondice člověka (Rothman 2008), tak byly provedeny studie, které poukazují na skutečnost, že normální BMI přispívá k vyšší diverzitě střevní mikrobioty u dětí a že obézní děti mají ve střevě vyšší přítomnost bakterií z čeledi *Enterobacteriaceae* nebo šířeji proteobakterií (Karlsson et al. 2012; Bai et al. 2019), přičemž zvýšený výskyt některých zástupců proteobakterií je u lidí spojován se střevní dysbiosou (Shin et al. 2015). Podobné změny ve složení mikrobioty způsobuje i podváha. Borgo et al. (2017) zjistili u skupiny anorektických pacientů, že jejich mikrobiota obsahovala vyšší počty zástupců čeledi *Enterobacteriaceae*, zatímco ve střevech běžné rody *Roseburia*, *Ruminococcus* a *Clostridium* vykazovaly nižší počty. Je možné, že vztah mezi střevní mikrobiotou a nesprávnou tělesnou hmotností je oboustranný. Bakterie totiž ve střevech produkují mimo jiné mastné kyseliny s krátkým řetězcem (SCFA), z nichž některé slouží jako zdroj energie a tím zvyšují

příjem energie metabolismem hostitele. Kromě toho je pravděpodobné, že SFCA hrají roli jako signální molekuly, které mohou ovlivňovat chuť k jídlu i insulinovou senzitivitu (Canfora et al. 2015). Střevní dysbiosa tak teoreticky může být jak příčinou, tak důsledkem změny tělesné hmotnosti.

Změny střevní mikrobioty jsou dobře popsány u různých střevních onemocnění. U lidí se syndromem dráždivého tračníku bylo vysledováno, že jejich stolice obsahuje méně laktobacilů a některých zástupců kmene Actinobacteria, mj. bifidobakterií, a naopak zvýšené množství proteobakterií (Krogius-Kurikka et al. 2009; Bhattarai et al. 2017). Crohnova choroba je charakteristická sníženým výskytem *Faecalibacterium prausnitzii*, významného producenta kyseliny máselné (Machiels et al. 2014; Sokol et al. 2008). Zároveň bylo pozorováno, že suplementace *F. prausnitzii* lidem trpícím Crohnovou chorobou má za následek zmírnění příznaků tohoto onemocnění. Stejně jako u některých dalších střevních onemocnění ale zatím není jasné, zda je dysbióza příčinou nebo následkem Crohnovy choroby (Rinninella et al. 2019). Snížení počtů bakterií, které jsou obecně považovány za zdraví prospěšné, jako laktobacily, bifidobakterie nebo *F. prausnitzii*, bylo pozorováno i u celiakie (Nadal et al. 2007; Collado et al. 2009). Pokles počtu butyrát produkujících bakterií je charakteristický i pro mikrobiotu pacientů s kolorektálním karcinomem, vyšších počtů naopak dosahují některé proteobakterie a někteří zástupci kmene Firmicutes, často jde o oportunní patogeny (Wang et al. 2012). Opět je ovšem třeba brát v úvahu, že tyto změny mohou být důsledkem kolorektálního karcinomu, ačkoli některé výzkumy poukazují na úzkou spojitost rozvoje kolorektálního karcinomu s výskytem *Fusobacterium* spp. (Kostic et al. 2012; Yang and Ji 2019).

Nejnovější vědecké poznatky ukazují, že střevní mikrobiota má zřejmě značný vliv na vývoj některých částí nervové soustavy díky takzvané ose střevo – mozek, což je spojení mezi centrální nervovou soustavou, střevní nervovou soustavou a trávicím ústrojím, díky kterému mohou střevní bakterie ovlivňovat celou řadu neurologických procesů prostřednictvím svých metabolitů (Mayer et al. 2015). Změny ve složení střevní mikrobioty byly pozorovány u pacientů s Alzheimerovou a Parkinsonovou chorobou a u dětí s poruchami autistického spektra. V případě Alzheimerovy choroby a poruch autistického spektra jsou u sledovaných subjektů pozorovány snížené počty bifidobakterií a některých zástupců kmene Firmicutes (Vogt et al. 2017; De Angelis et al. 2013).

Z výše citované literatury vyplývá, že některé nemoci jsou provázeny charakteristickými změnami ve střevní mikrobiotě. Které bakterie jsou naopak indikátory zdravé střevní mikrobioty? Ačkoli je střevní mikrobiota komplexní systém, ve kterém dochází k různorodým interakcím mezi jednotlivými druhy bakterií, dá se říci, že počty bifidobakterií a butyrát produkujících bakterií jako *F. prausnitzii*, *Eubacterium* spp. a *Roseburia* spp. jsou v negativní korelaci s výskytem zejména střevních onemocnění, je tedy možno s určitou opatrností říci, že tyto bakterie patří mezi indikátory zdravé střevní mikrobioty (Rivière et al. 2016).

2.2 Střevní mikrobiota drůbeže

Střevní mikrobiota drůbeže má některé aspekty shodné s lidskou střevní mikrobiotou, rozdíly jsou ovšem ve stavbě trávicí soustavy, která se, společně s výživou, promítá na jejím složení. Dominantní skupinou v trávicím traktu jsou laktobacily, které převažují především ve voleti a tenkém střevě (Lu et al. 2003), v tlustém a především slepém střevě je pak mikrobiota více různorodá, z hlediska zastoupení kmenů je dominantně zastoupen kmen Firmicutes, následovaný Bacteroidetes (Oakley et al. 2014). Pro člověka jsou významnými patogenní rody *Salmonella* a *Campylobacter*, jichž je drůbež významným rezervoárem (Shang et al. 2018).

2.3 Střevní mikrobiota prasat

Trávicí trakt prasete je v mnohém podobný lidskému trávicímu traktu, ať už z hlediska anatomie, fyziologie nebo imunologie (Wang and Donovan 2015). Prasečí duodenum a jejunum jsou osídleny zejména laktobacily a klostridiemi, zatímco tlusté střevo je dominantně osídleno rodem *Prevotella* (Crespo-Piazuelo et al. 2018).

2.4 Modely studia střevní mikrobioty

2.4.1 Gnotobiotické modely střevní mikrobioty

Jednou z nejlepších metod, jak studovat vliv výživy na střevní mikrobiotu a následný vliv mikrobioty na zdraví jedince, je pomocí gnotobiontů – zvířat s definovanou mikrobiotou. Gnotobionti se rodí jako bezmikrobní v přísně sterilních podmínkách. Kromě mikrobů chybí těmto zvířatům i imunitní odpověď, kterou mikroby přirozeně vyvolávají a také mají bezmikrobní zvířata pozměněné určité orgány, jako třeba větší cékum nebo pomalejší bazální metabolismus (Koopman 1998). Na gnotobiontech se studuje interakce mikrobioty s hostitelem. Gnotobiont je buď osídlený jedním určitým mikrobiálním kmenem (monoasociovaný gnotobiont), nebo celým komplexem mikrobů, jako je tomu například v případě humanizované myši, která je osídlena fekálním transplantátem od člověka. Tyto modely prokázaly, že mikrobiota je nezbytná pro dozrání imunitního systému, fyziologie zažívacího traktu a střevního nervového systému (Neumann et al. 2019). Vzhledem k tomu, že gnotobiotické modely jsou velmi náročné a nákladné (Lange et al. 2019), jsou vyvíjeny různé typy *in vitro* modelů.

2.4.2 *In vitro* modely pro studium střevní mikrobioty

Pro *in vitro* studium interakce mezi střevní mikrobiotou a hostitelskými buňkami jsou nejčastěji využívány buněčné linie získané z lidského kolorektálního karcinomu, jako třeba Caco-2 nebo HT-29. Jde o velké nediferenciované lidské buňky, které se spontánně množí a které napodobují enterocyty epitelu tenkého střeva. Caco-2 buňky se využívají například pro studium protektivních efektů probiotických bakterií vůči zánětům nebo pro studium vlivu různých molekul na adhezi bakterií na buněčné linie (Pearce et al. 2018; Volštátová et al.

2015). Buněčné linie HT-29 obsahují gobletovy buňky produkující mukosu, čehož bylo využito například při studiu vlivu oligosacharidů na adhezi různých bakterií na mukosní vrstvu (Altamimi et al. 2016). Kombinace Caco-2 a HT-29 buněčných linií byla použita například pro studium protektivních vlastností probiotik vůči patogenním bakteriím (Resta-Lenert and Barrett 2003).

In vitro modely jsou vhodné pro výzkum základních vztahů mezi jedním bakteriálním druhem a hostitelskými buňkami, jsou relativně levné a nenáročné na použití. Nenapodobují ale dostatečně buněčnou diverzitu střevního epitelu (Pearce et al. 2018).

2.4.3 *Ex vivo* modely pro studium střevní mikrobioty

Jedná se v podstatě o *in vitro* systémy, které mají napodobit trávicí soustavu a její pufrující a absorpční vlastnosti. Jsou koncipovány tak, že pracují s inokulem střevní mikrobioty. TNO model TIM-2 dovede sledovat produkci metabolitů, mechanismy rozkladu živin, metabolismus biologicky aktivních látek a také pracovat s izotopově značenými substráty (Venema 2015). TNO InTESTine™ model je navíc obohacen o komplexní tkáňový systém napodobující *in vivo* prostředí (Pearce et al. 2018).

2.5 Probiotika

FAO/WHO definuje probiotika jako živé mikroorganismy, které, jsou-li zkonzumovány v dostatečném množství, mají příznivý vliv na zdraví hostitele. Jedná se o bakteriální kmeny, které jsou taxonomicky jasně definované a jejichž příznivé vlastnosti na zdraví hostitele byly dostatečně ověřeny vědeckými studiemi. Probiotika musí být bezpečná, nesmí obsahovat virulentní faktory nebo být nositeli genů kódujících rezistenci vůči antibiotikům. Nejedná se o živé kultury tradičně využívané při fermentaci potravin. Fekální transplantáty také nejsou považovány za probiotika (Hill et al. 2014; Guarner and Malagelada 2003). I přes mnoho provedených studií Evropská unie stále nepovoluje používání výrazu probiotikum pro kterýkoli mikroorganismus používaný v lidské výživě (Salminen and van Loveren 2012), probiotika pro zvířata jsou vedena jako krmná aditiva (Anadon et al. 2006).

„Otcem“ konceptu probiotik byl Ilja Mečnikov, který nemalou část svého výzkumu zaměřil na studium příčin stárnutí a zdraví člověka a vytvořil teorii podle které je stárnutí důsledkem toxických bakterií ve střevě, jejichž produkty otravují organismus. Proti působení těchto škodlivých mikrobů doporučoval pít kyselé mléko obsahující bakterie mléčného kvašení (Rettger 1915).

2.5.1 Mechanismus účinku probiotik

Probiotické bakterie působí na lidské zdraví různými mechanismy, které jsou druhově či dokonce kmenově specifické. Každá probiotická bakterie musí mít vědecky dokázaný mechanismus účinku (Guarner and Malagelada 2003).

Střevo zdravého člověka je osídleno vyváženou populací střevních mikrobů, která se brání zásahům z vnějšku, proto je velmi obtížné podáváním probiotik zdravému jedinci docílit

jakékoli změny. Naopak, pokud je střevní mikrobiota narušena zánětlivým onemocněním nebo antibiotickou léčbou, využití probiotik je smysluplné (Sanders et al. 2018).

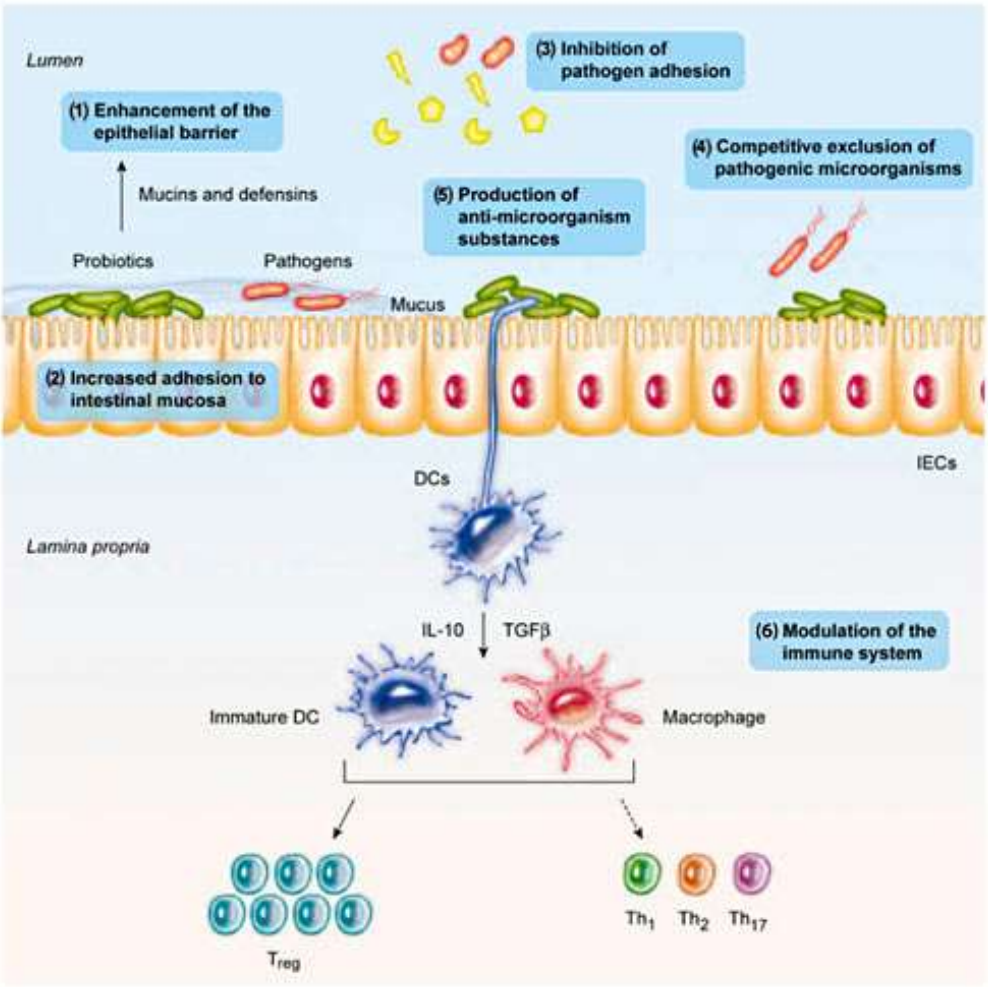
Jednou z předpokládaných schopností probiotik je posílení bariérového efektu, což je komplexní systém složený z mukózní vrstvy, antimikrobiálních peptidů, imunoglobulinů a spojů mezi střevními buňkami (Hooper and Gordon 2001). Je-li tato bariéra narušena, potenciálně patogenní bakterie a potravní antigeny mohou přijít do kontaktu se submukosní vrstvou a tam indukovat zánětlivou odpověď, která může vyústit až v zánětlivé onemocnění střeva (Sartor 2008).

Probiotické bakterie by měly být schopny adherovat na střevní mukózní vrstvu. Tato schopnost je důležitá pro zesílení imunitního systému (Schiffrin and Blum 2002) a rovněž kvůli efektu takzvané kompetitivní exkluze, při kterém dochází k antagonistickému působení adherovaných probiotických bakterií proti případné kolonizaci nežádoucími bakteriemi. Probiotická bakterie zabraňuje kolonizaci patogenu různými způsoby – vytvořením nevhodných podmínek produkcí metabolitů a antimikrobiálních látek, zablokováním vazebného místa na epitelu nebo vyčerpáním esenciálních živin (Nurmi et al. 1992).

Další typickou vlastností pro probiotické bakterie je produkce antimikrobiálních látek, ať už jde o mastné kyseliny s krátkým řetězcem (kyselina octová, kyselina mléčná), které mají silný inhibiční účinek na gramnegativní bakterie nebo antimikrobiální peptidy (např. nisin), které mají užší spektrum účinku. Častým mechanismem účinku antimikrobiálních látek produkovaných probiotiky je narušení buněčné stěny konkurenční bakterie (Russell and Diez-Gonzalez 1998; Bierbaum and Sahl 2009).

Je známo, že probiotické bakterie mají schopnost stimulovat imunitní systém hostitele. Probiotické bakterie jsou schopné interagovat s různými typy receptorů ať už zmírňováním přehnané imunitní odpovědi vedoucí k zánětlivému onemocnění nebo naopak aktivací některých receptorů (Lebeer et al. 2010).

Mezi probiotické bakterie patří zástupci následujících rodů: Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Bacillus, Escherichia coli a Clostridium. Probiotické jsou rovněž některé kvasinky rodů Saccharomyces a Kluyveromyces a také plísně rodu Aspergillus (Fijan 2014; Gaggia et al. 2010). V dnešní době se probiotika zpravidla podávají ve formě kombinace více různých kmenů.



Obr. 3: Mechanismus účinku probiotik ve střevě (Bermudez-Brito et al. 2012).

2.5.2 Bifidobakterie jako probiotické bakterie

Bifidobakterie jsou typickými obyvateli trávicího traktu lidí i zvířat, v nejvyšších počtech se vyskytují u malých dětí, u kterých patří mezi první kolonizátory jejich trávicího traktu. Jejich přítomnost je spojována s dobrým zdravotním stavem střeva (Rada et al. 2006), absence bifidobakterií naopak předznamenává hrozící výskyt potravinových alergií a atopického ekzému (Isolauri et al. 2012). Bifidobakterie jsou sacharolytické, jejich metabolismus produkuje kyselinu octovou a kyselinu mléčnou jako konečné metabolity v poměru 3:2, v malém množství mohou produkovat i etanol a kyselinu mravenčí (Amaretti et al. 2007). Typickou vlastností čeledi Bifidobacteriaceae je specifická metabolická dráha, pomocí které štěpí cukry. Klíčovým enzymem je fruktoso-6-fosfát fosfoketolasa (F6PPK), která štěpí z hexosy vytvořený fruktoso-6-fosfát na acetylfosfát a erytrosu-4-fosfát. Ačkoli jsou bifidobakterie schopny štěpit jednoduché cukry, tak je o nich známo, že preferují rozklad oligosacharidů (Ventura et al. 2007).

U bifidobakterií byla popsána schopnost potlačovat růst celé řady patogenů, jako třeba *E. coli*, salmonel, rotavirů, *Listeria monocytogenes* nebo *Helicobacter pylori* (Fujiwara et al. 2001; Chenoll et al. 2011; Moreno Muñoz et al. 2011), například pomocí produkce

bakteriocinů nebo mastných kyselin (O'Shea et al. 2012; Lievin et al. 2000). Bylo pozorováno, že podávání *Bifidobacterium longum* ssp. *infantis* zmírňovalo příznaky syndromu dráždivého tračníku u pacientů s tímto onemocněním (O'Mahony et al. 2005; Whorwell et al. 2006). *B. bifidum* v kombinaci se *Streptococcus thermophilus* snížilo výskyt akutních průjmů u kojenců (Saavedra et al. 1994). Podávání bifidobakterií se rovněž osvědčilo při snižování mortality u nekrotizujících enterokolitid u předčasně narozených dětí (Deshpande et al. 2010).

2.5.3 Laktobacily jako probiotické bakterie

Nejvíce kmenů s popsanými probiotickými účinky patří do rodu *Lactobacillus*. Mezi nejčastěji používané patří *L. casei*, *L. rhamnosus* a *L. acidophilus*, ale i mnohé další. Byl proveden nespočet studií na téma laktobacilů a jejich vlivu na zdraví, kupříkladu Olivares et al. (2006) tvrdí, že laktobacily stimulují vrozenou imunitní odpověď u dětí, některé druhy laktobacilů mají zase schopnost posilovat střevní bariéru (Martín et al. 2019; Schepper et al. 2019).

2.5.4 Klostridie jako probiotické bakterie

Klostridie jsou známy jako hojně rozšířené sporulující tyčinky, vyskytující se přirozeně v půdě a trávicím traktu lidí i zvířat. Jsou významnými producenty kyseliny máselné, která je ve střevě důležitá, jako zdroj energie pro kolonocyty (Pryde et al. 2002). Ačkoli rod *Clostridium* zahrnuje některé patogenní druhy, většina klostridií je komensální a patří mezi významné bakterie tlustého střeva, kde se podílejí na udržení střevní homeostázy (Lopetuso et al. 2013). Jsou ovšem známy i klostridie, u kterých byly popsány probiotické vlastnosti. *Clostridium butyricum* CBM 588 snížilo výskyt průjmů způsobených antibiotiky u dětí (Seki et al. 2003). Příznivý vliv na střevní mikrobiotu přisuzuje CBM 588 i výzkum Kuroiwa et al. (1990), podle kterých dokázal zmíněný kmen potlačit produkci toxinů *Clostridium difficile* u pacientů, kteří podstoupili antimikrobiální terapii.

2.5.5 Probiotika ve výživě zvířat

Probiotika mají široké využití i ve výživě zvířat ať už chovaných pro živočišnou produkci, tak i v hobby chovech. Důvodem je jednak stabilizace střevní mikrobioty zvířete mechanismy, které byly popsány v kapitole 2.5.1. U zvířat chovaných pro živočišnou produkci jsou ale probiotika podávána i z důvodu zvýšení využitelnosti živin a tím dosažení vyšších výnosů (Bajagai et al. 2016). Modulace střevní mikrobioty má i u zvířat dlouhou tradici. Nurmi and Rantala (1973) dokázali popsat princip tzv. kompetitivní exkluze neboli perorálního podání trusu od zdravého dospělce, kterým se zabrání salmonelové infekci čerstvě vylíhlých kuřat. V dnešní době se ve výživě zvířat využívá široké spektrum probiotik, často jde o preparáty, které obsahují kombinaci různých kmenů bakterií, což je považováno za výhodnější z hlediska synergického působení obsažených bakterií (Timmerman et al. 2005). Někteří výzkumníci doporučují, aby podávaný probiotický kmen pocházel ze stejného živočišného druhu vzhledem

k tomu, že účinky probiotik mohou být hostitelsky specifické (Gardiner et al. 2004; Vemuri et al. 2018).

2.6 Prebiotika

V předchozích kapitolách bylo zmíněno, že střevní mikrobiota je zásadním způsobem ovlivněna výživou. Dlouhodobé stravovací návyky ovlivňují druhovou skladbu střevních mikrobů. Nestravitelné látky z potravy se dostávají do dolních částí trávicího traktu, kde jsou metabolizovány přítomnými bakteriemi. Šířeji se tyto látky řadí mezi potravní vlákninu. Pokud ale selektivně podporují mikroorganismy, které mají příznivý vliv na zdraví hostitele, nazývají se prebiotika. Většina prebiotik je sacharidové povahy, avšak definici prebiotik mohou naplnit i určité polyfenoly nebo konjugované mastné kyseliny (Gibson et al. 2017). Výzkumné práce přisuzují jak potravní vláknině, tak prebiotikům mnohé účinky na zdraví; namátkou jde o modulaci imunity, ochranu před rakovinou tlustého střeva, zkrácení doby průjmového onemocnění, omezení adheze patogenů na střevní sliznici, snížení krevního cholesterolu, zvýšení absorpce minerálních látek a v neposlední řadě stabilizaci střevní mikrobioty (Tuohy et al. 2015). Bylo prokázáno, že lidé, kteří přijímají vysoké množství vlákniny v potravě, mají vyšší množství butyrátu a vyšší počty bifidobakterií a laktobacilů ve střevě. Rozdíly v počtech bifidobakterií a laktobacilů jsou ještě markantnější při konzumaci prebiotik (So et al. 2018). Stejně jako v případě probiotik, ani prebiotika nejsou prozatím oficiálně uznávána Evropskou unií (Salminen and van Loveren 2012).

2.6.1 Fruktany

Mezi fruktanová prebiotika patří inulin, oligofruktosa a fruktooligosacharidy (FOS). Chemicky jde o oligo- a polymery fruktosy propojené vazbou β(2-1) a většinou zakončené molekulou glukosy, která je napojena vazbou α(1-2). Pokud glukosu na konci neobsahují, jde o tzv. F_n fruktany, pokud ji obsahují, tak jsou to tzv. GF_n fruktany[3]. Inulin je polysacharid se stupněm polymerace (DP) 20 – 60, oligofruktosa a fruktooligosacharidy mají DP 2 – 20, nicméně nomenklatura je v této oblasti značně nejednotná a můžeme se setkat s publikacemi, ve kterých autoři tyto názvy zaměňují (Kelly 2008). Inulin a FOS jsou přírodní sacharidy, jejichž hlavními zdroji jsou kořen čekanky (*Cichorium intybus*), hlíza topinamburu (*Helianthus tuberosus*), česnek a cibule. Dále je možno připravit oligofruktosu enzymatickým naštěpením inulinu za vzniku produktu od DP 2 –20. Vyrábí se i syntetické FOS o DP 2 – 4 transfruktosylací sacharózy (Carlson et al. 2018).

Vzhledem ke strukturní různorodosti fruktanů se liší i jejich probiotické vlastnosti. Van de Wiele et al. (2007) pozorovali *in vitro*, že fermentace inulinu vynesla více SCFA, než oligofruktosy, pravděpodobně díky tomu, že delší řetězec inulinu dosahoval i distálních částí modelu střeva. Mnoho studií prokázalo schopnost fruktanů podporovat růst bifidobakterií ve střevě člověka. Gibson et al. (1995) pozorovali významný nárůst bifidobakterií a pokles

[3] „n“ v dolním indexu značí počet fruktosových jednotek.

fusobakterií, klostridií a *Bacteroides* spp. u lidí konzumujících 15 g FOS denně po dobu 45 dní. Podávání 8 gramů oligofruktosy denně po dobu pěti týdnů taktéž zvýšil počty bifidobakterií; počty laktobacilů, *Bacteroides* spp., koliformů a *C. perfringens* zůstaly nezměněny (Menne et al. 2000). Vysoké dávky inulinu 20 g/den po dobu osmi dnů a následně 7 dní 40 g/den měly za následek nárůst počtů bifidobakterií a pokles enterokoků a bakterií z čeledi *Enterobacteriaceae* u starších pacientů se zácpou. Navíc došlo ke zmírnění příznaků zácpy. Klostridie, *Bacteroides* spp. a *F. prausnitzii* zůstaly nezměněny (Kleessen et al. 1997). Některé studie ale poukazují na nedostatečnou selektivitu fruktanů. Euler et al. (2005) přidávali FOS do umělé výživy, kde ale měly zanedbatelný vliv na střevní mikrobiotu kojenců.

Fruktany nacházejí i uplatnění v potravinářství. Inulin se využívá jako plnidlo a díky své schopnosti tvořit s vodou tuku podobnou texturu i jako náhražka tuku. Nízkomolekulární oligofruktosa a FOS se pak dají využívat jako nízkoenergetické náhražky cukru se sladivostí 30 – 50 % sladivosti cukru (Niness 1999).

2.6.2 Galaktooligosacharidy

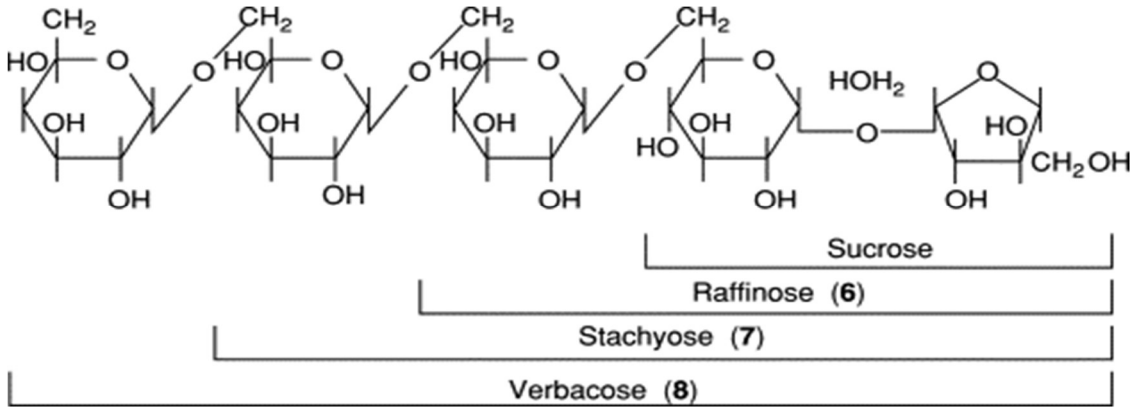
Jedná se o směs oligosacharidů, které jsou enzymaticky vyráběny transgalaktosylací laktosy. Vzniklé produkty mají DP 2 – 10. Galaktooligosacharidy (GOS) se přidávají ve směsi s FOS v poměru 9:1 do mléčných náhražek jako prebiotická složka. Bylo prokázáno, že přídavek této směsi do mléčné náhražky v množství 8 – 10 g/l vede ke zvýšení počtů bifidobakterií ve stolici kojenců, čímž přibližuje mikrobiotu takto živených dětí mikrobiotě kojených dětí (Boehm et al. 2005; Haarman and Knol 2005). Bouhnik et al. (1997) pozorovali nárůst počtů bifidobakterií při podávání GOS samostatně, počty enterobakterií se nezměnily. Alles et al. (1999) zkoumali vliv GOS na mikrobiotu dospělců, avšak nenašli ve stolici žádný významný rozdíl v počtu bifidobakterií v porovnání s placebem, ani změny důležitých biochemických ukazatelů jako SCFA nebo amoniaku. Oproti tomu, směs GOS a maltodextrinu v poměru 9:1 vedla při 5denním podávání ke zvýšení bifidobakterií ve stolici dospělců a naopak ke snížení počtů *E. coli* (Musilova et al. 2015a). Vulevic et al. (2013) podávali GOS lidem s nadváhou a metabolickým syndromem. Kromě zvýšení počtů bifidobakterií a snížení počtů gramnegativních bakterií byly u pokusné skupiny potlačeny i některé biochemické markery metabolickému syndromu.

2.6.3 Oligosacharidy rafinosové řady

Tyto oligosacharidy patří spolu s fruktany rovněž mezi přírodní látky. Oligosacharidy rafinosové řady (RSO) je název pro skupinu strukturně podobných oligosacharidů, které jsou charakteristické přítomností α(1-6) vazby mezi galaktosovými jednotkami napojenými α(1-6) vazbou na molekulu sacharózy. RSO se v odborné literatuře objevují pod různými označeními, jako α-galaktosidy, sójové oligosacharidy, nebo galactosyl-sucrose oligosaccharides[4]. Skupina RSO zahrnuje trisacharid rafinosu, tetrasacharid stachyosu a pentasacharid verbaskosu (van

[4] Pojem galactosyl-sucrose oligosaccharides nemá, na rozdíl od předchozích, český překlad.

der Riet et al. 1989). Vazba α(1-6) dělá z RSO nestravitelné sacharidy, jelikož lidská trávicí soustava nedisponuje enzymy, které by tuto vazbu mohly štěpit, RSO tak procházejí nestráveny do prostředí střeva, kde jsou fermentovány bakteriemi, které α-galaktosidasu produkují (Liebl et al. 1998).



Obr. 3: Porovnání jednotlivých RSO z hlediska chemické struktury (Liener 2003).

Zatímco většina výzkumníků nahlíží na Fruktany a GSO, jako na prebiotika, RSO jsou některými považovány za antinutriční látky podporující flatulenci (Suarez et al. 1999), existuje ale mnoho studií, které poukazují na bifidogenní faktory RSO srovnatelné s jinými prebiotiky. Bouhnik et al. (2004) pozorovali bifidogenní efekt in vivo u dobrovolníků ve studii, kde například inulin počty bifidobakterií nezvyšoval, zatímco RSO ano. Hayakawa et al. (1990) porovnávali *in vitro* růst různých bakterií na RSO a zjistili, že všechny testované druhy bifidobakterií, vyjma *B. bifidum*, využívaly RSO ve vysoké míře v porovnání s ostatními střevními izoláty, jako třeba *Bacteroides* spp., *Eubacterium* spp. nebo *Clostridium* spp. s výjimkou *C. butyricum*. Podobného výsledku bylo dosaženo při pokusu Saito et al. (1992), RSO při kultivaci s lidskou stolicí významně podporovaly růst bifidobakterií.

2.6.4 Isomaltooligosacharidy

Tyto oligosacharidy se přirozeně vyskytují ve fermentovaných výrobcích jako saké, nebo sójová omáčka, uměle jsou produkovány enzymatickou přeměnou škrobových hydrolyzátů, jsou tedy složeny z glukosových jednotek propojených α(1-6) glukosidiskou vazbou (Crittenden and Playne 1996). Typ vazby totožný s RSO napovídá, že i isomaltooligosacharidy (IMO) budou neštěpitelné enzymy lidského trávicího traktu. Kohmoto et al. (1988) testovali příjem IMO u dospělců a zjistili, že IMO podpořily výskyt bifidobakterií a *Bacteroides fragilis* v jejich stolici, zatímco počty *E. coli* se nezměnily. Podobných výsledků dosáhli i Kaneko et al. (1994), v jejich studii podporovaly některé frakce IMO bifidobakterie při dávkách 5 – 10 g/den. IMO jsou populární hlavně v Číně a Japonsku, kde jsou přidávány do funkčních i jiných potravin

jako mražený jogurt[5], sušenky, pečivo aj. V Evropské unii IMO zatím nebyly kvůli nedostatkům vědeckých důkazů prohlášeny za prebiotické (NDA 2010).

2.6.5 Laktulosa

Hojně využívána v lékařství jako laxativum, i laktulosa ukázala v některých studiích určitou prebiotickou aktivitu. Přídavek laktulosy do umělé výživy v množství 0,5 – 1 % zvýšil počty bifidobakterií na množství podobná u kojených dětí (Nagendra et al. 1995), zvýšení počtu bifidobakterií pozorovali i Tuohy et al. (2002) u dospělých dobrovolníků.

2.6.6 Ostatní prebiotika

Prebiotické účinky jsou popsány i u dalších rostlinných látek, které jsou běžně klasifikovány jako potravní vláknina, jako třeba rezistentní škrob, arabská guma nebo arabinoxylany. Resistentní škrob byl bifidogenní společně s FOS, GOS a RSO podle Bouhnika et al. (2004). Arabská guma zvyšovala počty bifidobakterií a bakterií mléčného kvašení u dobrovolníků v dávkách 10 a 15 g/den po dobu 10 dnů (Cherbut et al. 2003). Arabinoxylany se vyskytují v obilovinách a ve studii Walton et al. (2012) prokázaly schopnost zvyšovat počty bifidobakterií i množství fekálního butyrátu u dobrovolníků, kteří konzumovali arabinoxylany obohacený chléb.

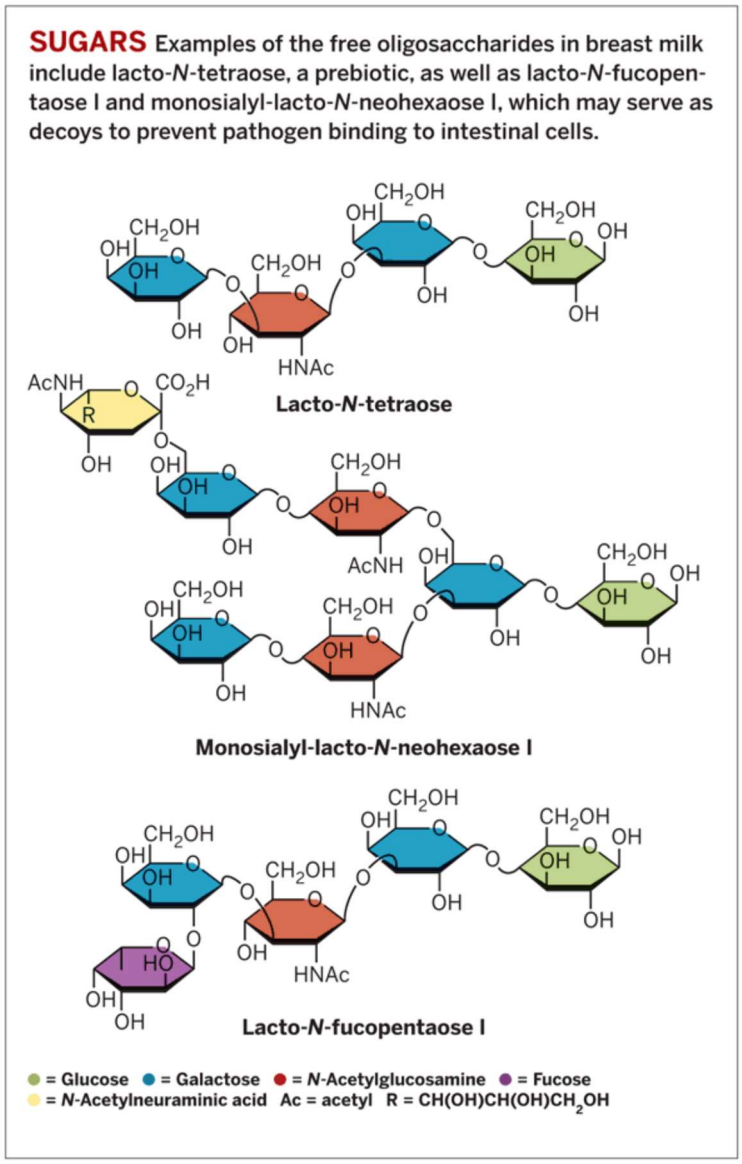
2.6.7 Oligosacharidy mateřského mléka

V mateřském mléce se nachází celé řada biologicky aktivních látek, které mají příznivý vliv na zdraví kojence. Kromě laktosy jsou zde v podstatném množství i oligosacharidy (HMO), a to v množství okolo 12 – 14 g/l v závislosti na jedinci a fázi laktace (Coppa et al. 1999). Je známo, že HMO se podílí na řadě funkcí, jsou spoluzodpovědné za imunitní vývoj kojence, prevenci patogenních onemocnění a ovlivňují i mikrobiální složení trávicího traktu (Kuntz et al. 2008; Martin-Sosa et al. 2002; Newburg et al. 2005).

Strukturně jsou HMO lineární nebo rozvětvené řetězce 3 až 14 monosacharidů (Wu et al. 2011b), téměř všechny mají na terminálním konci laktosu a dále mohou obsahovat N-acetylglukosamin, fukosu nebo kyselinu sialovou (Ruhaak and Lebrilla 2012). Celkově bylo v mateřském mléce identifikováno více, než 200 strukturních izomerů HMO (Wu et al. 2011a; 2011b). Fukosa a kyselina sialová dávají HMO řadu specifických vlastností, přičemž až 70 % HMO v mateřském mléce je fukosylovaných a 20 – 50 % sialylovaných. Fukosa se na N-acetylglukosamin váže prostřednictvím vazeb α(1-3), α(1-4), výjimečně α(1-2) na terminálním konci. Kyselina sialová se váže α(2-3), nebo α(2-6) vazbami (Jeong et al. 2012).

[5] V angličtině se tomuto výrobku říká „frogurt“. Český název „morgurt“ se prozatím neujal.

Mnoho studií prokázalo schopnost HMO ovlivnit střevní mikrobiotu kojence. HMO dovedou selektivně podpořit určité kmeny bifidobakterií, jako třeba *B. longum* ssp. *infantis* oproti jiným druhům jako třeba *B. breve* nebo *B. longum* ssp. *longum*, které rostou na HMO pouze v omezené míře vzhledem k tomu, že jsou schopny využívat některé oligosacharidy (Locascio et al. 2009). Kromě *B. longum* ssp. *infantis* dovedou HMO podpořit i růst *Bacteroides fragilis* a *Bacteroides vulgatus*, zatímco bakterie rodů *Enterococcus*, *Streptococcus*, *Veilonella*, *Eubacetrium*, *Clostridium* nebo *E. coli* HMO v růstu nepodporují (Marcobal et al. 2010).



Obr. 4: Struktura vybraných HMO (Sánchez 2010).

2.6.8 Prebiotika ve výživě drůbeže

U drůbeže se jako prebiotika využívají RSO, které jsou běžně součástí diety v komerčních chovech (Iji and Tivey 1998), které mají vliv na výskyt bifidobakterií (Bednarczyk et al. 2011). Dále mohou být využívány mananooligosacharidy, u nichž byla prokázána schopnost vyvazovat potenciální patogeny, jako *E. coli* nebo salmonely (Spring et al. 2000) a zároveň zvyšují počty celé řady laktobacilů (Teng and Kim 2018). Fruktany mají, stejně jako u lidí, vliv na střevní mikrobiotu i u drůbeže. Byl prokázán jejich pozitivní efekt na výskyt laktobacilů (Saminathan et al. 2011) i bifidobakterií (Perrin et al. 2002).

3 Hypotéza

Podávání probiotik a/nebo prebiotik bude mít příznivý efekt na střevní mikrobiotu hostitele, ať už člověka, nebo hospodářského zvířete. Vhodně zvolené probiotické mikroorganismy budou schopny přežívat v trávicím traktu. Dále předpokládáme, že probiotika budou hostitelsky specifická, tedy každý živočišný druh bude příznivě reagovat na jiné druhy bakterií, které by ovšem měly být ideálně izolovány ze stejného druhu, jako je cílový organismus.

4 Cíle práce

Hlavním cílem dizertační práce bylo ověřit schopnost probiotik a prebiotik ovlivnit střevní mikrobiotu. Testovány byly především netradiční zdroje, které nejsou doposud hojně využívány nebo u nich není dostatečně popsána probiotická nebo prebiotická aktivita. Dále byly testovány různé zdroje prebiotických oligosacharidů a byl hodnocen jejich potenciál ovlivnit střevní mikrobiotu hostitele.

5 Publikované práce

5.1 Seznam publikovaných prací

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Švejstil R., Plachý V., Joch M., Salmonová H., Dušková D., Hautekiet V., Vlková E. (2019): Effect of probiotic *Clostridium butyricum* CBM 588 on microbiota and growth performance of broiler chickens. Czech Journal of Animal Science, 64(9), 00-08. https://doi.org/10.17221/143/2019-CJAS.

5.2

Effect of probiotic Clostridium butyricum CBM 588 on microbiota and growth performance of broiler chickens

Švejstil R., Plachý V., Joch M., Salmonová H., Dušková D., Hautekiet V., E. Vlková E. (2019) Czech Journal of Animal Science 64:00-08.

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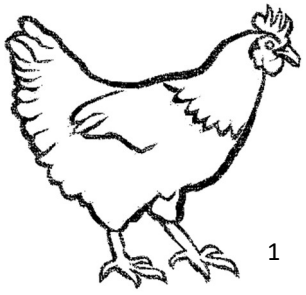

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Effect of probiotic *Clostridium butyricum* CBM 588 on microbiota and growth performance of broiler chickens

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Abstract: *Clostridium butyricum* CBM 588 is used as a probiotic in eastern Asian countries and has been recently approved as an animal feed additive in the European Union. The purpose of this study was to evaluate the effect of *C. butyricum* MIYAIRI 588 (CBM 588) on abundance of selected genera of caecal and crop bacteria, volatile fatty acids and growth performance of broiler chickens. We studied counts of anaerobic bacteria in caeca and crops of broiler chickens by plate-count method and evaluated their growth performance. CBM 588 significantly reduced *E. coli* counts in caeca of broiler chickens at days 10 and 42 and also enhanced their growth performance. Additionally, it significantly increased the amount of butyrate in the caeca that provides energy to enterocytes, resulting in increased weight gains. Out of the obtained results we conclude that *C. butyricum* CBM 588 influences caecal microbiota of broiler chickens and positively affects their growth performance.

Keywords: probiotic feed additive; *E. coli*; bifidobacteria; butyrate; feed efficiency

Intestinal microbiota is known to be an important factor influencing health of living beings by protecting the body from various diseases (Guarner and Malagelada 2003), transforming indigestible parts of food and feed, and synthesising several vitamins and metabolising some xenobiotics (Cummings and Macfarlane 1997). Avian gut is intensively inhabited by bacteria that possess the above-mentioned functions (Kohl 2012). The main role of bacteria in the avian gut is to utilise substrates that cannot be digested by the metabolic processes of the bird (Vispo and Karasov 1997). Providing good nutritional factors is, therefore, one of the ways to influence the composition of the gut microbiota.

Broiler chickens are produced on a large scale in developed countries – reared in an environment with strict hygienic standards, where they never get in contact with broody-hens (Fuller 2001). Therefore, the chickens are colonised by microbiota present in the environment (Lutful Kabir 2009; Varmuzova et al. 2016) in which they are reared. Thus, hatching conditions, hygiene, stress, and medication have major influence on the microbiota of the chickens as well as on their resistance to the colonisation of pathogenic bacteria (Barrow 1992).

The concept of modulation of intestinal microbiota is well-known in poultry production, ever since Nurmi and Rantala (1973) managed to pro-

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tect hatched chickens from *Salmonella enteritidis* infection by oral supplementation of faeces from healthy adult hens. Since then, many microorganisms belonging to the genera of *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, or *Saccharomyces* have been established in poultry production as probiotics. The effects of probiotics in poultry may possibly include modulation of intestinal microbiota, inhibition of pathogens, immunomodulation effects, and improvement of histological parameters of the gut. In addition, they may positively influence the growth performance as well as the meat quality, including its sensory aspects (Fuller 2001; Lutful Kabir 2009; Wang et al. 2017).

It has been shown that the bacteria of the genus *Clostridium* can also be employed as probiotics. While some strains of clostridia are well-known pathogens, some are a part of normal intestinal microbiota, and others are considered as probiotics in Asian countries (Cassir et al. 2016). *Clostridium butyricum* CBM 588 (also labelled as MIYAIRI 588 or FERM BP-2789) that is used in the present study has been used as a probiotic in Japan. It has been tested in several clinical trials, which indicate that the strain can decrease the incidence of antibiotics-associated diarrhoea in children together with stabilisation of the population of bifidobacteria (Seki et al. 2003). Shimbo et al. (2005) and Imase et al. (2008) observed stabilising effects on the intestinal microbiota when CBM 588 was administered as a supplement during the antibiotic eradication of *Helicobacter pylori* infection. Furthermore, it has been found that CBM 588 is able to suppress the production of *Clostridium difficile* toxins after antimicrobial therapy in humans and also to inhibit some strains of *Vibrio cholerae*, *Aeromonas hydrophila*, and *Shigella flexneri* in mixed cultures (Kuroiwa et al. 1990). CBM 588 was also tested in rats, wherein it was able to mitigate the symptoms of dextran sulphate sodium-induced colitis and also increased the counts of lactobacilli in their gut (Okamoto et al. 2000). Takahashi et al. (2004) found that CBM 588 decreased the amount of Stx1 and Stx2 toxins produced by enterohemorrhaegic *Escherichia coli* O157:H7 in mice. Study by Yang et al. (2012) revealed that *Clostridium butyricum* HJCB998 decreased the counts of *E. coli*, *C. perfringens*, and *Salmonella* spp., and increased the counts of lactobacilli and bifidobacteria in caeca of broiler chickens and also stimulated their immune functions. Multiple studies exhibited positive effects of HJCB998 on the growth performance of broiler chickens (Yang et al. 2012; Zhang et al. 2014, 2016).

Clostridium butyricum CBM 588 is a key substance in the composition of Miya-Gold®, a zootechnical feed additive, which is claimed to be a gut flora stabiliser, authorised in accordance with the Regulation EC No. 1831/2003 (EFSA, 2013).

Thus, the aim of this study is to evaluate the effect of feeding *Clostridium butyricum* CBM 588 in the form of Miya-Gold® on the growth performance and microbiota of broiler chickens.

MATERIAL AND METHODS

The feeding trial was carried out at the Demonstration and Experimental Centre of the Czech University of Life Sciences Prague, Czech Republic (DEC). A total of 160 ROSS 308 broiler chickens were divided into two groups per 80 animals in control and experimental groups. The protocol for this study was approved by the Ethics Committee of the Czech University of Life Sciences Prague (permission No. CZ 02225). The broilers were housed on German Horse Span Classic bedding under a 16 h light : 8 h darkness cycle. The control group was fed BR-2-based feed *ad libitum* throughout the whole experiment – from the 1st to the 49th day of life. The experimental group received the same feed mixture but enriched with 1 g Miya-Gold® S (Huvepharma®, Antwerp, Belgium) per 1 kg of the feed. According to the safety data sheet of Miya-Gold® S, it contains a minimum of 5×10^8 CFU/g, i.e. log 8.70 CFU/g of spores of *Clostridium butyricum* CBM 588. The mixture was pelleted under conditions not exceeding a temperature of 60°C at the DEC. The processing did not affect the counts of viable bacteria as verified by cultivation analysis (data not shown). The feed mixture based on BR-2 consisted of the following ingredients: 60.17% wheat, 29.50% extracted soybean meal, 6.30% rapeseed oil, 0.16% DL-methionine, 0.25% sodium chloride, 1.35% monocalcium phosphate, 1.15% limestone, and 0.12% sodium carbonate. One kilogram of the feed (as fed) provided an energy of 12.73 MJ, 211.88 g of crude proteins, and 11.84 g of lysine.

Throughout the experiment, the average weight of all the individuals, their daily weight gain, and feed conversion ratio were recorded at multiple timepoints (at days 1, 7, 10, 20, 35, and 49). The caecal

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and crop microbiota were analysed at the beginning of the experiment (day 1), as well as at day 10, and day 42 of the experiment in 5 individuals from each group; volatile fatty acids in the caeca and crops at day 42 were also analysed.

In the microbiological analysis, total counts of anaerobic bacteria as well as counts of bifidobacteria, lactobacilli, enterococci, and *E. coli* were determined by plate-count method using a ten-fold dilution of each sample up to 10^{-9} dilution. Prior to the analysis, the birds were slaughtered by stunning and cervical dislocation. Approximately 1 g of the caecum (faeces) and crop (chyme) content of each slaughtered chicken was immediately and aseptically transferred to the sterile tubes. The tubes were pre-weighed in order to determine accurate weight of collected caecal/crop content and to adjust the dilution to standardized volume of the sample. The CO_2-flushed sterile tubes contained Nutrient Broth No. 2 (5 g/l; procured from Oxoid, UK), tryptone (5 g/l; Oxoid), yeast extract (2.5 g/l; Oxoid), Tween 80 (0.5 ml/l; Sigma-Aldrich, USA), and L-cysteine (0.25 g/l; Sigma-Aldrich). The identical medium was used for dilution of the samples. The collected samples were homogenised by vortexing immediately after sampling, and underwent the analysis straight away. For microbiological analysis, 5 chickens from each group were sampled. To determine total anaerobes, Wilkins-Chalgren anaerobe agar (43 g/l; Oxoid) was used (Wilkins and Chalgren 1976; Rada and Petr 2000), with the addition of Veggietone Soya Peptone (5 g/l; Oxoid), L- cysteine (0.5 g/l; Sigma-Aldrich), and Tween 80 (1 ml/l; Sigma-Aldrich). An identical medium was enriched with the antibiotic, mupirocin (100 mg/l; Oxoid), and glacial acetic acid (1 ml/l) according to Rada and Petr (2000) and used for the determination of bifidobacteria. Culture plates for the growth of anaerobes and the bifidobacteria were incubated in anaerobic jars (Anaerobic Plus System; Oxoid) at 37°C for 48 h. Lactobacilli were cultured using Rogosa agar (82 g/l; Oxoid) adjusted to pH 5.4 by glacial acetic acid for 48 h under micro-aerophilic conditions (Corry et al. 2003). Counts of *E. coli* were determined using TBX medium (36.6 g/l; Oxoid) by incubating the plates aerobically at 37°C for 24 h (Verhaegen et al. 2015). Enterococci counts were determined using the Slanetz and Bartley medium (42 g/l; Oxoid) by incubating the plates aerobically at 37°C for 48 h (Niemi and Ahtiainen 1995). Total counts of anaerobes and bifidobacteria were cultured using a pour-plate method, lactobacilli were cultured using a double layered pour-plate method (Geigerova et al. 2016) and enterococci and *E. coli* were cultured using a spread-plate method.

Analysis of the volatile fatty acids of caeca and crops was performed by gas chromatography using a Stabilwax®-DA column (Restek, USA) with Flame-Ionisation detector (GC-FID) and H_2 as a mobile phase; the flow was 120 ml/min and the injection and detection temperature was 200°C. Briefly, the samples were vortexed and 0.1 ml of 3 M formic acid and 0.03 ml of internal standard (2-ethylbutyric acid) were added to 0.8 ml of each sample. After centrifugation, 1 µl of the sample was injected into the column (Joch et al. 2017).

Statistical evaluation was carried out by Statgraphics Centurion XV 15.2.05/2007 (Statpoint Technologies, USA) using two-sample *t*-test for comparison between the groups. The data were checked for normality by Shapiro-Wilk test prior to the statistical analysis.

RESULTS AND DISCUSSION

Weight gains of broiler chickens were significantly higher when they were fed a mixture containing Miya-Gold® than of those fed with regular feed throughout the whole trial, as shown in Table 1. There was a significant difference between the body weights of the individual chickens at day 7 ($P < 0.05$), day 10 ($P < 0.001$), day 20 ($P < 0.01$), and day 49 ($P < 0.001$); however, no significant difference ($P > 0.05$) between the body weights was observed at day 35. These findings are supported by values of daily weight gains (Table 2) and feed conversion ratio (Table 3). The results indicate that Miya-Gold® supported the growth of broiler chickens and their feed conversion, although there was an equalisation between the groups at day 35. This could be due to certain forms of social hierarchy that could result in aggressive pecking, thereby reducing access to the feed (Nicol et al. 1999). We found significantly more butyrate and isocapronate ($P < 0.05$) in the caeca of chickens from Miya-Gold® group than in those from control group (Table 4); and therefore, we assumed that it was the result of the metabolic activity of CBM 588. The increased weight gains in the experimental group could have been the result of butyrate production by CBM 588 (Hu and Guo 2007; Matis et al. 2013). Butyrate provides 60–70% of energy

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Table 1. Average weights of broiler chickens in the course of the experiment. Values are means ± standard error

Group	Average weight (g)					
	day 1	day 7*	day 10***	day 20**	day 35	day 49***
Control	44.10 ± 3.71	130.96 ± 13.19	195.04 ± 20.38	760.08 ± 79.59	1767.92 ± 242.09	2780.91 ± 445.41
Miya-Gold	44.71 ± 4.01	138.09 ± 15.85	217.25 ± 21.83	810.26 ± 73.56	1787.23 ± 215.09	3231.67 ± 509.15

significant differences between the groups:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Table 2. Average daily weight gains of broiler chickens. Values are means ± standard error

Group	Daily weight gains (g)				
	days 1–7	days 1–10	days 11–20	days 21–35	days 36–49
Control	14.48 ± 0.07	16.77 ± 0.11	56.56 ± 0.12	67.19 ± 0.17	72.36 ± 0.23
Miya-Gold	15.56 ± 0.10	19.17 ± 0.10	59.30 ± 0.14	65.13 ± 0.23	103.17 ± 0.28

Table 3. Average feed conversion ratio of broiler chickens

Group	Feed conversion ratio (kg)						
	days 1–7	days 1–10	days 11–20	days 21–35	days 36–49	days 1–35	days 1–49
Control	2.09	1.90	1.81	1.82	2.76	1.90	2.08
Miya-Gold	1.53	1.71	1.70	2.22	2.04	1.79	1.84

to the enterocytes present in the gut (Roediger 1995); thus, its production by gut bacteria could increase the overall energy intake, thereby enhancing the weights. Zhang et al. (2011) have reported that dietary inclusion of *C. butyricum* increased the heights of jejunal villi and relative lengths of the caeca. Similarly, Kotunia et al. (2004) found a proliferative effect of butyrate on the jejunum and ileum. Thus, we speculate that the production of butyrate by CBM 588 in our experimental chickens could have resulted in improved digestion and absorption of the nutrients, thereby leading to an increased energy intake. Improvement of the growth performance of broiler chickens has been found by multiple authors when these chickens were administered probiotic lactobacilli (Jin et al. 1998; Kalavathy et al. 2003; Apata 2008), such as *Enterococcus faecium* (Owings et al. 1990) and *Bacillus subtilis* (Khaksefidi and Ghoorchi 2006), or sodium butyrate alone (Zhang et al. 2011). Effects

Table 4. Analysis of volatile fatty acids (VFA) in the caeca and crops of broiler chickens by gas chromatography, day 42. Values (in mmol) are means ± standard error

VFA	Caecum			Crop	
	Control	*P*	Miya-Gold	Control	Miya-Gold
Acetate	441.52 ± 32.86		381.08 ± 63.11	59.42 ± 35.15	58.79 ± 29.03
Propionate	183.42 ± 41.20		196.96 ± 57.42	1.14 ± 2.20	0.27 ± 0.38
Isobutyrate	3.37 ± 0.68		2.14 ± 1.23	2.19 ± 2.85	1.85 ± 1.52
Buytrate	103.41 ± 14.78	*	132.12 ± 18.51	ND	ND
Isovalerate	7.51 ± 2.43		4.08 ± 3.46	0.48 ± 0.62	ND
Valerate	8.24 ± 2.33		4.80 ± 3.46	2.46 ± 3.71	1.25 ± 1.32
Isocapronate	0.91 ± 0.56	*	2.74 ± 1.63	4.64 ± 7.37	3.70 ± 4.45
Capronate	1.18 ± 2.63		0.28 ± 0.40	0.84 ± 0.91	2.78 ± 3.54
Heptanoate	ND		ND	ND	ND
∑VFA	749.57 ± 59.11		724.19 ± 96.92	71.16 ± 28.36	68.63 ± 25.95

ND = below detection limit

significant differences *$P < 0.05$

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of Miya-Gold® on growth performance of broiler chickens has already been demonstrated by several studies conducted for EFSA (2013), wherein two of three trials demonstrated a significantly higher final body weight. Additionally, the average daily weight gain in broilers receiving Miya-Gold® at feed doses of 2.5×10^8 CFU/kg in one trial and 5.0×10^8 CFU kg in the other one was significantly higher; the dosage administered in the latter is similar to that used in our experiment. Although Zhang et al. (2011), using a different strain of *C. butyricum*, found increased levels of butyrate in caeca but no effect on the growth performance of broilers. On the other hand, Yang et al. (2012) and Zhang et al. (2014, 2016) observed significant improvement in the broilers' growth performance after supplementation with *C. butyricum* HJCB998 strain.

An analysis of caecal and crop microbiota was carried out at days 1, 10, and 42 of the experiment; the results are shown in Table 5. We found that caecal counts of *Escherichia coli* were significantly lower in the experimental group at both days 10 ($P < 0.05$) and 42 ($P < 0.05$). No difference at the first time-point (day 1) was observed, since it was only the first day of feeding trial and the clostridia would not have been established at this point of time in the caecal milieu. Use of probiotics is usually recommended for several days (Shimbo et al. 2005; Islam 2016) to produce a significant effect. Our results are in accordance with the findings of Yang et al. (2012), who also observed a significant decrease of *E. coli* in caeca of broiler chickens, despite being supplemented by only 2×10^7 CFU/kg compared to 5×10^8 CFU/kg in our trial. The antagonistic effect of CBM 588 against pathogenic *E. coli* O157:H7 strain was shown in gnotobiotic mice by Takahashi et al. (2004), wherein the strain demonstrated a preventive as well as therapeutic effect. Contrastingly, Shimbo et al. (2005) did not observe any decrease of *E. coli* counts in humans receiving CBM 588 prior to antibiotic therapy, which could be due to the fact that some strains of *E. coli* are species-specific (McLellan et al. 2003; Zhi et al. 2015) and human patients in the above-mentioned study possessed the strains that were not susceptible to CBM 588.. These data suggest the possibility that broiler chickens may possess *E. coli* strains that are specifically susceptible to CBM 588. The hypothesis of the suppression of *E. coli* by CBM 588 is supported by the fact that we found significantly lower counts of these bacteria ($P < 0.01$) even in the crop (Table 5) at day 42. Unfortunately, we failed to detect *E. coli* at day 10 in the crops of both groups. The inhibitory effect of CBM 588 on *E. coli* can be attributed to the combined effect of its anti-adhesive properties and the production of butyrate. Takahashi et al. (2004) observed an inhibitory effect of CBM 588 on the adhesion of enteroheamorrhagic *E. coli* (EHEC) to Caco-2 cells and also observed an inhibitory effect of butyric acid on EHEC even at a neutral pH. Besides, Zhang et al. (2016) observed an increased immune response in broiler chicken challenged

Table 5. Analysis of caecal microbiota and crop microbiota of broiler chickens in the course of the experiment. Values are means log CFU/g ± standard error

Bacterial group	Day 1		Day 10			Day 42		
	Control	Miya-Gold	Control	*P*	Miya-Gold	Control	*P*	Miya-Gold
Caecal microbiota								
Total anaerobes	10.08 ± 0.26	10.06 ± 0.28	10.09 ± 0.20		10.25 ± 0.32	10.09 ± 0.26		10.13 ± 0.23
Bifidobacteria	4.92 ± 1.27	5.09 ± 1.09	9.18 ± 0.23		8.83 ± 1.35	9.92 ± 0.36		9.55 ± 0.19
Lactobacilli	5.91 ± 0.47	6.87 ± 0.91	9.04 ± 0.19		8.55 ± 0.56	8.53 ± 0.31		9.01 ± 0.40
Enterococci	9.55 ± 0.43	8.90 ± 1.75	8.64 ± 0.35		7.82 ± 0.61	8.10 ± 0.17	*	7.55 ± 0.39
E. coli	9.60 ± 0.06	9.03 ± 1.70	8.47 ± 0.81	*	7.29 ± 0.61	8.22 ± 0.64	*	7.00 ± 0.92
Crop microbiota								
Total anaerobes	8.95 ± 0.31	9.01 ± 0.12	9.32 ± 0.39		9.58 ± 0.46	8.74 ± 0.56		9.65 ± 0.33
Bifidobacteria	ND	5.40 ± 0.46	5.04 ± 0.39	*	4.29 ± 0.55	5.20 ± 0.32		4.73 ± 0.96
Lactobacilli	5.54 ± 1.72	7.29 ± 0.78	8.82 ± 0.11		8.69 ± 0.63	8.29 ± 0.59		9.04 ± 0.44
Enterococci	7.73 ± 0.78	7.84 ± 0.28	8.08 ± 0.33		8.34 ± 0.63	7.18 ± 0.43		6.94 ± 0.40
E. coli	7.90 ± 0.78	7.96 ± 0.75	ND		ND	6.73 ± 0.52	**	5.39 ± 0.49

ND = below detection limit; significant differences $^*P < 0.05$, $^{**}P < 0.01$

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with *E. coli* K88 when they were fed *C. butyricum* HJCB998. *E. coli* is a normal inhabitant of both mammalian and avian intestines; however, it has been reported that chickens can carry pathogenic strains that can cause diseases in humans and the birds themselves (Dho-Moulin and Fairbrother 1999; Manges 2016). Thus we consider the reduction of *E. coli* as a positive outcome and conclude that CBM 588 has similar effects on *E. coli* and broiler chicken performance as HJCB998.

In our experiment, counts of enterococci were significantly reduced ($P < 0.03$) in the caeca of experimental group at day 42 compared to those in the control group (Table 5). Enterococci are a part of normal microbiota of broiler chicken; however, they are not known to be infectious agents. Nevertheless, some strains can play the role of opportunistic pathogens (Stepien-Pysniak et al. 2016). Moreover, it has been reported that enterococci isolated from poultry often carry multiple resistance to antimicrobials administered in human medicine (Hayes et al. 2004). In this study, we observed that they were significantly less abundant only at the last time-point. The reduction at day 10 was not significant due to heterogeneity of the results; consequently, we cannot clearly deduce whether CBM 588 was able to suppress enterococci throughout the whole experiment.

Counts of bifidobacteria were significantly lower in the crops of experimental group at day 10 ($P < 0.05$), but were not significantly lower at day 42 than those in control group (Table 5). Additionally, bifidobacteria counts in the caeca of experimental chickens were also not significantly lower throughout the experiment. Yang et al. (2012) tested a different strain of *C. butyricum* and found that it increased the counts of bifidobacteria in caeca. Moreover, Zhang et al. (2014) found that *C. butyricum* HJCB998 increased the population of bifidobacteria and lactobacilli in broiler chickens. Our findings seem to be different in comparison with the data obtained by these investigators; however, most of the differences observed are non-significant. Although data on bifidobacteria in caeca is available, there is a lack of knowledge about bifidobacteria in the crop of broiler chickens.

It is an obvious fact that counts of *Clostridium* spp. should be determined when analyses of microbiota in feeding trials with clostridia added to a diet are performed. Unfortunately, it is very difficult to determine the clostridia in the faecal samples due to insufficient selectivity of media for the cultivation of clostridia for such types of samples. *Clostridium* spp. are able to grow in the mupirocin-containing medium that we used for the analysis of bifidobacteria (Vlkova et al. 2015). Nevertheless, due to high counts of bifidobacteria in the samples, we could not enumerate the counts of clostridia by the plate-count method and a ten-fold dilutions up to 10^{-9} used in this study. Thus, using alternative methods, such as microscopy and MALDI-TOF mass spectrometry (Bruker Daltonik, Germany) using MALDI Biotyper RTC with DB-5989 MSP library for identification of these bacteria (data not shown), we verified that the most abundant colonies on the agar plates were bifidobacteria.

CONCLUSION

In the present study we analysed the effect of *Clostridium butyricum* CBM 588 on the growth performance of broiler chickens *in vivo* and also its influence on caecal and crop microbiota. We found that CBM 588 was able to positively affect the growth performance of broiler chickens. Moreover, CBM 588 was able to increase the content of butyrate in the caeca by its metabolic activity and influence the composition of the intestinal microbiota by reducing the counts of *E. coli*. Elevated amount of butyrate can contribute to gut health and improve weight gain. Some strains of *E. coli* can act as opportunistic pathogens; thus, their decrease can be beneficial to the host. Since the administration of *C. butyricum* did not completely suppress *E. coli* or the other tested bacterial genera, it did not disrupt the microbial balance in the caecum. In conclusion, we consider *C. butyricum* CBM 588 as a potentially beneficial additive to the feeds of broiler chickens.

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5.3
Raffinose-Series Oligosaccharides in Soybean Products

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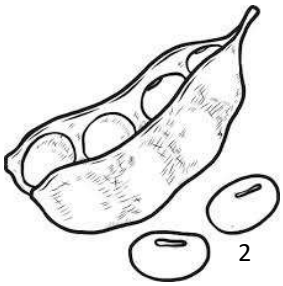

2

RAFFINOSE-SERIES OLIGOSACCHARIDES IN SOYBEAN PRODUCTS*

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Soybean foods forming a substantial part of Asian diet have still more expanded into European diet. Raffinose-series oligosaccharides (RSO) are important constituents of soya beans and they can be found also in soybean products. These oligosaccharides can be considered potentially prebiotic for their capability of influencing the composition of the host's intestinal microbiota. The aim of the present paper was to determine the oligosaccharide content in various soybean products. Enzymatic assay has been used for the determination of oligosaccharides. RSO have been found in all tested samples and their content varied from 0.66 g per 100 g in soybean beverage to 5.59 g per 100 g in first clear soybean flour. Generally, the highest content of RSO has been detected in soybean flour in the average amount of 4.83 g per 100 g. There was no statistically significant difference observed in the amount of oligosaccharides in all four types of soybean flour ($P < 0.01$). Considerably high amounts of RSO have been found in sweet soybean bars and textured soy protein. Foods as soybean flour and soybean bar 'Sójový suk' seem to be effective natural sources of prebiotic oligosaccharides for humans.

soya; saccharides; legumes; stachyose; intestinal microbiota

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INTRODUCTION

Soya beans (*Glycine max*) are a legume crop and excellent food and feed source all over the world. Dried soya beans contain approximately 40–45% of protein, 20% of total lipids, 30–35% of carbohydrates including 20% of total dietary fibre and around 5% of ash (Berk, 1992). Various types of carbohydrates present in soya beans are described in Table 1.

Soya beans are a rich source of oligosaccharides, namely stachyose, raffinose, and verbascose. Raffinose is a trisaccharide containing galactose linked by α-(1-6) bond to the glucose unit of sucrose, stachyose and verbascose are further elongations by α-(1-6) linked galactose unit bonded to tetrasaccharide and pentasaccharide, respectively. These oligosaccharides are called raffinose-series oligosaccharides (RSO), raffinose family oligosaccharides, galactosyl-sucrose oligosaccharides or α-galactosides. Other reported major sugar of soya beans is sucrose, with lower amounts of monosaccharides: fructose, rhamnose, and arabinose. Significant levels of glucose occur only in immature seeds (van der Riet et al., 1989).

A characteristic α-galactosidic bond between sucrose and galactose is very important as humans do not possess the α-galactosidase enzyme that is necessary for hydrolyzing the bond typical in these oligosaccharides, so that they cannot be digested when consumed. Intact oligosaccharides reach the colon, where they are fermented by microorganisms that contain α-galactosidase (Liu, 1997).

Soya beans are potential prebiotics (Roberfroid, 2007), but there are different views on the effects of soybean oligosaccharides on human intestinal microbiota. Bouhnik et al. (2004) observed significant increase of population of bifidobacteria *in vivo* by soybean oligosaccharides. Similar results were obtained both *in vitro* and *in vivo* in a previous study published by Hayakawa et al. (1990) and in *in vitro* study by Saito et al. (1992). Rada et al. (2008) tested effects

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Table 1. Classification of carbohydrates present in soybeans (Berk, 1992)

Total carbohydrates	30 - 35 %
• Soluble sugars	10 %
- Sucrose	5 %
- Stachyose	4 %
- Raffinose	1 %
• Insoluble fibre (cellulose, hemicelluloses, pectic polysaccharides)	25 %
• Starch	< 1 %

of raffinose and stachyose on infant bifidobacteria and clostridia *in vitro* and found that both oligosaccharides stimulated the growth of both bifidobacteria and clostridia, which raises the question regarding the selectivity of these oligosaccharides. Suarez et al. (1999) observed increased gas production in healthy volunteers when consuming conventional soybean flour which also supports the doubts about the selectivity taking into consideration that bifidobacteria are not gas-producing bacteria (Biavati, Mattarelli, 2012). Other study also concluded that low molecular constituents of soya bean are responsible for flatulence in men (Steggerda et al., 1966). On the other hand, Bouhnik et al (2004) did not observe increased symptoms of intestinal discomfort in volunteers when consuming soybean oligosaccharides.

A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health (Roberfroid, 2007). Although some authors do not consider RSO as prebiotic (Gibson et al., 2004; Roberfroid, 2007), there are strong suggestions that they are able to promote bifidobacteria at the expense of other intestinal bacteria and thus to promote the host's health (Saito et al., 1992; Mitsuoka, 1996).

Oligosaccharides can be removed from soya beans by various methods. Soaking is the easiest, but also most ineffective way to reduce oligosaccharides in whole grains with around 25% reduction. By the combination of soaking, dehulling, washing, and cooking, more than half of total oligosaccharides can be removed (Egounlety, Aworh, 2003). Bianchi et al. (1983) recommended cooking instead of soaking as a more useful method for reduction of RSO. Enzymatic degradation by α-galactosidase can be also used to reduce RSO from soybean products (Kotiguda et al., 2007) as well as aqueous ethyl alcohol extraction (Singh et al., 2008). Low oligosaccharide meal from genetically modified soya beans represents another way of reduction of RSO in the diet (Parsons et al., 2000).

Soya beans are primarily cultivated for edible oil and animal feeding. Considering high protein content, soya beans are potentially rich source of protein for human nutrition. There is a variety of products made from soya beans nowadays. Many soy products are traditionally used in eastern Asia as an important part of the diet (Berk, 1992), a great nutritional potential of soybeans has led to extension of soybean products to western world. A soy beverage, sometimes incorrectly called soymilk, is one of such products. It is a water extract of soya beans nutritionally comparable to bovine milk, but not of the same quality as it has a slightly different amino-acid pattern, significantly lower micronutrients content, and it lacks cholesterol and cobalamin in comparison to bovine milk. Moreover, it contains typical soybean compounds as phytic acid, saponins, phytoestrogens, and oligosaccharides (Dostálová, 2003). Textured soy protein or textured vegetable protein was analyzed within this study. It has often been used as a meat extender or a meat analogue (Berk, 1992).

The aim of the present study was to determine the amount of RSO in various kinds of soybean flour and several soybean products and to evaluate possible prebiotic effect on human intestinal microbiota.

MATERIAL AND METHODS

Soybean products selected for measurement of RSO content are shown in Table 2. The products were purchased from local Czech manufacturers.

Measurements were performed by commercial assay kit for determination of galactosyl-sucrose oligosaccharides (raffinose, stachyose, verbascose), K-RAFGL (Megazyme International Ireland Ltd., Bray, Ireland). The principle of the assay is that RSO are hydrolyzed to D-galactose and sucrose using α-galactosidase. Subsequently, sucrose is hydrolyzed to D-glucose and D-fructose using invertase. D-glucose is then determined using glucose oxidase/peroxidase reagent. The method does not distinguish between raffinose, stachyose, and verbascose, but rather, measures these as a group. Since one mole of each of the raffinose-series oligosaccharides contains one mole of D-glucose, the concentrations are presented on a molar basis. Free sucrose and D-glucose in sample extracts are determined concurrently in duplicates not containing α-galactosidase. With samples used in this assay, we considered stachyose as the main oligosaccharide contained in the samples. Therefore the amount of RSO is expressed as a stachyose equivalent. After the incubation, the colour change appears. All samples were measured spectrophotometrically with 510 nm absorbance by Infinite 200PRO microplate reader (Tecan Group Ltd., Männendorf, Switzerland).

STATISTICA (Version 12, 2013) software was used to perform one-way ANOVA analysis (Duncan test) with multiple range comparison at a confidence

Table 2. RSO content of soybean products.

Product	Manufacturer	mMol/100 g[1]	g/100 g[1,2]
Soybean flour – reference sample	-	7.44 ± 1.80	4.95 ± 1.20
Organic first clear soybean flour	Natural Jihlava, CZ	6.37 ± 0.66	4.24 ± 0.44
First clear soybean flour	Paleta, Lipnice, CZ	8.38 ± 2.20	5.59 ± 1.47
All-purpose soybean flour	Paleta, Lipnice, CZ	6.80 ± 1.22	4.53 ± 0.82
Soybean beverage 'Zajíc'	Morgador, Otrokovice, CZ	0.99 ± 0.40	0.66 ± 0.27
Sweet soybean bar 'Sójový suk'	Unimex, Prague, CZ	4.17 ± 0.80	2.78 ± 0.54
Sweet soybean bar 'Sójový řez'	Altis, Kolín, CZ	3.25 ± 0.32	2.16 ± 0.15
Soybean bar with coconut flavor 'Margot'	Nestlé, Prague, CZ	2.45 ± 0.50	1.63 ± 0.33
Textured soy protein 'Sojové vločky'	Natural Food, Prague, CZ	5.03 ± 1.03	3.36 ± 0.69
Soya creamer	Morgador, Otrokovice, CZ	2.73 ± 0.49	1.82 ± 0.33

[1]All values are means of three measurements ± standard deviation.

[2]Values are calculations of stachyose equivalent.

level of 99% for the differences among various types of soybean flour and among sweet soybean bars.

RESULTS

All soybean products contained detectable amounts of RSO. The largest amount of RSO was measured in first clear soybean flour. Other types of soybean flour contained similar amounts of oligosaccharides as in first clear soybean flour. Average amount of oligosaccharides in all kinds of soybean flour was 4.83 g per 100 g. These values correlate with those recorded by Karr-Lilienthal et al. (2005) and Choct et al. (2010) indicating that concentration of stachyose in soybean flour is 2–5% of dry matter (DM) and concentration of raffinose is 0.5–2% of DM. Textured soy protein contained the highest amount of oligosaccharides among non-flour samples. Two of soybean bars contained more than 2 g of RSO and soybean bar with coconut flavour contained around 1.5 g of RSO. Almost 2 g of RSO was found in soya creamer. Soybean beverage contained the lowest amount of RSO. No statistically significant difference ($P < 0.01$) in the amount of oligosaccharides present in all kinds of soybean flour and in both sweet soybean bars was observed. All results are shown in Table 2.

DISCUSSION

Concentrations of oligosaccharides determined in the four samples of soybean flour corresponded with those given in literature (Karr-Lilienthal et al., 2005) despite quite great standard deviations in some samples, probably due to nature of the method which is adequate for tentative determination of RSO (Vinjamoori et al., 2004). On the other hand, enzymatic methods are highly specific.

There are no limits and regulations for prebiotics or oligosaccharides in food whatsoever, so it is difficult to compare our results with official data. The exception is the Commission Directive of the European Union on infant formulae and follow-on formulae that recommends maximum content of 0.8 g per 100 ml of oligosaccharides in a combination of 90% of galactooligosaccharides and 10% of long chain fructooligosaccharides (Commission Directive 2006/141/EC).

Moreover, RSO can be included either in the category of carbohydrates or fibre in the product composition data sheet. It is recommended to consume 2.5–10 g of inulin type prebiotics per day to increase population of bifidobacteria in gut (Kelly, 2009). Bouhnik et al. (2004) reported that all dosages from 2.5 to 10 g of RSO per day increased the number of bifidobacteria in human gut. Considering these data, consummation of three slices (150 g) of soya bread per day is suitable for increasing the number of gut bifidobacteria as soya bread can contain up to 40% of soybean flour with no deterioration of sensory quality (Kopáčová, 2002; Řezáčová, 2010). Two pieces (100 g) of soybean bar 'Sójový suk' per day can also stimulate growth of bifidobacteria in human intestinal tract.

Textured soy protein also contains detectable amount of RSO and, moreover, it does not contain large amounts of monosaccharides and disaccharides unlike the sweet bars. However, it does not contain a sufficient amount of RSO per serving (up to 50 g) to stimulate the bacterial population of gut and thus cannot be considered as a potential prebiotic, the same as the soybean bars 'Sójový řez' and 'Margot' and soya creamer, because its recommended serving is only 5 g per cup of coffee. The lowest amount of

RSO, which has been found in soybean beverage, can be attributed to deliberate action during the processing. Recommended quantity of soybean beverage per serving is 25 g of powder into 250 ml of water. This amount is not enough for influencing of gut bacteria population and thus the soybean beverage cannot be considered as prebiotic.

The issue of prebiotic effect of RSO still needs to be resolved when we take into account that there are different opinions on prebiotic effects of RSO (Suarez et al., 1999; Bouhnik et al., 2004; Gibson et al., 2004).

CONCLUSION

Raffinose-series oligosaccharides are substantial components of soybean foods which may beneficially affect the host's health by influencing the composition of microbiota. We have determined amounts of raffinose-series oligosaccharides in 9 products used in human nutrition and it can be concluded that consummation of sweet soybean bar 'Sójový suk' and pastry containing sufficient amount of soybean flour can influence the host's intestinal microbiota due to perceptible amount of RSO.

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5.4

Effect of dietary lupin (Lupinus albus) on the gastrointestinal microbiota composition in broiler chickens and ducks

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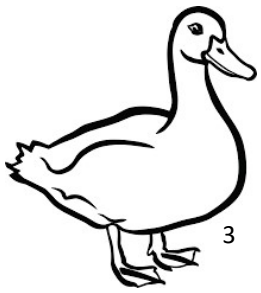

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Effect of Dietary Lupin (*Lupinus albus*) on the Gastrointestinal Microbiota Composition in Broiler Chickens and Ducks

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ABSTRACT

Geigerová M., Švejstil R., Skřivanová E., Straková E., Suchý P. (2017): **Effect of dietary lupin (*Lupinus albus*) on the gastrointestinal microbiota composition in broiler chickens and ducks**. Czech J. Anim. Sci., 62, 369–376.

The purpose of the study was to evaluate the amount of raffinose-series oligosaccharides (RSO) in soybean meal (SBM), whole white lupin seed meal (WLM), sunflower meal (SFM), and rapeseed oil meal (ROM) and to determine whether partial or complete dietary WLM replacement affected the numbers of bacteria in selected groups in the microbiota of broiler chickens and ducks without inducing any weight loss. Total counts of anaerobes, lactobacilli, bifidobacteria, and *Escherichia coli* in caecal samples from both ducks and broiler chickens, as well as in a crop chyme, in broiler chickens, were determined. Live weights before slaughter were determined. Both broiler chickens and ducks were fed a control diet with SBM (L_0) or diet containing 50% or 100% WLM as a substitute for SBM (groups L_{50} and L_{100}, respectively). In comparison with SBM, WLM contained significantly higher amounts of RSO, and the amounts of oligosaccharides in SFM (1.73 ± 0.26 g/100 g) and ROM (1.79 ± 0.14 g/100 g) were negligible compared to those in WLM (8.26 ± 0.14 g/100 g) and SBM (6.96 ± 0.21 g/100 g). The inclusion of lupin in chicken diets did not significantly affect the monitored bacterial groups in crop chyme, but a complete replacement of SBM with WLM (L_{100} group) in chicken diets significantly ($P \leq 0.05$) increased the counts of lactobacilli in caecal samples. Partial (L_{50} group) and complete (L_{100} group) lupin supplementation in the duck diet significantly ($P \leq 0.05$) increased counts of lactobacilli and bifidobacteria by at least one order of magnitude. *E. coli* counts in poultry were not affected by changes in diet. The results of our study indicate that partial dietary replacement of SBM with WLM did not significantly affect the live weight of broiler chickens and ducks, but that complete replacement of SBM with WLM may lead to weight loss.

Keywords: bacterium; poultry; prebiotic; white lupin; raffinose-series oligosaccharides

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From an ecological point of view, animal production and the nutrition feed required for these animals, represent an undeniable environmental burden. Therefore, there is an effort, especially in European countries, to select locally sourced feed ingredients, if possible. In the last decade, soybeans became the most common source of vegetable protein in monogastric animal diets (Chaudhary et al. 2015; Heger et al. 2016); however, a majority of soybeans are imported from overseas. In addition to reducing the need for these imports, feeds based on non-genetically modified plants are currently desirable in developed countries (Frewer et al. 2013). Potential alternative sources of dietary protein can be obtained from by-products of vegetable oil extracted from sunflower or rapeseed meal (Dadalt et al. 2016; Liermann et al. 2016). Other protein sources include a pea protein isolate and potato or corn protein concentrate (Froidmont et al. 2009; Wiltafsky et al. 2009; Dadalt et al. 2016). However, there are certain nutritional limitations (e.g., alkaloid content, trypsin inhibitors, and tannins) which must be considered. Based on these requirements, low-alkaloid varieties of sweet lupin (*Lupinus albus*, *Lupinus angustifolius*, *Lupinus luteus*) are considered promising for use in animal feed. Its advantage is that lupin can be used to completely replace soybean meal (Zraly et al. 2008; Hernandez and Roman 2016). The use of lupin as an alternative source of vegetable protein for the production of animal feed is increasing rapidly. There are many studies analyzing the impact of replacing soya with lupin in animal diets. Many authors confirmed that lupin is a suitable protein component for use in animal feed, based on production parameters and nutrient digestibility in animals (Zraly et al. 2008; Volek and Marounek 2009; Zdunczyk et al. 2016; Zwolinski et al. 2017). In addition to proteins, important growth-promoting factors in lupin seeds include their significant amounts of saccharides, including raffinose-series oligosaccharides (RSO). These oligosaccharides are not digested in the upper gastrointestinal tract (GIT) of monogastric animals. Without changing their structures, they pass to the intestine, where are fermented by gut microbiota to produce short-chain fatty acids and gas. This can lead to flatulence and abdominal discomfort (Guillon and Champ 2002). However, RSO have been identified as prebiotic agents. In *in vitro* studies, particularly, they have been shown to promote the growth of health-promoting bacteria such as bifidobacteria and inhibit the growth of *Escherichia coli* in the gut (Hernandez-Hernandez et al. 2011; Wongputtisin et al. 2015). Therefore, the aim of our study was to determine the amounts of oligosaccharides in selected meals serving as a potential source of protein and to assess whether the inclusion of *Lupinus albus* instead of soya in broiler chicken and duck diets could induce changes in selected bacterial groups.

MATERIAL AND METHODS

Quantitative determination of raffinose series oligosaccharides in experimental meals. Four meals were selected as a potential source of protein in animal nutrition including soybean meal (SBM), white lupin seed meal (WLM), sunflower meal (SFM), and rapeseed oil meal (ROM). The amount of RSO was determined by an enzymatic method – the Megazyme Raffinose/Sucrose/Glucose Assay Kit (Megazyme International, Ireland) using α-galactosidase and invertase was applied according manufacturer's instructions. The method does not distinguish between raffinose, stachyose and verbascose; their quantities were measured as a group. Three replicates were used to determine the amounts of RSO per meal.

Birds and housing. The study was conducted at an accredited experimental barn of the Department of Animal Nutrition at the University of Veterinary and Pharmaceutical Sciences Brno. The protocol for this study was approved by the local ethic committee.

In this study, a total of 240 one-day-old broiler chickens (ROSS® 308) and 180 one-day-old ducks (Cherry Valley) were purchased from International Poultry Testing MTD Ústrašice, Czech Republic. Animals were placed in pens with deep litter, and each experimental group was maintained separately. For broiler chickens and ducks, a 23 : 1 h light : darkness lighting regime was used throughout the experiment. The temperature was set at 21–31°C for broiler chickens and 8–30°C for ducks, depending on their ages.

Experimental design and diets. Broiler chickens were randomly assigned to three dietary treatments (80 replicates each), and each treated group was divided by sex for 40 males and 40 females. During the study period, broiler chickens were fed

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Table 1. Selected nutrients of experimental diets (g/kg, dry matter) for broiler chickens containing a different amount of white lupin seed meal

Nutrition value	1–14 days			15–29 days			30–35 days		
	L_0	L_{50}	L_{100}	L_0	L_{50}	L_{100}	L_0	L_{50}	L_{100}
Crude protein	254.5	265.1	248.7	226.9	217.3	223.3	205.1	215.5	203.6
Fat	63.9	57.7	60.5	66.4	68.1	69.8	81.4	66.5	80.9
Crude fibre	24.3	35.1	52.3	24.9	49.4	57.5	36.3	35.9	45.2

L_0 = diet with soybean meal (SBM), L_{50} = diet containing 50% of white lupin seed meal (WLM) as a substitute for SBM, L_{100} = diet with WLM

a control diet based on SBM (L_0) or one of two diets containing 50% or 100% WLM as a substitute for SBM (groups L_{50} and L_{100}, respectively). The chickens were fed for three experimental periods over 35 days (i.e. days 1–14, 15–29, and 30–35). The composition and calculated nutritional values of these diets are shown in Table 1. Analogously, ducks were separated by dietary treatment and sex into six groups (30 ducks per group) and were fed diets containing SBM meal as a control or diets with 50% or 100% WLM as a replacement for SBM (groups L_0, L_{50}, and L_{100}, respectively). The ducks were fed for 40 days in four periods (i.e. days 1–10, 11–19, 20–35, and 36–40). The composition of their diets is presented in Table 2.

The control feed mixture was prepared by ZZN Pelhřimov, Czech Republic, and the test feed mixtures containing whole white lupin seed were prepared by MTD Ústrašice, Czech Republic. The poultry had free access to water and feed mixtures and were fed through feeder drop tubes *ad libitum*. At the end of the experiment, the poultry were weighed. To monitor intestinal bacteria, 18 broiler chickens and 18 ducks (6 birds per group) from each treatment group (L_0, L_{50}, and L_{100}) were randomly selected. Immediately after slaughter, samples from caeca from both kinds of birds and crop chyme from broiler chickens were collected directly into tubes containing Wilkins-Chalgren broth (Oxoid, UK). Samples were kept on ice until the microbiological analysis.

Microbiological analysis. Counts of total anaerobic bacteria, bifidobacteria, lactobacilli, and *Escherichia coli* were determined by cultivation. The obtained samples were homogenized and serially diluted in Wilkins-Chalgren broth (Oxoid) under anaerobic conditions. Wilkins-Chalgren agar (50 g/l; Oxoid) supplemented with soya peptone (5 g/l; Oxoid), L-cystein (0.5 g/l; Sigma-Aldrich, USA), and Tween 80 (1 ml/l; Sigma-Aldrich) was used for enumeration of total anaerobic bacteria. Bifidobacteria were enumerated on the same agar as total anaerobes with the addition of glacial acetic acid (1 ml/l) and the antibiotic mupirocin (100 mg/l; Oxoid), according to a method reported by Rada and Petr (2000). These plates were incubated in anaerobic jars (Anaerobic Plus System, Oxoid) at 37°C for 72 h. To enumerate lactobacilli, Rogosa agar (82 g/l; Oxoid) adjusted to pH 5.4 ± 0.2 with glacial acetic acid was used. Lactobacilli were cultivated for 72 h under micro-aerophilic conditions using the double-layered pour-plate method. Counts of *E. coli* were determined using TBX-agar (Oxoid), with plates incubated aerobically at 37°C for 24 h.

Statistical analysis. The amounts of RSO in meals, live weight, and bacteria enumeration were analyzed statistically using STATISTICA software

Table 2. Selected nutrients of experimental diets (g/kg, dry matter) for ducks containing a different amount of white lupin seed meal

Nutrition value	1–10 days			11–19 days			20–35 days			36–40 days		
	L_0	L_{50}	L_{100}	L_0	L_{50}	L_{100}	L_0	L_{50}	L_{100}	L_0	L_{50}	L_{100}
Crude protein	238.2	247.9	265.8	213.7	214.5	229.8	180.1	182.8	175.8	178.7	170.4	180.9
Fat	39.5	42.9	57.7	37.9	44.5	55.5	37.6	43.0	52.4	43.3	46.3	49.4
Crude fibre	26.1	48.3	53.3	30.3	38.0	63.5	31.4	39.3	46.1	36.1	35.1	37.7

L_0 = diet with soybean meal (SBM), L_{50} = diet containing 50% of white lupin seed meal (WLM) as a substitute for SBM, L_{100} = diet with WLM

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(Version 12.0, 2013). Amounts of RSO and numbers of bacteria are presented as mean values ± standard deviations (SD). Live weights are presented as mean values with pooled standard errors of the mean (SEM). A one-way analysis of variance (ANOVA) was performed to determine whether values differed among the treatment groups, and $P \leq 0.05$ was considered statistically significant. Prior to the statistical analysis data were checked for normality (Shapiro-Wilk test).

RESULTS

Amounts of RSO in experimental meals. The quantities of RSO in experimental meals are shown in Table 3. The amounts of oligosaccharides found in meals ranged from 1.73 to 8.26 g/100 g. Relatively low amounts of RSO were found in SFM and ROM compared to those in WLM and SBM. WLM contained the highest amount of RSO of all the tested meals.

Growth performance. The live weights of broiler chickens and ducks were determined. The final body weights of broiler chickens are shown in Table 4. No statistical differences were found in the live weights of male broiler chickens fed different diets. The means of live weights of broiler chickens in the L_0 group and L_{50} group were almost identical. The average live weight of female broiler chickens in the L_{100} group (2.19 kg) was significantly lower than that in the L_{50} group (2.33 kg), but no statistical difference was found between live weights in the L_0 and L_{100} groups. Complete lupin replacement in duck diets also negatively affected their final body weights (Table 5). Significant differences in live weights were found between the L_{100} group and L_{50} group of female ducks, and between the L_{100} group and L_{50} group of male ducks, and moreover between the L_{100} group and L_0 group of male ducks.

Table 3. The amount of raffinose series oligosaccharides (RSO) (g/100 g) in soybean meal (SBM), white lupin seed meal (WLM), sunflower meal (SFM), and rapeseed oil meal (ROM)

Meal	Amount of RSO
SBM	6.96 ± 0.21^{A}
WLM	8.26 ± 0.14^{B}
SFM	1.73 ± 0.26^{C}
ROM	1.79 ± 0.14^{C}

$^{A–C}$means with different superscripts significantly differ ($P \leq 0.05$)

Table 4. Live weight (kg) before slaughter of broiler chickens fed diets based on soybean meal (SBM) and/or white lupin seed meal (WLM)

	L_0	L_{50}	L_{100}	SEM
Male	2.53^{A}	2.55^{A}	2.46^{A}	0.32
Female	2.30^{AB}	2.33^{A}	2.19^{B}	0.23

L_0 = diet with SBM, L_{50} = diet containing 50% of WLM as a substitute for SBM, L_{100} = diet with WLM, SEM = standard error of the means

^{A,B}means in the same row with different superscripts significantly differ ($P \leq 0.05$)

Bacteria enumeration. Counts of selected bacterial groups in caecum and crop samples collected from broiler chickens are shown in Table 6. The average numbers of total anaerobic bacteria, bifidobacteria, lactobacilli, and *E. coli* isolated from crop chyme in all three experimental groups were not significantly different. The amount of lupin in diets did not affect the number of these bacteria. Although statistically significant differences were not found, counts of bifidobacteria and lactobacilli were the highest in the group in which soya was completely replaced with lupin. Conversely, in the same group (L_{100}), the counts of *E.coli* were the lowest. In the caeca of broiler chickens, lactobacilli counts were significantly higher in the L_{100} group than in the L_{50} and L_0 groups. This is the only statistically significant difference that was found in the faecal microbiota of broiler chickens. The highest counts of bifidobacteria as well as *E. coli* were detected in the L_{50} group.

Considerably higher bacterial diversity was observed in the faecal microbiota of ducks than that of chickens (Table 7). Bifidobacteria and lactobacilli

Table 5. Live weight (kg) before slaughter of ducks fed diets based on soybean meal (SBM) and/or white lupin seed meal (WLM)

	L_0	L_{50}	L_{100}	SEM
Male	3.26^{A}	3.14^{A}	2.94^{B}	0.31
Female	3.11^{AB}	3.19^{A}	2.98^{B}	0.25

L_0 = diet with SBM, L_{50} = diet containing 50% of WLM as a substitute for SBM, L_{100} = diet with WLM, SEM = standard error of the means

^{A,B}means in the same row with different superscripts significantly differ ($P \leq 0.05$)

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Table 6. Bacterial counts (log CFU/g ± SD, n = 6) in caecal samples and crop chyme of broiler chickens fed diets based on different amounts of white lupin seed meal (WLM)

		Total anaerobes	Bifidobacteria	Lactobacilli	*E. coli*
Caecum	L_0	9.33 ± 0.38^A	8.34 ± 1.34^A	8.76 ± 0.68^A	7.99 ± 0.39^A
	L_{50}	9.56 ± 0.27^A	9.14 ± 0.27^A	8.21 ± 0.68^A	8.57 ± 0.65^A
	L_{100}	9.77 ± 0.29^A	8.95 ± 0.46^A	9.55 ± 0.49^B	8.22 ± 0.23^A
Crop	L_0	8.72 ± 0.54^A	5.66 ± 0.37^A	8.28 ± 0.51^A	6.20 ± 1.14^A
	L_{50}	8.31 ± 0.54^A	5.42 ± 0.56^A	8.04 ± 0.69^A	6.29 ± 1.38^A
	L_{100}	9.21 ± 0.56^A	6.29 ± 1.24^A	8.93 ± 0.69^A	5.44 ± 0.50^A

L_0 = diet with soybean meal (SBM), L_{50} = diet containing 50% of white lupin seed meal (WLM) as a substitute for SBM, L_{100} = diet with WLM

^{A,B}means with different superscripts in columns from the same type of sample significantly differ ($P \leq 0.05$)

counts were significantly higher in both experimental groups in which soya was replaced with lupin (L_{50} and L_{100}), as compared to the control (L_0) group. The numbers of bifidobacteria in the L_{50} and L_{100} groups were higher by at least one order of magnitude. The number of lactobacilli in the L_{50} group was higher by two orders of magnitude. No statistically significant differences were found among counts of total anaerobic bacteria, and the amounts of *E. coli* were approximately equal in all three groups.

DISCUSSION

Members of the raffinose family of oligosaccharides are present in various plant sources (Andersen et al. 2005). High amounts of RSOs are mainly found in legumes, and their levels in seeds vary by species and based on environmental factors (Martiinez-Villaluenga et al. 2005). The aforementioned oligosaccharides are not digested by the monogastric animals, and are therefore available for bacterial fermentation to produce short-chain fatty acids and gas (Guillon and Champ 2002). High levels of oligosaccharides, in particular α-galactosides, can by certain means be even considered as antinutritional factors, as their fermentation in monogastric animals can lead to the increased fluid retention, hydrogen production, and can impair the utilization of nutrients (Saini and Gladstones 1986). Therefore, the particular source and concentration of these compounds should always be tested *in vivo*.

Generally, all lupin species are good sources of RSOs and can be used for the isolation of oligosaccharides. According to Martiinez-Villaluenga et al. (2005), white lupin seeds contain RSO amounts ranging from 5.46 to 8.51% dry matter (DM). In our experiment, WLM contained comparatively high levels of RSO (8.26 ± 0.14 g/100 g). High amounts of RSOs were also found in SBM, but these levels were lower than those in lupin, which corroborates the findings of other authors (Kumar et al. 2010; Svejstil et al. 2015). According to Zdunczyk et al. (2014), the inclusion of blue lupin seeds (20%) to a layer diet can increase the RSO content (from 0.77 to 2.08% DM). The amount of oligosaccharides in SFM and ROM was similar, and RSO contents in these meals were negligible compared to those with WLM and SBM.

Table 7. Bacterial counts (log CFU/g ± SD, n = 6) in caecal samples of ducks fed diets based on different amounts of white lupin seed meal (WLM)

		Total anaerobes	Bifidobacteria	Lactobacilli	*E. coli*
Caecum	L_0	9.54 ± 0.45^A	6.93 ± 0.74^A	4.53 ± 0.40^A	7.21 ± 0.29^A
	L_{50}	9.96 ± 0.42^A	8.55 ± 0.44^B	6.55 ± 0.98^B	6.97 ± 0.86^A
	L_{100}	9.93 ± 0.32^A	8.15 ± 0.53^B	6.06 ± 0.46^B	7.13 ± 0.28^A

L_0 = diet with SBM, L_{50} = diet containing 50% of WLM as a substitute for SBM, L_{100} = diet with WLM

^{A,B}means with different superscripts in columns significantly differ ($P \leq 0.05$)

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The commensal microbial community plays a major role in poultry health and digestion and its composition can be influenced by diet. Currently, there is limited information available in the literature on whether crop microbial composition can be affected by feed. The crop is the first major defence against pathogens in broiler chickens. One of the barriers of the crop against pathogens is an acidic pH. A lower pH can be promoted by lactic acid fermentation performed by lactobacilli. Lactobacilli are the dominant bacterial group in the crops of broiler chickens (Kieronczyk et al. 2016), as shown also in our results. The numbers of lactobacilli in crops were similar to the numbers of total anaerobic bacteria in all three groups (L_0, L_{50}, and L_{100}). Besides lactobacilli, among the health-promoting bacteria belong also bifidobacteria. In our study, bifidobacteria were found at approximately 10^6 CFU/g in poultry crops, which was a lower order of magnitude than described by Petr and Rada (2001). The highest numbers of lactobacilli and bifidobacteria were found in the L_{100} group, relative to those in the L_{50} and L_0 groups, but these differences were not significant. *E. coli* counts in the crops of broiler chickens were not affected by diet. Undigested oligosaccharides in the upper part of the GIT are fermented in the intestines of birds by the gut microbiota (Patterson and Burkholder 2003). The presence of RSO in diets may result in increased numbers of bacteria in certain populations (Jozefiak et al. 2004). Higher counts of total anaerobes in the caeca of broiler chickens and ducks were found in the L_{100} group, relative to that in the L_0 group. However, these differences were not significant because both diets contained some RSO. Dietary RSO has been shown to increase the numbers of lactic acid bacteria, as well as to increase visible bacteria attached to cell walls in the caecum (Lan et al. 2007). Complete replacement of SBM with WLM in the diets of broiler chickens affected the numbers of lactobacilli in caeca samples; however, the other investigated bacterial groups were not affected. Similarly, differences in the composition of duck diets positively affected lactobacilli and bifidobacteria counts. The inclusion of whole white lupin seeds in the experimental diets caused appropriate changes in the amounts of probiotic bacteria. Increased numbers of bifidobacteria and lactobacilli can have a positive effect on poultry by regulating the intestinal microbial balance (Buclaw 2016). Similar results were described by Zdunczyk et al. (2014), who observed increased counts of bifidobacteria and lactobacilli in laying hens fed a diet supplemented with 20% blue lupin seeds. In contrast, the addition of yellow lupin seed meal to the feed of turkeys did not increase the numbers of lactobacilli (Zdunczyk et al. 2016). *E. coli* is a common intestinal bacterium, and most of strains are commensal; however, some strains can cause disease. The counts of *E. coli* in faecal samples of both types of poultry were not affected by differences in the composition of diets.

In addition to monitoring quantitative changes in selected bacterial groups, final body weights were determined. As suggested in the introduction, the replacement of SBM with lupin meal in the diets of various monogastric animals, including rabbits, turkeys, chickens, and pigs, does not necessarily reduce weight gain (Wu et al. 2004; Zraly et al. 2008; Volek and Marounek 2009; Zdunczyk et al. 2016). However, there have been some reports of weight loss with this replacement (Olkowski et al. 2005; Smulikowska et al. 2014). Our results showed that partial inclusion of lupin in diets did not significantly affect the body weights of broiler chickens or ducks, but that complete replacement of SBM with WLM reduced their live weights.

CONCLUSION

The present study shows that WLM contains higher levels of RSO than SBM, and supplementation of diets had a positive influence on the intestinal microbiota composition of broiler chickens and ducks. Partial and complete replacement of SBM with lupin in duck diets significantly increased counts of lactobacilli and bifidobacteria. Further, a significant increase in the numbers of lactobacilli in broiler chicken caecum was observed only when SBM was fully replaced with WLM. The obtained data showed that a diet containing 50% whole white lupin had a positive effect on the composition of the intestinal microbiota in ducks, and that this addition had neither negative nor positive effects on the live weights of ducks and broiler chickens.

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5.5

Assessment of the synbiotic properites of human milk oligosaccharides and Bifidobacterium longum subsp infantis *in vitro* and in humanised mice

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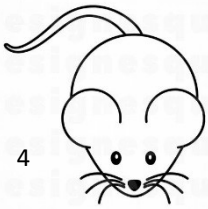

Assessment of the synbiotic properites of human milk oligosaccharides and *Bifidobacterium longum* subsp. *infantis in vitro* and in humanised mice

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RESEARCH ARTICLE

Abstract

The mode of delivery plays a crucial role in infant gastrointestinal tract colonisation, which in the case of caesarean section is characterised by the presence of clostridia and low bifidobacterial counts. Gut colonisation can be modified by probiotics, prebiotics or synbiotics. Human milk oligosaccharides (HMOs) are infant prebiotics that show a bifidogenic effect. Moreover, genome sequencing of *Bifidobacterium longum* subsp. *infantis* within the infant microbiome revealed adaptations for milk utilisation. This study aimed to evaluate the synbiotic effect of *B. longum* subsp. *infantis*, HMOs and human milk (HM) both *in vitro* and *in vivo* (in a humanised mouse model) in the presence of faecal microbiota from infants born by caesarean section. The combination of *B. longum* and HMOs or HM reduced the clostridia and G-bacteria counts both *in vitro* and *in vivo*. The bifidobacterial population *in vitro* significantly increased and produce high concentrations of acetate and lactate. *In vitro* competition assays confirmed that the tested bifidobacterial strain is a potential probiotic for infants and, together with HMOs or HM, acts as a synbiotic. It is also able to inhibit potentially pathogenic bacteria. The synbiotic effects identified *in vitro* were not observed *in vivo*. However, there was a significant reduction in clostridia counts in both experimental animal groups (HMOs + *B. longum* and HM + *B. longum*), and a specific immune response via increased interleukin (IL)-10 and IL-6 production. Animal models do not perfectly mimic human conditions; however, they are essential for testing the safety of functional foods.

Keywords: human milk, bifidobacteria, synbiotics, short chain fatty acids, cytokine

1. Introduction

Breast milk is a complex source of nutrients for infants due to a balanced composition of proteins, fats and carbohydrates, particularly oligosaccharides. This is a result of millions of years of evolution and not only supplies the nutritional needs of new-borns, but also protects them from disease while their own immune system matures (Kramer *et al.*, 2008; Lawrence and Pane, 2007; McVea *et al.*, 2000; Reilly *et al.*, 2005; Smith *et al.*, 2003). This protective effect is most frequently associated with human milk oligosaccharides (HMOs), which are the first prebiotics for infants (Kunz *et al.*, 2000). These molecules guide the development of the infant immune system and shape the intestinal microbiota towards a health-promoting community enriched with bifidobacteria, which are the predominant gut bacteria in healthy breast-fed infants and are often used as probiotics (Euler *et al.*, 2005; Haarman and Knol, 2006). *Bifidobacterium longum* subsp. *infantis* is one of the most abundant species within the genus *Bifidobacterium* present in the gut microbiota of healthy breast-fed infants, and is one of the best utilisers of HMOs (Sela *et al.*, 2008).

The present study aimed to examine how the synbiotic properties of HMOs and *B. longum* subsp. *infantis* affect

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the composition of the new-born gut microbiota both *in vitro* and *in vivo* (in a mouse model). We hypothesised that the synbiotic mixture would replace clostridia and shift the composition of the gut microbiota towards a more health-promoting community enriched with bifidobacteria. To test this, mice were fed with HMOs or human milk (HM) *ad libitum* for 14 days. Stool samples were collected at three time points and intestinal contents were collected at the end of the study to characterise and compare the bacterial composition using the plate technique and fluorescence *in situ* hybridisation (FISH). Targeted metabolomic and cytokine analyses were conducted to determine the impact of the synbiotics on the production of inflammatory markers.

2. Material and methods

In vitro competition experiments

Preparation of rifampicin-resistant bifidobacterial mutant

Probiotic strain *B. longum* subsp. *infantis* was isolated from the probiotic product Infloran (Societa' Laboratorio Farmaceutico S.I.T. Srl, Mede, Italy) by culture on modified TPY agar (Sharlau, Spain) and identified by subspecies-specific PCR (Rada and Petr, 2000; Sakata *et al.*, 2002). A rifampicin-resistant bifidobacterial mutant (RRBM) was prepared using the gradient-plate technique (highest antibiotic concentrations: rifampicin, 100 mg/l; mupirocin, 100 mg/l). Antibiotic resistance enabled the administered microorganisms to be distinguished from the wild-type strains because resistance to rifampicin is rare among bifidobacteria (Marounek *et al.*, 1995; Rada *et al.*, 1995).

Growth of rifampicin-resistant bifidobacterial mutant on human milk oligosaccharides

The ability of RRBM to utilise various fractions of HMOs, including sialyllactose and fucose as monomers of HMOs, which were added to the medium as the sole carbon source at concentrations of 2 g/l, was tested. The HMOs were isolated and purified from HM samples obtained from volunteer mothers, as described by Rockova *et al.* (2011). Sialyllactose and fucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). RRBM were cultivated on the selective medium (Wilkins-Chalgren anaerobe broth; Oxoid, Basingstoke, UK) supplemented with rifampicin (100 mg/l), mupirocin (100 mg/l) and glacial acetic acid (1 ml/l) under anaerobic conditions at 37 °C for 24 h. The initial bacterial concentration was 4 log cfu/ml. After incubation, bacteria were plated on Wilkins-Chalgren anaerobe agar (Oxoid) supplemented with rifampicin (100 mg/l), mupirocin (100 mg/l) and glacial acetic acid (1 ml/l) and counted.

Faecal samples

Eight fresh faecal samples were collected from fully breast-fed infants of both sexes [5 male and 3 female; 33.25±7.89 days (4-6 weeks) old] born by caesarean section and receiving no antibiotic or probiotic treatment. Immediately after collection, the samples were transferred to a tube with an anaerobic atmosphere and subsequently analysed using the plate technique (Vlková *et al.*, 2005) and a biochemical assay (API & ID 32; BioMérieux, Craponne, France) to confirm that they were bifidobacteria-free.

Testing of synbiotic: bifidobacteria and human milk oligosaccharides presence in infant faeces

Simultaneously with the reanalysis of infant stools, faecal samples were diluted to a concentration of 6 log cfu/g bacteria, and 2 µl was inoculated into a microtiter plate containing 88 µl of medium [HMOs; HM-pasteurised at 62.5 °C/30 min (lysozyme content of HM = 90.11±9.06 µg/ml); Control = Wilkins-Chalgren anaerobe broth] along with 5 log cfu/ml RRBM. The microtiter plate was then incubated under anaerobic conditions at 37 °C for 24 h before samples were analysed by the plate technique and by FISH, as described previously (Musilova *et al.*, 2015). Bacteria were isolated from the RRBM-selective medium and subsequently re-identified for the confirmation of RRBM by a biochemical assay and by PCR. The isolates were identified at the genus level by detecting fructose-6-phosphate phosphoketolase activity (Orban and Patterson, 2000) and at the subspecies level by PCR (Sakata *et al.*, 2002).

Measurement of short chain fatty acid and lactate levels

After the *in vitro* competition experiments, primary metabolites in all samples were determined by ion-exchange chromatography with suppressed conductivity using an ion chromatograph ICS 1600 (Dionex, Sunnyvale, CA, USA) equipped with an IonPac AS11-HC (Dionex) guard and analytical columns. The eluent comprised 1-37.5 mM KOH, with a gradient of 1-50 min and a flow rate of 1 ml/min. The ASRS 300-4 mm suppressor (Dionex) and the Carbonate Removal Device 200 (Dionex) were used to suppress eluent conductivity.

In vivo competition experiments

Faecal samples

Two bifidobacteria-free faecal samples from infants born by caesarean section (4-6 weeks old) were analysed using the plate technique and the API & ID 32 assay (BioMérieux). After analysis, both samples were collected again, mixed and used to prepare a faecal mixture (bacterial concentration, 9 log cfu/g), which was then used to humanise the gut

of germ-free (GF) mice and for reanalysis to confirm the absence of bifidobacteria. This faecal mixture was transferred to the Laboratory of Gnotobiology in Nový Hrádek and again analysed to confirm that the samples were still bifidobacteria-free.

Humanisation of germ-free mice

GF immunocompetent Balb/c mice were reared in plastic isolators and fed the 1414 diet (Altromin Spezialfutter GmbH, Lage, Germany). The mice were colonised by oral gavage (9 log cfu/g infant faecal mixture). Subsequently (after 14 days), the bacterial communities in freshly collected faeces were determined using the selective plate method after serial dilution on selective media and by FISH. Appropriate sample dilutions were transferred to sterile Petri dishes, which were immediately filled with selective media to detect the following: total anaerobes (Wilkins-Chalgren anaerobe agar); RRBM (Wilkins-Chalgren anaerobe agar), modified by addition of mupirocin (100 mg/l), rifampicin (100 mg/l) and acetic acid (1 ml/l); G-bacteria [Wilkins-Chalgren anaerobe agar supplemented with G-N Anaerobe Selective Supplement (Oxoid)]; *Enterococcus* spp. (Slanetz-Bartley medium; Oxoid); and *Escherichia coli* (TBX medium; Oxoid). FISH kits specific for the *Clostridium butyricum* group – specific probe (RiboTechnologies, The Netherlands) were used to detect and count clostridia. Total anaerobes, G-bacteria and RRBM were incubated in anaerobic jars (Anaerobic Plus System; Oxoid) at 37 °C for 48 h. *Enterococcus* spp. and *E. coli* were cultivated aerobically at 37 °C for 48 h and 24 h, respectively. Sample analysis was performed to confirm colonisation of GF mice by the infant faecal microbiota.

Assay of synbiotic mixture in humanised mice

The ability of RRBM to compete with bacteria in faecal samples from bifidobacteria-free infants born by caesarean section was tested in three different media: medium containing HMOs, medium containing HM and a Control medium (Control). Survival of RRBM was monitored by cultivation on Wilkins-Chalgren anaerobe agar supplemented with mupirocin, rifampicin and acetic acid. RRBM was then re-identified by species-specific PCR.

Two weeks after colonisation, the humanised mice were divided into three groups (each group contained four mice, 2 male + 2 female). The first group (HMOs+RRBM) received water supplemented with HMOs (7 g/l) and RRBM as described by Mielcarek *et al.* (2011), whereas the second group (HM+RRBM) received HM instead of water and RRBM. The third group was used as the Control (no HMOs, HM or RRBM). HM was obtained from The Institute for the Care of Mother and Child, Prague, and was donated by volunteer mothers. The milk was treated using the Holder method (heated to 62.5 °C for 30 min), which has minimal impact on lactoferrin, sIgA and lysozyme levels. The bifidobacterial probiotic strain was grown in Wilkins-Chalgren anaerobe broth supplemented with 5 g/l soya peptone and centrifuged at 14,100×*g* for 5 min. The supernatant was discarded, and the bacterial cells were flushed with saline and resuspended in saline to prepare the bacterial suspension (approximately 10^7 cfu/ml). The first (HMOs+RRBM) and the second (HM+RRBM) group received the bifidobacterial probiotic strain, RRBM, on every third or fourth day via oral gavage (200 µl; 2×10^6 cfu/ml RRBM). On every third day, fresh faecal samples were aseptically collected from the mice, transferred to tubes containing Wilkins-Chalgren anaerobe broth and analysed within 2 h. The samples were serially diluted in Wilkin-Chalgren anaerobe broth under anaerobic conditions. Media were prepared in an oxygen-free CO_2 environment. Faecal bacteria were detected on selective media and by FISH (clostridia) as mentioned above. After 14 days, mice were anesthetised 3-4 h after the removal of the drinking solution. Organs were aseptically removed in the following order to avoid cross bacterial contamination: peripheral blood, mesenteric lymph nodes, spleen and intestine.

Measurement of short chain fatty acid and lactate levels

In all faecal samples, primary metabolites were determined by ion-exchange chromatography with suppressed conductivity.

Cytokine analysis

Spleens were removed aseptically, and single cell suspensions were prepared. Spleen cells (6×10^5/well) were cultured in medium (RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin) (all Sigma-Aldrich) at 37 °C in CO_2 for 48 h. The concentrations of interleukin (IL)-4, IL-6, IL-10 and interferon (IFN)-γ in the cell supernatants were measured using the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore Corporation, Bedford, MA, USA). Tumour necrosis factor (TNF)-α was measured using an ELISA kit (Ready-Set-Go!; eBioscience, San Diego, CA, USA), as per the manufacturer's instructions.

Statistical analyses

Differences in log bacterial counts were evaluated using a two-tailed *t*-test with equal variance and by multiple range comparisons using Duncan's multiple range test (this procedure was used as a precaution against the known type I error inflation problem that arises when many (necessarily interdependent) comparisons are tested). Significance was set at $P\leq0.05$. All statistical analyses were performed using STATGRAPHICS Centurion XV.II (Manugistics, Rockville, MD, USA).

3. Results

In vitro competition experiments

Growth of rifampicin-resistant bifidobacterial mutant on human milk oligosaccharides

The growth of RRBM was tested in the presence of three mixtures containing HMOs isolated from pooled samples of HM. RRBM was able to utilise all the test mixtures. However, weak growth was observed on sugar substrates such as sialyllactose and fucose. We found that, even if the data for the three mixtures were significantly different at $P<0.01$ (8.17±0.04; 8.27±0.04; 8.65±0.02 log cfu/ml), the order was very similar, suggesting that there was slight statistical difference in growth between mixtures 1 to 3 because RRBM was able to grow by four orders of magnitude. However, RRBM growth on medium containing sialyllactose (6.58±0.02 log cfu/ml) and fucose (6.43±0.16 log cfu/ml) was weaker than that in the presence of a mixture of HMOs (Table 1).

Table 1. Growth of rifampicin-resistant bifidobacterial mutant (RRBM) on human milk oligosaccharides (HMOs).[1]

Carbon source	Log cfu/ml
Mixture of HMOs 1	8.17±0.04^{b}
Mixture of HMOs 2	8.27±0.04^{b}
Mixture of HMOs 3	8.65±0.02^{c}
Fucose	6.58±0.02^{a}
Sialyllactose	6.43±0.16^{a}

[1] Initial concentration of RRBM = 4 log cfu/ml. Data are expressed as the mean ± standard deviation of three independent measurements. Values denoted by different superscripts are significantly different ($P\leq0.05$). The differences among log bacterial counts were evaluated by multiple range comparison using multiple range tests.

Competition

The combination of RRBM and HMOs reduced the clostridia counts to below the detection limit (3 log cfu/g) when compared with the Control (7.43±1.02 log cfu/g), resulting in replacement of these bacteria by probiotic bifidobacteria at counts of 8.27±0.57 log cfu/g. The results for the remaining groups of detected bacteria were non-significant. The best synbiotic effect was exhibited by a combination of HM and probiotics. The combination of RRBM and HM also reduced the counts of clostridia to below the detection limit (3 log cfu/g) when compared with the Control (7.73±1.76 log cfu/g), resulting in the replacement of these bacteria by RRBM at counts of 8.23±1.40 log cfu/g. Moreover, we detected reduced *E. coli* (7.13±1.71 log cfu/g) counts when compared with the Control (8.72±0.36 log cfu/g) in all infant faecal samples in the presence of HM. We observed significantly lower counts of G-bacteria and *E. coli* after incubation of faecal samples with HM+RRBM (*in vitro* competition) when compared with Control samples, and after incubation with HMOs+RRBM (Table 2).

Significant differences were observed between the HMOs group and HMOs+RRBM group with respect to lactate concentration (HMOs, 4.31 mmol/l; HMOs+RRBM, 7.96 mmol/l). There were also significant differences between the HM and HM+RRBM groups in terms of lactate (22.34 mmol/l and 45.78 mmol/l, respectively), acetate (27.37 mmol/l and 45.66 mmol/l, respectively) and butyrate (0.93 mmol/l and 0.26 mmol/l, respectively) concentrations (Figure 1). There were also significant differences in lactate concentrations between the HM+RRBM and Control (45.78

Table 2. Bacterial counts after *in vitro* incubation of infant faeces on different substrates with/without rifampicin-resistant bifidobacterial mutant (RRBM) (log cfu/ml).[1]

Microbiota	Human milk oligosaccharides		Human milk		Control	
	Infant faeces	Infant faeces + RRBM	Infant faeces	Infant faeces + RRBM	Infant faeces	Infant faeces + RRBM
Total bacteria	9.00±0.23^{a}	9.04±0.34^{a}	8.97±0.25^{a}	9.16±0.37^{ab}	9.48±0.29^{b}	9.36±0.29^{b}
G-bacteria	7.27±0.90^{bc}	7.54±0.86^{bc}	6.52±1.26^{b}	5.18±0.77^{a}	8.11±0.57^{c}	7.91±0.74^{c}
Clostridium spp.	7.43±1.02^{a}	ND[2]	7.73±1.76^{a}	ND	7.24±1.19^{a}	ND
Bifidobacterium spp.	ND	8.27±0.57^{a}	ND	8.23±1.40^{a}	ND	8.16±0.36^{a}
Escherichia coli	9.28±1.05^{b}	9.01±0.89^{b}	8.72±0.36^{b}	7.13±1.71^{a}	9.41±0.72^{b}	9.13±0.48^{b}
Enterococcus spp.	8.71±0.42^{bc}	8.15±1.20^{bc}	7.33±1.31^{ab}	6.69±1.66^{a}	8.99±0.28^{c}	8.69±0.63^{bc}

[1] Data are expressed as means ± standard deviation (SD) of three independent measurements. Values denoted by different superscripts are significantly different ($P\leq0.05$). The differences between log bacterial counts were evaluated by multiple range comparison using multiple range tests.
[2] ND = not detected.

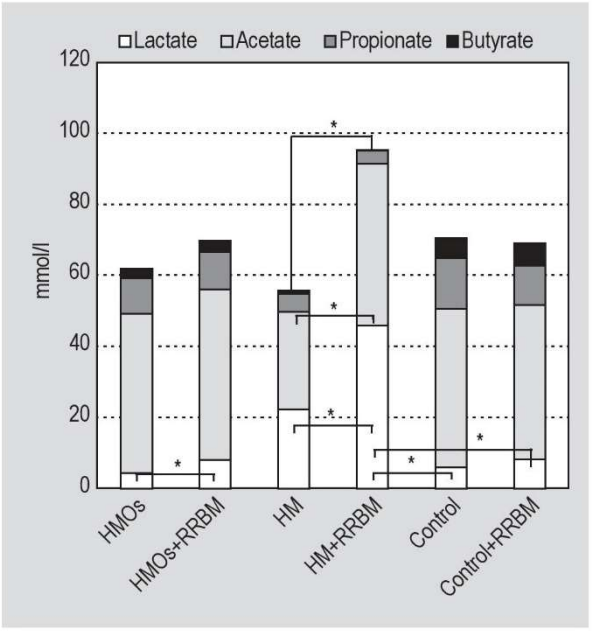

Figure 1. Lactate and short chain fatty acid concentrations were analysed after 24 h of incubation in the presence of infant faecal samples with synbiotic mixture HMOs with/without RRBM, HM with/without RRBM and Control with/without RRBM (mmol/l). Error bars indicate standard deviations (*$P \leq 0.05$). HM = human milk; HMOs = human milk oligosaccharides; RRBM = rifampicin-resistant bifidobacterial mutant.

mmol/l and 5.85 mmol/l, respectively) and also between HM+RRBM and Control+RRBM (45.78 mmol/l and 8.12 mmol/l, respectively). The results for short chain fatty acids (SCFA) correlated with the bacterial counts obtained using the plate technique.

Re-identification of isolates

Biochemical and molecular genetic tests were used to validate the presence of RRBM after the competitive growth assays. Samples confirmed as microscopically pure cultures (12/12, 25/25, 18/21, 13/13, 13/13, 8/13, 14/15, 15/15) were examined at the genus level (F6PPK); a positive result was obtained for all samples subjected to this biochemical assay. Subsequently, the isolates were identified at the subspecies level (PCR). The result for all isolates was the same, indicating that all positive samples were rifampicin-resistant mutants of *B. longum* subsp. *infantis*. From these results (Supplementary Table S1), we were able to confirm the efficacy of the selective media from which the purified isolates originated.

In vivo competition experiments

After the 14 days of the experiment, total counts for anaerobic bacteria in mice after humanisation (9.99±0.78 log cfu/g) were similar to those in the mixed infant stool used for colonisation (8.95±0.17 log cfu/g) in all experimental groups. The *E. coli* counts (9.43±0.73 log cfu/g) were not different between the experimental groups throughout the entire experiment (Figure 2). After 15 days, the counts for G-bacteria were unchanged in the second experimental (HM+RRBM) and Control groups (8.50±1.52 log cfu/g), whereas those in HM+RRBM decreased from 8.48 to 5.60 log cfu/g.

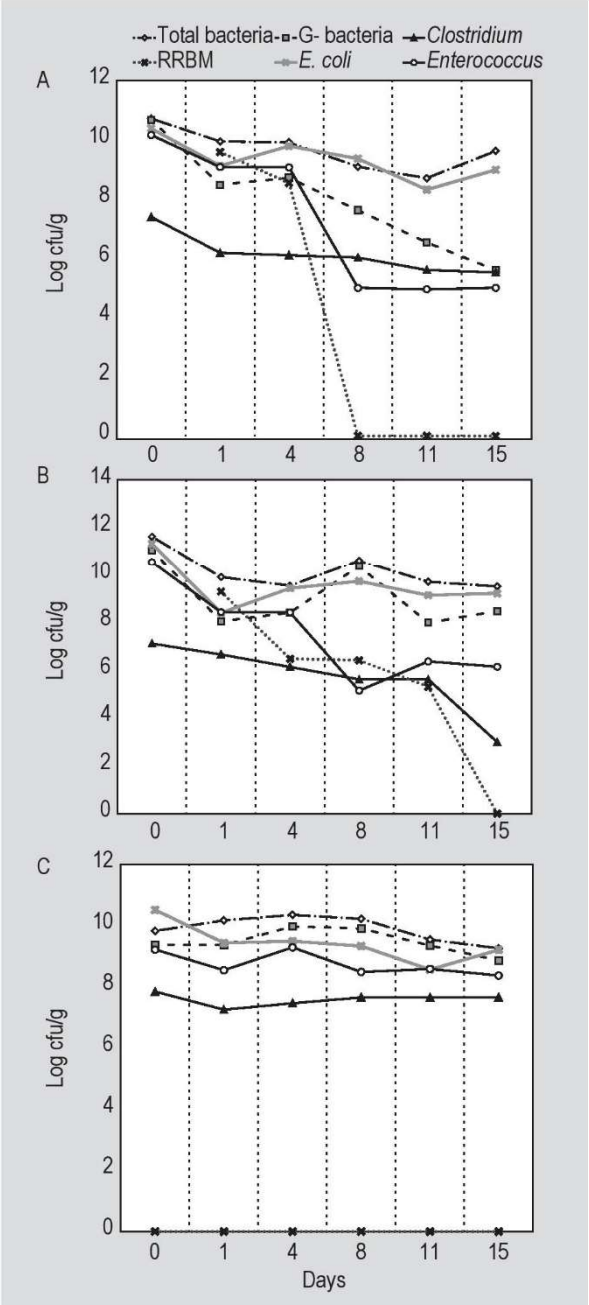

Figure 2. Bacterial counts (log cfu/g) in faeces from humanised Balb/c mice. (A) human milk oligosaccharides (HMOs) and rifampicin-resistant bifidobacterial mutant (RRBM); (B) human milk and RRBM; (C) Control group.

In both experimental groups (HMOs+RRBM and HM+RRBM), *Enterococcus* spp. counts decreased from 10.36 log cfu/g to 5.58 log cfu/g when compared with the Control group. The counts in the Control group (8.74±0.40 log cfu/g) did not change during the experiment. RRBM was able to colonise the intestinal tract of mice in just 4 days in the presence of HMOs and in 8 days in the presence of HM (Figures 2A and 2B), after which it disappeared. RRBM was not detected in the HMOs+RRBM group (limit of detection, 3 log cfu/g) even after the second administration of probiotics (8 days after the first) (Figure 2A). RRBM counts in the HM+RRBM group decreased from 9.30 to 5.31 log cfu/g, and disappeared completely 11 days after the first administration (Figure 2B).

The mouse faeces contained high clostridia counts after humanisation (9.15 log cfu/g). The clostridia counts decreased across all experimental groups (Figure 2A and 2B) when compared with the Control group. There were significant differences in the clostridia counts for the first (HMOs+RRBM) and second (HM+RRBM) groups when compared with those for the Control group. Administration of HMOs reduced the clostridia counts from 7.39 to 5.52 log cfu/g over 15 days (first group HMOs+RRBM; Figure 2A). No clostridia were detected after 15 days in the presence of HM; they disappeared when RRBM was administered. Clostridia counts decreased from 7.12 log cfu/g (on the first day of the experiment) to below the limit of detection (<5 log cfu/g). By contrast, counts in the Control decreased slightly, from 7.84 to 7.65 log cfu/g (Figure 2C).

Significant differences were observed between the groups in terms of the concentration of butyrate: HMOs+RRBM, 4.07 mmol/kg and Control group, 7.80 mmol/kg; and HM+RRBM, 3.68 mmol/kg and Control group, 7.80 mmol/kg (Figure 3). These reductions correlated with decreased clostridia counts. Production of the anti-inflammatory cytokine IL-10 is associated with a protective effect against intestinal inflammatory diseases (Kole and Maloy, 2014). We found that the HMOs+RRBM group had significantly higher IL-10 levels in the supernatant of cultured spleen cells than the Control group. Treatment with RRBM increased IL-10 levels. In addition, the HMOs+RRBM group also showed significantly higher levels of IL-6, TNF-α and IFN-γ in the supernatant of spleen cells than the other groups, even though increased levels were also detected in the HM+RRBM group (Figure 4).

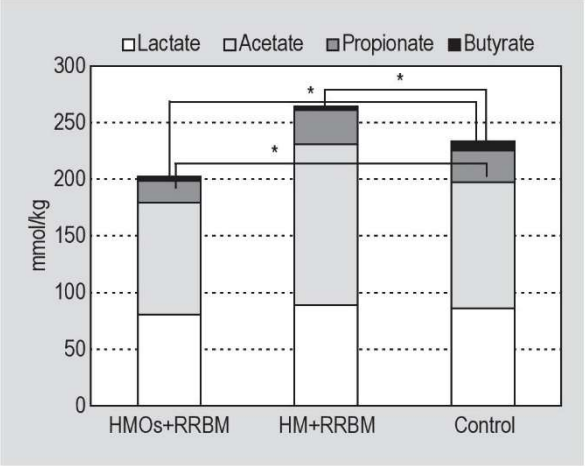

Figure 3. Lactate and short chain fatty acid concentrations in faecal samples from humanised mice after *in vivo* competition with a synbiotic mixture of human milk oligosaccharides (HMOs) and rifampicin-resistant bifidobacterial mutant (RRBM), human milk (HM) and RRBM, or Control (mmol/kg). Error bars indicate standard deviations (*$P \leq 0.05$).

4. Discussion

This study aimed to evaluate the impact of the synbiotic properties of HMOs and bifidobacteria compared to HM and bifidobacteria in the gut microbiome of new-borns delivered by caesarean section. We found no significant differences were observed in the starting values on day 1. The duration of treatment was based on previously published reports of experiments performed in humans and mice: treatment ranged from 7 days (Nakayama and Oishi, 2013) to 14 days (Monteagudo-Mera *et al.*, 2016). We observed colonisation by RRBM and reduced numbers of clostridia in HM+RRBM mice by day 8, which is in agreement with previous studies (Monteagudo-Mera *et al.*, 2016; Nakayama and Oishi, 2013) reporting that a 1 week treatment was sufficient to induce changes in the gut microbiota of mice, resulting in decreased clostridia and G-bacteria counts. After administration of HMOs, we observed flatulence or abdominal bloating at autopsy. Most studies of prebiotics in humans used a recommended dose of 8 to 15 g/day (0.11 to 0.21 g/kg weight/day) (Macfarlane *et al.*, 2008), although a bifidogenic effect was observed at doses as low as 5 g/day (Anthony *et al.*, 2006; Callaway and Ricke, 2011). Here, we used HMOs at concentrations that approximate those in HM (i.e. 7 g/l). Increased abundance of bifidobacteria and lactobacilli has been reported in mice fed with 1 g galactooligosacharides (GOS)/kg body weight (Pan *et al.*, 2009). Pan *et al.* (2009) also examined the influence of prebiotic oligosaccharides on changes in SCFA concentrations, which we confirmed in our *in vitro* and *in vivo* experiments. It was recently shown that some *Bifidobacterium* spp. have a significant immunomodulatory effect (Srutkova *et al.*, 2015). Here, we found increased levels of both IL-6 and IL-10 antagonistic cytokines, in the HMOs+RRBM mouse group.

Some studies have investigated the influence of pro- or prebiotics *in vivo*, but data from synbiotic studies are limited. Recent studies assessed the effect of synbiotic supplementation, and found promising results for three types of synbiotics (*B. longum* BL999 plus GOS/

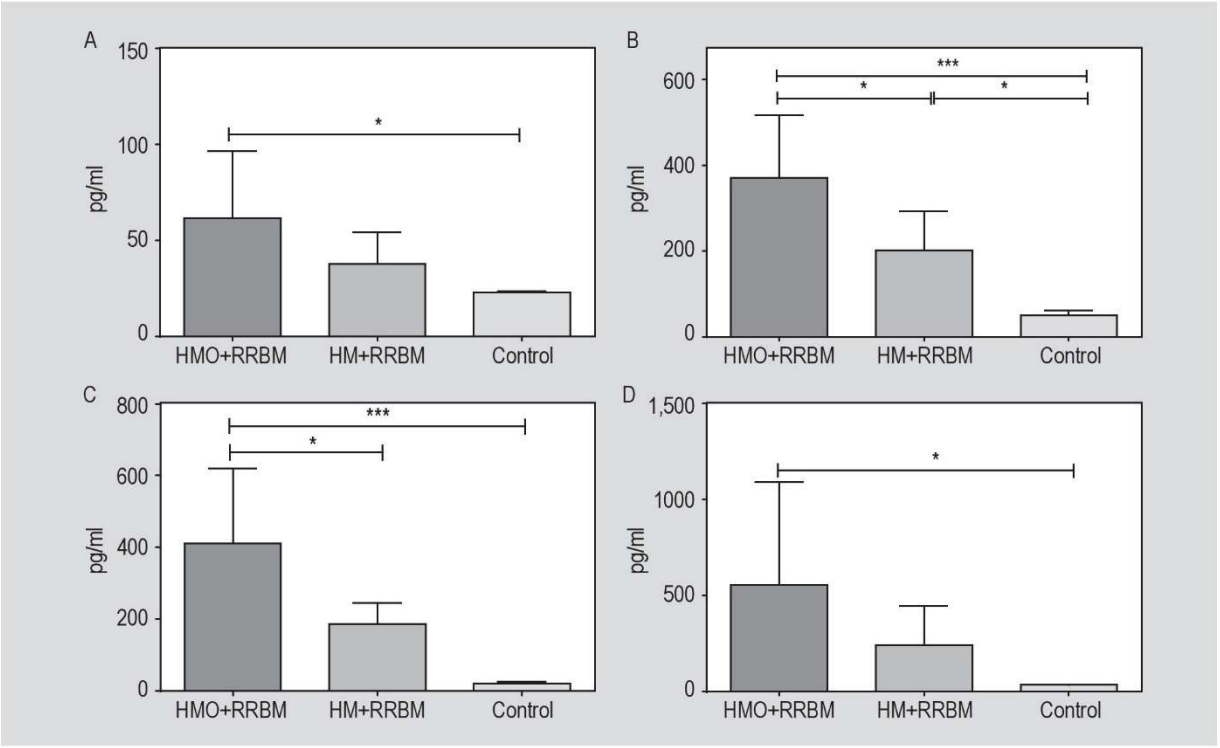

Figure 4. Cytokine levels in spleen cell supernatants from Balb/c mice after *in vivo* administration of human milk oligosaccharides (HMOs) + rifampicin-resistant bifidobacterial mutant (RRBM), human milk (HM) + RRBM or Control. Values are expressed as pg/ml. Error bars indicate standard deviations (*$P \leq 0.05$). (A) Interleukin 10; (B) interleukin 6; (C) tumour necrosis factor α; (D) interferon γ.

fructooligosaccharides (FOS), *B. longum* BL999 plus *Lactobacillus rhamnosus* LPR plus GOS/FOS, and *Lactobacillus paracasei* subsp. *paracasei*) in terms of increased stool frequency (Mugambi *et al.*, 2012); however, data regarding the effect of synbiotics on the composition of the gut microbiota are lacking. Some *in vitro* studies identified potential candidates [*Bifidobacterium breve* in combination with Vivinal® (GOS) and Actilight®950P (FOS)] for use in a synbiotic product targeted to infants (Mazzola *et al.*, 2015); however, their *in vivo* effects are unknown. Here, we obtained promising *in vitro* results with the synbiotics HMOs and RRBM; therefore, this combination was tested *in vivo* in an animal model. RRBM was able to colonise the intestinal tract of mice in just 4 days in the presence of HMOs and in 8 days in the presence of HM, even after a second administration of probiotics. The *in vitro* tests suggested that the probiotic bifidobacterial strains used herein were the best utilisers of HMOs. Unfortunately, this characteristic was not sufficient to enable colonisation of the gastrointestinal tract. Grmanova *et al.* (2010) examined the survival of nine bifidobacterial strains in the gastrointestinal tract, where one of the nine strains also disappeared after 7 days. In a previous study, we found that infants had bifidobacteria in the faeces after delivery; however, the bifidobacteria disappeared within 4-24 days (Rockova *et al.*, 2012).

We only observed the synbiotic effects *in vitro*. The greatest synbiotic effect was exhibited by HM (as a prebiotic) along with RRBM (as a probiotic). A reduced synbiotic effect was exhibited when RRBM was combined with HMOs. The maximum reduction in G-bacteria counts was recorded in medium containing HM and RRBM. RRBM counts increased in all media, which were initially inoculated with almost equal numbers of bacteria. The bifidogenic effect of HMOs was not as high as that of HM because HMOs (when provided as the sole carbon source) are complex sugars (oligosaccharides), whereas breast milk also contains simpler and easily fermentable sugars (glucose, galactose and lactose). It also contains antimicrobial factors such as lactoferrin, lactoferricin, and lysozyme, and antimicrobial peptides that inhibit the growth of potentially pathogenic microorganisms (Field, 2005). An *in vivo* study showed that HM protects suckling mice from the diarrhoeagenic effects of a heat-stabile enterotoxin produced by *E. coli* (Newburg *et al.*, 1990).

The synbiotic effect of HMOs and probiotics was demonstrated only in the *in vitro* assay. *In vivo* mouse models have several limitations and are the subject of much criticism. Although *in vivo* models may provide accurate results, these do not always agree with those from humans. For example, adult humans are not the equivalent of 70 kg mice (Leist and Hartung, 2013; Seok *et al.*, 2013)

or 3.5 kg new-borns. However, mouse models can still provide important information. Modification of the gut microbiota is possible via the use of prebiotics, probiotics and synbiotics. Animal models have been used to test the safety of some HMOs and to clarify their functions in the new-born gut, along with lacto-*N*-tetraose (Coulet *et al.*, 2013, 2014).

5. Conclusions

In vitro competition assays confirmed that the RRBM strain isolated from the probiotic Infloran is suitable for babies and can be used in combination with HM or HMOs; therefore, it might be a potential synbiotic that can inhibit potentially pathogenic bacteria in infants. The synbiotic effect was not confirmed *in vivo*. However, we did observe significantly decreased counts of clostridia in both experimental groups (HMOs+RRBM and HM+RRBM), and of G-bacteria in the HMOs+RRBM group. However, there remains a lack of *in vivo* studies. Unfortunately, not all animal models are suitable for testing HMOs. Animal models cannot perfectly mimic human conditions, but are necessary for testing the safety of functional foods such as prebiotics, probiotics and synbiotics. *In vivo* testing of HMOs and probiotic bacteria such as bifidobacteria seems to be a challenge for the future. Based on this study, we conclude that the probiotic RRBM has an immunostimulatory effect on the host immune system, which is more pronounced when using HMOs as a prebiotic than when using HM. A specific cytokine response is evoked, as evidenced by increased levels of IL-10 and IL-6 in the group of mice treated with RRBM.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2016.0138.

Table S1. Re-identification of rifampicin-resistant bifidobacterial mutants using biochemical and molecular tests.

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5.6

Influence of human milk oligosaccharides on adherence of bifidobacteria and clostridia to cell lines

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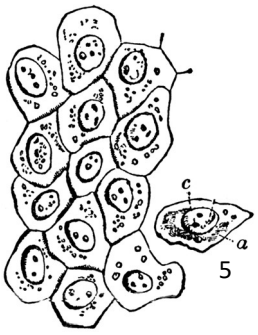

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INFLUENCE OF HUMAN MILK OLIGOSACCHARIDES ON ADHERENCE OF BIFIDOBACTERIA AND CLOSTRIDIA TO CELL LINES

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Adhesion of gut bacteria to the intestinal epithelium is the first step in their colonization of the neonatal immature gut. Bacterial colonization of the infant gut is influenced by several factors, of which the most important are the mode of delivery and breast-feeding. Breast-fed infants ingest several grams of human milk oligosaccharides (HMOs) per day, which can become receptor decoys for intestinal bacteria. The most abundant intestinal bacteria in vaginally delivered infants are bifidobacteria, whereas infants born by cesarean section are colonized by clostridia. The influence of HMOs on the adhesion of five strains of intestinal bacteria (three bifidobacterial strains and two clostridial strains) to mucus-secreting and non-mucus-secreting human epithelial cells was investigated. *Bifidobacterium bifidum* 1 and *Bifidobacterium longum* displayed almost the same level of adhesion in the presence and absence of HMOs. By contrast, adhesion of *Clostridium butyricum* 1 and 2 decreased from 14.41% to 6.72% and from 41.54% to 30.91%, respectively, in the presence of HMOs. The results of this study indicate that HMOs affect bacterial adhesion and are an important factor influencing bacterial colonization of the gut. Adhesion of the tested bacteria correlates with their ability to autoaggregate.

Keywords: human milk oligosaccharides, bifidobacteria, clostridia, cell lines, autoaggregation

Introduction

Bacterial colonization of the infant gut is influenced by several factors, of which the most important are the mode of delivery and breast-feeding. Breast milk is a complex source of nutrients for infants and has protective effects against

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diseases while their immune system matures [1–3]. This defensive function is attributed to antimicrobial peptides and proteins and human milk oligosaccharides (HMOs). Infants ingest several grams of HMOs daily and these are the first prebiotics they are exposed to [4]. These carbohydrates guide the development of the neonatal gut and shape the intestinal microbiota toward a health-promoting community of bifidobacteria, which are the predominant gut bacteria of vaginally delivered and breast-fed infants [5]. However, the predominant bacteria in infants delivered by cesarean section are clostridia, which can be gradually replaced by bifidobacteria during the breast-feeding period [6]. HMOs assist implantation of bifidobacteria into the intestines of infants. Microbial adhesion is the first step in colonization of the neonatal intestinal tract. It has been explored whether auto-aggregation of bacteria increases their ability to adhere. Del Re et al. [7] investigated the relationship between autoaggregation and the adhesion ability of *Bifidobacterium longum*. The aim of this study was to evaluate the influence of HMOs on the adhesion of bifidobacteria and clostridia to mucus-secreting HT29-MTX and non-mucus-secreting Caco-2 cell lines. We hypothesized that bifido-bacteria would adhere better than clostridia in the presence of HMOs and that their ability to adhere would correlate with their autoaggregation properties.

Materials and Methods

Bacterial strains

Bifidobacterium longum subsp. *infantis* was isolated from the probiotic product Infloran (Laboratory Pharmaceutical S.I.T., S.r.l., Italy). *Bifidobacterium bifidum* 1 and 2 were isolated from fecal samples of 3-month-old vaginally delivered breast-fed infants. Bifidobacteria were isolated using selective TPY agar (Scharlau, Spain) modified by the addition of mupirocin (100 mg/L) and acetic acid (1 mL/L) according to the method described by Rada and Petr [8] and identified by subspecies-specific polymerase chain reaction [9, 10]. *Clostridium butyricum* 1 and 2 were isolated from fecal samples of 2-month-old breast-fed infants born by cesarean section using reinforced clostridial medium agar (Oxoid, UK) according to Vlková et al. [10]. Fresh fecal samples of infants were aseptically transferred to tubes containing Wilkins–Chalgren broth (Oxoid), transported to the laboratory, and analyzed within 2 h. The samples were serially diluted in the Wilkins–Chalgren broth under anaerobic conditions and analyzed using selective media. All strains were identified by fluorescence *in situ* hybrid-ization kits with specific probes (*C. butyricum* group and *Bifidobacterium* spp.; Ribo Technologies, the Netherlands). They were further characterized by the API

50 CHL biochemical test (bioMérieux, Marcy l'Etoile, France) and their auto-aggregation properties were determined according to Vlková et al. [10].

Aggregation assay

Bacteria were cultivated in Wilkins–Chalgren broth at 37 °C for 24 h. The tubes were shaken and absorbance at 600 nm (A_{600}) was measured in the upper part of the suspension at 0, 0.5, 1, 2, 3, 4, and 5 h [10]. Aggregation ability (Agg) was expressed as the Agg percentage after 5 h of incubation, which was calculated using the following formula: Agg % = [1 − (A_{600} of upper suspension at time t/A_{600} of total bacterial suspension at time 0)] × 100 at different time points [7].

Cell culture

The human colorectal adenocarcinoma cell line Caco-2 and the mucus-secreting cell line HT29-MTX-E12 were obtained from Sigma-Aldrich (Prague, Czech Republic). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, 1% non-essential amino acids, 100 µg/mL penicillin, and 100 µg/mL streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 and 95% air. The medium was changed every 2 days, and the cells were subcultured at 80% confluency every week. All solutions were purchased from Sigma-Aldrich.

HMOs preparation

HMOs were isolated and purified from breast milk samples obtained from volunteer mothers as described by Rockova et al. [11]. Stock solution of HMOs (c = 23.5 g/L) were prepared using phosphate-buffered saline (PBS) and syringe microfilters (pore size 0.2 µm; Whatman® Anotop® IC and LC; Whatman GmbH, Germany) and used immediately after preparation.

Adhesion assays

The adhesion assay was performed using a previously described method [12] with slight modifications. Caco-2 and HT29-MTX cells were seeded in NUNC 24-well culture plates at a density of 3.6×10^4 and 0.4×10^4 cells per well, respectively, and grown for 14 ± 1 days until confluent at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 and 95% air. The culture medium was changed

every 2 days. Before adding the bacterial suspension, the cell monolayers were washed with PBS to remove antibiotics. Thereafter, 900 µL of HMO solution was added to the washed cell monolayers, whereas 900 µL of DMEM (without supplements) was added to the control wells. The final concentration ($c = 21.5$ g/L) of HMOs was the same as in the human colostrum. Subsequently, 100 µL of the bacterial suspension was added. For each strain, controls and treated wells were prepared in triplicate. Then, the plates were incubated at 37 °C for 1 h in an atmosphere of 5% CO_2. After incubation, the cell layers were gently washed three times with PBS to remove non-adherent bacteria. Finally, the cell layers were lysed by the addition of 300 µL of 1% Triton X-100 (Sigma-Aldrich) per well for 1 min, followed by the addition of 700 µL of PBS. The lysed suspension of cells with viable adhered bacteria was diluted to a bacterial concentration of 1×10^7 cfu/mL.

The bacterial counts of the collected samples were determined using the plate method after serial dilution on cultivation media (Wilkins–Chalgren anaerobe agar, Oxoid). All plates were anaerobically incubated at 37 °C for 48 h and then bacteria were counted.

Adhesion of bacteria was evaluated according to the following formula:

$$\text{Adhesion} = (\text{bacterial count of sample}/\text{bacterial count of control}) \times 100$$

where the control represents 100% adhesion.

Statistical analyses

Differences in adherence and autoaggregation of bacteria were evaluated by performing multiple range comparisons using Duncan's multiple range test [this procedure was used as a precaution against the known type I error inflation problem that arises when many (necessarily interdependent) comparisons are tested]. Significance was set at $P \leq 0.05$. All statistical analyses were performed using STATGRAPHICS Centurion XVII (Manugistics, Rockville, MD, USA).

Results

Autoaggregation of bacteria was examined on the basis of their sedimentation characteristics (Table I). The tested strains either autoaggregated (Agg+) or did not autoaggregate (Agg−) according to the phenotypes described by Del Re et al. [7]. *B. bifidum* 1 autoaggregated, had a high Agg percentage of nearly 50% (49.90%) after 4 h and formed a precipitate, resulting in a clear solution, which correlated with its ability to adhere to cell lines in the presence of HMOs.

Table I. Autoaggregation (Agg %) of the tested strains (measured after 5 h)

Strain	Agg %
Bifidobacterium bifidum 1	49.90 ± 1.33
Bifidobacterium bifidum 2	8.55 ± 1.18
Bifidobacterium longum subsp. *infantis*	1.51 ± 0.09
Clostridium butyricum 1	11.90 ± 1.64
Clostridium butyricum 2	5.70 ± 0.34

Note: Values are means of triplicates ± standard deviations.

C. butyricum 1 demonstrated weak aggregation (Agg+, 11.90%). The other tested strains (two bifidobacterial strains and one clostridial strain) were unable to aggregate (Agg−, ≤10%) and constant turbidity was observed in the tubes. The adhesion of all five strains to human Caco-2/HT29-MTX cells was examined. The ability of *B. bifidum* 1 and *B. longum* to adhere did not differ significantly between medium containing HMOs and medium lacking of HMOs (Figure 1). *B. bifidum* 1 and *B. longum* displayed almost the same level of adhesion in the presence of HMOs (32.59% ± 4.81% and 37.59% ± 4.97%, respectively) and the absence of HMOs (38.91% ± 6.83% and 39.43% ± 5.47%, respectively) (Figure 1). *B. bifidum* 2 was not able to autoaggregate (8.55 ± 1.18) and its ability to adhere

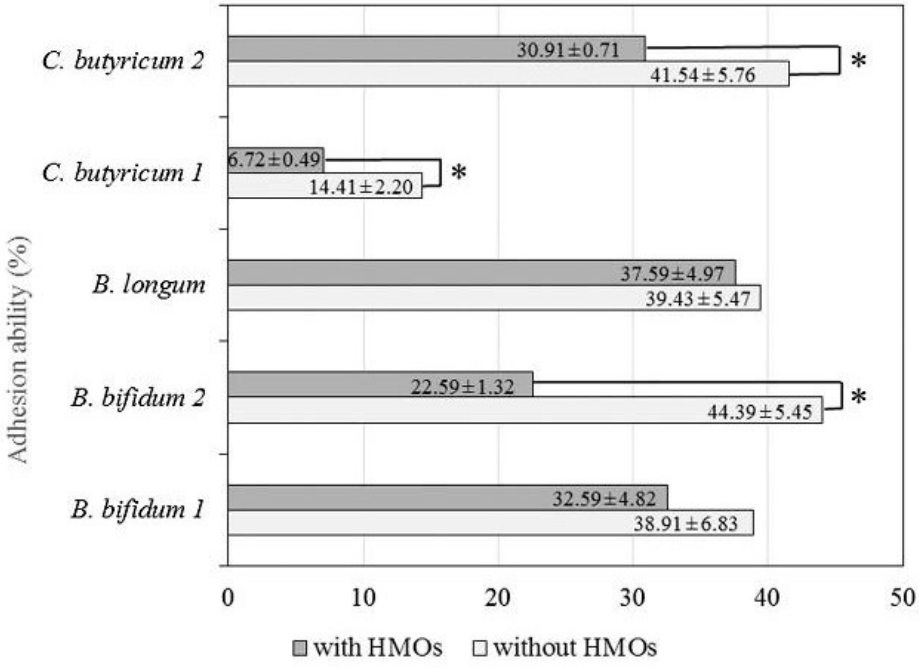

Figure 1. Ability of the tested strains to adhere to cell lines with HMOs and without HMOs. *Values are significantly different ($P < 0.05$)

to cell lines was similar as to *B. bifidum* 1 without HMOs and lower with HMOs. By contrast, *C. butyricum* 1 and 2 and *B. bifidum* 2 adhered better in medium lacking of HMOs than in medium containing HMOs.

Discussion

The Agg test is often used for preliminary screening to identify potentially adherent bacteria [13]. One of the tested strains (*B. bifidum* 1) had a high Agg and was able to adhere equally well in the presence and absence of HMOs. On the other hand, *B. longum* did not aggregate and its adhesion ability was similar in the presence and absence of HMOs, as in the case of *B. bifidum* 1. Bacterial adhesion to the intestinal mucosa is important for colonization of the infant gut. The bacterial adhesion to intestinal mucosa is one of the most important properties for colonization of the infant gut. It belongs to many health benefits of probiotic bacteria [14]. Oligosaccharides can influence the adhesion of bacteria. Inconsistent results have been reported between tested oligosaccharides and bacterial strains. In general, oligosaccharides have antiadhesive effects on gut bacteria. Chitooligosaccharides inhibit the adhesion of pathogenic *Escherichia coli* [15].

Kadlec and Jakubec [16] also tested several prebiotic oligosaccharides and reported that fructooligosaccharides and galactooligosaccharides generally decrease adhesion of the tested probiotic strains (*Lactobacillus, Lactococcus, Enterococcus*, and *Bifidobacterium* spp.). It is well known that bifidobacteria are also able to utilize different carbon source such are raffinose-series oligosaccharides present in soya beans and other legumes oligosaccharides [17]. Altamimi et al. [18] first investigated the antiadhesive effect of oligosaccharides on mixed bacteria imitating the gut microbiota *in vitro*. They reported that clostridial strains are the most strongly influenced bacteria in mixed cultures. In this study, clostridial strains were most influenced by HMOs.

In addition to their prebiotic effect, HMOs also have inhibitory effects on adhesion of pathogens, such as *Campylobacter jejuni*, enteropathogenic *E. coli, Salmonella enterica* serovar Fyris, and *Pseudomonas aeruginosa* to the intestinal human cell line Caco-2 [19]. Some HMOs act as molecular decoys by inhibiting the potential pathogens.

Therefore, we tested the influence of HMOs on common bacteria of the infant gut microbiota. Adhesion of some bifidobacterial strains was not influenced by prebiotic HMOs, while other bacteria were less able to adhere in the presence of HMOs. The ability of four of the five tested strains to adhere to epithelial cells correlated with their ability to autoaggregate, similar to the findings of a previous study [7].

Conclusions

HMOs may act as antiadhesive molecules and therefore prevent adhesion not only of potential pathogens but also of potential probiotic microorganisms. The ability to adhere to the epithelial surface of the gut seems to be strain-specific. The impact of HMOs is also strain-specific, but bacteria that can autoaggregate probably adhere better. Aggregation is an advantage, but not a requirement, for bacterial adhesion to the mucus layer.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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5.7

Impact of purified human milk oligosaccharides as a sole carbon source on the growth of lactobacilli in in vitro model

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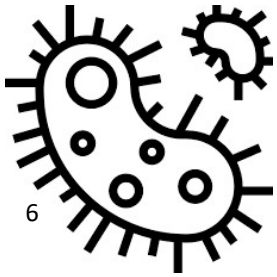

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Impact of purified human milk oligosaccharides as a sole carbon source on the growth of lactobacilli in *in vitro* model

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Recently, there is a growing interest in the use of oligosaccharides as prebiotics in order to modulate the growth of beneficial gut microbiota. It is known that human milk is a rich source of complex oligosaccharides. This paper reports the *in vitro* growth of six strains of lactobacilli in media containing purified human milk oligosaccharides (HMOs) obtained from breast milk. Based on the evaluation of bacterial densities in the growth media, together with the evaluation of pH values and bacterial metabolite detection, we concluded that the lactobacilli tested did not appear to be active HMO consumers. In the case of four strains (*Lbc. fermentum*, *Lbc. animalis* and two strains of *Lbc. delbrueckii* subsp. *bulgaricus*), no increase in bacterial density was detected. Two strains (*Lbc. acidophilus* and *Lbc. casei* subsp. *paracasei*) showed a slight, but insignificant increase in bacterial densities during 24 h of incubation.

Key words: Bifidobacteria, human milk oligosaccharides, lactobacilli, utilization.

INTRODUCTION

Human milk is a dynamic biological system (Bertino et al., 2009) containing nutrients such as proteins, lactose, fatty acids, and others, as well as biomolecules having prebiotic, immunomodulatory, or antimicrobial effects. From this group, human milk oligosaccharides (HMOs) are thought to have an important role, especially in infant nutrition. HMOs represent the third most abundant component in human milk (Casado et al., 2009), after lactose and lipids. The content of HMOs is estimated to make between 5 to 23 g/l (Ninonuevo and Lebrilla, 2009) depending on the lactation phase, genetic factors, dietary, geographical factors, and individual determinants (German et al., 2008).

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Maximum concentrations are present in colostrums, while in mature milk, contents of approximately 12 to 14 g/l are detected (Coppa et al., 2006). HMOs are composed by the following monosaccharides: glucose, galactose, sialic acid, fucose and N-acetylglucosamine (Garrido et al., 2012). Many diverse combinations and compositions of these monosaccharides, as well as several combinations of glycosidic bonds, contribute to the complexity of HMO structures (Ninonuevo and Lebrilla, 2009).

Various functions of HMOs are described in literature. They seem to have important functions in the development of the intestinal epithelium of infants (Lara-Villoslada et al., 2006), in establishing a healthy microbiota (Ninonuevo and Lebrilla, 2009), in acting as pathogen receptors (Barile and Rastall, 2013), and in having immunomodulatory properties (Venema, 2012). They are also an important source of monosaccharides, – as they provide glucose as an energy source (Venema, 2012), and sialic acid for neural tissue and brain development. One of the most important functions of HMOs is the prebiotic (bifidogenic) effect. They seem to play a key role in promoting a bifidobacteria-dominant microbiota in newborns (Coppa et al., 2006). Prebiotics influence the host by stimulating the growth and/or activity of beneficial microbiota already established in the colon (Roberfroid, 2007). The potential bifidogenic effect of breast milk was already observed and published by György et al., in 1954 (Ward et al., 2007). Since then, many other works have supported this hypothesis, and further specified that this bifidogenic effect is linked especially to oligosaccharides present in human milk (Han et al., 2012). HMOs have been proved to selectively stimulate the growth of specific bifidobacterial strains, preferentially *Bif. longum* biovar *infantis* and *Bif. bifidum,* which grew successfully on purified HMOs as the sole carbon source (Ward et al., 2006, 2007; LoCascio et al., 2007; Marcobal et al., 2010; Rockova et al., 2011a,b). It is generally accepted that HMOs have prebiotic effects, selectively serving as a source of energy for desired bacteria in the infant intestine (Bode, 2009). However, research on the capability of utilizing HMOs is mainly focused on bifidobacteria – as the predominant bacterial group in the infants' gut. Data on the utilization of HMOs by other intestinal microorganisms, among others also lactobacilli, as beneficial bacteria is scarce. As demonstrated by Marcobal et al. (2010), aside from bifidobacteria, some other intestinal bacteria are able to metabolize HMOs, including *Bacteroides fragilis* and *Bacteroides vulgatus*. These strains were proved to metabolize HMOs with high efficiency in *in vitro* conditions.

From the genus *Lactobacillus*, only strains *Lbc. gasseri* ATCC33323 (Ward et al., 2006) and *Lbc. acidophilus* NCFM (Marcobal et al., 2010) were tested for their ability to grow on HMOs. In the case of *Lbc. gasseri,* no growth was observed, whereas *Lbc. acidophilus* showed weak, but noticeable growth. No more information on the ability of lactobacilli to utilize HMOs is available according to our knowledge. The aim of this study was to investigate the ability of several strains of lactobacilli to ferment HMOs as a sole carbon source in *in vitro* conditions, thus furthering our knowledge regarding the selectivity of HMOs.

MATERIALS AND METHODS

Bacterial strains

The list of strains (six strains of lactobacilli and one strain of bifidobacteria) used in this work is shown in Tables 1 and 2. The strains were procured from the Culture Collection of Dairy Microorganisms Laktoflora® - CCDM (Prague, Czech Republic), from the Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences in Prague. Human isolates of lactobacilli were obtained from biopsy samples (Dairy Research Institute Tábor, Czech Republic).

Isolation and purification of HMOs

Human milk samples obtained from three different donors, kindly provided by the Gynecology and Obstetrics Clinic of Charles University and the General Faculty Hospital in Prague, were used for the isolation and purification of HMOs. Oligosaccharides were extracted according to the methodology described by Gnoth et al. (2000), with a few modifications. In the first step, milk (100 ml) was centrifuged at 1800 *g* for 30 min at 4°C, thus, lipids, proteins and cells were partially removed. Subsequently, proteins were precipitated by the addition of ethanol (2:1, v/v). The solution was stored at 4°C for 24 h. After centrifugation (under above mentioned conditions), the solvent was removed by rotatory evaporation, and the remainder of the solution was dissolved in deionized water. The whole process of precipitation was repeated twice. Gel filtration chromatography on a column filled with Toyopearl HW40F in 1% acetic acid (flow rate 0.1 ml/min) was used. The eluate was collected in 2.5 ml fractions and screened for the presence of oligosaccharides by thin-layer chromatogramy using isopropanol : water : 25% ammonia solution (5:1:2, by volume) as a mobile phase, and was then visualized by spraying with 10% sulphuric acid in ethanol and heating. Carbohydrate containing fractions (a total volume of 50 ml) were dispensed into vials and cooled at a temperature of 4-8°C for 30 min., and frozen at – 70°C for 90 min. Samples were subsequently lyophilized using Cryodos device (Telstar, Spain). The yield from 100 ml of milk made 0.5 g of purified oligosaccharides.

Bacterial growth on HMOs

Basal medium (tryptone, 10 g; peptone, 10 g; yeast extract, 5 g; Tween 80® 1 ml, distilled water 1 L) was autoclaved (121°C, 15 min). Purified oligosaccharides (1 % w/w) were added as a sole carbon source to the cooled medium after sterile filtration (Puradisc FP 30 filter 0.2 µm, Whatman, Germany). As a negative control, a medium devoid of carbohydrate was used. As a positive control, Wilkins Chalgren broth (Oxoid, Basingstoke, UK) was used. Overnight bacterial cultures were centrifuged (5000 *g*, 7 min) and re-suspended in saline. Bacterial suspensions were inoculated into

Table 1. Utilization of human milk oligosaccharides.

Strain	Density of lactobacilli (change in A_{540})		
	HMO	BM	WCH
Lbc. fermentum RL 25	0.13 ± 0.06^{ab}	0.17 ± 0.12^{b}	5.20 ± 0.20^{c}
Lbc. delbrueckii subsp. *bulgaricus* CCDM 66	0.07 ± 0.06^{a}	0.03 ± 0.06^{a}	5.03 ± 0.21^{bc}
Lbc. acidophilus CCDM 151	0.53 ± 0.06^{c}	0.07 ± 0.06^{ab}	4.87 ± 0.06^{b}
Lbc. delbrueckii subsp. *bulgaricus* CCDM 767	0.03 ± 0.06^{a}	0.03 ± 0.06^{a}	4.77 ± 0.21^{b}
Lbc. casei subsp. *paracasei* PE1TB-P	0.43 ± 0.06^{bc}	0.17 ± 0.06^{b}	4.97 ± 0.15^{bc}
Lbc. animalis CCDM 382	0.17 ± 0.06^{ab}	0.07 ± 0.06^{ab}	5.17 ± 0.15^{c}
Average	$0.23 \pm 0.20^{\beta}$	$0.09 \pm 0.08^{\alpha}$	$5.00 \pm 0.21^{\gamma}$
Bif. bifidum JKM	2.01 ± 0.47^{d}	0.07 ± 0.02^{ab}	3.01 ± 0.11^{a}

Data are expressed as increase in turbidity of bacterial suspension estimated from increase in A540 during 24 h of incubation; values are means from triplicate determination ± standard deviation (SD). HMO, medium containing purified human milk oligosaccharides as a carbon source; WCH, Wilkins Chalgren broth (control medium); BM, basal medium without carbohydrate source (negative control). a-d data in columns with different superscripts differ ($P < 0.05$). αβγ data in lines with different superscripts differ ($P < 0.05$).

Table 2. pH values of media.

Strain	Origin	Final pH values after 24 h of incubation	
		HMO	WCH
Lbc. fermentum RL 25	human faeces	6.20 ± 0.03^{b}	4.86 ± 0.04^{f}
Lbc. delbrueckii subsp. *bulgaricus* CCDM 66	yogurt, Turkey	6.50 ± 0.04^{cd}	4.56 ± 0.04^{b}
Lbc. acidophilus CCDM 151	pill Biolacta	6.14 ± 0.02^{b}	4.66 ± 0.05^{d}
Lbc. delbrueckii subsp. *bulgaricus* CCDM 767	yogurt, Switzerland	6.55 ± 0.03^{d}	4.86 ± 0.04^{f}
Lbc. casei subsp. *paracasei* PE1TB-P	biopsy sample (colon)	6.20 ± 0.05^{b}	4.54 ± 0.05^{a}
Lbc. animalis CCDM 382	raw goat milk	6.45 ± 0.05^{c}	4.68 ± 0.05^{e}
Average		$6.34 \pm 0.17^{\alpha}$	$4.69 \pm 0.14^{\beta}$
Bif. bifidum JKM	infant faeces	5.00 ± 0.20^{a}	4.65 ± 0.15^{c}

Values are means ± standard deviation (SD) of three measurements. $^{a-f}$ data in columns with different superscripts differ ($P < 0.05$). $^{\alpha\beta}$ data in lines with different superscripts differ ($P < 0.05$). HMO – medium containing purified human milk oligosaccharides as a carbon source. WCH, Wilkins Chalgren broth (control medium). Initial pH values of HMO and WCH media were 6.60 and 6.40, respectively.

a medium containing HMOs and then incubated at 37°C for 24 h under anaerobic conditions. All strains were grown in triplicate. The growth of lactobacilli was evaluated as the change in absorbance A_{540} during 24 h of incubation by measuring transmitted light using densitometer DEN-1 (Dynex, Czech Republic). Results were expressed as increase in turbidity of the bacterial suspension estimated from increase in A_{540}. For the determination of pH values, pH meter HACH sension 1 (HACH, USA) was used. The results were evaluated using MS Excel 2007 (Microsoft, Redmond, USA).

Determination of bacterial metabolites

To determine organic acids concentration, the isotachophoretic (ITP) method was used. The samples after fermentation by lactobacilli were subjected to isotachophoretic separations using IONOSEP 2003 device (Recman, Czech Republic). The change in the content of lactic acid as the major metabolite of lactobacilli as well as the content of acetic, butyric, propionic, formic and succinic acids was monitored. Prior to analysis, the samples were diluted with 150 volumes of deionized water, and then purified using the Puradisc FP 30 filter with a pore size of 0.2 µm (Whatman, Germany). Solution containing 10 mM HCl, 22 mM ε-aminocaproic acid and 0.1 % 2-hydroxy-ethylcellulose (pH 4.5) as leading electrolyte (LE) was used. As trailing electrolyte (TE), 5 mM caproic acid was used. All chemicals were obtained from Sigma-Aldrich (Czech Republic). The values of the initial and final stream used were 80 and 30 µA, respectively.

Statistical analyses

For evaluation of the results Statgraphics® Centurion XV (StatPoint, Inc., Warrenton, USA), the multiple range comparison - LSD test was used. A significant difference was statistically considered at the level of $P < 0.05$.

RESULTS AND DISCUSSION

In this work, 6 strains of lactobacilli of different origin were tested for their ability to ferment HMOs as a sole

carbohydrate source. The growth of strains tested is summarised in Table 1. In the case of the four strains (*Lbc. fermentum* RL25, *Lbc. animalis* CCDM 382 and two strains of *Lbc. delbrueckii* subsp. *bulgaricus* CCDM 66 and CCDM 767), no increase in bacterial density in the medium with HMOs was observed. The change in the absorbance A_{540} after 24 h of incubation in these groups of strains ranged from 0.03 to 0.17. In the rest of the strains tested (*Lbc. acidophilus* CCDM 151 and *Lbc. casei* subsp. *paracasei* PE1TB-P), a slight increase in bacterial densities in HMO-containing medium was observed (0.53 for *Lbc. acidophilus*, 0.43 for *Lbc. casei* subsp. *paracasei*). As a positive control, Wilkins Chalgren (WCH) broth was used. In this medium, high cell densities (from 4.77 to 5.20) in all strains were obtained (Table 1). The strain *Bif. bifidum* JKM was used as a positive control, too. This strain is able to effectively utilize HMOs, as demonstrated previously (Rockova et al., 2011a). As a negative control, a basal medium without any added sugar was used. A marginal increase in absorbance $A_{540,}$ even in the absence of sugar, was seen (Table 1). Increased cell numbers for bacterial species like *Lactobacillus, Enterococcus, Enterobacteriaceae* or *Staphylococcus* in media without carbohydrate supplementation were also observed by other authors (Marcobal et al., 2010; Satoh et al., 2013).

The strain PE1TB-P began to grow in WCH broth after the first hour of incubation (Figure 1), while growth in the HMO-containing medium was noticeable after three hours. Instead of exponential growth, a slight steady growth during 24 h of incubation was observed. A very similar trend was noticed for the strain *Lbc. acidophlilus* (data not shown).

To precisely evaluate the fermentation ability, besides measuring the bacterial density, it is important to analyse the changes in pH of growth media, and possibly to analyse metabolite concentration produced by bacteria. Final pH values (Table 2) are consistent with the change in A_{540} measured after 24 h of incubation. The pH of the medium containing purified HMOs decreased from the initial value of 6.60 to 6.34 on average, while in the control medium (WCH), the pH decrease was much more apparent (from 6.40 to 4.69 on average). Anaerobic intestinal microbiota convert carbohydrates to lactic acid and short-chain fatty acids (Loo et al., 1999) such as acetic, propionic and butyric acids. Lactic acid has a role in maintaining lower intestinal pH (Satoh et al., 2013), while butyric acid, sometimes produced by heterofermentative lactic acid bacteria, provides nutrition of the colonic epithelium and has an important role in gut maintenance (Venema, 2012). The results of bacterial metabolite analysis are presented in Figures 2 and 3. The medium with HMOs produced significantly lower concentrations of lactic acid compared to the control medium (WCH broth) after 24 h of fermentation. The production of lactic acid in WCH broth rose to 225 mg/100 ml (in the strain PE1TB-P), while the maximum concentration of lactic acid detected in the medium with HMOs made no more than 40 mg/100 ml (in the strain CCDM 151). To a somewhat lower extent also in the strain PE1TB-P a slight increase in lactic and acetic acids was visible, which indicates some bacterial growth. Concentrations of succinic and formic acids rose marginally (up to 16 and 11 mg/100 ml, respectively), and in the case of propionic and butyric acids, non-detectable concentrations, even lower than 2 mg/100 ml (data not shown), were obtained.

The strain *Bif. bifidum* JKM, used as a positive control, showed very good growth in the medium with HMOs compared to the growth of lactobacilli. The increase in the absorbance A_{540} made 2.01 (Table 1). The growth was accompanied by a decrease in pH values (Table 2) and by an increase of acids produced (Figure 2).

Direct fermentation of HMOs by intestinal microbiota has not yet been well described and there is a lack of information regarding their utilization by specific bacterial species (lactobacilli). The majority of information, that exists on HMO fermentation refers to bifidobacteria as the predominant bacterial group in a healthy infants's gut. Many *in vitro* studies were conducted on the capability of bifidobacteria to ferment HMOs with positive results (Ward et al., 2006, 2007; LoCascio et al., 2007; Marcobal et al., 2010; Satoh et al., 2013), but growth in the presence of HMOs is not a property of all representatives of the genus *Bifidobacterium*. Preferential growth of *Bif. longum* subsp. *infantis,* a species often occurring in infants, was noticed in the aforementioned studies. This strain preferentially utilized oligosaccharides with a degree of polymerization (DP) ≤ 7. These oligosaccharides form a significant part of breastmilk (LoCascio et al., 2007). In the study conducted by Rockova et al. (2011a), bifidobacterial strains of human origin (*Bif. bifidum* and *Bif. longum*) were proved to utilize HMOs with high efficiency in comparison with bifidobacteria of animal origin (*Bif. animalis).* Utilization capability is closely related to the enzymatic equipment that specific bacteria possess. Enzyme lacto-N-biose I phosphorylase was recently proved to be responsible for the cleavage of lacto-N-biose I, which is an important component of HMOs (Satoh et al., 2013). The presence of this enzyme was detected in species *Bifidobacterium bifidum* and *Bifidobacterium longum* occuring in infants' gut (Wada et al., 2008). Conversely, in other bacterial groups like lactobacilli, clostridia or bacteroides, this enzyme was not observed (Wada et al., 2008). The strain *Bif. longum* subsp. *infantis* also possesses other enzymes involved in the cleavage of HMOs, such as fucosidase or sialidase (LoCacio et al., 2007). Additionally, between certain bifidobacterial strains, commensal activities were described, where strains able to cleave long-chain HMOs (*Bif. bifidum*) can provide monosaccharides for other strains

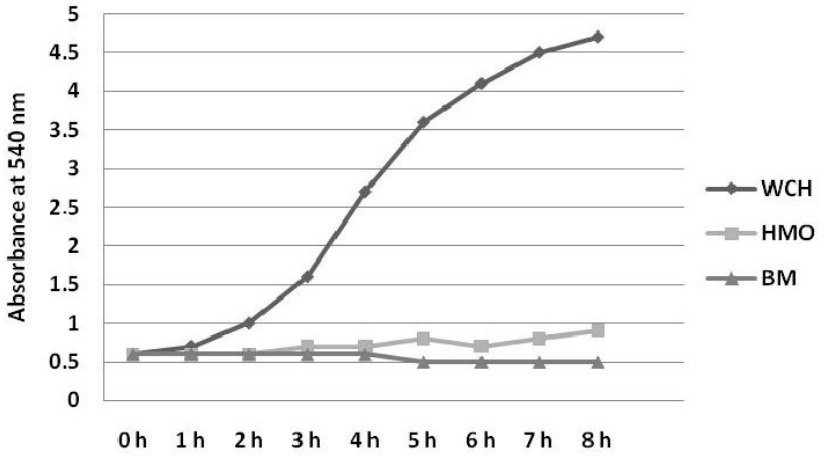

Figure 1. Growth of *Lbc. casei* subsp. *paracasei* PE1TB-P in the medium containing HMOs as a sole carbon source. WCH, Wilkins Chalgren medium as a positive control; BM, basal medium without any carbohydrate as a negative control.

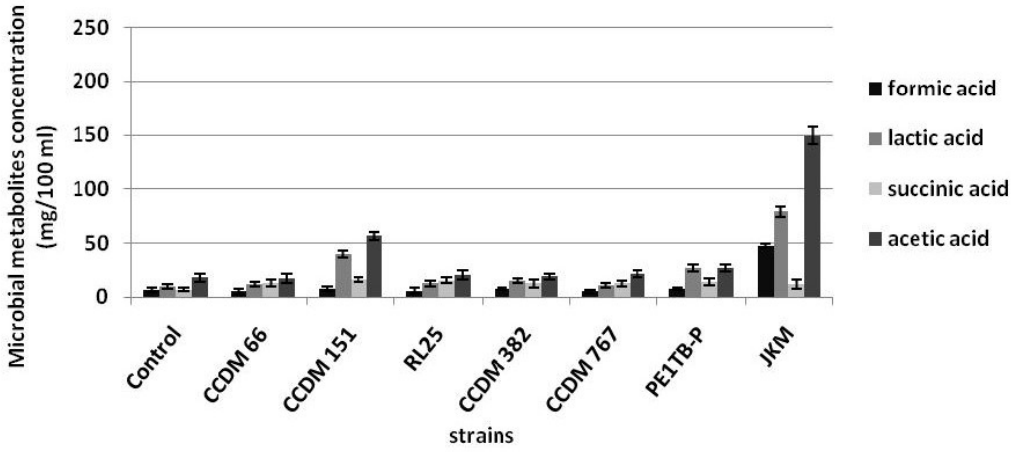

Figure 2. Concentrations of microbial metabolites in the medium containing purified human milk oligosaccharides after 24-h fermentation by lactobacilli. Concentrations of butyric acid and propionic acid made less than 2 mg/100 mL of the media (data not shown). Bars present mean ± SD.

(*Bif. breve*, Ward et al., 2007). Marcobal et al. (2010) demonstrated that HMO fermentation is not an exclusive property of specific strains of bifidobacteria. In the study conducted by this group, apart from *Bif. longum* subsp. *infantis*, for the first time, *Bacteroides fragilis* and *Bacteroides vulgatus* were proved to be able to metabolize HMOs with high efficiency. Either weak or no fermentation was exhibited by genera *Clostridium, Eubacterium, Enterococcus, Streptococcus*, *Veillonella* and *E. coli* strains. From the group of lactobacilli, a strain *Lbc. acidophilus* NCFM was tested which showed some growth ability on this substrate (Marcobal et al., 2010). In another *in vitro* study (Ward et al., 2006), a strain *Lbc. gasseri* ATCC33323 was tested in which the ability to ferment HMOs was not proved.

The major part of HMOs reach the colon in unhydrolyzed form, where they may be utilized by intestinal microbiota into short chain fatty acids (Lasrado and Gudipati, 2013) and thus serve as nutrients – prebiotics (Loo et al., 1999; Ninonuevo and Lebrilla, 2009). A prebiotic effect is proven when the growth of beneficial bacteria is stimulated, while potentially harmful bacteria

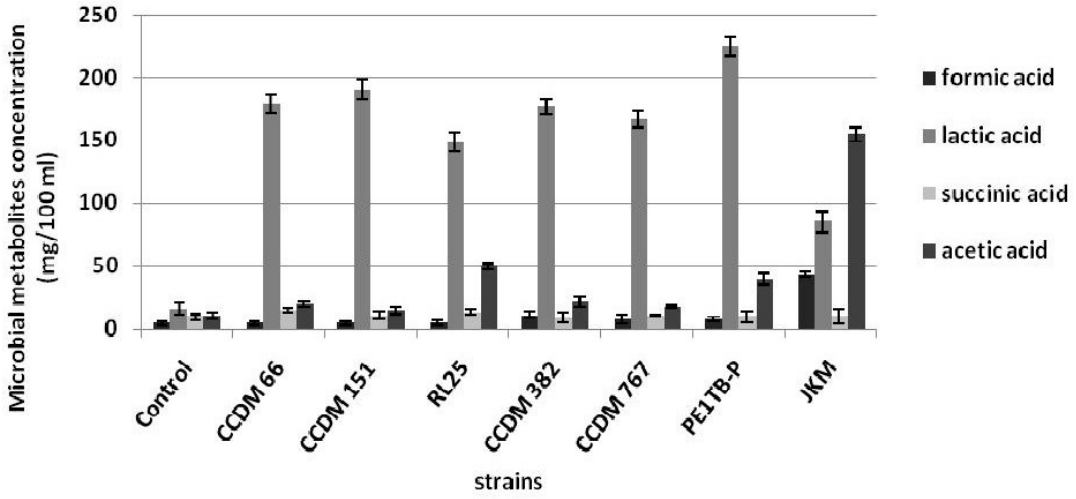

Figure 3. Concentrations of microbial metabolites in the medium containing Wilkins Chalgren broth after 24-h fermentation by lactobacilli. Concentrations of butyric acid and propionic acid made less than 2 mg/100 mL of the media (data not shown). Bars present mean ± SD

are inhibited (Boehm et al., 2004). In this study we conducted an *in vitro* testing on direct fermentation of purified HMOs by lactobacilli. The results of this work support the hypothesis that utilisation of HMOs may be species- and strain specific. Based on the evaluation of the results obtained by absorbance $A_{540,}$ measured together with bacterial metabolite detection and the evaluation of pH values, we concluded that the lactobacilli tested did not appear to be active HMO consumers. This fact supports the hypothesis that HMOs may selectively enhance the growth of specific bacterial groups (particularly bifidobacteria) present in the colon of newborns.

Conflict of Interests

The authors did not declare any conflict of interests.

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5.8

Colonization of Germ-Free Piglets with Commensal Lactobacillus amylovorus, Lactobacillus mucosae, and Probiotic E. coli Nissle 1917 and Their Interference with Salmonella Typhimurium

Šplíchal I., Donovan S. M., Šplichalová Z., Neužil Bunešová V., Vlková E., Jenišťová V., Killer J., **Švejstil R.**, Skřivanová E., Šplíchalová A. (2019). Microorganisms. 7(8), 273.

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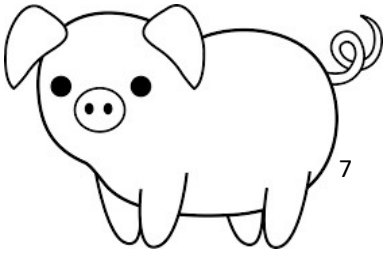

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Colonization of Germ-Free Piglets with Commensal *Lactobacillus amylovorus, Lactobacillus mucosae,* and Probiotic *E. coli* Nissle 1917 and Their Interference with *Salmonella* Typhimurium

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Abstract: Non-typhoid *Salmonellae* are worldwide spread food-borne pathogens that cause diarrhea in humans and animals. Their multi-drug resistances require alternative ways to combat this enteric pathogen. Mono-colonization of a gnotobiotic piglet gastrointestinal tract with commensal lactobacilli *Lactobacillus amylovorus* and *Lactobacillus mucosae* and with probiotic *E. coli Nissle* 1917 and their interference with *S.* Typhimurium infection was compared. The impact of bacteria and possible protection against infection with *Salmonella* were evaluated by clinical signs, bacterial translocation, intestinal histology, mRNA expression of villin, claudin-1, claudin-2, and occludin in the ileum and colon, and local intestinal and systemic levels of inflammatory cytokines IL-8, TNF-α, and IL-10. Both lactobacilli colonized the gastrointestinal tract in approximately 100× lower density compare to *E. coli* Nissle and *S.* Typhimurium. Neither *L. amylovorus* nor *L. mucosae* suppressed the inflammatory reaction caused by the 24 h infection with *S.* Typhimurium. In contrast, probiotic *E. coli* Nissle 1917 was able to suppress clinical signs, histopathological changes, the transcriptions of the proteins, and the inductions of the inflammatory cytokines. Future studies are needed to determine whether prebiotic support of the growth of lactobacilli and multistrain lactobacilli inoculum could show higher protective effects.

Keywords: *Lactobacillus amylovorus*; *Lactobacillus mucosae*; *E. coli* Nissle 1917; *Salmonella* Typhimurium; food-borne pathogen; intestine; cytokine; gnotobiotic piglet

1. Introduction

A microbiota consists of a pool of microorganisms that harbor a host body. The vast majority of these microorganisms colonize the gastrointestinal tract (GIT) [1]. This resident microbiota shapes physiology of the host via digestion and assimilation of nutrients, stimulation, and maturation of host tissues,

regulation of the host immune response, and keeps status quo to prevent a deleterious appearance of opportunistic and obligatory pathogens [2]. The colonization of the GIT and establishment of the balanced microbiota is sequential. In newborns, it is influenced by the mode of delivery, nutrition, and exposition to antibiotics [3]. Low concentrations of antibiotic supplements used as growth promoters in feed for livestock cause antibiotic resistance of microorganisms. Alternative supplements of the feed as acidifiers, zinc, copper, and tungsten, yeast products, nucleotides, plant extracts, prebiotics, and probiotics replace antibiotics and increase the growth of animals and feed utilization, reduce mortality and morbidity, and improve reproduction parameters without development of the antibiotic resistance [4,5].

Pioneer strains that first settled the GIT are facultative anaerobes that consume oxygen and create conditions in this environment suitable for strictly anaerobic bacteria [6]. The interactions between different members of microbial consortia and the host can extend from mutualism to pathogenesis; the interactions between different members of microbial consortia and the host can extend from mutualism to pathogenesis in dependence on the balanced microbiota composition that prevents to dysbiosis is a basic prerequisite of the host health [7].

The genus *Lactobacillus* comprises more than 200 species of Gram-positive facultative anaerobic bacteria that occupy nutrient-rich niches in humans, animals, plants, and food [8]. They are a frequent component of probiotic preparations for humans, fish, livestock, and pets [9]. *E. coli* Nissle 1917 belongs to the most used and studied probiotic bacteria. It has several siderofores and other iron acquisition systems, produce colicins and microcins, induce defensins in the host, and modulates its intestinal barrier [10,11]. In contrast to lactobacilli and probiotic *E. coli* Nissle 1917 that are considered beneficial microbes, the genus Salmonella comprises obligatory enteric pathogens [12]. Non-typhoid *Salmonellae* (NTS) are major agents of food-born infectious diarrhea and cause 200,000 deaths annually worldwide [13]. The infection with NTS commonly causes self-limiting enterocolitis (salmonellosis). However, it can cause life-threatening invasive diseases such as meningitis, osteomyelitis, septic arthritis, deep soft-tissue infection, and pneumonia in immunocompromised individuals [14,15]. The serovar Typhimurium belongs to the most commonly spread *Salmonella* serovars in human and pigs [16,17]. Due to its genetics, physiology, and anatomy, the pig is commonly used in biomedical research and it is a suitable model of human gastrointestinal [18] and infectious diseases [19]. Gnotobiotic animals, with their simple and defined microbiota, enable investigations of interactions among different bacterial species and strains and interactions between microbiota and its host [20–22].

The aim of this research was to evaluate the ability of the commensal *Lactobaccilus* strains, *L. amylovorus* or *L. mucosae*, to colonize the GIT of newborn germ-free piglets, their impact to the host, and their ability to suppress infection with *S.* Typhimurium. The probiotic *E. coli* Nissle 1917, which has been previously shown to reduce diarrhea in different host species, served as a positive control for protective effects against *S.* Typhimurium infection.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were reviewed and approved by the Animal Care and Use Committee of the Czech Academy of Sciences, protocol #117/2012.

2.2. Isolation, Characterization, and Identification of Commensal Lactobacilli

Two commensal lactobacilli were isolated from a fresh pig fecal sample using Rogosa Agar (Oxoid, Basingstoke, UK) and cultivated in enriched Wilkins–Chalgren broth (Oxoid), characterized by function

tests, and identified by MALDI-TOF MS and 16S rRNA gene sequencing as described in detail in Appendix A.

2.3. Bacterial Strains and Bacterial Suspensions

Lactobacillus amylovorus, strain P1 (LA), and *Lactobacillus mucosae*, strain P5 (LB), were isolated from pig feces, characterized by function tests, identified by MALDI-TOF MS and 16S rRNA gene sequencing, and used in the experiments. A probiotic *E. coli* Nissle 1917 (EcN) is a biologically active compound of a probiotic preparation Mutaflor® (Ardeypharm, Herdecke, Germany). *Salmonella enterica* subsp. enterica serovar Typhimurium, strain LT2 (*S*. Typhimurium, ST) was from a collection of the microorganisms of the Institute of Microbiology of the Czech Academy of Sciences (Novy Hradek, Czech Republic).

Fresh bacterial cultures were prepared for each experiment by cultivation for 16 h at 37 °C. Lactobacilli were cultivated in 10 mL MRS broth (Oxoid). The cells were harvested by centrifugation at 4000 × *g* for 10 min. The pellet was washed twice with 0.05 M phosphate buffer and resuspended to an approximate density of 8.5 log colony forming units (CFU)/mL. EcN and ST were cultivated overnight on meat-peptone agar slopes (blood agar base; Oxoid), and both resuspended to 8.5 log CFU/mL. The number of CFU was verified by cultivation methods.

2.4. Gnotobiotic Piglets

Miniature Minnesota-derived germ-free piglets were obtained by hysterectomy under inhalation isoflurane anesthesia (Isoflurane; Piramal Healthcare UK, Morpeth, UK) on the 112th day of gestation as described in details elsewhere [23]. The gnotobiotic piglets were reared in positive-pressure fiberglass isolators with heated floor. They were fed to satiety 6–7 times per day with an autoclave-sterilized cow's milk-based formula (Mlekarna Hlinsko, Hlinsko, Czech Republic) by a nipple. Specimens taken at hysterectomy (amniotic membranes, umbilical cords, meconium, mouth, and isolator surface smears) and twice a week during rearing of the piglets (mouth, surface body and isolator smears, and stool) were cultivated for the presence of aerobic and anaerobic bacteria, and mold. Additionally, Gram-stained rectal swabs were inspected under a light microscope.

2.5. Experimental Design

Each piglet group was created from three hysterectomies (Figure 1). A total of 55 gnotobiotic piglets were assigned to eight groups: i) Germ-free for the whole experimental period (GF, $n = 6$); ii) one-week-old GF piglets orally infected with 6.0 log CFU of *S*. Typhimurium for 24 h (ST, $n = 7$); iii) orally colonized with 8.0 log CFU of *L. amylovorus* 4 h after hysterectomy (LA, $n = 7$); iv) one-week-old piglets LA-colonized (since 4 h after hysterectomy) and orally infected with 6.0 log CFU of *S*. Typhimurium for 24 h (LA+ST, $n = 7$); v) orally colonized with 8.0 log CFU of *L. mucosae* 4 h after hysterectomy (LM, $n = 7$); vi) one-week-old piglets LM-colonized (since 4 h after hysterectomy) and orally infected with 6.0 log CFU of *S*. Typhimurium for 24 h (LM+ST, $n = 7$); vii) orally colonized with 8.0 log CFU of *E. coli* Nissle 1917 4 h after hysterectomy (EcN, $n = 7$); and viii) one-week-old piglets EcN-colonized (since 4 h after hysterectomy) and orally infected with 6.0 log CFU of *S*. Typhimurium for 24 h (EcN+ST, $n = 7$). The bacterial inoculums were applied in 5 mL of a milk diet, and the control GF piglets obtained 5 mL of milk without any bacteria. Twenty-four hours after the challenge with *Salmonella* (ST, LA+ST, LM+ST, and EcN+ST), the piglets were euthanized by exsanguination via cardiac puncture under isoflurane anesthesia, and samples were collected. Their non-infected counterparts (GF, LA, LM, and EcN) were proceeded in the same way at the same age.

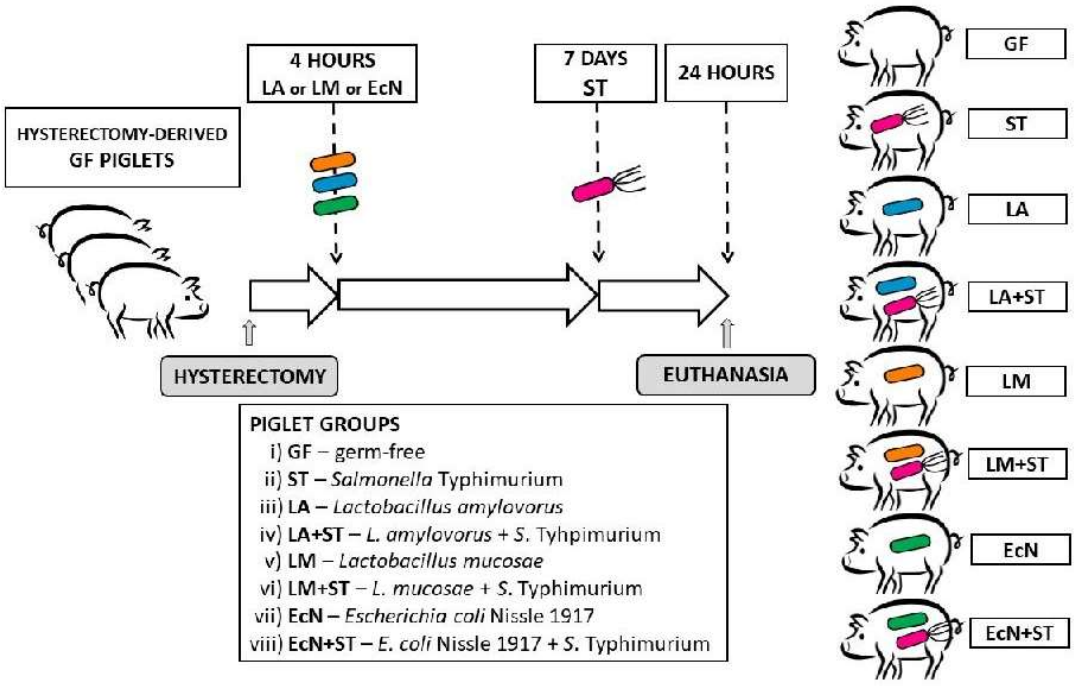

Figure 1. Schema of the experiment. The gnotobiotic piglets (n = 55) were assigned into eight groups: (i) Germ-free (GF, n = 6); (ii) infected with *S.* Typhimurium for 24 h (ST, n = 7); (iii) colonized with *L. amylovorus* (LA, n = 7); (iv) LA-colonized and infected with *S.* Typhimurium for 24 h (LA+ST, n = 7); (v) colonized with *L. mucosae* (LM, n = 7); (vi) LM-colonized and infected with *S.* Typhimurium for 24 h (LM+ST, n = 7); (vii) colonized with *E. coli* Nissle 1917 (EcN, n = 7); and (viii) EcN-colonized and infected with *S.* Typhimurium for 24 h (EcN+ST, n = 7).

2.6. Clinical Signs

The piglets were observed for fever, anorexia, somnolence, and diarrhea during each feeding.

2.7. Bacterial Colonization of the GIT and Translocation

Samples of peripheral blood were cultivated log 10 and diluted by PBS. Jejunum (40 cm of the proximal part of the jejunum) and ileum (40 cm segment of a terminal part of the small intestine containing the ileum and part of the distal jejunum) lavages were cut off, filled with 2 mL of Dulbecco's PBS (DPBS; Life Technologies, Carlsbad, CA), gently kneaded, and rinsed. Colon lavage was obtained by placing the whole colon in a 90 mm Petri dish, cut into small pieces in 4 mL of DPBS. All lavages were vigorously vortexed. Further, 0.2 g of mesenteric lymph nodes, liver, and spleen were homogenized in 0.8 mL deionized water in a 2 mL Eppendorf tube containing two 3.2 mm stainless-steel beads in a TissueLyser LT beadbeater (Qiagen, Hilden, Germany) shaken for 3 min at 50 Hz. The intestinal lavages, tissue homogenates, and blood were serially diluted in PBS and cultivated in 90 mm Petri dishes with MRS agar for lactobacilli (Oxoid), MacConkey agar (Merck, Darmstadt, Germany) for *E. coli* or Brilliant green agar (Oxoid) for *S.* Typhimurium. The plates were incubated aerobically at 37 °C for 48 h for lactobacilli or 24 h for *E. coli* or *S.* Typhimurium. The CFU were counted from dishes optimally containing 20–200 colonies.

2.8. Blood Plasma and Intestinal Lavage Supernatants

A citrated blood was spun at 1200 × *g* for 10 min at 8 °C, and protease inhibitor cocktail (Roche Diagnostics, Manheim, Germany) was added to the collected plasma. The intestinal lavages were spun at 2500 × *g* for 30 min at 8 °C, supernatants were filtered through 0.2 µm nitrocellulose filter (Sartorius, Goettingen, Germany), and protease inhibitor cocktail (Roche Diagnostics) was added. Both the plasma and the lavage supernatants were immediately frozen and stored at –45 °C until cytokines were measured.

2.9. Histologic Assessment

Terminal ileum specimens were fixed in Carnoy's fluid for 30 min, dehydrated and embedded in paraffin. Cross-sections of tissue (5 µm) were stained with hematoxylin-eosin and examined under blinded conditions under an Olympus BX 40 microscope with an Olympus Camedia C-2000 digital camera (Olympus, Tokyo, Japan). Ten measurements for each parameter were taken per piglet to assess ileal villus length and crypt depth. Thirty evenly spaced radial lamina mucosalis propria widths per each piglet were measured. Histological scoring was evaluated as described elsewhere [21]. Briefly: i) Submucosal edema (0–2); ii) PMNs (polymorphonuclear neutrophils) infiltration into the lamina propria (0–2); iii) villus atrophy (0–3); iv) exudate in lumen (0–2); v) vessels dilatation (0–2); vi) inflammatory cellularity in lymphatic vessel lumen (0–2); vii) hyperemia (0–2); viii) hemorrhage (0–2); ix) peritonitis (0–1); or x) erosion of the epithelial layer (0–3). The total score of 0–21 points was obtained.

2.10. Total RNA Isolation and Reverse Transcription

Cross-sections (1–2 mm) of terminal ileum and transversal colon were stored in RNAlater (Qiagen, Hilden, Germany) at −20 °C until RNA purification. Slices of the intestine were moved from RNAlater to 600 µL RLT buffer of the RNeasy Plus Mini kit (Qiagen) containing an antifoaming reagent DX (Qiagen) and 2 mm zirconia beads (BioSpec Products, Bartlesville, OK) in 2 mL Eppendorf tube. The tissue was homogenized in TissueLyser LT beadbeater (Qiagen) at 50 Hz for 5 min at RT. The next steps of the total RNA purification followed the manufacturer's instructions. Total RNA (500 ng) with ratio absorbances $A_{260}-A_{320}/A_{280}-_{A320} \geq 2.0$ measured in 10 mM Tris-HCl buffer pH 7.5 were reverse transcribed by QuantiTect Reverse Transcription kit (Qiagen) with initial 2 min genomic DNA wipeout at 42 °C, 20 min reverse transcription at 42 °C, and 3 min terminating step at 95 °C according to manufacturer's instructions. Then, 180 µL of PCR quality water (Life Technologies, Carlsbad, CA) was added to 20 µL of the synthesized cDNA, and these 1/10 diluted PCR templates were stored at −25 °C until the following real-time PCR.

2.11. Real-Time PCR

First, 2 µL of the PCR template was added to 18 µL of the FastStart Universal Probe Master (Roche Diagnostics) containing 100 nM LNA (locked nucleic acid) probe (Universal ProbeLibrary; Roche Diagnostics) and 500 nM each of the forward and reverse primers (Generi-Biotech, Hradec Kralove, Czech Republic) (Table 1). Ten minutes initial heating at 95 °C followed 45 cycles at 95 °C for 15 s and 60 °C for 60 s. The mixtures were incubated and measured in duplicates on an iQ cycler with iQ5 Optical System Software 1.0 (Bio-Rad, Hercules, CA, USA). Cq for villin, claudin-1, claudin-2, and occludin were normalized to β-actin and cyclophilin A and their relative mRNA fold change expressions were calculated by $2^{-\Delta C_T}$ method [24] by GenEx 6.1 software (MultiD Analyses AB, Gothenburg, Sweden).

Table 1. LNA probe-based real-Time PCR systems.

Gene	5′-forward primer-3′	5′-reverse primer-3′	#LNA Probe
BACT [1]	TCCCTGGAGAAGAGCTACGA	AAGAGCGCCTCTGGACAC	9
CYPA [2]	CCTGAAGCATACGGGTCCT	AAAGACCACATGTTTGCCATC	48
VILLIN	GCATGAAGAAGGTGGAGACC	ACGTTCCTCTTGCCCTTGA	42
CLD-1 [3]	CACCACTTTGCAAGCAACC	TGGCCACAAAGATGGCTATT	3
CLD-2 [4]	CTCGCGCCAAAGACAGAG	ATGAAGATTCCACGCAACG	77
OCLN [5]	AAAGAGCTCTCTCGACTGGATAAA	AGCAGCAGCCATGTACTCTTC	42

[1] β-actin, [2] cyclophylin A, [3] claudin-1, [4] claudin-2, [5] occludin.

2.12. Luminex xMAP Technology

Intestinal lavage and plasma levels of IL-8, TNF-α, and IL-10 were measured by a paramagnetic sphere-based xMAP technology (Luminex Corporation, Austin, TX, USA) with a Porcine ProcartaPlex kit (Affymetrix, Santa Clara, CA, USA). The frozen samples were slowly melted at 6 °C, centrifuged at 10,000 × *g* for 5 min at 6 °C, and 25 µL of the samples were incubated with the beads according to the manufacturer's instructions. The cytokine levels were measured on the Bio-Plex Array System and were evaluated by Bio-Plex Manager 4.01 software (Bio-Rad).

2.13. Statistical Analysis

Differences among the groups in parameters with normal distribution according to the Kolmogorov–Smirnov test were evaluated with one-way analysis of variance (ANOVA) with Sidak's multiple comparisons post-hoc test or one-way ANOVA with Tukey's multiple comparisons post-hoc test. Values that did not meet the normal distribution were evaluated with Kruskal–Wallis with Dunn's multiple comparisons post-hoc test. The statistical comparisons were performed at $P < 0.05$ by GraphPad 6 software (GraphPad Software, La Jolla, CA, USA) and differences depicted in figures by a letter system.

3. Results

3.1. Characterization and Identification of Lactobacilli

The lactobacilli suitable for the experiment were chosen according to their resistance to low pH and bile extract and strain affiliation. Strains, later identified as *L. amylovorus*, strain P1 (LA), and *L. mucosae*, strain P5 (LM), did not decrease in counts after 3 h of incubation in oxygen-free phosphate buffered saline (OF-PBS) supplemented with bile extract even at the concentration of 3% of bile extract. Strain LA was also fully resistant to pH 3, and strain LM to pH 2. LM showed a slight decrease in viability about 0.5 log CFU/mL. MALDI-TOF MS identification determined the first strain as the species *L. amylovorus* (LA) and the second one as *L. mucosae* (LM) and they were marked P1 and P5 strains for storing purposes, respectively. The identity of the selected strains was confirmed based on the 16S rRNA gene sequencing. The assigned GenBank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequences are MK377165 (*L. amylovorus*, P1 strain) with 99.78% of 16S rRNA gene similarity to *L. amylovorus* DSM 20531^T and MK377166 (*L mucosae*, strain P5) to *L. mucosae* DSM 13345^T with 100%.

3.2. Clinical Signs

All monocolonized non-infected gnotobiotic piglets (LA, LM, and EcN) thrived and were comparable with the GF controls. The *Salmonella*-infected ST piglets suffered from fever, anorexia, somnolence, and non-bloody diarrhea. In the piglets previously colonized with LA, LM, or EcN, the infection with *Salmonella* provoked clinical signs similar to the ST group in the LA+ST and LM+ST only, but not in the EcN+ST group. The EcN+ST piglets thrived as non-infected ones.

3.3. Colonization of the Intestine and Translocation of L. amylovorus, L. mucosae, E. coli Nissle 1917, and Their Interference with S. Typhimurium in the Gnotobiotic Piglets

The influence of *S.* Typhimurium on the growth of *L. amylovorus*, *L. mucosae*, and *E. coli* Nissle 1917 was studied by comparison of the groups LA vs. LA+ST, LM vs. LM+ST, and EcN vs. EcN+ST (Figure 2). No differences between LA, LM, or EcN alone and LA, LM or EcN in the presence of *S.* Typhimurium were found in the jejunum (Figure 2A) and ileum (Figure 2B). In the colon (Figure 2C), the presence of *S.* Typhimurium decreased numbers of LA and LM but not EcN. Lactobacilli were not cultivated, or in some cases, seldom with relatively low numbers from MLN (Figure 2D). However, EcN translocated to MLN in all cases up to log 4 CFU/g, but *Salmonella* diminished these counts. Neither commensal lactobacilli nor probiotic *E. coli* were found in the liver (Figure 2E), spleen (not shown here), and blood (Figure 2F).

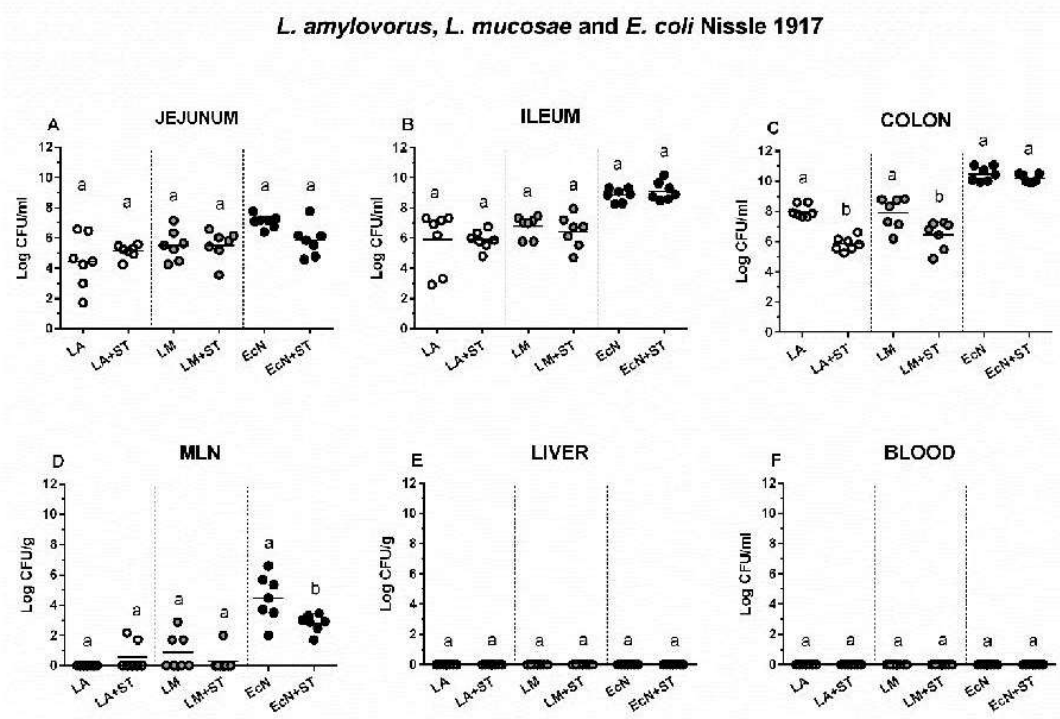

Figure 2. Colonization and translocation of *L. amylovorus*, *L. mucosae*, and *E. coli* Nissle 1917 in the gnotobiotic piglets. *L. amylovorus* (LA), *L. mucosae* (LM), and *E. coli* Nissle 1917 (EcN) colony forming units (CFU) were counted in the jejunum (**A**), ileum (**B**), colon (**C**), mesenteric lymph nodes (MLN; (**D**)) the liver (**E**), and blood (**F**) in monocolonized piglets (LA, LM, and EcN) and monocolonized piglets infected with *S.* Typhimurium (LA+ST, LM+ST, and EcN+ST). Interferences between LA, LM, EcN, and ST as LA vs. LA+ST, LM vs. LM+ST, and EcN vs. EcN+ST, respectively were evaluated by one-way ANOVA with Sidak's multiple comparisons post-hoc test. Statistical differences were marked by a letter system at $P < 0.05$. The same letter means no statistical significance. Log CFU are depicted as individual spots with mean as a horizontal line and $n = 7$ for all groups.

3.4. S. Typhimurium in the Intestine, Its Translocation, and Interference with L. amylovorus, L. mucosae, and E. coli Nissle 1917

Salmonella was completely suppressed in the jejunum in the piglets previously colonized with EcN (Figure 3A). This suppression was much lower but still statistically significant in the ileum (Figure 3B), but it did not occur in the colon (Figure 3C). Lower numbers of *Salmonella* CFU were found in MLN (Figure 3D) of the EcN+ST piglets (approximately 3 log CFU/g) but not in the cases of LA+ST or LM+ST (approximately 5 log CFU/g) that were comparable with the ST group. EcN also lowered *Salmonella*

translocation into the liver (Figure 3E), but neither previous colonization with LA nor with LM did it. The lower number of bacteremia occurrence was found in LM+ST and EcN+ST groups (both in two of seven cases), but this lowering was not statistically significant (Figure 3F).

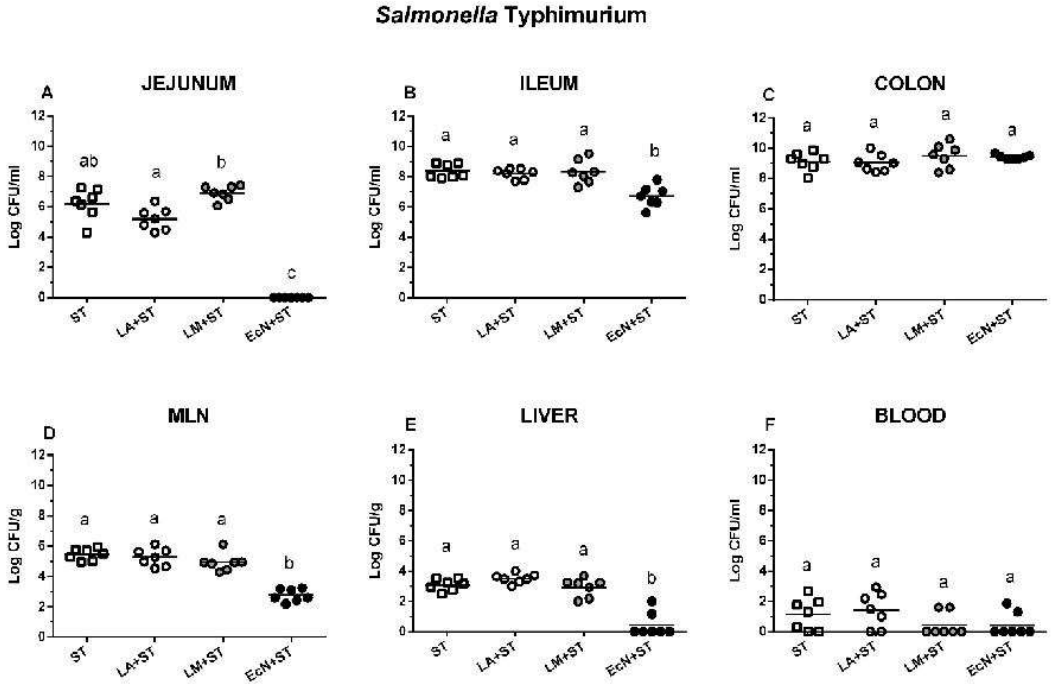

Figure 3. *S.* Typhimurium counts in the intestine, mesenteric lymph nodes, liver, and blood. *S.* Typhimurium (ST) colony forming units (CFU) were counted in the jejunum (**A**, proximal jejunum), the ileum (**B**), the colon (**C**), mesenteric lymph nodes (MLN; (**D**)), the liver (**E**), and blood (**F**). *S.* Typhimurium (ST) and its interferences with *L. amylovorus* (LA+ST), *L. mucosae* (LM+ST), and *Escherichia coli* Nissle 1917 (EcN+ST) were evaluated by one-way ANOVA with Tukey's multiple comparisons post-hoc test. Statistical differences were marked by a letter system at $P < 0.05$. The same letter means no statistical significance. Log CFU are depicted as individual spots with mean as a horizontal line and $n = 7$ for all groups.

3.5. Histological Assessment in the Ileum of the Gnotobiotic Piglets

Histological assessment was performed on hematoxylin- and eosin-stained ileum slices. The villus height and muscular thickness were comparable among GF, LA, LM, and EcN groups (Table 2). Crypts were statistically significantly deeper and the ratio of villus height/crypt depth was lower in the EcN group compare to GF piglets.

Table 2. Villus height, crypt depth, ratio of villus height/crypt depth, and muscularis thickness in the terminal ileum in the gnotobiotic piglets.

	GF	LA	LM	EcN
Villus height (μm)	705.2 ± 76.2 ^{a}	610.0 ± 289.0 ^{a}	679.7 ± 82.9 ^{a}	450.2 ± 146.5 ^{a}
Crypt depth (μm)	74.1 ± 3.4 ^{a}	74.5 ± 9.2 ^{a}	78.8±6.6 ^{a}	94.5 ± 5.0 ^{b}
Height/Depth (ratio)	10.2 ± 2.3 ^{a}	8.6 ± 5.3 ^{ab}	8.7 ± 1.4 ^{ab}	4.8 ± 1.6 ^{b}
Muscularis thickness (μm)	54.2 ± 12.1 ^{a}	54.2 ± 17.9 ^{a}	47.6 ± 10.1 ^{a}	71.2 ± 27.3 ^{a}

Differences among villus height, crypt depth, ratio of villus height/crypt depth, and muscularis thickness in the terminal ileum in the germ-free (GF) piglets and the piglets mono colonized with *L. amylovorus* (LA), *L. mucosae* (LM), and *E. coli* Nissle 1917 (EcN) were evaluated by one-way analyses of variance (ANOVA) with Tukey's multicomparison post-hoc test. The results are presented as mean ± S.D. The values with different letters significantly differ ($P < 0.05$). Six piglets in each group were compared.

The ileum showed villi with the presence of vacuolated enterocytes along the whole length of the villus from the villus tips to the top of crypts in the GF, LA, and LM piglets (Figure 4A,B). The representative picture of the LA group is shown only because no obvious differences among the GF, LA, and LM groups were found. The vacuolated enterocytes were found on the apical half of villus in the EcN piglets (Figure 4E,F). *Lamina propria* cellularity in the EcN group was higher, and Peyer's patches were 2–3-fold larger than in the GF, LA, and LM groups (Figure 4A,B). No inflammation was found in the ileum of the non-infected piglets. In contrast, the ileal sections in the piglets infected with *Salmonella* (ST, LA+ST, and LM+ST) represented by the LA+ST group (Figure 4C,D) showed signs of acute inflammation such as submucosal edema, PMN infiltration into the *lamina propria*, villus atrophy, exudate in the lumen, vessel dilatation, inflammatory cellularity in the lymphatic vessel lumen, hyperemia, hemorrhage, and multiple erosion. Using our scoring system (Figure 4I), the total histological score was between 10–12 and was comparable among infected groups except the EcN+ST group, which was without the inflammation (Figure 4G,H).

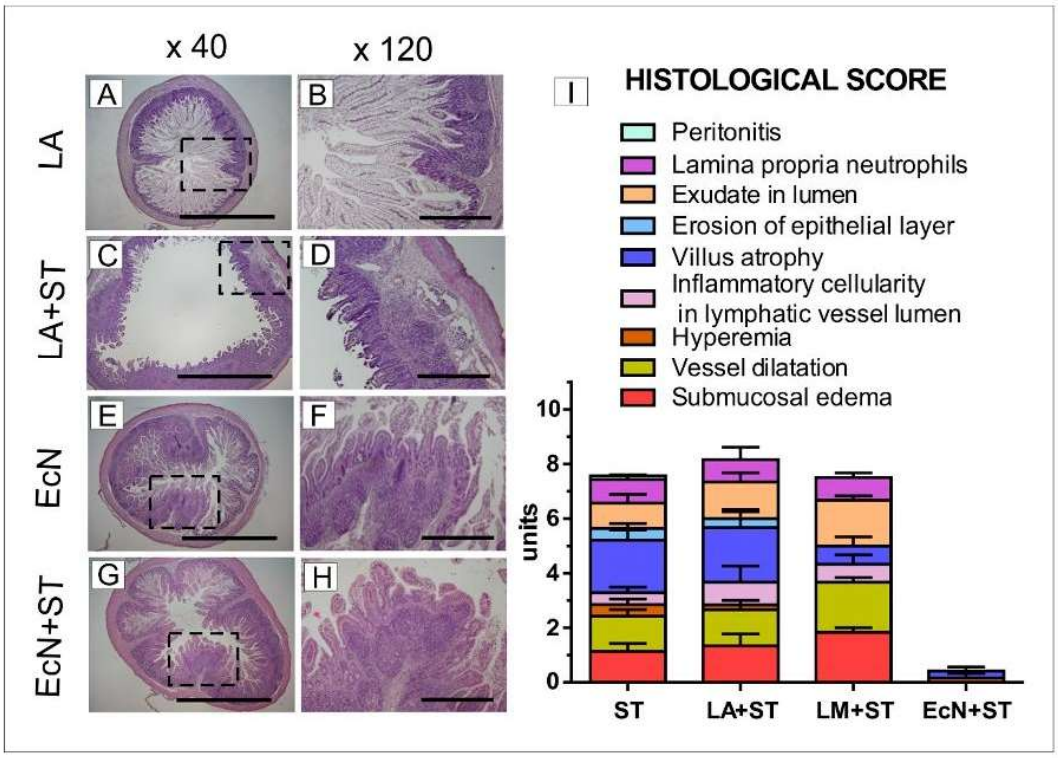

Figure 4. Representative hematoxylin- and eosin-stained cross sections of the ileum in the gnotobiotic piglets and a histological score. The piglet colonized with *L. amylovorus* (LA; **A**,**B**), the piglet colonized with *L. amylovorus* and infected with *S.* Typhimurium for 24 h (LA+ST; **C**,**D**), the piglet colonized with *E. coli* Nissle 1917 (EcN; **E**,**F**), and the piglet colonized with *E. coli* and infected with *S.* Typhimurium for 24 h (EcN+ST; **G**,**H**). No obvious differences were observed between the GF, LA, and LM groups; thus, LA represents these three groups. Bars represent 1 mm (**A**,**C**,**E**,**G**) and 500 µm (**B**,**D**,**F**,**H**) cross sections, respectively. Histological scores from the ileum of six piglets per group are depicted (**I**).

3.6. Transcriptions of Villin, Claudin-1, Claudin-2, and Occludin in the Intestine of the Gnotobiotic Piglets

Relative transcriptions of villin (5A,E), claudin-1 (5B,F), claudin-2 (5C,G), and occludin (5D,H) in the ileum (5A–D) and colon (5E–H) are depicted on Figure 5. Villin mRNA in the ileum was significantly downregulated by *Salmonella* (Figure 5A), but in the case of the piglets colonized with *E. coli* Nissle 1917 and infected with *S.* Typhimurium (EcN+ST), this decrease was not statistically significant. All bacteria

downregulated villin transcription in the colon, and the presence of *L. mucosae* and *Salmonella* made this downregulation statistically significant (Figure 5E). In contrast, claudin-1 transcription (Figure 5B) was statistically significantly upregulated in the ileum in all *Salmonella*-infected piglets (ST, LA+ST, LM+ST) except the piglets colonized with EcN and later infected with ST (EcN+ST). In the colon (Figure 5F), the infection with *Salmonella* (ST, LA+ST, LM+ST) showed a similar trend as in the ileum and again with the exception of EcN of little increase transcription in comparison with GF, LA, and LM groups, but this transcription was not statistically significantly upregulated by the following infection with *Salmonella* (EcN+ST). Claudin-2 did not show any obvious trend in the ileum (Figure 5C), but in the colon (Figure 5G), *Salmonella* significantly downregulated it in the majority of *Salmonella*-infected piglets (ST, LA+ST, and LM+ST) except EcN colonized piglets (EcN+ST). In this group, the decrease was not statistically significant. As in the case of claudin-2, the down/upregulation of occludin in the ileum (Figure 5D) was not obvious and usually not statistically significant. However, in the colon (Figure 5H) the downregulation by *Salmonella* in all *Salmonella*-infected groups was observed.

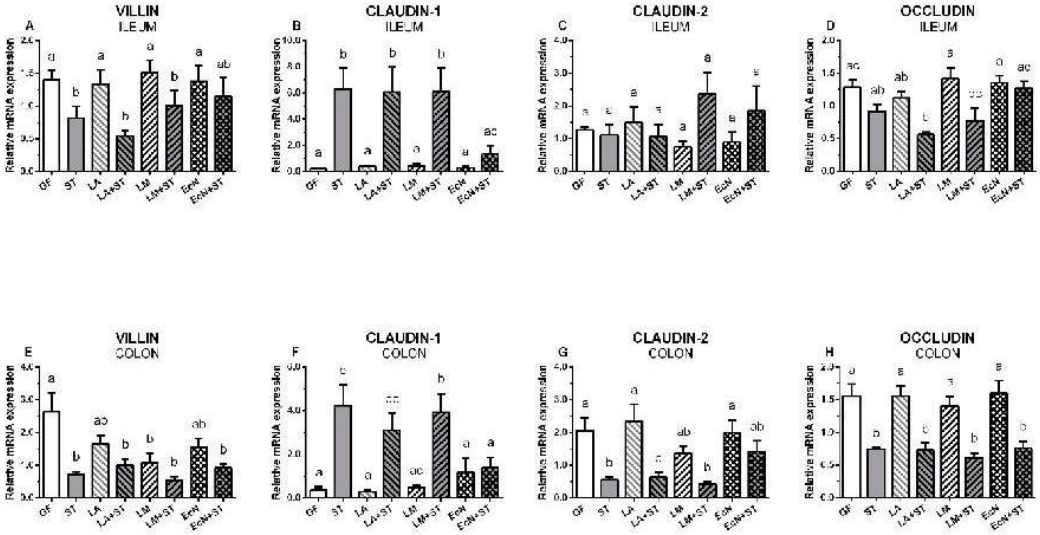

Figure 5. Transcriptions of villin, claudin-1, claudin-2, and occludin in the intestine of the gnotobiotic piglets. The relative mRNA expressions (fold change) were evaluated in the germ-free piglets (GF) and the piglets colonized/infected with *L. amylovorus* (LA and LA+ST), *L. mucosae* (LM and LM+ST), *E. coli* Nissle 1917 (EcN and EcN+ST), and *S.* Typhimurium (ST, LA+ST, LM+ST, and EcN+ST). Villin (**A**,**E**), claudin-1 (**B**,**F**), claudin-2 (**C**,**G**), and occludin (**D**,**H**) mRNA in the ileum (**A**–**D**) and colon (**E**–**H**) of the gnotobiotic piglets were normalized to β-actin and cyclophilin A. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons post-hoc test was used to compare differences among the groups. The values are presented as mean + SEM. Statistical differences $P < 0.05$ are denoted with different letters above the columns, and the same letter shown above the column indicates no statistically significant differences. Six samples in each group were analyzed.

3.7. Local and Systemic Levels of IL-8, TNF-α, and IL-10 in the Gnotobiotic Piglets

The levels of IL-8, TNF-α, and IL-10 were measured in the ileum and colon lavages and plasma of the gnotobiotic piglets. IL-8 in the ileum and colon were comparable in the GF, LA, and LM groups and they were statistically different from the groups infected with *Salmonella* (ST, LA+ST, and LM+ST) except the EcN+ST group (Figure 6A,D). The colonization with EcN slightly increased IL-8 values in both parts of the intestine, but this increase was not significantly different from GF or from the *Salmonella*-infected piglets. A similar situation was in the cases of TNF-α levels in the ileum (Figure 5B) and colon (Figure 5E). However, TNF-α in the GF, LA, LM, and EcN groups were under the detection limit of the method. The previous colonization with EcN in the EcN+ST group was able to completely

suppress TNF-α levels that were also under detection limit as in the case of the GF and other the groups mentioned above. While in the ileum the levels in the groups ST, LA+ST, and LM+ST increased significantly from the GF group (Figure 6B), in the colon, the increase in the LM+ST group was not significant (Figure 6E). The local levels of IL-10 in the intestine (Figure 6C,F) reached much lower levels than in the case of IL-8 and TNF-α, but it was again possible to observe an induced increase in the ST, LA+ST, and LM+ST groups. Additionally, in the ileum (Figure 6C), EcN induced IL-10 levels, and its previous colonization kept this level on the comparable values. However, these values were non-significant increases from the GF and other piglets infected with *Salmonella*. This increase was not observed in the colon (Figure 6F).

IL-8 in plasma was detected and significantly differed from other groups in the ST group only (Figure 6G). TNF-α was increased in ST, LA+ST, and LM+ST groups and this increase in the groups ST and LA+ST was statistically significant (Figure 6H). IL-10 showed similar results as TNF-α (Figure 6I).

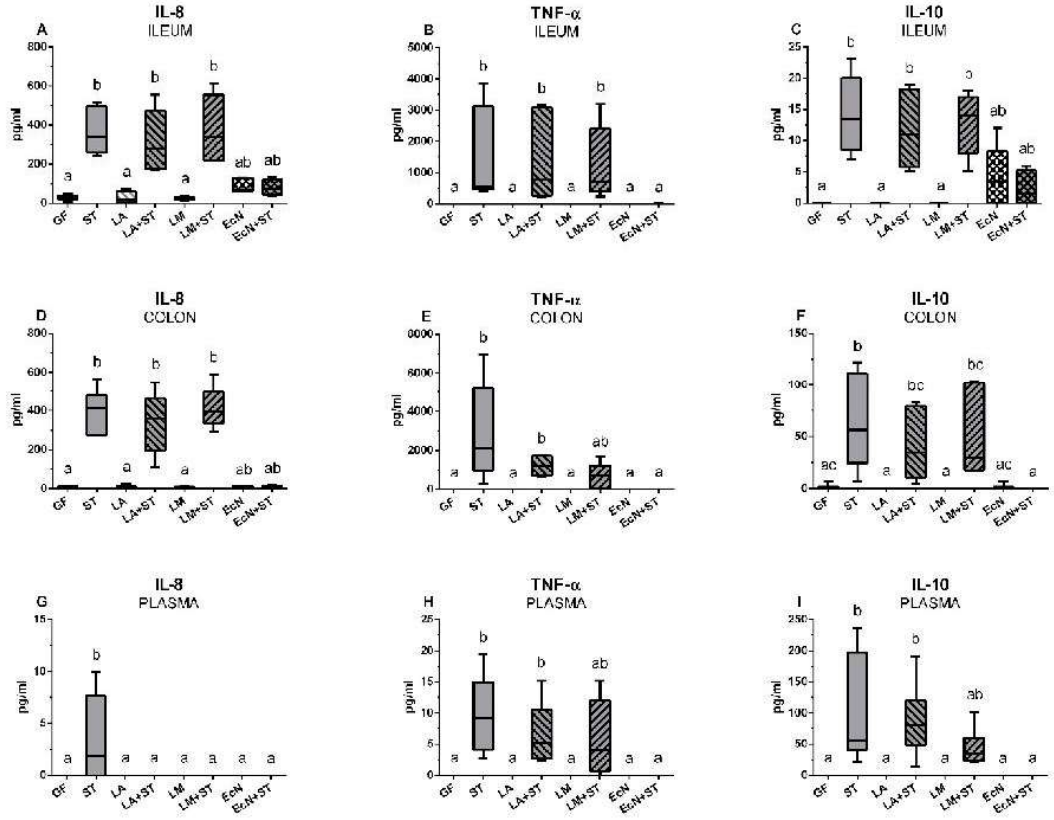

Figure 6. Local and systemic levels of IL-8, TNF-α, and IL-10 in the gnotobiotic piglets. The levels of IL-8 (**A**,**D**,**G**), TNF-α (**B**,**E**,**H**), and IL-10 (**C**,**F**,**I**)) in the ileum (**A–C**), colon (**D–F**), and plasma (**G–I**) in the germ-free piglets (GF) and the piglets colonized/infected with *L. amylovorus* (LA and LA+ST), *L. mucosae* (LM and LM+ST), *E. coli* Nissle 1917 (EcN and EcN+ST), and *S.* Typhimurium (ST, LA+ST, LM+ST, and EcN+ST) are depicted. Kruskal–Wallis test with Dunn's multiple comparison post-hoc test was used to compare differences among the groups. The cytokine levels were measured in six piglets per group. The values are presented as median and range. Statistical differences $P < 0.05$ are denoted with different letters above the columns and the same letter shown above the column indicates no statistically significant differences.

4. Discussion

This study aimed to evaluate the growth of *L. amylovorus* or *L. mucosae* isolated from pig feces and their ability to interfere with infection by *S.* Typhimurium LT2 strain, which is virulent for

gnotobiotic piglets [25]. *Lactobacillus* spp. are facultative anaerobes that are the most abundant bacteria in conventional pigs, reaching approximately 40% of the bacterial population in the small intestine [26,27]. Their percentage decreases in the colon despite increased bacterial density and species variability [26,28].

L. amylovorus is the most abundant species among *Lactobacillus* spp. as determined by 16S rDNA amplification and cloning, followed by *L. johnsonii*, *L. reuteri*, *L. vaginalis*, and *L. mucosae* [29,30]. It positively influenced the growth of lactobacilli in the ileum and colon of weaned piglets [30] and prevents membrane damage of an epithelial cell line infected with enterotoxigenic *E. coli* K88 [31]. *L. mucosae* is another frequent inhabitant of the swine GIT that has been tested for its possible probiotic properties [28]. It expresses a mucus-binding protein (Mub) that is typical for this species, which meditates its binding to mucus in vitro [32]. We expected that the ability of *L. mucosae* to adhere to the mucus increases the ability of this strain to colonize the intestine and create a biofilm, which limits the growth of the enteric pathogens. The commonly used probiotic *E. coli* Nissle 1917 showed a protective effect against diarrhea in infants and toddlers [33] and pigs [34]. We and others have shown that *E. coli* Nissle 1917 reduced the invasion rate of enteric pathogens in vitro [35] and alleviated signs of experimental enteric infections in the gnotobiotic piglets [20,36–38], including of infections with *S.* Typhimurium [25]. In the present work, *E. coli* Nissle 1917 was used for comparison of the effects of lactobacilli in the *Salmonella*-infected gnotobiotic piglets. At the same time, both *L. amylovorus* and *L. mucosae* vs. *E.* coli Nissle 1917 served for comparison of the effect of Gram-positive and Gram-negative bacteria on the host [39]. *E. coli* Nissle 1917 colonized the ileum and colon in the cell density 8 to 10 log CFU/mL. In contrast, both lactobacilli only reached 6 to 8 log CFU/mL in the ileum and colon, which appeared to be too low to effectively combat the *Salmonella* infection. While *E. coli* Nissle 1917 counts were comparable to *Salmonella* counts, *Salmonella* outnumbered lactobacilli by 100-fold. Thus, colonization with one *Lactobacillus* strain showed lower lactobacilli cell density than that found in conventional pigs, where multistrain *Lactobacillus* settlement exceeded counts of *Enterobacteriaceae* [40,41]. Infection with *Salmonella* diminished lactobacilli populations in a few gnotobiotic piglets. *E. coli* Nissle 1917 was able to suppress the growth *Salmonella* in the jejunum and ileum and suppress its translocation to the mesenteric lymph nodes and the liver; whereas neither *L. amylovorus* nor *L. mucosae* suppressed the growth of the *Salmonella* in any observed organ. In the monocolonized piglets, both lactobacilli translocated to the MLN, where they were likely trapped and eliminated. In contrast, *E. coli* Nissle 1917 showed higher ability to translocate to the MLN. However, this *E. coli* strain has semi-rough chemotype LPS that makes it complement-sensitive [42] and cannot be spread via the blood to the liver or other organs.

To investigate potential mechanisms of epithelial damage, we investigated the abundance on villin, a cytoskeletal protein that associates with the microvillar actin filaments localized in the apical border of the intestinal epithelial cells [43]. Villin also participates in the epithelial restitution after damage through its actin-severing activity [44]. Herein, transcription of villin was downregulated in *Salmonella*-infected groups with the exception of EcN+ST group, which was in accordance with the erosion of the epithelial layer in the ileum evidenced by histopathological assessment. In contrast to previous findings [37], we did not observe upregulated transcription of the villin in the intestine in the piglets colonized with *E. coli* Nissle 1917. Villin participates in the balance between actin polymerization and actin severing and facilitates the initial steps of *Salmonella* invasion [44], and *Salmonella* can spread via lymph vessels to the liver, spleen, and other organs, or it can reach these sites via blood [45].

S. Typhimurium causes enterocolitis (salmonellosis) in the human and pig manifested by diarrhea [16]. In this case, a movement of ions through the intestinal barrier either transporters or the lateral spaces among cells via tight junctions is altered [46]. Mammalian claudins consist of a large group of tight junction proteins. Some claudins are barrier-forming and prevent against the loss of electrolytes (e.g., claudins 1 and 4), whereas claudins 2 and 10 are pore-forming and transmit electrolytes from the intestine [47]. Therefore, we evaluated claudin-1 and claudin-2 expression with *Salmonella* to evaluate changes in the intestinal barrier. Transcription of claudin-1 was upregulated in

the ileum and colon of *Salmonella*-infected piglets. Neither previous colonization with *L. amylovorus* nor *L. mucosae* prevented these increased levels of mRNA in the infected piglets.

In contrast, the previous colonization with *E. coli* Nissle 1917 prevented increased claudin-1 expression. These findings were closely correlated with diarrhea in piglets in the ST, LA+ST, and LM+ST groups, but not in the EcN+ST group. We propose that upregulated transcription of claudin-1 is an attempt to reconstitute the disrupted intestinal barrier and prevent loss of electrolytes [47]. A similar trend was found in the colon, but not in the ileum. While the small intestine is the main site of the nutrient transport, water and electrolyte transport occurs primarily in the colon [48]. The downregulated transcription of claudin-2 may be an attempt to restore the physiological function of the intestine. In contrast to claudins, much less is known about occluding [49]. While claudins participate in the transfer of low molecular compounds, occludin participate in the transport of large molecules and cells [50] and requires the proper function of the intestinal barrier. Decreased transcription of occludin in *Salmonella*-infected piglets likely reflects disrupted intestinal epithelial barrier function. This disruption was potentially to prevent ileal colonization with *E. coli* Nissle 1917, but not by the commensal lactobacilli.

Cytokines mediate communication among cell populations. They fulfill various physiological activities, but excessive secretion ('cytokine storm') can cause multiple organ dysfunction syndrome [51]. We focused on chemotactic cytokine (chemokine) IL-8, pro-inflammatory cytokine TNF-α, and regulatory cytokine IL-10, which are suitable diagnostic markers in enteric infections in the pig [52]. *S.* Typhimurium can facilitate its bacterial translocation either by disruption of tight junctions by direct contact with epithelium or via cytokine induction [53]. Physiologically, cytokines mainly act locally, and upregulated mRNA levels in the pig intestine infected with *S.* Typhimurium have been reported [25,54,55]. IL-8 mRNA expression in the ileum and colon of the gnotobiotic piglets were not statistically significant, however, previous colonization with *E. coli* Nissle 1917 prevented *Salmonella*-induced IL-8 mRNA in the ileum and the colon. In contrast, neither *L. amylovorus*, nor *L. mucosae* increased IL-8 mRNA expression, and their previous colonization did not prevent the increase of IL-8 mRNA in the intestine of *S.* Typhimurium-infected piglets. IL-8 induction in the ileum of the gnotobiotic piglets colonized with rough *S.* Infantis 1326/28 or *S.* Typhimurium 1591 was believed to be responsible for the protection against subsequent infection with virulent *S.* Typhimurium strains 98 [56] or LT2 [57], respectively. Intestinal expression of TNF-α in non-infected piglets or piglets previously colonized with *E. coli* Nissle 1917 (EcN+ST) was below the detection limit. While induction of TNF-α with *Salmonella* infection in the ileum was significant, expression in the colon of the piglets previously colonized with both lactobacilli was reduced, suggesting that lactobacilli partially suppressed local expression of TNF-α in the colon. We believe that increased expression of IL-8 and TNF-α in the intestine of ST, LA+ST, and LM+ST piglets exceed their physiological functions and indicate a detrimental cytokine storm [51]. The *Salmonella*-induced levels of IL-10 were much lower than IL-8 and TNF-α and probably fulfilled their regulatory functions.

IL-8 in plasma was detected in the ST group only, but values were very low. Concordantly, IL-8 levels in plasma was found in the experiment with preterm less mature gnotobiotic piglets infected with *S.* Typhimurium [21]. Lower levels of plasma TNF-α compared with our previous results [58] were found. The dose of *Salmonella* CFU used in the present experiments was two orders lower than in our former study. It may be the reason for the prolonged innate immune response and lower cytokine levels, because the maximal counts of the *Salmonella* CFU were probably reached several hours later in the piglets infected with a lower dose of *S.* Typhimurium and so these piglets were exposed to the maximal number of *Salmonella* shorter time. Increased plasma IL-10 was observed in the ST, LA+LM, and LM+ST groups. Increased sytemic levels of TNF-α and IL-10 indicated the severity of infection and poor prognosis for survival in preterm infants [59] and adults [60]. Similar prediction validity of these cytokines was reported from experiments with gnotobiotic piglets infected with necrotoxigenic *E. coli* O55 [61], in which piglets with lower clinical symptoms had significantly lower plasma TNF-α and IL-10 concentrations compared to their counterparts that suffered from a

severe infection. We hypothesize that the lack of measurable systemic TNF-α and IL-10 in blood plasma in the present experiments might be due to time delayed infection, which made it possible to mobilize the innate immune response to combat to infection.

5. Conclusions

In the present experiments, we compared mono-colonization of the gnotobiotic piglet GIT with commensal lactobacilli, *L. amylovorus* and *L. mucosae,* and with probiotic *E. coli Nissle* 1917 and their interference with *S.* Typhimurium. Neither lactobacillus strains suppressed the inflammatory reaction caused by infection with *S.* Typhimurium, whereas, probiotic *E. coli* Nissle 1917 suppressed reduced the clinical signs and inflammatory response. Future studies are needed to determine whether higher beneficial bacteria counts could by achieved in the GIT by supporting of their growth by administering prebiotics and multistrain/species inoculum, which may more effectively inhibit enteric infections.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Methods of Isolation, Characterization, and Identification of Lactobacilli

Two different lactobacilli species were isolated from a fresh fecal sample of a one-year-old Peitrain female pig (*Sus scrofa* f. *domestic*) using Rogosa Agar (Oxoid, Basingstoke, UK) supplemented with 1.32 mL/L acetic acid (Sigma-Aldrich, St. Louis, MO, USA). Cultivation was performed in microaerophilic conditions at 37 °C for 48 h. The Wilkins–Chalgren broth (Oxoid) supplemented with 5 g/L soya peptone (Oxoid), 0.5 g/L L-cysteine (Sigma-Aldrich), and 1 mL/L Tween 80 (Sigma-Aldrich) was used for isolation of the strains and routinely sub-cultivation.

Properties predicting survival of lactobacilli during passage through the gastrointestinal tract (acid and bile tolerance) were tested as described elsewhere [62]. Briefly, overnight bacterial suspension free of cultivation media was mixed with oxygen-free phosphate buffered saline (OF-PBS) of pH 2 or 3 (pH adjusted with HCl), with OF-PBS buffer (pH 7.2) containing 1%, 2%, or 3% bile extract, or with OF-PBS buffer (pH 7.2) for control. The bacterial suspensions were incubated at 37 °C for 2 h (acid tolerance) or 3 h (bile tolerance). After the incubation, viable counts were determined by the standard plate counts methods and decrease in counts was calculated.

Both lactobacilli species were identified using MALDI-TOF MS (ethanol-formic acid extraction procedure and mixed with HCCA matrix) according to the manufacturer's instructions (Bruker Daltonik, Bremen, Germany). Analysis of protein spectra was processed by Microflex LT MALDI-TOF MS (Bruker Daltonics) using FlexControl 3.4 software (Bruker Daltonics). Obtained spectra were analyzed using MALDI Biotyper OC version 3.1, and flexAnalysis version 3.4 (both Bruker Daltonics).

The identities of the lactobacilli strains were confirmed by 16S rRNA gene sequencing. Briefly, genomic DNA was extracted using the PrepMan® Ultra Sample Preparation Reagent protocol (Thermo Fisher Scientific, Waltham, MA, USA). Fragments of the 16S rRNA gene were amplified using primers

285F (5′-GAGGGTTCGATTCTGGCTCAG-3′) and 261R (5′-AAGGAGGTGATCCAGCCGCA-3′) with PCR conditions as described elsewhere [63]. Concentrations of template DNA for PCRs ranged from 10 to 100 ng. All PCR amplifications consisted of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) and primers (Eurofins Genomics, Ebersberg, Germany) at 0.5 mol concentrations. Amplicons were purified using an E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Norcross, GA, USA) and then sequenced by the company Eurofins Genomics (Eurofins Genomics). Amplicons were sequenced in both directions to achieve almost complete 16S rDNA coverage. The resulting sequences (~1450 bp) have been aligned in the Geneious version 7.1.7 software (Biomatters, Auckland, New Zealand) using Geneious Alignment. The EzBioCloud database was used to find the closest species based on pairwise similarities (%) and then deposited at GenBank database through the NCBI BankIt tool (https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank).

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6 Souhrnná diskuze

S přihlédnutím k publikačním výstupům je diskuse rozdělena na tři části:

- Prebiotické vlastnosti oligosacharidů rafinosové řady
- Prebiotické vlastnosti oligosacharidů mateřského mléka
- Testování potenciálně probiotických bakterií

6.1 Prebiotické vlastnosti oligosacharidů rafinosové řady (RSO)

Fruktany a GOS jsou nejvíce probádanými prebiotiky, existuje celá řada studií podporující jejich vliv na růst bifidobakterií ať už samostatně (Gibson et al. 1995; Menne et al. 2000; Kleessen et al. 1997; Bouhnik et al. 1997; Vulevic et al. 2013), nebo ve vzájemné kombinaci GOS 9:1 FOS, která je přidávána do umělých kojeneckých výživ (Boehm et al. 2005; Haarman and Knol 2005). Existují ale i práce, které poukazují na nedostatečnou selektivitu komerčně používaných fruktanů a GOS (Euler et al. 2005; Alles et al. 1999). Rada et al. (2008) tvrdí, že na komerčně dostupných oligosacharidech včetně fruktanů a GOS rostou bifidobakterie a klostridie, a je tudíž diskutabilní podávat prebiotika dětem, které nemají ve střevech bifidobakterie. Bouhnik et al. (2004) pozorovali pozitivní podporu bifidobakterií u RSO ve stejné míře jako u GOS a FOS a i další studie naznačují skutečnost, že RSO podporují růst bifidobakterií (Saito et al. 1992; Hayakawa et al. 1990). Ve **článku 5.3** byla stanovena množství RSO v sójových moukách a v sójových výrobcích dostupných na českém trhu. Testované typy sójových mouk obsahovaly v průměru 4,83 g RSO/100 g, texturovaný sójový protein 3,36 g RSO/100 g a sójové tyčinky průměrně 2,47 g RSO/100 g. Vzhledem k nedostatečnému příjmu vlákniny v Česku (Stephen et al. 2017) by bylo dobré, kdyby se sójové výrobky více zařadily do stravy Čechů. Přídavkem sójové mouky do pečiva a občasnou konzumací sójových výrobků, jako třeba texturovaného sójového proteinu jako náhražky masa nebo sójové tyčinky ve formě dezertu by mohl být zvýšen příjem vlákniny i RSO u populace, což by se mohlo příznivě podepsat na jejím zdravotním stavu. Konzumace vlákniny má totiž jednoznačně příznivý dopad na lidské zdraví a zvýšení příjmu sójových výrobků, které obsahují relativně vysoké množství vlákniny i RSO by mohlo zvýšit počty bifidobakterií a producentů butyrátu v trávicí soustavě konzumentů (Holscher 2017; Bouhnik et al. 2004; Hayakawa et al. 1990). RSO tak lze považovat za účinnou a zároveň levnou alternativu komerčně dostupných prebiotik.

Množství RSO bylo stanoveno i v lupině v **článku 5.4**, a zároveň byl proveden i *in vivo* pokus na brojlerových kuřatech a kachnách. Lupina je krmná plodina, která je považována za alternativu sóji vzhledem k tomu, že EU ročně dováží kolem 14 milionů tun sóji ze států mimo EU (European Commission 2018) a také proto, že v porovnání se sójou obsahuje podobně vysoké množství živin důležitých ve výživě zvířat (Sedláková et al. 2016; Froidmont and Bartiaux-Thill 2004). Ve studii byla porovnána mouka lupiny bílé (*Lupinus albus*) se sójovou moukou a v lupině bylo dokonce naměřeno významně vyšší množství RSO, než v sóji. Následný krmný pokus na brojlerových kuřatech a kachnách pak ukázal významně vyšší počty

laktobacilů v céku kuřat krmených výhradně lupinou a významně vyšší počty bifidobakterií v céku kachen krmených 50 % i 100 % lupiny v dietě. K podobným výsledkům došli i Zdunczyk et al. (2014), kteří zjistili vyšší počty laktobacilů a bifidobakterií ve střevě nosnic přijímajících krmivo s přídavkem 20 % lupiny modré. Je tedy možné, že zastoupení lupiny v dietě může mít příznivý vliv na množství bifidobakterií i laktobacilů v trávicím traktu některých zvířat, stejně jako bylo vypozorováno u lidí. Úplné nahrazení sóji lupinou v dietě nicméně mělo nepříznivý vliv na přírůstky, kachny (samci i samice) krmené výhradně lupinou měly významně nižší porážkovou hmotnost, u kuřat byl stejný efekt pozorován jen u samic. K podobným výsledkům došli i při nižších přídavcích lupiny do diety Smulikowska et al. (2014) a Olkowski et al. (2005) u brojlerových kuřat. Naopak, u prasat (Zralý et al. 2008) nebo králíků (Volek and Marounek 2009) tento negativní efekt pozorován nebyl, stejně jako při přidávání lupiny žluté do krmiva pro krocany (Zdunczyk et al. 2016), u kterých došlo dokonce k nárůstu SCFA ve střevech. Stabilní a vysoké počty bakterií produkujících SCFA jako acetát a laktát zabraňují proliferaci patogenních bakterií ve střevě (Buclaw 2016; Zdunczyk et al. 2014), RSO nejsou u monogastrů rozkládány trávicími enzymy a tudíž se dostávají do dolních částí trávicího traktu, kde jsou k dispozici bakteriím, které jejich rozkladem dovedou produkovat SCFA a plyn, z tohoto hlediska je možné považovat RSO v luštěninách jako probiotické, nicméně je třeba brát na zřetel i antinutriční vlastnosti RSO u některých monogastrů, jako třeba u selat po odstavu (Choct et al. 2010). Vezmeme-li v potaz vliv lupiny na střevní mikrobiotu a na přírůstky, považujeme za optimální pouze částečný přídavek lupiny do diety a to pouze pro některé druhy a kategorie monogastrů.

6.2 Prebiotické vlastnosti oligosacharidů mateřského mléka (HMO)

Oligosacharidy jsou významnou součástí mateřského mléka, v této práci jsou jim věnovány hned tři publikace. Je známo, že HMO podporují růst bifidobakterií, zejména *B. longum* ssp. *infantis* (Marcobal et al. 2010; Locascio et al. 2009), proto se také bifidobakterie pravidelně vyskytují ve vysokých počtech u kojených dětí, stejně jako laktobacily (Guaraldi and Salvatori 2012; Haarman and Knol 2005). Námi provedené studie měly za cíl prohloubit informace ohledně využívání HMO čistými kulturami bifidobakterií a laktobacilů a dále byl rovněž zkoumán synbiotický efekt HMO s *B. longum* ssp. *infantis in vivo*.

K izolaci HMO byla použita upravená metoda podle Finke et al. (1999) a Ročkové et al. (2011), kdy bylo mléko nejprve zbaveno tuku pomocí centrifugace a následně byl odstraněn protein v několika krocích za pomoci etanolu a dichlormetanu. Výsledný roztok obsahoval směs laktosy a HMO, které byly rozděleny na jednotlivé frakce pomocí gelové permeační chromatografie. Identita frakcí byla poté analyzována tenkovrstvou chromatografií. Frakce obsahující pouze HMO byly poté využity pro následující pokusy, jejichž design byl mimo jiné postaven na předchozích výsledcích Ročkové et al. (2011a) a (2011b), kteří zjistili, že HMO podporují *in vitro* růst bifidobakterií lidského původu, zejména *B. bifidum* a nepodporují růst klostridií a bifidobakterií původu zvířecího (*B. animalis* ssp. *lactis*).

Článek 5.7 se zaměřil na růst laktobacilů na HMO. Ačkoli většina dosavadních publikací dokazuje využití HMO především bifidobakteriemi (Ward et al. 2006; Locascio et al. 2009), bylo popsáno, že i bakterie rodu *Bacteroides* dovedou metabolizovat HMO (Marcobal et al. 2010). Z vybraných kmenů laktobacilů dokázaly využívat HMO jako zdroj uhlíku pouze *L. casei* ssp. *paracasei* a *L. acidophilus*, avšak pouze v omezené míře v porovnání s kontrolním médiem s glukosou. *L. fermentum*, *L. delbrueckii* ssp. *bulgaricus a L. animalis* nevyužívaly HMO vůbec. Rozklad HMO byl odečítán nejen ze schopnosti bakterií množit se v médiu obsahujícím HMO (měřeno densitometricky při 540 nm), ale také schopností snižovat pH produkcí SCFA, která je v anaerobním prostředí charakteristická pro sacharolytické bakterie (Van Loo et al. 1999). Vzhledem k tomu, že HMO jsou komplexní skupina oligosacharidů (Wu et al. 2011b; Jeong et al. 2012), tak výsledky naznačují, že *L. casei* ssp. *paracasei* a *L. acidophilus* využívají pouze některé z obsažených HMO. Thongaram et al. (2017) pozorovali schopnost laktobacilů využívat různé HMO a prokázali, že *L. acidophilus* rostl na lakto-N-neotetraose. Lakto-N-neotetraosa je dobře popsaná frakce HMO. Vzhledem k tomu, že v dnešní době je možné ji v určitém množství syntetizovat a již se pomalu zavádí její přidávání do umělých kojeneckých výživ (Hegar et al. 2019; Vandenplas et al. 2018), je nasnadě, že umělé kojenecké výživy obsahující tento HMO budou mimo jiné podporovat růst *L. acidophilus*, který je považován za člověku přirozenou a probiotickou bakterii (Liévin-Le Moal and Servin 2014).

Článek 5.5 je zaměřen na analýzu synbiotického efektu HMO a *B. longum* subsp. *infantis*, což je dobře popsaný konzument HMO (Marcobal et al. 2010; Locascio et al. 2009). Ve studii byl sledován synbiotický efekt *in vitro* za 24 h a *in vivo* v humanizovaných myších po dobu 15 dnů. Synbiotický efekt se podařilo prokázat *in vitro*, když kombinace HMO a pokusné bifidobakterie potlačovala klostridie ve stolici kojenců porozených císařským řezem. Stejného výsledku bylo dosaženo v kombinaci pokusné bifidobakterie a mateřského mléka, tato kombinace navíc významně potlačovala i gramnegativní bakterie, a tudíž prokázala nejsilnější synbiotický efekt, což bylo pravděpodobně důsledkem toho, že mateřské mléko obsahuje celou řadu antimikrobiálních faktorů, jako laktoferin, lysozym a antimikrobiální peptidy, které potlačují růst potenciálních patogenů (Field 2005). Synbiotický efekt se bohužel nepodařilo potvrdit v *in vivo* experimentu, po podání sice postupně vymizely klostridie, avšak ani pokusné bifidobakterie po pár dnech nebyly detekovány. I tak byl ale zřejmý částečně pozitivní dopad HMO a především mateřského mléka na střevní mikrobiotu i v *in vivo* pokusu. HMO byly rovněž schopny ovlivnit imunitní odpoveď, konkrétně byly u pokusných myší zvýšené hladiny IL–6 a IL–10, což jsou prozánětlivý a protizánětlivý cytokin (Sapan et al. 2016). Schopnost indukovat produkci cytokinů pozorovali u některých kmenů *B. longum* i Šrůtková et al. (2015). Lane et al. (2013) pozorovali schopnost HMO zvýšit produkci IL–17. Zajímavým zjištěním byl nález plynatosti u myší, které přijímaly HMO, to by naznačovalo, že ani HMO, stejně jako třeba fruktany, nejsou zcela selektivní a ve střevě je využívaly i jiné rody bakterií vzhledem k tomu, že bifidobakterie plyny neprodukují (De Vuyst and Leroy 2011). Synbiotický efekt probiotik a prebiotik není *in vivo* příliš prozkoumán. Byly testovány především GOS nebo FOS v kombinaci s bifidobackteriemi nebo laktobacily a tato kombinace neprokázala synbiotický efekt (Krumbeck et al. 2018). Jena et al. (2018) pozorovali u myší synbiotický efekt kombinace *B.*

longum ssp. *infantis* a HMO, myši měly ve výkalech vyšší množství SCFA a nižší počty *Biophilia* spp. Skutečnost, že pokusný kmen *B. longum* ssp. *infantis* přežíval u myší pouze 4 dny při suplementaci HMO a 8 dní při suplementaci mateřským mlékem, je porovnatelná s výsledky Grmanové et al. (2010), kde pokusné bifidobakterie vymizely u lidí po 14 dnech. Vzhledem k tomu, že vztahy mezi bakteriemi ve střevě jsou komplikované a že byl popsán cross-feeding mezi jednotlivými bifidobakteriemi i jinými rody bakterií přítomnými ve střevě (Belenguer et al. 2006; Turroni et al. 2018), je možné, že krátká životnost bifidobakterií ve střevě v našem pokusu byla zapříčiněna podstatou modelu, který, ač mnohem bližší *in vivo* modelům v porovnání s *in vitro* modely, stále obsahuje určité limitace, například skutečnost, že v podávané vzorky stolice byly prosté dalších druhů bifidobakterií, které se ale jinak ve střevě kojence přirozeně vyskytují. Myší model je rovněž nepřesný i z celkem logického hlediska, že metabolismus myši je v některých ohledech jiný, než metabolismus lidský (Leist and Hartung 2013; Seok et al. 2013).

Jednou ze zajímavých vlastností střevních bakterií je adhezivita k buňkám střevního epitelu. Schopnost adherovat na střevní epitel je důležitá pro setrvání ve střevě a tím i jeho kolonizaci, jde o významnou vlastnost probiotických bakterií (Collado et al. 2009). Bernet et al. (1993) prokázali schopnost lidských bifidobakterií inhibovat adherenci patogenních *E. coli* a salmonel, což je považováno za jeden z probiotických ukazatelů. Podobné vlastnosti mají i HMO, některé z nich mohou připomínat buněčný povrch a tím na sebe navázat bakteriální buňku, čímž zabrání patogenní bakterii k navázání na epitel (Newburg et al. 2005). Tyto vlastnosti byly u HMO pozorovány v případě adherence *Campylobacter jejuni* (Morrow et al. 2005) nebo *E. coli* (Martin-Sosa et al. 2002). V **článku 5.6** byla sledována antiadhezivní schopnost HMO vůči bifidobakteriím a klostridiím. Z výsledků vyplývá, že HMO jsou schopny snižovat adhezivitu klostridií, avšak i některých kmenů bifidobakterií. Schopnost adherovat na buněčné linie Caco-2 a HT29-MTX byla v pozitivní korelaci se schopností autoagregace, což je ve shodě s poznatky Del Re et al. (2000). Antiadhezivní schopnost HMO vůči testovaným klostridiím může být dalším ukazatelem jejich selektivity a tím i prebiotické aktivity, výsledky jsou v souladu s poznatky Bezirtzoglou et al. (2011), kteří pozorovali nižší počty klostridií u kojených dětí. Weichert et al. (2013) testovali antiadhezivní vlastnosti u dvou různých HMO a zjistili, že tyto byly schopny snižovat adhezivitu *Campylobacter jejuni*, enteropatogenní *E. coli*, *Salmonella enterica* serovar *fyris* a *Pseudomonas aeruginosa* k Caco–2 buněčným liniím. Costalos et al. (2008) zaznamenali nižší výskyt klostridií u dětí živených umělou výživou se směsí GOS:FOS, je tedy možné, že antiadhezivními vlastnostmi disponují i jiné prebiotické oligosacharidy, nicméně Kadlec a Jakubec (2014), zjistili, že GOS a FOS snižují adherenci probiotických bakterií.

Z námi získaných výsledků usuzujeme, že podpora růstu bakterií HMO je komplexně provázána s dalšími složkami mateřského mléka. Zároveň HMO podporují jen některé druhy bakterií, zejména ty, které se vyskytují u kojenců. Rozklad HMO střevními bakteriemi je provázen tvorbou SCFA, které mají jednoznačně pozitivní vliv na střevo hostitele (Van Loo et al. 1999), díky čemuž se dají HMO považovat za prebiotika. Byla pozorována schopnost HMO společně s bifidobakteriemi potlačovat klostridie a gramnegativní bakterie ve stolici *in vitro*,

synbiotický efekt pokusného kmene bifidobakterií a HMO nebyl prokázán *in vivo*, v této oblasti je nicméně zapotřebí dalších výzkumů, ideálně *in vivo* u lidí. HMO rovněž mohou působit jako antiadhezivní molekuly, které zabraňují přichycení potenciálně patogenních, ale i probiotických bakterií. Schopnost HMO zabraňovat adherenci na střevní stěnu je pravděpodobně druhově i kmenově specifická, přičemž autoagregace je výhodu, nikoli však podmínkou pro bakterie, které chtějí ve střevě setrvávat i v přítomnosti antiadhezivních molekul. Je pravděpodobné, že výskyt některých kmenů bifidobakterií u kojených dětí je dán nejen jejich schopností využívat HMO jako prebiotikum, ale rovněž i jejich rezistencí vůči antiadhezivním účinkům HMO. Ty naopak navazováním jiných bakterií vytvářejí těmto bifidobakteriím lepší prostředí pro růst, což lze popsat jako synbiotický efekt.

6.3 Testování potenciálně probiotických bakterií

Bylo napsáno mnoho prací o probiotických vlastnostech bifidobakterií nebo laktobacilů pro člověka, méně pozornosti se však věnuje netradičním druhům probiotických bakterií, jako jsou klostridie. Bylo již zmíněno, že butyrát produkující bakterie jsou nesmírně důležité pro správnou proliferaci střevních buněk (Rivière et al. 2016; Pryde et al. 2002), avšak klostridie jsou někdy i přes tuto svou vlastnost opomíjeny, ačkoli zahrnují mnohé komensální druhy (Lopetuso et al. 2013) a existují studie, které naznačují jejich pozitivní význam pro zdraví hostitele (Kuroiwa et al. 1990; Seki et al. 2003) například při zmírňování dysbiosy při léčbě antibiotiky. **Článek 5.2** se zabývá vlivem *C. butyricum* CBM 588, japonského kmene izolovaného z půdy (EFSA 2009), na střevní mikrobiotu a užitkovost brojlerových kuřat. Při krmném pokusu bylo podáváním CBM 588 dosaženo významného snížení počtů *E. coli*, které u drůbeže představují potenciálně patogenní bakterie a mohou představovat i riziko pro spotřebitele (Dho-Moulin and Fairbrother 1999; Manges 2016). Navíc podávané klostridie byly v céku i metabolicky aktivní, což dokazuje zvýšené množství butyrátu. Počty bifidobakterií nebyly suplementací *C. butyricum* pozměněny, jejich počty byly stabilně vyšší než počty klostridií. Zvýšená produkce butyrátu ve střevě mohla mít za následek významně vyšší přírůstky pokusných kuřat, což je v souladu s jinými studiemi podávání probiotik, většinou ale byly použity laktobacily (Jin et al. 1998; Kalavathy et al. 2003; Apata 2008) nebo *Enterococcus faecium* (Owings et al. 1990), případně *Bacillus subtilis* (Khaksefidi and Ghoorchi 2006). Jiný kmen *C. butyricum* byl použit ve studiích Yang et al. (2012) a Zhang et al. (2016) a tito zjistili, že pokusná kuřata měla nejen vyšší přírůstky, ale také byly podávané klostridie schopny snížit počty *E. coli*, salmonel a *C. perfringens* (Yang et al. 2012) nebo podpořit imunitní odpověď a zlepšit funkci střevní propustnosti (Zhang et al. 2016). Klíčovou roli butyrátu při zvýšení přírůstků naznačuje studie Zhang et al. (2011), kteří podávali brojlerovým kuřatům butyrát samotný a taktéž pozorovali jeho pozitivní vliv na přírůstky. CBM 588 bylo testováno i u lidí, Seki et al. (2003) zjistili, že CBM 588 snížilo výskyt průjmů způsobených antibiotiky u dětí, Kuroiwa et al. (1990) pozorovali schopnost CBM 588 potlačit produkci toxinů *C. difficile* u pacientů, kteří podstoupili antimikrobiální terapii. Počty *E. coli* nebyly u lidí, kteří přijímali CBM 588 před léčbou antibiotiky, nijak ovlivněny. Naopak, jejich počty byly zachovány oproti

skupině bez suplementace CBM 588. Tento jev může být důsledkem toho, že výskyt *E. coli* ve střevní mikrobiotě je druhově specifický (McLellan et al. 2003; Zhi et al. 2015), to znamená, že člověk je hostitelem jiných kmenů *E. coli*, než drůbež a tyto kmeny nejsou na CBM 588 citlivé. Zároveň je třeba podotknout, že drůbež hostí potenciálně patogenní kmeny *E. coli* (Dho-Moulin and Fairbrother 1999; Manges 2016). Takahashi et al. (2004) pozorovali antiadhezivní efekt CBM 588 vůči enterohemorhagické *E. coli* na Caco–2 buněčné linie.

Vezmeme-li v úvahu relativně nízký zájem o *C. butyricum* jak ve výživě lidí, tak zvířat, představuje tato bakterie velký potenciál pro budoucí využití jako probiotická bakterie. Předností *C. butyricum* CBM 588 bezesporu je, že je podávána ve formě spor, což usnadňuje jak manipulaci, tak to vede k vyšší odolnosti při průchodu trávicím traktem a teoreticky i k vyšší odolnosti v případě nepříznivých podmínek ve střevě, kde testované *C. butyricum* dokáže být metabolicky aktivní.

Výběr nových probiotik je zvláště ve výživě zvířat aktuální problematika, zejména díky přechodu na alternativní způsoby léčby a zvýšení preventivních opatření po zákazu užívání antibiotik pro krmné účely. Někteří výzkumníci doporučují, aby podávaný probiotický kmen pocházel ze stejného živočišného druhu vzhledem k tomu, že účinky probiotik mohou být hostitelsky specifické (Gardiner et al. 2004; Vemuri et al. 2018). **Článek 5.8** porovnává kolonizaci bezmikrobních selat komensálními kmeny laktobacilů *L. amylovorus* a *L. mucosae* získanými z výkalů prasete domácího s probiotickým kmenem *E. coli* Nissle 1917 (EcN) a jejich schopnost potlačit infekci patogenní bakterií *Salmonella typhimurium* LT2, která u lidí i selat způsobuje enterokolitidu projevující se průjmy (Kim and Isaacson 2017). EcN je dobře prozkoumaným probiotickým druhem, který má protektivní efekt proti výskytu průjmů u kojenců i prasat (Schroeder et al. 2006; Henker et al. 2007) a byly u něj prokázány i antiinvazivní vlastnosti proti patogenním bakteriím (Altenhoefer et al. 2004). Laktobacily jsou hojně se vyskytujícím rodem bakterií u prasat, přičemž u nich byly popsány i probiotické vlastnosti, jako třeba schopnost *L. sobrius* ochránit buňky střevní sliznice před působením patogenní *E. coli* K88 (Roselli et al. 2007), *L. rhamnosus* GG byl schopen u selat napadených *E. coli* K88 zmírnit průjmy (Zhang et al. 2010), podobných výsledků dosáhl u selat i *L. fermentum* I5007 (Liu et al. 2014). Předpokládali jsme podobné vlastnosti u námi získaných kmenů laktobacilů. Výsledky monokolonizací potvrdily schopnost EcN potlačovat *S. typhimurium* a zabraňovat ji ve vyvolání zánětu, zatímco oba komensální laktobacily byly v potlačení neúspěšné a kolonizovaná selata prodělala infekci způsobenou *S. typhimurium*. Použité kmeny *L. amylovorus* a *L. mucosae* nepůsobily protektivně vůči patogenní bakterii, naopak, výsledky naznačují, že jde o běžné komensální druhy, které nejsou schopny chránit hostitele před infekcí salmonelou. EcN je známa svou účinností proti průjmům jak u kojenců (Henker et al. 2007), tak u selat (Schroeder et al. 2006) a i v této studii dokázala potlačit salmonelovou invazi, což je v souladu s dříve získanými výsledky (Splichalova et al. 2011). Jedním z důvodů, proč testované laktobacily nedokázaly potlačit nastávající invazi, by mohla být skutečnost, že byly schopny osídlit střeva selat jen v počtech 6 až 8 log KTJ/ml, zatímco počty EcN dosahovaly 8 až 10 log KTJ/ml. Po kolonizaci tak byly počty EcN vůči protivníkovi vyrovnané, zatímco salmonela přečíslila laktobacily zhruba stonásobně. Námi stanovené počty

laktobacilů tak byly nízké i v porovnání s běžně naměřenými počty laktobacilů u prasat (Pieper et al. 2008), což může být následkem toho, že za normálních okolností se ve střevě prasete vyskytují v komunitě dalších druhů laktobacilů i jiných bakterií, což může podporovat jejich růst a celkově rezistenci celé bakteriální komunity vůči invazivním patogenům.

Námi získané výsledky naznačují, že *C. butyricum* CBM 588 je schopné ovlivnit střevní mikrobiotu i užitkovost brojlerových kuřat, což potvrzují i údaje z vědecké literatury jak z pokusů na kuřatech, tak na lidech (u lidí nebyla užitkovost sledována). Tento netradiční kmen je tedy potenciálně probiotický a měla by mu být věnována pozornost i v budoucích výzkumech. Naopak, námi testované komensální druhy laktobacilů neprokázaly protektivní efekt vůči invazi patogenní *S. typhimurium* a na rozdíl od jiných kmenů laktobacilů tak neprokázaly probiotický efekt.

Zároveň se nepodařilo prokázat, že by druhová specifita hrála roli v probiotickém efektu testovaných bakterií. Je nutno podotknout, že pro prokázání této hypotézy by bylo potřeba vzít v úvahu více výsledků z různých studií, nicméně testované *C. butyricum* CBM 588, které v naší studii i studiích jiných autorů vykazuje probiotické vlastnosti je izolováno z půdy (EFSA 2009), tudíž není vůbec jisté, zda je tato bakterie přirozená trávicímu traktu lidí nebo zvířat.

7 Závěr

- Oligosacharidy rafinosové řady jsou schopné pozitivně ovlivnit střevní mikrobiotu konzumenta. Vzhledem k jejich přirozenému výskytu v luštěninách skýtají potenciál dostupného prebiotika. Hypotéza prebiotického efektu byla potvrzena.

- Oligosacharidy mateřského mléka podporují růst určitých druhů bifidobakterií a v omezení míře i laktobacilů. Jejich selektivita ovšem není stoprocentní, čímž se podobají jiným prebiotickým oligosacharidům. Jejich synergie s dalšími látkami mateřského mléka pravděpodobně hraje podstatnou roli při podpoře zdravé mikrobioty kojence. Hypotéza prebiotického efektu byla potvrzena jen částečně.

- *Clostridium butyricum* je potenciálně probiotickou bakterií, která by se mohla stát alternativou běžných probiotik. Hypotéza probiotického efektu byla potvrzena.

- Námi testované komensální druhy laktobacilů pocházející ze stejného živočišného druhu neukázaly protektivní efekt proti kolonizaci patogenem. *Bifidobacterium longum* ssp. *infantis* nebylo schopné dlouhodobě přetrvávat ve střevě humanizované myši. Naopak, klostridie izolované z půdy ukazují probiotický efekt u různých živočišných druhů i člověka. Hypotéza hostitelské specifičnosti jednotlivých probiotik nebyla potvrzena.

- Schopnost probiotik přežívat v trávicím traktu rovněž nebyla potvrzena. Podávané bifidobakterie a laktobacily časem vymizely z trávicího traktu, klostridie sice ve střevě brojlerů přetrvávat mohly, avšak tyto byly každý den doplňovány společně s krmivem.

- Podáváním probiotik je většinou obtížné ovlivnit střevní mikrobiotu zdravého dospělého jedince. Lepší výsledky jsou zpravidla dosaženy podáváním prebiotik ve stravě. Z tohoto důvodu doporučuji zvýšit množství prebiotik ve stravě, případně je kombinovat s probiotiky ve formě synbiotik. Doporučení by měla být personalizovaná nejen pro jednotlivé druhy zvířat a člověka, ale i pro jednotlivé skupiny v rámci druhu.

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10 Seznam použitých zkratek a symbolů

BMI – Body Mass Index

CBM 588 – Clostridium butyricum CBM 588

DP – stupeň polymerace

EcN – Escherichia coli Nissle 1917

FOS – fruktooligosacharidy

GOS – galaktooligosacharidy

HMO – oligosacharidy mateřského mléka

IMO – isomaltooligosacharidy

RSO – oligosacharidy rafinosové řady

SCFA – mastné kyseliny s krátkým řetězcem