ČESKÁ ZEMĚDĚLSKÁ UNIVERZITA V PRAZE

Fakulta agrobiologie, potravinových a přírodních zdrojů

Katedra mikrobiologie, výživy a dietetiky

Fenotypová a genotypová charakterizace bifidobakterií

Doktorská disertační práce

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Praha 2021

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V Praze dne 18. 8. 2021 _____

Poděkování

Ráda bych touto cestou poděkovala své školitelce doc. Ing. Věře Neužil Bunešové, Ph.D., která byla mou skvělou vedoucí, oporou a inspirací po celé mé studium, a díky které jsem poznala vědecký svět, plno zajímavých lidí a navštívila mnoho nových míst. Dále bych chtěla poděkovat prof. Ing. Evě Vlkové, Ph.D. za podporu, vedení a přátelské prostředí, které na katedře máme. Velké díky patří také kolegům z naší laboratoře Katedry mikrobiologie, výživy a dietetiky, se kterými byla radost spolupracovat, a také kolegům z laboratoře Lebensmittelmikrobiologie und Lebensmittelhygiene na univerzitě BOKU ve Vídni za skvělou pracovní i životní zkušenost.

Za finanční podporu děkuji České zemědělské univerzitě v Praze (projekt CIGA 20182018), Ministerstvu školství, mládeže a tělovýchovy České republiky (projekt mezinárodní česko-rakouské spolupráce 8J19AT02), Evropskému regionálnímu rozvojovému fondu (NutRisk Centre No. CZ.02.1.01/0.0/0.0/16_019/0000845) a METROFOOD-CZ (MEYS Grant No: LM2018100).

Dále bych chtěla poděkovat rodině, přátelům a všem těm, kteří mi byli během studia oporou a podporovali mě na této krásné cestě.

Obsah

1 Úvod

Mikrobiota střevního traktu tvoří hojný a komplexní ekosystém, ve kterém dochází k nespočtu interakcí mezi mikroorganismy a hostitelem, ale také mezi mikroorganismy navzájem. Bifidobakterie jsou jednou z důležitých komenzálních skupin střevního mikrobiálního společenstva. Většinou jsou dominantně zastoupeny ve střevní mikrobiotě v raných fázích života hostitele, především u mláďat savců na mléčné výživě. Přestože se jejich počty se stárnutím hostitelského organismu postupně snižují, zachovávají si ve střevním mikrobiomu řadu důležitých funkcí a vlastností. Je zajímavé, že byly popsány i takové ekologické niky, ve kterých je vysoké zastoupení bifidobakterií stálé až do dospělosti daného hostitele. Nejtypičtějším případem je střevní mikrobiota novosvětských opic. Souvisí to i se současných trendem izolace mnoha nových druhů bifidobakterií z tohoto prostředí.

Přítomnost bifidobakterií ve střevě je obecně spojována s podporou zdraví svého hostitele. Bifidobakterie jsou schopny utilizovat širokou škálu variabilních sacharidových substrátů, které sám hostitel není schopen využít. Jako finální produkty svého metabolismu produkují těkavé mastné kyseliny a laktát. Řada z těchto metabolitů a meziproduktů metabolismu je poté zpřístupněna jako zdroj energie pro další členy střevní mikrobioty, kterým bifidobakterie v tomto prostředí umožňují prospívat. Dále také podporují udržení homeostáze střeva a podílejí se na modulaci imunity. Právě na základě těchto a mnoha dalších vlastností jsou bifidobakterie široce využívány jako probiotické mikroorganismy. Přestože je jejich historie použití poměrně dlouhodobá, mnoho jejich vlastností, interakcí ve střevě a mechanismů účinku na zdraví hostitele stále není zcela známo. Řada z nich dokonce velmi často bývá druhově, kmenově či hostitelsky specifická. Právě proto je monitoring výskytu bifidobakterií u různých živočišných druhů, nejlépe kombinací kultivačně závislých a kultivačně nezávislých metod, s následnou izolací a fenotypovou a genotypovou charakterizací, nezbytný pro pochopení principu jejich adaptace k hostiteli a prostředí. Zároveň je také velmi důležitý pro detekci specifických funkčních vlastností bifidobakterií, výběr vhodných probiotických kmenů a testování jejich účinku na hostitelský organismus. Souvisí s tím i nalezení možností jejich cílené podpory formou bifidogenních prebiotik.

2 Literární přehled

2.1 *Bifidobacterium* spp.

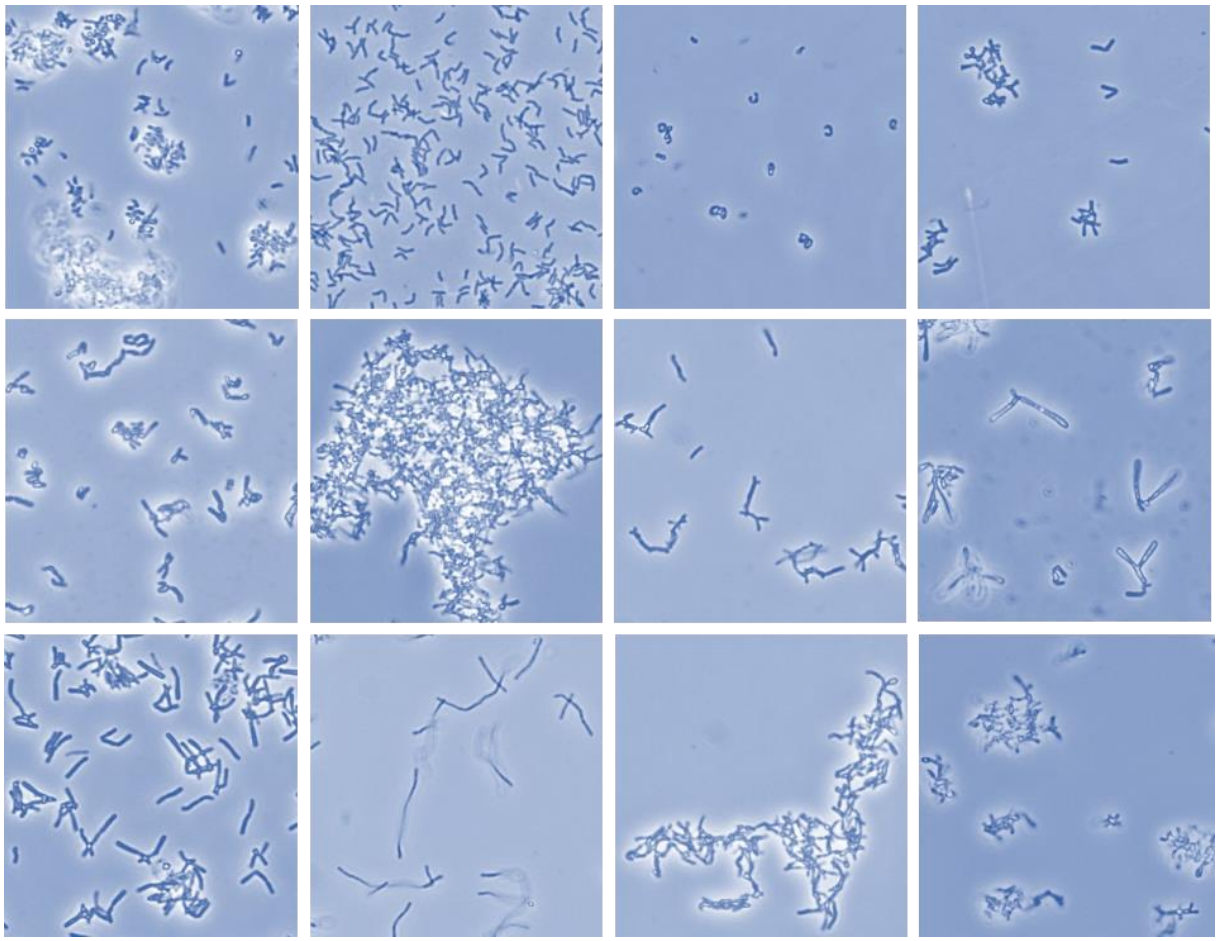
2.1.1 Obecná charakteristika

Francouzský pediatr Henry Tissier jako první popsal nepravidelné buňky bifidobakterií. Detekoval je ve sníženém množství ve stolici kojenců s gastrointestinálními potížemi ve srovnání s jejich množstvím u kojenců zdravých. V roce 1900 je pojmenoval jako *Bacillus bifidus communis* (Tissier 1900). Ve dvacátých letech 20. století bylo poukazováno na možnou existenci bifidobakterií jako samostatného rodu (Orla-Jensen 1924). Již po roce 1960 se bifidobakterie dostaly do popředí zájmu řady významných vědců (Reuter 1963; Mitsuoka 1969; Scardovi et al. 1970). Pro bakteriální taxonomii této doby bylo využíváno hlavně popisu fenotypových vlastností bakterií, jako je morfologie kolonií a buněk, spolu s jejich fermentačními profily. Zlomovým okamžikem pro identifikaci bifidobakterií bylo zahrnutí detekce přítomnosti specifického enzymu fruktóza-6-fosfát fosfoketolázy (F6PPK) (Scardovi 1965; de Vries & Stouthamer 1968) a měření procentuálního obsahu guaninu a cytosinu (G+C) (Sebald et al. 1965), zejména pro druhy, které vykazovaly netypickou morfologii bifidobakteriálních buněk a odlišné fermentační schopnosti. DNA-DNA hybridizace (DDH) byla další významnou metodou nezbytnou pro rozlišení již popsaných druhů a nových izolátů umožňující porovnávat podobnost jejich DNA (Scardovi et al. 1970). Přestože bifidobakterie byly zájmem mnoha výzkumných týmů, teprve až v roce 1974 byly odloučeny od rodu *Lactobacillus* a byly klasifikovány jako samostatný rod *Bifidobacterium* (Buchanan & Gibbons 1974). Po přelomovém zavedení sekvenační analýzy genu 16S rRNA byl dokonce hierarchicky přeskupen i celý kmen Actinobacteria (Stackebrandt et al. 1997).

Podle *Bergey's Manual of Systematic Bacteriology* je rod *Bifidobacterium* taxonomicky řazen do čeledi *Bifidobacteriaceae,* řádu Bifidobacteriales, podtřídy Actinobacteridae, třídy Actinobacteria, kmene Actinobacteria a domény Bacteria (Whitman et al. 2012). Do konce července roku 2021 bylo podle LPSN-PNU (*List of Prokaryotic Names with Standing in Nomenclature-Prokaryotic Nomenclature Up-to-Date*; https://lpsn.dsmz.de/) platně popsáno 87 druhů a 10 poddruhů bifidobakterií a v současné době je stále popisováno poměrně velké množství nových druhů. Do čeledi *Bifidobacteriaceae* patří dále ještě rody *Alloscardovia, Aeriscardovia, Bombiscardovia, Gardnerella, Neoscardovia, Parascardovia, Pseudoscardovia* a *Scardovia*, které jsou bifidobakteriím fylogeneticky velmi blízké (Biavati & Mattarelli 2018).

Bifidobakterie jsou Gram-pozitivní, nepohyblivé, nesporulující, nehemolytické, F6PPK pozitivní, kataláza, oxidáza a indol negativní tyčinkovité bakterie (Kawasaki et al. 2018; Mattarelli & Biavati 2018). Jejich buňky jsou morfologicky velmi variabilní. Mohou být krátké i dlouhé, rovné, pravidelné i nepravidelné, kyjovitě ztluštělé, rozdvojené, stočené, prstencovité či tvarem připomínající písmena Y, X a V. Vyskytují se samostatně, ve shlucích nebo řetězcích (obr. 1). Podmínky kultivace nicméně často jejich morfologii významně ovlivňují. Při kultivaci na polotuhých médiích obvykle tvoří lesklé konvexní krémově bíle až bílé kolonie s hladkým okrajem mající měkkou konzistenci (Biavati & Mattarelli 2015).

Obr. 1: Variabilita morfologie bifidobakteriálních buněk



Pozn.: Foceno fázově kontrastním mikroskopem Nikon Eclipse E 200LED MV RS (Japan); 400krát zvětšeno (autorka Nikol Modráčková).

Bifidobakterie jsou většinou striktně anaerobní. Některé kmeny tolerují kyslík za přítomnosti CO_2 (Biavati & Mattarelli 2015). Přítomnost kyslíku je nicméně často spojována se ztrátou jejich životaschopnosti ve fermentovaných mléčných výrobcích (Li et al. 2010). Rozmezí 36–38 °C je teplotní optimum pro růst bifidobakterií lidského původu, zatímco 41–43 °C pro růst bifidobakterií animálního původu (Ventura et al. 2004). Optimální pH pro jejich růst je

v rozmezí hodnot 6,5–7,0. Je pro ně typický vysoký obsah G+C bází v rozmezí 47–67 mol% (Biavati & Mattarelli 2015). Fylogenetickou analýzou sekvencí 233 core genů a odvozených proteinů typových kmenů Lugli et al. (2019b) definovali deset fylogenetických skupin bifidobakterií, konkrétně *B. adolescentis, B. boum, B. pullorum, B asteroides, B. longum, B. psychraerohilum, B. bifidum, B. pseudolongum, B. bombi* a *B. tissieri*.

Bifidobakterie se vyskytují v různých ekologických nikách. Gastrointestinální trakt savců, zejména v období mléčné výživy, ptáků a hmyzu žijícím sociálním způsobem života je běžným prostředím jejich výskytu (Bunesova et al. 2014; Milani et al. 2017b). Jsou spojovány i s prostředím dutiny ústní (Toh et al. 2015), krví (Hoyles et al. 2002), odpadními vodami (Dong et al. 2000) a fermentovanými potravinami (Laureys et al. 2016; Eckel et al. 2020). Všechny zmíněné niky jsou přímo nebo nepřímo spojeny s lidských či zvířecím střevem. Jejich výskyt v dalších prostředích je pravděpodobně důsledkem fekální kontaminace z jejich původního přirozeného zdroje. Existuje předpoklad, že k distribuci bifidobakterií dochází mezi živými organismy, které pečují o své potomky. Jedná se tedy pravděpodobně o důsledek přímého přenosu buněk bifidobakterií z rodiče/pečovatele na svého potomka (Turroni et al. 2011; Milani et al. 2015b; Turroni et al. 2018a).

B. animalis subsp. *lactis/animalis*, *B. adolescentis, B. dentium, B. catenulatum, B. longum* a *B. pseudolongum* jsou typickými multi-hostitelskými druhy bifidobakterií vyskytujícími se u různých savců (Lamendella et al. 2008; Bunesova et al. 2014; Bunesova et al. 2017; Lugli et al. 2019a). Se střevní mikrobiotou člověka je spojována řada typických bifidobakteriálních druhů. *B. breve*, *B. bifidum, B. longum* subsp. *infantis* a *B. longum* subsp. *longum* jsou běžnými dominantními druhy/poddruhy bifidobakterií osídlujícími střevo kojence (Turroni et al. 2012a; Turroni et al. 2014a), zatímco ve střevě dospělých dominují druhy *B. adolescentis, B. catenulatum, B. pseudocatenulatum* a *B. longum* (Ishikawa et al. 2013; Arboleya et al. 2016). Bifidobakterie v lidské mikrobiotě však nelze striktně rozdělit na dospělé a kojenecké druhy. Příkladem je *B. adolescentis*, který je přenášen z matky na potomka již během vertikálního transferu a osídluje tak běžně tímto způsobem také novorozenecké střevo (Duranti et al. 2014; Turroni et al. 2018a).

Příkladem dalších hostitelsky specifických druhů jsou *B. magnum, B. cuniculi* a poddruh *B. gallinarum* subsp. *saeculare*, které byly izolovány z výkalů králíka (Scardovi & Zani 1974; Scardovi et al. 1979; Biavati et al. 1991). Výhradně u hlodavců byly detekovány druhy *B. dolichotidis* (mara stepní), *B. criceti* (křeček polní) a *B. castoris* (bobr evropský) (Lugli et al. 2018; Duranti et al. 2019b), zatímco pouze u drůbeže to byly druhy *B. anseris* (husa domácí), *B. pullorum* a poddruh *B. gallinarum* subsp. *gallinarum* (kur domácí) (Trovatelli et al. 1974;

Watabe et al. 1983; Lugli et al. 2018), u divokého prasete *B. apri* (Pechar et al. 2017), u kaloně egyptského *B. rousetti* a *B. vespertilionis* (Modesto et al. 2021) a u lenochoda dvouprstého *B. choloepi* (Modesto et al. 2020). V posledním desetiletí bylo mnoho nových druhů bifidobakterií objeveno u nehumánních primátů. Velké množství těchto druhů, které byly izolovány a popsány například u gorily (Tsuchida et al. 2014), kosmana bělovousého s tamaríny (Modesto et al. 2014; Michelini et al. 2016a; Michelini et al. 2016c; Modesto et al. 2018c; Duranti et al. 2020) a u lemurů (Modesto et al. 2015; Michelini et al. 2016b), nebylo nikdy znovu nalezeno u jiného hostitele, ať už člověka nebo dalších zvířat (Mattarelli & Biavati 2018). Výjimkou byla detekce typicky lidských druhů *B. adolescentis* a *B. dentium* u orangutana a šimpanze (D'Aimmo et al. 2014).

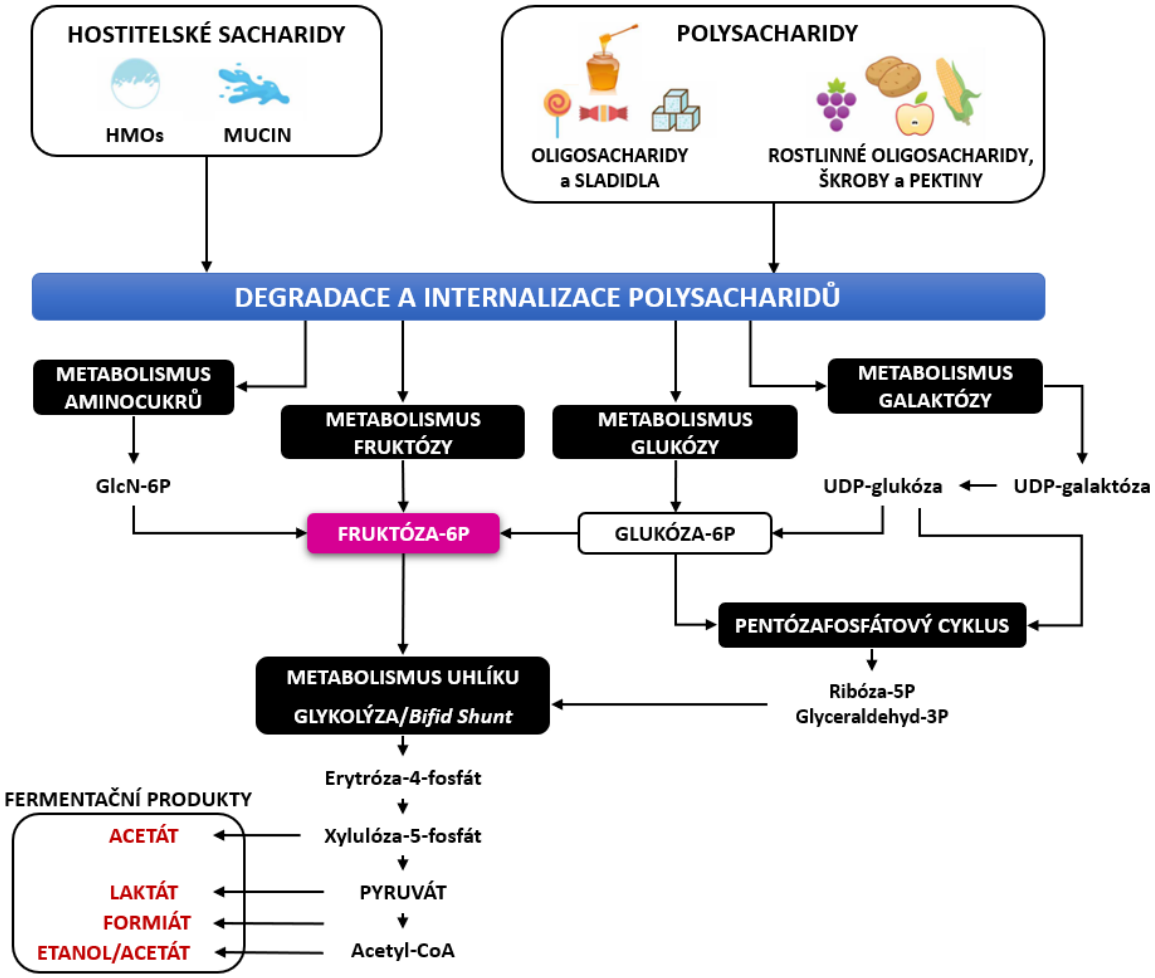
Prapůvodní ekologická nika, která je dodnes obývána bifidobakteriemi, je pravděpodobně střevo hmyzu žijícím sociálním způsobem, zejména blanokřídlých. Druhy *B. asteroides, B. commune* a *B. indicum* jsou striktně vázány na svého hostitele včelu medonosnou (Scardovi & Trovatelli 1969; Praet et al. 2015), zatímco *B. actinocoloniiforme, B. bohemicum* a *B. bombi* na čmeláka (Killer et al. 2009; Killer et al. 2011). Je zajímavé, že přestože jsou bifidobakterie většinou striktně anaerobní, *B. asteroides* stále disponuje geny pro respirační dráhy ve svém metabolismu. Tato výbava byla s velkou pravděpodobností u bifidobakteriálních druhů navázaných na savčí hostitele evolučně ztracena (Bottacini et al. 2012). Právě tento symbiotický vztah hmyzích bifidobakterií s jejich hostiteli odráží jejich společnou dlouhodobou koevoluci (Mattarelli & Biavati 2018). Je zajímavé, že bifidobakterie nebyly detekovány jen pouze u zmiňovaného hmyzu se sociálním způsobem života. Alberoni et al. (2019) totiž popsali další dva nové druhy bifidobakterií, *B. aemilianum* a *B. xylocopae,* také u drvodělky fialové, která je řazena mezi včely samotářky.

Hostitelská specifita bifidobakterií nicméně nemusí být vždy zcela jednoznačná. Distribuce bifidobakterií u 291 fekálních vzorků od 67 savčích druhů byla sledovaná metodou ITS profilování, cílené amplikonové sekvenování *Internal Transcribed Spacer* podle Milani et al. (2014b), která je kultivačně nezávislým přístupem pro screening širokého spektra vzorků. Jednotlivé druhy bifidobakterií byly rozsáhle rozšířeny mezi všemi jednotlivci bez ohledu na taxonomii nebo dietu hostitele. Výskyt a zastoupení bifidobakterií je pravděpodobně značně ovlivněn domestikací zvířat. U domestikovaných králíků, prasat a psů ve srovnání s populací bifidobakterií u divokých zajíců, divočáků a vlků byla zjištěna jejich vyšší diverzita, která je pravděpodobně způsobena jejich interakcemi s lidmi, se kterými žijí v úzkém kontaktu, a s tím souvisejícím modifikovaným životním prostředím (Milani et al. 2017b).

2.1.2 Metabolismus

Bifidobakterie mají sacharolytický typ metabolismu a při kolonizaci střevního traktu jsou přímo závislé na své schopnosti využívat složité sacharidy v tomto prostředí přítomné (Egan & Van Sinderen 2018). Sacharidy, které nejsou hydrolyzovány hostitelskými enzymy a nejsou tedy ani absorbovány v tenkém střevě, jsou možnými substráty pro růst bifidobakterií (Fushinobu 2010), které je využívají za účelem zisku energie a uhlíku. Tyto sacharidy mohou být dietárního nebo hostitelského původu (obr. 2) (Milani et al. 2015a).

Obr. 2: Metabolické dráhy bifidobakterií při utilizaci sacharidů (Bottacini et al. 2017)



Pozn.: Schématické znázornění fermentačních drah bifidobakterií při utilizaci hostitelských a dietárních sacharidů. HMOs – oligosacharidy mateřského mléka, P – fosfát, Acetyl-CoA – acetylkoenzym A, GlcN-6P – glukosamin-6-fosfát, UDP – uridindifosfát.

Oligosacharidy (rafinóza, melezitóza, maltotrióza, stachyóza), polyoly (mannitol, sorbitol) a dietární vlákninu (rezistentní škroby, maltodextriny, fruktany, pektiny, inulin, celulóza, galaktany, xylany, arabinany, arabinogalaktany, arabinoxylany) lze zařadit mezi dietární sacharidy (Bottacini et al. 2017). Běžně se vyskytují v buněčné stěně rostlin (Koropatkin et al.

2012), jsou součástí obilovin (Shewry & Hey 2015), ovoce a zeleniny (Posé et al. 2018; Klaassen & Trindade 2020). Zatímco oligosacharidy mateřského mléka (HMOs), O-glykany a N-glykany patří mezi hostitelské sacharidy, které má řada bifidobakterií schopnost utilizovat (Bottacini et al. 2017). Schopnost fermentace těchto substrátů je často druhově či kmenově specifická (Palframan et al. 2003; Liu et al. 2021).

V genomu bifidobakterií je kódováno velké množství specifických enzymů (Cummings & Englyst 1995; Cronin et al. 2011; Pokusaeva et al. 2011). Ve srovnání s ostatními komenzálními bakteriemi ve střevě má pangenom bifidobakterií jeden z největších predikovaných glykobiomů. Až 13,7 % identifikovaných genů se totiž podílí na metabolismu sacharidů (Milani et al. 2014a; Milani et al. 2016). Na základě dat z *Carbohydrate Active Enzymes (CAZy)* databáze bylo zjištěno, že v pangenomu bifidobakterií je celkem 3 385 predikovaných genů kódujících enzymy podílející se na metabolismu sacharidů, které zahrnují 57 rodin glykosyl hydroláz (GH), 13 glykosyl transferáz a 7 sacharidových esteráz (Lombard et al. 2014; Milani et al. 2016). Nejhojněji zastoupené predikované GH byly detekovány v genomech kmenů *B. scardovii* JCM12489 (126 GH) a *B. dentium* Bd1 (87 GH), zatímco nejméně jich bylo zjištěno u kmene *B. indicum* LMG 22698 (25 GH) (Egan & Van Sinderen 2018).

Obecně je dominantně zastoupenou skupinou enzymů bifidobakterií rodina GH13 (α-glukosidázy a sacharóza fosforylázy), která katalyzuje hydrolýzu širokého spektra sacharidů s α-glukopyranózovými jednotkami jako je škrob, glykogen, amylóza, amylopektin, pullanan, maltodextrin, cyklomaltodextrin a izomaltulóza (Pokusaeva et al. 2011), které jsou běžnou součástí diety savců (El Kaoutari et al. 2013a). GH36 (α-galaktosidázy) je další rodinou enzymů běžně se vyskytující u bifidobakterií s funkcí hydrolýzy α-galaktooligosacharidů, jako je stachyóza, rafinóza a melibióza, přítomných v sóje a dalších rostlinách (Goulas et al. 2009; O'Connell Motherway et al. 2013). GH2 a GH42 (β-galaktosidázy) patří mezi další charakterizované a běžně zastoupené GH bifidobakterií (Pokusaeva et al. 2011) umožňující jim utilizovat laktózu, galaktany a galaktooligosacharidy a zároveň také odstraňovat galaktózu z oligosacharidů mléka a mucinů (O'Connell Motherway et al. 2011; James et al. 2016). S utilizací dalších rostlinných substrátů jsou spojovány GH1 a GH3 (β-glukosidázy), pomocí nichž bifidobakterie mohou využívat celobiózu, cellodextrin a bioaktivní fenoly a flavonoidy (Youn et al. 2012), a GH43 (arabinofuranosidázy a xylanázy) štěpící oligosacharidy buněčné stěny rostlin (O'Callaghan et al. 2015). Přítomnost GH32 (β-fruktofuranosidázy – invertázy a sacharázy) poté umožňují utilizaci sacharózy a fruktooligosacharidů s krátkým řetězcem (Ryan et al. 2005). Pro *B. longum* je například typická přítomnost genů kódujících GH36 a GH27 (α-galaktosidázy), GH2 a GH42 (β-galaktosidázy), GH1 a GH3 (β-glukosidázy), GH32

(β-fruktofuranosidázy) a GH127 (L-arabinofuranosidázy), které katalyzují utilizaci sacharidů rostlinného původu (Arboleya et al. 2018). Naopak GH33 (exo-sialidázy), GH29 (fukosidázy), GH20 (hexoaminidázy, lakto-*N*-biosidázy), GH38 (α-mannosidázy) a GH101 (α-*N*-acetyl-galaktosaminidázy) jsou příkladem GH katalyzujících degradaci hostitelských sacharidů (Milani et al. 2015a).

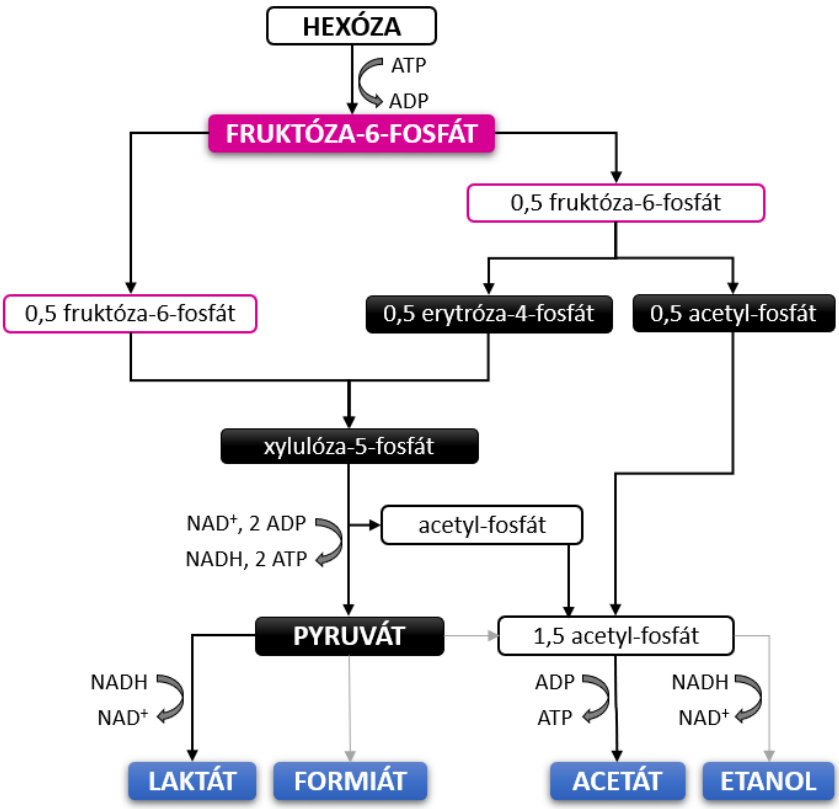
Bifidobakterie jsou schopny metabolizovat nejrůznější monosacharidy, disacharidy a polysacharidy nacházející se v prostředí střeva. Extracelulární GH degradují komplexní sacharidy na oligosacharidy a monosacharidy, které jsou dále importovány do cytoplazmy internalizací specifickými transmembránovými proteiny, a to zejména ABC transportéry (*ATP-Binding Cassette Transporters*) nebo méně častěji také MFS transportními systémy (*Major Facilitator Superfamily Transport Systems*), jako jsou protonové symportéry, permeázy a fosfoenolpyruvát fosfotransferázovým systémem, za spotřeby ATP (Mazé et al. 2007; Pokusaeva et al. 2011; Turroni et al. 2012b; Bottacini et al. 2017). Některé cukry mohou po vstupu do cytoplazmy podléhat dalším procesům, jako například epimerizaci, deacetylaci, deaminaci nebo fosforylaci, za účelem produkce fosforylovaných monosacharidů, které poté vstupují do hlavní fermentační dráhy bifidobakterií (Pokusaeva et al. 2011).

Hlavní a specifickou metabolickou dráhou bifidobakterií je *Bifid Shunt Pathway* (obr. 3) s klíčovým enzymem F6PPK (De Vries & Stouthamer 1967). Detekcí F6PPK je umožněna rodová identifikace bifidobakterií (Orban & Patterson 2000; Vlkova et al. 2002). Tento enzym je navíc považován za taxonomický marker čeledi *Bifidobacteriaceae* (Felis & Dellaglio 2007). Touto metabolickou dráhou jsou asimilovány hexózové i pentózové cukry. Fruktóza-6-fosfát je enzymem F6PPK rozkládána na acetyl-1-fosfát a erytróza-4-fosfát s teoretickým ziskem 1,5 mol acetátu, 1 mol laktátu a 2,5 ATP z 1 mol glukózy, zatímco pentózy do této metabolické dráhy vstupují jako ribulóza-5-fosfát nebo xylulóza-5-fosfát s teoretickým ziskem acetátu s laktátem v poměru 1:1. Tyto poměry metabolitů se nicméně mohou v závislosti na kmenu bifidobakterie, jeho růstové buněčné fázi, zdroji uhlíku a pH prostředí značně lišit (De Vries & Stouthamer 1967; Macfarlane & Macfarlane 2003; Palframan et al. 2003). Bifidobakterie totiž na základě dostupnosti sacharidů poměrně snadno modifikují své metabolické dráhy (Palframan et al. 2003). Část uhlíkového toku je navíc přímo oddělena od fermentace pro anabolickou produkci biomasy (Amaretti et al. 2007). Při testování fermentačního profilu 22 kmenů různých druhů bifidobakterií byla zjištěna variabilní produkce hlavních metabolitů v závislosti na kmenu bifidobakterie a sacharidovém substrátu. Většina testovaných kmenů produkovala acetát jako hlavní fermentační produkt, nicméně 3 kmeny, konkrétně

B. pseudocatenulatum NCIMB 8811, *B. longum* NCIMB 8809 a *B. bifidum* NCIMB 8810, produkovaly laktát jako svůj hlavní metabolit (McLaughlin et al. 2015).

Kromě acetátu a laktátu mohou v rámci štěpení pyruvátu vznikat i dodatečná množství formiátu. Navíc mohou být vyprodukována i malá množství etanolu a sukcinátu, zatímco k produkci butyrátu a propionátu u bifidobakterií nikdy nedochází (Whitman et al. 2012). Produkce laktátu z pyruvátu totiž může být odchýlena k produkci acetyl-fosfátu a formiátu, kdy acetyl-fosfát může být redukován až na etanol ve výsledný neprospěch produkce acetátu (de Vries & Stouthamer 1968). Variabilní poměry konečných produktů metabolismu bifidobakterií pravděpodobně souvisí také se specifickou rychlostí růstu při utilizaci sacharidů. Rychlejší růst bifidobakterií v přítomnosti snadno dostupného substrátu v růstovém médiu je většinou spojován s významnou produkcí laktátu a relativně menším množstvím vyprodukovaného acetátu, formiátu a etanolu. Pokud je zdroj energie spotřebováván pomalu a růst daného kmene bifidobakterie je z tohoto důvodu zpomalen, dochází naopak ke zvýšené produkci acetátu, formiátu a etanolu v neprospěch laktátu (Van der Meulen et al. 2006; Falony et al. 2009).

Obr. 3: *Bifid Shunt Pathway* – metabolická dráha štěpení hexóz (Amaretti et al. 2007)



Pozn.: Hexóza je degradována na 1 pyruvát a 1,5 acetyl-fosfát, který umožňuje vznik alternativních konečných produktů (minoritní dráhy jsou znázorněny šedými šipkami). ATP – adenosintrifosfát, ADP – adenosindifosfát, NAD^+, NADH – nikotinamidadenindinukleotid.

Obecně jsou mastné kyseliny s krátkým řetězcem (SCFAs), acetát (C2), propionát (C3) a *n*-butyrát (C4), hlavními konečnými produkty bakteriálního metabolismu prebiotických substrátů ve střevě člověka. Jejich produkce a metabolická aktivita je spojována se zdravím střev a udržením střevní homeostáze, správnou funkcí kolonocytů a imunitního systému, poskytnutím využitelného uhlíku pro zisk energie pro hostitele, správnou fyziologií ledvin a chutí k jídlu (Roberfroid et al. 2010; O'Keefe 2016; Pluznick 2016). Řada těchto metabolických produktů je limitujícím faktorem pro mnoho dalších mikroorganismů ve střevním ekosystému (Bottacini et al. 2017). Produkce bifidobakteriálního acetátu byla například spojena se zprostředkováním střevní epiteliální ochrany hostitele proti infekci (Ventura et al. 2014). Dalšími produkty metabolismu bakterií jsou také plyny (H_2, H_2S, CO_2, metan), které ale bifidobakterie netvoří (Whitman et al. 2012).

Zatímco u většiny mikroorganismů je v případě degradace galaktózy využívána specifická dráha *Leloir Pathway*, ve které je galaktóza přeměněna na glukózu-6-fosfát, u bifidobakterií byla popsána alternativní energeticky úspornější varianta *Galacto-N-biose/Lacto-N-biose (GNB/LNB) Pathway* (Frey 1996; Nishimoto & Kitaoka 2007; Kitaoka 2012; De Bruyn et al. 2013). Tato specifická dráha umožňuje bifidobakteriím růst na substrátech od galaktózy odvozených (Pokusaeva et al. 2011; Bottacini et al. 2014b). Během degradace uvolněných aminocukrů (např. *N*-acetylglukosamin) z hostitelských sacharidů dochází ke konverzi na fruktózo-6-fosfát (Plumbridge & Vimr 1999; Egan et al. 2014b). Pro bifidobakterie je nicméně z důvodu vyššího zisku energie vždy výhodná a upřednostňovaná fermentace cukrů jejich hlavní metabolickou dráhou *Bifid Shunt Pathway* (Palframan et al. 2003), do které jsou přímo či nepřímo přiváděny jednotlivé substráty a meziprodukty dílčích souvisejících metabolických drah (James et al. 2016).

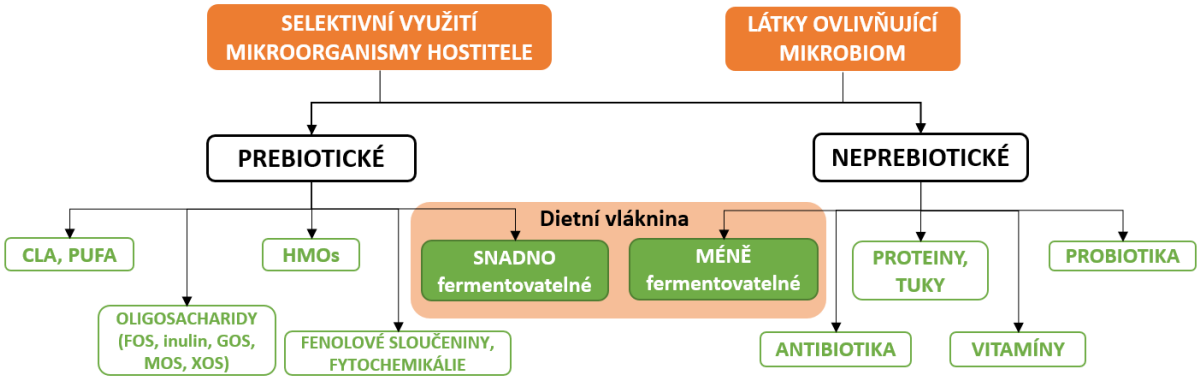
Znalost metabolismu bifidobakterií se schopností utilizace širokého spektra sacharidů je nezbytná pro zajištění jejich růstových výhod oproti konkurenční střevní mikrobiotě a zvýšení jejich životaschopnosti při realizaci komerčního využití, skladování a jejich podávání jako probiotik (Ventura et al. 2007).

2.1.3 Prebiotika pro bifidobakterie

Prebiotika jsou substráty, které jsou selektivně využívány mikroorganismy hostitele. Tyto látky nemohou být degradovány cílovými hostitelskými enzymy a jejich příjem musí být spojován se zdravotním přínosem pro hostitele. Koncept prebiotik byl nově Mezinárodní vědeckou asociací pro probiotika a prebiotika (ISAPP; *International Scientific Association of Probiotics and Prebiotics*) rozšířen také o další látky, kromě již stávajících definovaných sacharidů

(obr. 4), a jejich aplikace byla aktualizována o podávání těchto substrátů i jinými cestami než pouze gastrointestinálním traktem hostitele. Přestože je většina současných prebiotik podávána orálně, lze je aplikovat i přímo do jiných mikrobiálně kolonizovaných míst těla, jako je urogenitální trakt a kůže (Gibson et al. 2017).

Obr. 4: Schéma rozšířené definice prebiotik (Gibson et al. 2017)



Pozn.: Na obrázku jsou znázorněny již uznaná a potenciálně uznatelná prebiotika. Substráty, které sice složení mikrobioty ovlivňují (antibiotika, minerální látky, vitamíny a bakteriofágy), ale pouze neselektivním způsobem, nemohou být považovány za prebiotika (Gibson et al. 2017). CLA – konjugovaná linolová kyselina, PUFA – polynenasycené mastné kyseliny, FOS – fruktooligosacharidy, GOS – galaktooligosacharidy, MOS – mannanooligosacharidy, XOS – xylooligosacharidy, HMOs – oligosacharidy mateřského mléka.

Přestože je prebiotický účinek řady látek na zdraví hostitele stále zkoumán, byla zjištěna řada pozitivní vlivů prebiotik na zdraví jedince. Jako některé z nich lze zmínit například inhibici patogenů v gastrointestinálním traktu (Morrow et al. 2005; Abd El-Hack et al. 2021), stimulaci imunity (Kulinich & Liu 2016) a prevenci propuknutí alergií (Osborn & Sinn 2013), snížení hladiny lipidů v krvi u obézních lidí (Beserra et al. 2015), podporu mikrobiální rovnováhy urogenitálního traktu (Coste et al. 2012), pokožky (Kano et al. 2013; Miyazaki et al. 2014) a správné funkce metabolismus kostní tkáně (Holloway et al. 2007).

Pro správnou strategii podávání prebiotik s cílem zlepšení zdraví lidí a zvířat by neměl být používán izolovaný přístup. Je důležité zvážit i zdravou výživu a životní styl jedince. Do budoucna mají prebiotika terapeutický i preventivní potenciál pro podporu zdraví a snížení rizika propuknutí onemocnění, nicméně je nezbytné další testování a prokázání jejich prebiotického účinku, bezpečnosti a vhodného dávkování pro konkrétního cílového hostitele (Gibson et al. 2017).

Přestože dnes je cíleno prebiotiky leckdy na řadu různorodých mikroorganismů, původně a stále signifikantně prebioticky podporovými členy střevní mikrobioty jsou hlavně

bifidobakterie a laktobacily (Tochio et al. 2018; Zhu et al. 2018; Murakami et al. 2021). Bifidobakterie totiž disponují enzymatickým vybavením nezbytným k degradaci a následné utilizaci nejběžněji používaných prebiotických substrátů, které snadno fermentují (Rastall 2010; Sarbini & Rastall 2011). Metabolická aktivita a perzistence bifidobakterií ve střevě je pak přímo závislá na dostupnosti dietárních a hostitelských sacharidů (Turroni et al. 2016). Některé druhy bifidobakterií, jako je například *B. breve,* jsou dokonce schopny utilizovat jak dietární, tak i hostitelské sacharidy. Tato metabolická schopnost je přisuzována k perzistenci určitých bifidobakteriálních druhů ve střevní mikrobiotě bez ohledu na dietu a věk hostitele (Bottacini et al. 2014a; Kelly et al. 2016).

2.1.3.1 Dietární sacharidy

Existuje řada fermentovatelných sacharidů, u kterých byl popsán prebiotický účinek. Jako nejlépe prostudovaná dietární prebiotika pro člověka s prozkoumanými účinky jsou nejčastěji popisovány nestravitelné oligosacharidy **fruktany** a **galaktany** (Rastall & Gibson 2015), které jsou přednostně využívány probiotickými bifidobakteriemi (Roberfroid et al. 2010). Chemické vazby mezi fruktooligosacharidy a galaktooligosacharidy jsou relativně snadno štěpeny pomocí bifidobakteriálních β-fruktanosidáz a β-galaktosidáz (Rastall 2010; Sarbini & Rastall 2011). Bifidobakterie zároveň disponují také vhodným transportním systémem pro zachycení a distribuci těchto substrátů do mikrobiální cytoplazmy, čímž je zajištěna další selektivita schopnosti utilizace těchto látek v konkurenčním mikrobiálním prostředí komplexního ekosystému střeva (Goh & Klaenhammer 2015). Některé kmeny bifidobakterií jsou navíc schopny utilizovat i řadu dalších podobných sacharidů, jako jsou například xylooligosacharidy (Berger et al. 2021), arabinoxylan (Lagaert et al. 2010), inulin (Ramirez-Farias et al. 2008) a mannooligosacharidy (Singh et al. 2018).

Po prokázání selektivního využívání žádoucími mikroorganismy hostitele a zajištění prokazatelného zdravotního benefitu, mohou být **některé typy dietární vlákniny** považovány také za prebiotické. Nicméně vzhledem k individualitě a různým schopnostem hostitelské mikrobioty při utilizaci širokého spektra substrátů, nelze přesně definovat které. Celulóza je například vhodným prebiotikem pro přežvýkavce, nikoliv pro člověka. Lidská střevní mikrobiota totiž není schopna zcela využít β(1→4) vazby mezi jednotkami D-glukózy (Ben David et al. 2015; Delcour et al. 2016). Při definici prebiotik je neméně důležité i místo účinku. Za prebiotikum pro mikrobiotu dutiny ústní je například považován xylitol (Gibson et al. 2004; Roberfroid et al. 2010).

Rezistentní škrob skládající se z amylózy a amylopektinu je dalším dietárním sacharidem s potenciálně prebiotickými vlastnostmi. Maltodextrin, maltotrióza a amylopektin jsou deriváty od něj odvozené (Van der Maarel et al. 2002). Bifidobakterie, i když jsou minoritní skupinou dospělé lidské mikrobioty, se také podílení na štěpení těchto substrátů (Milani et al. 2015a). *B. adolescentis* například disponuje příslušnými enzymy pro degradaci škrobu (Duranti et al. 2014), jako jsou α-glukosidázy zahrnující například konkrétně amylázy, pullanázy a cyklomaltodextrinázy, pomocí nichž vykazuje výborný růstový potenciál na rostlinných sacharidech ve srovnání s dalšími lidskými druhy bifidobakterií (Milani et al. 2016).

2.1.3.2 Hostitelské sacharidy

Mezi hostitelské sacharidy patří HMOs a muciny, které jsou důležitými substráty pro kolonizaci střeva kojence bifidobakteriemi (Bottacini et al. 2017). Bifidobakterie totiž disponují adekvátní sadou enzymů GH, které utilizaci hostitelských sacharidů umožňují (Katoh et al. 2020).

2.1.3.2.1 HMOs

HMOs jsou komplexní sacharidy, které jsou produkovány mléčnou žlázou všech savců, nicméně jejich profil mezi druhy je velmi variabilní (Tao et al. 2011). Jejich přítomnost v savčím mléce je spojována s přirozenou selekcí (Hinde & Milligan 2011; Hinde & German 2012). Jsou prvními prebiotiky ve výživě člověka (Oozeer et al. 2013). Laktací jsou totiž selektivně vyživovány geneticky kompatibilní bakterie kojenců prebiotickým komplexem volných oligosacharidů (Zivkovic et al. 2011; Turroni et al. 2018b). Přítomnost HMOs je spojována s modulací imunitní odpovědi, prevencí adheze patogenů na střevní epitel a vytvořením konkurenčního prostředí ve střevě novorozence, čímž je chráněn před propuknutím řady infekčních onemocnění (Morrow et al. 2005; Oliveira et al. 2015; Kulinich & Liu 2016; Plaza-Díaz et al. 2018). Mateřské mléko je svým unikátním složením považováno za přirozený a optimální způsob výživy novorozence a bylo evolučně formováno výhradně pro tento účel. Je komplexní výživou bohatou na laktózu, mastné kyseliny a proteiny, které kojenci přímo dodávají potřebnou energii (Thomson et al. 2018).

Přestože pro novorozence mají HMOs malou nutriční hodnotu, jsou v mateřském mléce přítomny volně a ve vysokých koncentracích (Smilowitz et al. 2013; Garrido et al. 2016). Po laktóze a lipidech jsou HMOs třetí nejvíce zastoupenou složkou mateřského mléka (5–15 g L^{-1}), kde až stokrát převyšují koncentrace v mléce kravském a jsou považovány za jeho klíčový komponent (Bode 2015; Plaza-Díaz et al. 2018). Jejich zastoupení v lidském mléce je zároveň rozmanitější (Hinde & German 2012) a vzhledem k jejich komplexnosti je stále nemožné je

zcela dokonale uměle syntetizovat (Musilova & Rada 2015; Sprenger et al. 2017). Hlavními složkami HMOs jsou galaktóza, glukóza, *N*-acetylglukosamin, fukóza a deriváty sialové kyseliny. Laktózové jádro je na redukujícím konci fukosylací a/nebo sialylací prodlužováno o *N*-acetyllaktosaminové jednotky (fukóza a sialová kyselina) s větší strukturální diverzitou (Zivkovic et al. 2011; Bode 2015). HMOs lze dále rozdělit na neutrální fukosylované, neutrální nefukosylované (*N*-acetylglukosamin) a kyselé sialylované (Bode 2015). Stupeň polymerace se pohybuje mezi 3–32 a jejich složitost spočívá v rozmanitosti glykosidických vazeb a vytváření velkého množství potenciálních kombinací (Wu et al. 2010; Wu et al. 2011).

HMOs nejsou štěpeny enzymatickým systémem savců, a proto procházejí gastrointestinálním traktem až do tlustého střeva, kde jsou degradovány střevní mikrobiotou (De Leoz et al. 2015). Schopnost využívat HMOs je známá u zástupců z čeledí *Bifidobacteriaceae* a *Bacteroidaceae* (De Leoz et al. 2015; Hirvonen et al. 2019). Obecně je utilizace oligosacharidů spojena se zvýšeným zastoupením *Bifidobacteriaceae* ve střevní mikrobiotě, vyššími hladinami mastných kyselin s krátkým řetězcem a sníženým pH (Matsuki et al. 2016). Tvorba střevního acetátu bifidobakteriemi je navíc spojována s vyvoláním příznivých účinků na hostitele, včetně zlepšení funkce střevní bariéry (Fukuda et al. 2011). Bylo prokázáno, že v genomech *B. longum, B. bifidum* a *B. breve* jsou obsaženy specifické soubory genů kódující enzymy hydrolyzující určité HMOs (Marcobal & Sonnenburg 2012; Underwood et al. 2015; James et al. 2016). Příkladem těchto GH jsou fukosidázy, sialidázy, β-hexosaminidázy, β-galaktosidázy a lakto-*N*-biosidázy (Sela et al. 2008; Kitaoka 2012). Právě tato enzymatická výbava je spojována s vysokým zastoupením bifidobakterií ve střevě kojenců (Matsuki et al. 2016), což je spolu s výživou kojence klíčové z hlediska dlouhodobého vlivu na jeho zdraví (Turroni et al. 2018a). Schopnost bifidobakterií využít HMOs je druhově specifická (Sela 2011).

U bifidobakterií jsou známé dvě strategie degradace HMOs. Intaktní HMOs jsou pomocí řady transportérů a vazebných proteinů transportovány do buňky, kde jsou degradovány na monosacharidy, které jsou dále utilizovány. Druhou strategií je extracelulární štěpení HMOs na monosacharidy a disacharidy, které jsou až poté transportovány do buňky a dále metabolizovány (Garrido et al. 2012; Shani et al. 2018). Velmi homogenní skupinou jsou kmeny poddruhu *B. longum* subsp. *infantis*, které jsou schopny využít všechny HMOs přítomné v mateřském mléce. Z tohoto důvodu jsou způsoby přenosu sacharidů přes buněčnou membránu značně rozmanité. ABC transportéry přenáší HMOs, laktózu, lakto-*N*-biózu, *N*-acetylglukosamin a sialovou kyselinu, permeázy poté fukózu, glukózu, galaktózu a laktózu a méně častěji pak fosfotransferázový systém může přenášet glukózu a *N*-acetylglukosamin. Intracelulární GH poté katalyzují rozklad sacharidů na jednotlivé cukerné složky, které jsou

dále degradovány specifickými metabolickými dráhami bifidobakterií (Sela et al. 2008). Tímto způsobem degradují HMOs také *B. longum* subsp. *longum* a *B. breve* (Shani et al. 2018).
Odlišný, velmi rozmanitý a kmenově specifický je v utilizaci HMOs druh *B. bifidum*, který štěpí a do své cytoplazmy přenáší pouze některé frakce HMOs (Garrido et al. 2015). Oproti *B. longum* subsp. *infantis*, *B. bifidum* disponuje řadou různých extracelulárních GH (Kitaoka 2012). Je schopen štěpit HMOs a zpřístupňovat je tak v prostředí jako substrát pro růst dalším bakteriím střevní komunity kojence. Jeho přítomnost je navíc spojována se zvýšeným zastoupením dalších druhů bifidobakterií (Gotoh et al. 2018). Některé druhy jsou totiž schopny využívat pouze malé frakce strukturně odlišných HMOs. Například některé kmeny *B. breve* disponují enzymy pouze pro štěpení neutrálních HMOs (Ruiz-Moyano et al. 2013) a sialové kyseliny (Egan et al. 2014b), ale nejsou schopny přímo využít mucinové sacharidy a sialyllaktózu. Zmíněné sacharidy jsou nejprve extracelulárně degradovány druhem *B. bifidum* na fukózu a sialovou kyselinu, kterou je poté *B. breve* již schopen využít (Egan et al. 2014a; Centanni et al. 2019). Kooperativní vlastnosti v rámci ekosystému kojeneckého střeva při využívání HMOs vykazovaly také poddruhy/druhy *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. breve* a *B. pseudocatenulatum*, které tak maximalizovaly spotřebu poskytnutých živin (Lawson et al. 2020).

2.1.3.2.2 Mucin

Glykoprotein mucin tvoří většinu ochranné vrstvy mukózního povrchu střevní sliznice, která je fyzickou bariérou chránící epitel před adhezí patogenů, antigenů a toxinů. Genová exprese, glykosylace a sekrece mucinu je ovlivňována střevními mikroorganismy (Comelli et al. 2008; Wrzosek et al. 2013). Symbióza hlenové vrstvy spolu se střevní mikrobiotou je spojována s udržením homeostáze hostitele (Holzapfel et al. 1998). Narušená vrstva sliznice a mikrobiální dysbióza jsou totiž v úzké korelaci s propuknutím řady onemocnění, jako jsou například mukositida (van Vliet et al. 2010), zánětlivá střevní onemocnění (Merga et al. 2014), kolorektální karcinom (Baxter et al. 2014) a metabolický syndrom (Everard et al. 2013). Včasná modulace interakcí mezi mukózou a mikrobiotou je proto klíčová (Rokhsefat et al. 2016).
Mucin je produkován hlavně mukózními a pohárkovými buňkami. V mucinových granulích pohárkových buněk jsou muciny ukládány intracelulárně, odkud jsou vylučovány do prostředí (Brockhausen et al. 2009). Prolin, serin a threonin jsou obsaženy v polypeptidové části mucinu s O-glykosylovanými řetězci. *N*-acetylgalaktosamin, *N*-acetylglukosamin, fukóza, galaktóza a sialová kyselina jsou hlavními složkami těchto heterogenních O-glykanů. Schopnost metabolizovat mucinové oligosacharidy je pravděpodobně klíčovým faktorem při bakteriální

kolonizaci sliznice střeva. Pro jejich degradaci je nicméně nezbytná přítomnost specifických enzymů GH (Tran & Ten Hagen 2013; Pelaseyed et al. 2014; Tailford et al. 2015).

Schopnost využívat hostitelské sacharidy jako zdroj uhlíku a energie je pravděpodobně výsledkem koevoluce lidského střeva a střevních komenzálních bakterií (Turroni et al. 2010). Pouze některé z nich jsou schopny mucin využít. V rámci rodu *Bifidobacterium* byla tato schopnost zjištěna výhradně u druhu *B. bifidum* (Duranti et al. 2015). Endo-α-*N*-acetylgalaktosaminidázy katalyzují rozklad O-glykosidických vazeb mezi galaktosyl-β-1,3-*N*-acetylgalaktosaminem a serinovými nebo threoninovými zbytky u většiny mucinových glykoproteinů (Fujita et al. 2005). Fukosidázy poté uvolňují L-fukózu z oligosacharidového jádra mucinové struktury (Ashida et al. 2009). *N*-acetyl-β-hexosaminidázy, β-galaktosidázy a sialidázy se na metabolizaci mucinu také podílejí (Nishimoto & Kitaoka 2007). Oligosacharidové jádro mucinu skládající se z *N*-tetraózy je poté degradováno na *N*-biózu (Wada et al. 2008), která je do cytoplazmy transportována specifickými ABC transportéry, fosforylována a nakonec metabolizována v glykolytických a aminosacharidových cestách (Nishimoto & Kitaoka 2007).

2.1.3.3 Další prebiotické zdroje

Rostlinné glykosidy jsou dalšími potenciálně prebiotickými zdroji pro bifidobakterie. Jedná se o deriváty sacharidů skládající se ze dvou chemicky a funkčně nezávislých částí, glykonu a aglykonu, které jsou spojeny glykosidickou vazbou (Bartnik & Facey 2017). Některé glykosidy přirozeně se vyskytující v rostlinách jsou používány v tradiční medicíně již po celá staletí. Jejich biologické účinky nicméně nejsou připisovány glykosidickým formám, ale jejich aglykonům (de Arriba et al. 2013; Biernat et al. 2018). Obecně je zhruba 90–95 % těchto substrátů odolných vůči absorpci v tenkém střevě a dostává se tak v nezměněné formě až do tlustého střeva (Clifford 2004), kde jsou pomocí střevních mikroorganismů biotransformovány na bioaktivní metabolity (Dueñas et al. 2015). Bifidobakterie mají širokou škálu enzymů GH, včetně β-glukosidáz (Bottacini et al. 2018). Kmen *B. pseudocatenulatum* IPLA 36007 například disponuje geny kódujících deglykosylaci sójových isoflavonových glykosidů (Alegría et al. 2014). Konkrétně čtyři tyto β-glukosidázy byly dále charakterizovány jako enzymy uvolňující aglykony daidzein a genistein (Guadamuro et al. 2017). Kromě druhu *B. pseudocatenulatum*, také *B. catenulatum*, *B. adolescentis* a *B. breve* disponují β-glukosidázami, které využívají například k degradaci fazolových isoflavonových glykosidů daidzinu, genistinu a kemferolu (Marotti et al. 2007). β-glukosidázovou aktivitu u štěpení anthokyaninových glykosidů

delfinidinu a malvidinu poté vykazoval také kmen *B. animalis* subsp. *lactic* BB12 (Avila et al. 2009).

Potenciální prebiotické účinky s cílenou stimulací růstu mikroorganismů byly zjištěny také u katechinů, anthokyanů a proanthokyanů (Alves-Santos et al. 2020). Růst bifidobakterií podporují například i katechiny zeleného čaje (Dey et al. 2019), lignan syringaresinol (Cho et al. 2016) a polyfenoly z tradičního asijského rostlinného nápoje Kudingcha vyráběného z listů cesmíny (Xie et al. 2018).

Alternativou příjmu prebiotických rostlinných sacharidů v lidské dietě by mohlo být začlenění **chitinu** a jeho derivátů z jiných zdrojů, jako je hmyz, exoskeletony korýšů a houby (Lopez-Santamarina et al. 2020). Jednou z možností je například biopolymer chitin-glukan, který je obvykle extrahován z *Aspergillus niger* a skládá se z chitinu a β-1,3-D-glukanu (Neyrinck et al. 2012). Na základě *in vitro* a *in vivo* testování byl shledán novou potenciální bifidogenní prebiotickou sloučeninou, jejíž přidání do lidské diety by mohlo podpořit kolonizaci střeva bifidobakteriemi, zejména druhy *B. breve* a *B. bifidum* (Alessandri et al. 2019). Chitin je také významnou složkou jedlého hmyzu, kdy například konzumace cvrčků podpořila růst druhu *B. animalis* (Stull et al. 2018).

2.1.4 Cross-feeding

Cross-feeding představuje ekologickou strategii získávání živin. Složité sloučeniny, například dietární nebo hostitelské polysacharidy, jsou kompetentními bifidobakteriemi degradovány na jednodušší oligosacharidy a monosacharidy, které se tím stávají dostupnými i dalším střevním mikroorganismům (De Vuyst & Leroy 2011; Egan et al. 2014a; Egan et al. 2014b). Poskytnutí konečných produktů bakteriálního metabolismu, zejména bifidobakteriálního acetátu a laktátu, jako substrátů například pro butyrát produkující bakterie, je dalším z příkladů cross-feedingu (Moens et al. 2017). Přítomnost bifidobakterií je obecně spojována s modulací střevního mikrobiomu a rozšiřováním jeho sacharolytických schopností (Turroni et al. 2016). Právě vzájemné zpřístupňování sacharidů ve střevě je ukazatelem sociálního způsobu chování mezi bifidobakteriemi a lze tím pravděpodobně vysvětlit jejich dominanci v rané mikrobiotě (Turroni et al. 2018b; Luo et al. 2020).

Kolonizace konvenčních myší *in vivo* kojeneckými druhy *B. bifidum, B. adolescentis, B. breve* a poddruhem *B. longum* subsp. *infantis* obohatila střevní glykobiom myší v enzymatických schopnostech degradovat rostlinné a hostitelské sacharidy. *B. bifidum* se ve srovnání s *B. longum* subsp. *infantis* aktivně účastnil extracelulárního rozkladu hostitelských sacharidů a zpřístupňoval tak jednoduché cukry ostatním členům bifidobakteriální komunity (Turroni et

al. 2016). *B. bifidum* kooperuje také s *B. breve* v rámci cross-feedingových aktivit při utilizaci mucinu. Enzymy GH kmene *B. bifidum* PRL2010, zejména endo-α-*N*-acetylgalaktosaminidázy, lakto-*N*-biosidázy, exo-α-sialidázy a α-fukosidázy, které jsou zodpovědné za degradaci mucinu, zpřístupňují tento substrát také druhu *B. breve* (Turroni et al. 2010; Turroni et al. 2014a).

Dalším z příkladů cross-feedingu bifidobakterií s dalšími členy střevní mikrobioty je metabolizace L-fukózy druhy *B. longum* subsp. *infantis*, *B. longum* subsp. *suis* a *B. breve* na acetát, formiát, laktát a 1,2-propandiol (Bunesova et al. 2016; Schwab et al. 2017), který je dále metabolizován *Eubacterium hallii* za vzniku střevního propionátu (Engels et al. 2016). Cross-feeding byl například objeven i mezi mucin degradujícím druhem *B. bifidum* a 1,2-propandiol utilizujícím *E. hallii* (Bunesova et al. 2018), dále mezi HMOs degradujícím *B. longum* subsp. *infantis* a jeho metabolity utilizujícím *Anaerostipes caccae* (Chia et al. 2021), *B. adolescentis* a laktát utilizujícími a butyrát produkujícími *E. halii* a *A. caccae* (Belenguer et al. 2006), *B. adolescentis* a *Faecalibacterium prausnitzii* (Moens et al. 2016), *B. breve* a *Bacteroides cellulosilyticus* (Munoz et al. 2020).

2.1.5 Bifidobakterie jako probiotika

Přítomnost bifidobakterií v gastrointestinálním traktu hostitele je velmi důležitá, zejména v raných fázích života. Přispívá k zachování mikrobiální rovnováhy ve střevě, s čímž je spojeno udržení zdraví v pozdějších fázích života jedince (Rodríguez et al. 2015). Vzhledem k pozitivním vlastnostem bifidobakterií jsou některé kmeny komerčně používány jako probiotika. Probiotika jsou živé mikroorganismy, které pokud jsou podávány hostiteli v adekvátním množství, pozitivně ovlivňují jeho zdravotní stav (FAO/WHO 2001; Hill et al. 2014). Právě proto jsou za tímto účelem probiotika používána jako doplňky léčby specifických infekčních a zánětlivých onemocnění. Řada probiotických vlastností je nicméně druhově nebo kmenově specifická (Hill et al. 2014). Z důvodu běžné kmenové specifity, kdy různé kmeny stejného druhu mohou vykazovat různé účinky, je proto nezbytná charakterizace vlastností, účinků a také potvrzení jejich bezpečnosti vždy až na úroveň kmene (Jungersen et al. 2014).

Přestože mnoho probiotických kmenů bakterií bylo shledáno jako bezpečné, Evropský úřad pro bezpečnost potravin (EFSA; *European Food Safety Authority*) a Úřad pro kontrolu potravin a léčiv (FDA; *Food and Drug Administration*) nepřisuzují podávání probiotik schopnost předcházet nemoci nebo ji léčit. V mnoha zemích jsou tedy probiotika dostupná jako doplňky stravy, které podléhají jiným standardům výroby a kontroly kvality než schválená léčiva, a musí

tak dodržovat aktuální pravidla dané tržní politiky (Sanders et al. 2016; EFSA 2017; Plaza-Diaz et al. 2019).

Všechny mikroorganismy v probiotickém preparátů musí být vždy identifikovány až na kmenovou úroveň. Pro testování potenciálně probiotických kmenů jsou poté v první fázi obecně doporučovány *in vitro* testy simulující nepříznivé podmínky gastrointestinálního traktu hostitele. Příkladem je testování odolnosti kmene vůči žaludeční kyselosti a žlučovým kyselinám, antimikrobiální aktivity proti potenciálně patogenním mikroorganismům, schopnosti snížit adhezi patogenů, hodnocení hydrofobicity a schopnosti autoagregace. Druhou fázi by měla být preklinická validace na vhodných zvířecích modelech a jako poslední poté klinické studie na lidských subjektech. V rámci testování bezpečnosti by vždy měly být provedeny testy citlivosti na antibiotika a testování hemolytické aktivity (Ganguly et al. 2011; Somashekaraiah et al. 2019; Kim et al. 2020; Nath et al. 2020). Při podávání probiotik je také velmi důležitá vhodná dávka, hostitel a cílený efekt (Sanders 2008).

Bifidobakterie a laktobacily jsou nejčastěji dostupnými probiotiky uvedenými na trh s dlouhou historií použití. Původně pocházejí ze střev nebo tradičních fermentovaných potravin, jako je například jogurt, nakládaná zelenina a kefírová zrna (O'Toole et al. 2017). Zejména bifidobakterie jsou dominantně zastoupenými mikroorganismy ve střevě zdravých kojených novorozenců (Di Gioia et al. 2014), mají vysokou schopnost kolonizovat kojenecké střevo, jsou bezpečné, a i proto jsou široce používány jako probiotika s preventivním a terapeutickým účelem u novorozenců a kojenců (Sanders et al. 2010). Působí jako doplněk k prevenci komplikací a zlepšení celkového zdravotního stavu svého hostitele (Bozzi Cionci et al. 2018).

Světově a historicky nejdiskutovanější a v rámci potravinářství nejpoužívanější bifidobakterií je poddruh *B. animalis* subsp. *lactis* (Milani et al. 2013a; Jungersen et al. 2014). Jedním z nejčastěji aplikovaných kmenů do mléčných výrobků a probiotických suplementů je konkrétně kmen *B. animalis* subsp. *lactis* BB12, u kterého byly popsány probiotické vlastnosti se schopností stimulace imunitní funkce (Jungersen et al. 2014). *B. animalis* subsp. *lactis* AD011 je dalším z příkladů nově popsaného probiotického kmene bifidobakterie, který je bezpečný a vhodný při použití jako startovací kultura s probiotickými vlastnostmi (Ku et al. 2019). Přestože jsou bifidobakterie poměrně hojně zastoupeny v každodenně konzumovaných mléčných výrobcích (Raeisi et al. 2013), nelze nicméně předpokládat, že touto konzumací dojde k trvalému osídlení střeva. A to zejména z toho důvodu, že vzhledem k animálnímu původu běžně používaných kmenů bifidobakterií je pravděpodobný pouze jejich transientní průchod střevem hostitele (Sanders 2011; Xiao et al. 2021). U podávání bifidobakterií by tedy mělo být

vždy cíleno na výběr kmenů, které jsou typické pro daného hostitele (Maldonado-Gómez et al. 2016).

Podávání probiotických bifidobakterií je spojováno s řadou příznivých vlivů na zdraví člověka. Jedná se například o snížení výskytu a délky průjmů souvisejících s antibiotickou léčbou (de Vrese et al. 2011; Trallero et al. 2019), zmírnění zažívacích obtíží při syndromu dráždivého tračníku (O'Mahony et al. 2005; Whorwell et al. 2006), snížení výskytů ekzémů u kojenců (Kim et al. 2010), zmírnění průběhu a urychlení rekonvalescence při akutním infekčním průjmu u dětí (Di Gioia et al. 2014; Cruchet et al. 2015; Escribano et al. 2018) a o prevenci nekrotizující enterokolitidy u předčasně narozených dětí bez zvýšené incidence sepse (Underwood 2019; Zhu et al. 2019). Do budoucna je možná i probiotická intervence bifidobakteriemi v souvislosti s redukcí váhy u zdravých jedinců (Minami et al. 2018) a u jedinců s neurologickými problémy (Kobayashi et al. 2017; Tian et al. 2019).

Mikroorganismy, které indukují pozitivní účinky na neuropsychické funkce jsou definovány jako psychobiotika (Dinan et al. 2013). Tato definice byla rozšířena také o prebiotika, která zprostředkovávají tento bakteriální účinek (Sarkar et al. 2016). Obecně je interakce mezi mikroorganismy uskutečňována prostřednictvím uvolněných aminokyselin, peptidů, biogenních aminů a SCFAs, které jim slouží jako signální molekuly. Tyto látky jsou zároveň vlastní i pro hostitelský organismus, kde fungují jako neurochemikálie, hormony a cytokiny. Interakce mezi mikrobiotou a hostitelem je tedy obousměrná (Evrensel & Ceylan 2015; Oleskin & Shenderov 2019). Sekrecí neuroaktivních metabolitů psychobiotik, například serotoninem, katecholaminem, gamma-aminomáselnou kyselinou a acetylcholinem, může být indukována signalizace v enterálním nervovém systému, která následně může regulovat také funkce mozku a samotné chování hostitele (Wall et al. 2014). Příkladem psychobiotika podávaného štěňatům, kterým byl vyvolán chronický stres separací od matek, je kmen *B. pseudocatenulatum* CECT 7765. Jeho administrací u nich byla oslabena akutní stresová reakce a úzkost (Moya-Perez et al. 2017). Také kmen *B. breve* CCFM1025 signifikantně snížil úzkostné a depresivní stavy u myší (Tian et al. 2020). Dalším potenciálně psychobiotickým kmenem je *B. longum* subsp. *infantis* 35624, který u lidí se syndromem dráždivého tračníku normalizoval poměr interleukinů (O'Mahony et al. 2005). Stejný kmen navíc vykazoval antidepresivní aktivitu také u krys, která byla srovnatelná s podávaným antidepresivem (Desbonnet et al. 2008; Desbonnet et al. 2010). Podávání kmene *B. longum* 1714 zdravým lidem poté snížilo reakci na stres a mírně zlepšilo jejich kognitivní funkce (Allen et al. 2016).

Probiotické bifidobakterie nejsou podávány pouze lidským hostitelům. Probiotický potenciál s potvrzeným vlivem na snížení výskytu průjmů u selat byl potvrzen například u *B. animalis*

subsp. *lactis* (Shu et al. 2001), jehož probiotickou intervencí v kombinaci spolu s *B. longum* subsp. *infantis* byla snížena zátěž způsobená expozicí patogenu *Salmonella* Typhimurium (Barba-Vidal et al. 2017). *B. animalis* subsp. *animalis, B. thermophilum, B. longum* subsp. *suis* a *B. choerinum* jsou poté příkladem potenciálně probiotických druhů/poddruhů bifidobakterií pro telata (Bunešová et al. 2012a; Geigerová et al. 2016), zatímco *B. breve* a *B. longum* subsp. *infantis* pro brojlery (Santini et al. 2010; El-Sharkawy et al. 2020) a *Bifidobacterium animalis* pro psy (Kelley et al. 2009; O'Mahony et al. 2009).

Pro kategorizaci mezi probiotika je nezbytné, aby daný mikroorganismus byl životaschopný. V případě již odumřelých mikrobiálních buněk, jejich fragmentů a metabolitů zahrnujících jejich signální molekuly jsou tyto struktury a látky definovány jako postbiotika (Aguilar-Toalá et al. 2018), někdy označována jako metabiotika (Oleskin & Shenderov 2019). Existuje i dílčí detailnější rozdělení těchto látek na postbiotika a parabiotika. V rámci tohoto dělení je do postbiotik řazena komplexní směs metabolitů sekretovaných probiotiky v bezbuněčných supernatantech (enzymy, proteiny, SCFAs, vitamíny, biosurfaktanty, aminokyseliny, peptidy a organické kyseliny), zatímco do parabiotik jsou zahrnuty inaktivované buňky probiotik, ať už intaktní, nebo narušené, obsahující buněčné komponenty (peptidoglykan, teichové kyseliny a povrchové proteiny) (Martín & Langella 2019; Nataraj et al. 2020). Výhodou výše zmíněných látek je znalost jejich chemické struktury a dostupnosti v čisté formě, také snadnost výroby a skladování, bezpečnost, cílenost a možnost stanovení dávky. Při podání hostiteli mají navíc potenciál optimalizovat regulační, metabolické a behaviorální reakce organismu související s aktivitou přítomné mikrobioty. Zároveň mají potenciál se vyrovnat probiotikům první generace (Nataraj et al. 2020; Shenderov et al. 2020). Příkladem možnosti cílené produkce postbiotik v praxi je fermentace syrovátky kmenem *B. animalis* subsp. *lactis* BB12 s cílenou produkcí potenciálně postbiotické konjugované linolové kyseliny, exopolysacharidů a bakteriocinů (Amiri et al. 2021). Dalším příkladem postbiotika je lipoteichová kyselina vyprodukovaná kmenem *B. animalis* subsp. *lactis* BPL1, která má potenciál v redukci ukládání tuku (Balaguer et al. 2021), nebo například také různé frakce buněk *B. bifidum* BGN4, které mají pravděpodobně imunoregulační schopnosti (Lee et al. 2002).

2.1.6 Význam bifidobakterií ve střevní mikrobiotě

Mikroorganismy jsou všudypřítomné. Lidské tělo, stejně jako těla zvířat, je obýváno velkým množstvím mikrobiálních druhů, které mohou se svým hostitelem koexistovat po celou dobu jeho života (Milani et al. 2017a). Většina mikroorganismů žije v komplexních komunitách tvořících mikrobiotu, která je složena z bakterií, archaea, mikroskopických hub a jejich virů

a fágů. Mikrobiota střevního traktu není výjimkou a tvoří hojný ekosystém, ve kterém dominují především bakterie (Rajilić-Stojanović & de Vos 2014). Je důležitým environmentálním faktorem pro lidské zdraví (Clemente et al. 2012), který má evolučně zachované role v metabolismu, imunitě, vývoji a chování hostitele (Cabreiro & Gems 2013; Erkosar et al. 2013). Způsob porodu a výživy mají významný vliv na složení a její funkci (Bäckhed et al. 2015).

Od narození se střevní mikrobiota dynamicky vyvíjí spolu se svým hostitelem, a proto má její rozvoj zásadní význam pro zdraví v pozdějším životě (Maynard et al. 2012; Gensollen et al. 2016). Je regulována komplexní souhrou mezi hostitelem a faktory prostředí zahrnujícími dietu a životní styl (Rothschild et al. 2018). Právě dostupnost substrátů v dietě během prvního roku života je stěžejním faktorem při vývoji střevní mikrobioty (Derrien et al. 2019). Stewart et al. (2018) rozdělují vývoj mikrobiomu dětí do věku 46 měsíců do tří fází: vývojová (3–14 měsíců), přechodná (15–30 měsíců) a stabilní (≥31 měsíců). Během první fáze dochází k postupné změně α-diverzity a k detekci nejvíce zastoupených kmenů Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria a Verrucomicrobia s dominancí bifidobakterií. Při přechodné fázi dochází k dalšímu rozvoji hlavně Bacteroidetes a Proteobacteria spolu s přetrvávající postupnou změnou α-diverzity. Alfa-diverzita a přítomné kmeny pak zůstávají nezměněné ve stabilní fázi, ve které je zároveň charakteristická vyšší bakteriální diverzita a predominance Firmicutes. Další studie nicméně naznačují, že k úplnému ustálení střevní mikrobioty může docházet i o mnohem déle (Hollister et al. 2015; Cheng et al. 2016), což by mohlo být zohledněno a dále využito při cílených mikrobiálních intervencí pro podporu zdraví a prevenci nežádoucího vývoje střevního mikrobiomu i u starších jedinců (Derrien et al. 2019).

Střevní mikrobiota dospělých je komplexní, relativně stabilní a velmi specifická pro každého jednotlivce (Claesson et al. 2011; Faith et al. 2013). Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria a Verrucomicrobia jsou jejími dominantními kmeny, kdy Firmicutes a Bacteroidetes tvoří až 90 % (Arumugam et al. 2011; Ringel-Kulka et al. 2013; Rinninella et al. 2019). Struktura střevní mikrobioty kojenců je naopak obecně nestabilní a vysoce dynamická s nízkou diverzitou jednotlivých druhů (Arrieta et al. 2014) se signifikantní dominancí kmene Actinobacteria, konkrétně rodu *Bifidobacterium* (Turroni et al. 2012a).

Bifidobakterie jsou dominantní mikrobiální skupinou mikrobioty zdravých kojenců a mláďat (Milani et al. 2016; O'Callaghan & van Sinderen 2016; Turroni et al. 2018b). Jejich zastoupení postupně klesá až do dospělosti, kde představují kolem 5 % typické střevní mikrobioty (Arumugam et al. 2011), která je relativně stabilní (Arboleya et al. 2016; Derrien et al. 2019). K tendenci jejich snižování dochází až při stárnutí organismu (Salazar et al. 2013). Zároveň

také druhové zastoupení bifidobakterií se mění v průběhu života (Arboleya et al. 2016). Druhy *B. longum*, *B. breve*, *B. bifidum* a druhy skupiny *B. catenulatum* jsou prvními bifidobakteriálními kolonizátory střeva, které jsou následovány *B. dentium* a *B. adolescentis.* Okolo třetího roku života je v mikrobiotě kojenců detekován i druh *B. angulatum* (Nagpal et al. 2017). Distribuce bifidobakterií přítomných v tlustém střevě hostitelů různého věku zdůrazňuje variabilitu mezi jedinci, nicméně také ale vysokou stabilitu v různých místech tlustého střeva u jedince stejného. Toto odpovídá variabilitě střevní mikrobioty na intraindividuální a interindividuální úrovni (Eckburg et al. 2005; Turroni et al. 2009).

Vzhledem k relativně nízkému zastoupení bifidobakterií v tlustém střevě dospělého jedince je zajímavé, že i malé snížení jejich počtu je spojeno s řadou nežádoucích stavů organismu, jako jsou například zánětlivá střevní onemocnění (Prosberg et al. 2016), obezita (Nakayama et al. 2015; Christensen et al. 2018) a autismus (Wang et al. 2011). Jejich přítomnost ve střevě je naopak spojována s produkcí mnoha metabolitů, jako jsou vitamíny (LeBlanc et al. 2013), antioxidanty (Gagnon et al. 2015), konjugovaná linolová kyselina (Gorissen et al. 2012), bakteriociny (Martinez et al. 2013) a SCFAs (Rivière et al. 2016). Zároveň se bifidobakterie, vzhledem ke své enzymatické výbavě, významně podílejí na rozšíření střevního glykobiomu a účastní se aktivně na rozkladu nestravitelných polysacharidů (Milani et al. 2015a). Většina těchto funkcí je nicméně druhově nebo kmenově specifická (Rivière et al. 2016).

Bifidobakterie mají geny, které kódují syntézu vitamínů thiaminu (B1), riboflavinu (B2), niacinu (B3), pyridoxinu (B6), listové kyseliny (B11) a neméně důležitých 19 různých aminokyselin (Ventura et al. 2009; Cronin et al. 2011). Kmenově specifickou schopností je dále také syntéza folátu (B9), která je spojována s adaptací bifidobakterií na dietu hostitele (Pompei et al. 2007; D'Aimmo et al. 2014). Bakteriocin bifidocin A poté působí antimikrobiálně proti řadě patogenů, jako je například *Listeria monocytogenes, Staphylococcus aureus* a *Escherichia coli* (Liu et al. 2015a). Produkcí acetátu spolu s laktátem bifidobakterie navíc přispívají k udržení střevní homeostáze, snižování luminálního pH a tyto substráty poté dále poskytují k přeměně na butyrát jinými bakteriemi tlustého střeva (Rivière et al. 2016). Bifidobakteriální acetát navíc chrání organismus hostitele před infekcemi způsobené enterohemoragickými kmeny *E. coli* (Fukuda et al. 2011).

Obecně je přítomnost bifidobakterií v pozitivní korelaci s podporou zdraví svého hostitele. Bifidobakterie totiž zabraňují adhezi nežádoucích mikroorganismů na sliznici střeva a jsou schopny vyvazovat železo na úkor střevních patogenů (Vazquez-Gutierrez et al. 2016), čímž podporují správnou funkci epiteliální bariéry střeva (Martín et al. 2016; Westermann et al. 2016). Bifidobakterie dále produkují také exopolysacharidy modulující imunitní odpověď

hostitele (Schiavi et al. 2016; Ruiz et al. 2017), podílejí se na stabilizaci přirozené mikrobioty (Binda et al. 2018) a snižují riziko vzniku rotavirových průjmů (Moreno Muñoz et al. 2011). Neméně důležitou vlastností některých kmenů bifidobakterií je schopnost produkovat extracelulární struktury, které ovlivňující nejen proces kolonizace gastrointestinálního traktu. Biosyntetizované exopolysacharidy zvyšují odolnost bifidobakterií vůči žaludečním šťávám a žlučovým kyselinám a zlepšují tak jejich perzistenci ve střevě hostitele (Hidalgo-Cantabrana et al. 2014a; Hidalgo-Cantabrana et al. 2014b). Vláskové přívěsky pili a fimbrie, spolu s extracelulární exopolysacharidovou vrstvou na povrchu kapsuly, se podílejí na adhezi buněk bifidobakterií na střevní mukózu a zároveň zprostředkovávají interakce mezi mikroorganismy, jako je například agregace, a podílejí se také na imunomodulaci (Filloux 2010; Foroni et al. 2011; Turroni et al. 2013; Turroni et al. 2014b; Milani et al. 2017c).

2.2 Metody identifikace a charakterizace bifidobakterií

Přestože již bylo objeveno skoro 90 druhů bifidobakterií, jsou v současné době stále ještě izolována a identifikována velká množství bifidobakteriálních izolátů z řady ekologických stanovišť s velkým potenciálem popisu nového druhu. Pro popis nových druhů bifidobakterií je nezbytná jejich fenotypová a genotypová charakterizace. Je velmi důležitá také pro identifikaci nových probiotických kmenů, u kterých musí být potvrzena jejich identita a vztah s již dříve platně popsanými druhy (Mattarelli & Biavati 2018). Mattarelli et al. (2014) uvádí soubor pokynů nezbytných pro popis nových druhů bifidobakterií, při kterém je důležité dodržení polyfázové charakterizace. Tyto pokyny byly schváleny *Subcommittee on the Taxonomy of Bifidobacterium, Lactobacillus and related organisms of the International Committee on the Systematics of Prokaryotes* a byly publikovány v časopise *International Journal of Systematic and Evolutionary Microbiology*.

2.2.1 Fenotypová charakterizace

Fenotypová charakterizace zahrnuje popis morfologických, fyziologických, biochemických a nutričních vlastností daného bakteriálního kmene. Obvykle se jedná o popisné mikroskopické charakteristiky jako je popis tvaru, uspořádání buněk, jejich schopnosti motility, Gramovy reakce a schopnosti tvořit endospory spolu s makroskopickým popisem morfologie kolonií. Součástí charakterizace je dále stanovení fermentačních profilů běžně dostupných substrátů a stanovení hlavních produktů metabolismu s jejich konečnými poměry při fermentaci glukózy. Při popisu musí být dále uvedeno také vhodné růstové médium a fyziologické vlastnosti daného kmene, včetně jeho vztahu ke kyslíku pro zajištění optimálních kultivačních podmínek,

a detekce přítomnosti enzymu katalázy. Doplňující charakteristikou může být testování citlivosti k antibiotikům. Součástí charakterizace je také uvedení informace o zdroji a habitatu daného kmene (Mattarelli et al. 2014). Charakterizace těchto znaků může být dále rozšířena o chemotaxonomický popis složení buňky, jako je například analýza mastných kyselin, polárních lipidů a aminokyselin peptidoglykanu buněčné stěny (Tindall et al. 2010).

Vzhledem k možnosti ovlivnění fenotypu kultivačními a testovacími podmínkami během analýzy je doporučeno zahrnutí také typových kmenů příslušných referenčních taxonů. Nezbytné je to zvláště u kmenů, které jsou si blízce příbuzné na základě sekvence genu 16S rRNA a u kterých musí být prokázané rozdílné fenotypové charakteristiky, aby daný nový druh bylo možné odlišit (Mattarelli et al. 2014).

2.2.2 Genotypová charakterizace

Genotypové metody pro identifikaci mikroorganismů jsou založeny na složení nukleových kyselin a nejsou tedy ovlivňovány prostředím. Některé geny, které jsou zakódovány v genomu bakterií, totiž nemusí být exprimovány, a tudíž výsledky fenotypové charakterizace mohou být leckdy značně variabilní (Mattarelli et al. 2014).

Procentuální obsah guaninu a cytosinu jsou základní informací o složení DNA bází (Mattarelli et al. 2014). První fází genotypové charakterizace je většinou sekvenace genu 16S rRNA v minimální délce 1500 párů bází (Mattarelli & Biavati 2018). Obecně je gen 16S rRNA nejpoužívanějším molekulárním markerem pro stanovení fylogenetických vztahů bakterií, jehož sekvence jsou dostupné u většiny typových kmenů a může tak být používán k identifikaci většiny izolátů (Mattarelli et al. 2014). Mezní hodnota pro odlišení dvou druhů byla stanovena na 98,65 % (Kim et al. 2014). V případě shody genu 16S rRNA u dvou srovnávaných kmenů z více než 97 % je nezbytné provedení DDH. Aby totiž mohly být kmeny v rámci taxonu považovány za jeden druh, je nutné potvrdit jejich dostatečný stupeň podobnosti (Tindall et al. 2010).

Pokud jsou tyto metody nedostačující k druhové diskriminaci, je nezbytné osekvenování také komplementárních fylogenetických markerů. Protein kódující geny (*Housekeeping Genes*) a nekódované regiony jsou mnohem variabilnější a umožňují proto vyšší stupeň rozlišení (Santos & Ochman 2004). Vždy by měl být také osekvenován *hsp*60 a alespoň jeden z *clp*C, *rpo*B, *rpo*C, *dna*K nebo *dna*G genů (Mattarelli & Biavati 2018). Při popisu nového druhu bakteriálního kmene je vždy nezbytné uložení těchto sekvencí do veřejně dostupné databáze (Mattarelli et al. 2014). Dalšími aplikovatelnými genotypovými metodami jsou například *Multilocus Sequence Typing* (MLST), *Multiple Locus Variable Number of Tandem Repeats*

Analysis (MLVA), profilování plazmidů a genomické fingerprintování (Mattarelli et al. 2014). Velmi významnou metodou je poté celogenomové sekvenování (Mattarelli & Biavati 2018). Vzhledem ke snížení ceny a dostupnosti této metody, má srovnávání podobnosti genetických sekvencí potenciál stát se rutinním taxonomickým nástrojem (Kim et al. 2014). Jednou z nejpoužívanějších zavedených metod pro srovnání dvou genomů je stanovení průměrné nukleotidové identity (ANI; *Average Nucleotide Identity*), která je používaná jako další nástroj pro odlišení bakteriálních druhů (Konstantinidis & Tiedje 2005), a to v případě hodnot ANI 95–96 %, které odpovídají 70 % DDH (Goris et al. 2007; Richter & Rosselló-Móra 2009).

2.2.3 MALDI-TOF hmotnostní spektrometrie

Hmotnostní spektrometrie s laserovou desorpcí a ionizací za účasti matrice s průletovým analyzátorem (MALDI-TOF MS; *Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry*) je rutinně používanou analytickou metodou pro identifikaci bakterií v klinické diagnostice (Croxatto et al. 2012; Hou et al. 2019), potravinářském průmyslu (Wenning et al. 2014) a environmentální mikrobiologii (Santos et al. 2016). Je také běžně používána v některých sbírkách mikroorganismů pro taxonomickou kontrolu (Mattarelli et al. 2014). Umožňuje identifikaci s diskriminační sílou běžně až na úroveň druhu (Zeller-Péronnet et al. 2013; Yang et al. 2018). MALDI-TOF MS má navíc potenciál být funkční náhradou za rutinní sekvenování genu 16S rRNA pro identifikaci anaerobních mikroorganismů (Bizzini et al. 2011). Přes velkou počáteční přístrojovou investici je metoda MALDI-TOF MS při identifikaci mikroorganismů velmi rychlá, přesná a nákladově efektivnější než konvenční fenotypové a molekulární techniky (Pavlovic et al. 2013). Novou perspektivou v MALDI-TOF MS mikrobiální identifikaci je navíc také typizace mikroorganismů až na úroveň poddruhu a kmene (Florio et al. 2018), detekce antimikrobiální rezistence (Oviaño & Bou 2018) a generování MS profilů k hodnocení bakteriálních funkčních znaků (Clark et al. 2018).

Principem MALDI-TOF MS identifikace mikroorganismů je analýza malých molekul proteinů pocházejících z buněčných povrchů, intracelulárních membrán a ribozomů (Giebel et al. 2010). Jedná se o měkkou ionizační techniku, kdy je analyt zabudován do krystalů matrixu (slabá organická kyselina s nízkou molekulovou hmotností). Následnou ionizací laserem poté dochází k vaporizaci sloučeniny matrice s analytem. Tímto způsobem jsou generovány jednotlivé nabité ionty (Karas et al. 2000; Lewis et al. 2000), které jsou následně zrychleny v elektrickém poli při průletu letovou trubicí. Hmotností analyzátor poté měří čas potřebný k pohybu iontu od zdroje k detektoru (Doroshenko & Cotter 1999). K separaci iontů dochází na základě jejich poměru molekulové hmotnosti a náboje (m/z). Tento proces je mnohokrát opakován a vede

k řadě molekulárních detekcí, jejichž vizualizací je hmotnostní spektrum daného analytu. Naměřená spektra jsou poté skórovacím algoritmem srovnávána s referenčními spektry přítomnými v databázi (Jang & Kim 2018; Yang et al. 2018). Jejich diferenciace je založena na přítomnosti nebo nepřítomnosti píků a jejich intenzity (Spitaels et al. 2016).

Přestože jsou MALDI-TOF MS referenční databáze velmi komplexní, vyžadují pravidelnou aktualizaci neustále se měnící mikrobiální taxonomie a také přidávání nových druhů popsaných mikroorganismů (Welker et al. 2019).

3 Vědecké hypotézy a cíle práce

3.1 Hypotézy

Bifidobakterie jsou sacharolytické bakterie, které disponují celou řadou genů pro utilizaci nejrůznějších komplexních substrátů. Přítomnost těchto genů je nicméně často druhově a kmenově specifická a jejich exprese je podmíněna dietou a prostředím výskytu hostitele. Hostitelská strava a evoluce ovlivňují bifidobakteriální rozmanitost, která se zvyšuje od masožravců, všežravců až po býložravce. Monitoring jejich výskytu u různých druhů živočichů je předpokladem pro popis nových druhů rodu *Bifidobacterium* a bakterií z čeledi *Bifidobacteriaceae*, jehož nezbytnou součástí je genotypová a fenotypová charakterizace. Předpokládáme, že bifidobakterie jsou schopny se adaptovat a obohatit své funkční vlastnosti, a to zejména v případě hostitelských a dietetických změn, nebo dysbiózy, a tudíž izoláty multi-hostitelských druhů mohou vykazovat značnou fenotypovou a genotypovou variabilitu.

3.2 Cíle

Hlavním cílem disertační práce je poukázat na genetické a funkční vlastnosti bifidobakterií pomocí genomických a ekologicky podložených informací se zaměřením především na jejich adaptaci a speciální funkční vlastnosti. Prvotním cílem této práce je monitoring výskytu bifidobakterií u různých živočišných druhů zahrnujících především člověka, primáty, psy a jedince s dysbiózou, jako je Crohnova choroba, a popis nových druhů rodu *Bifidobacterium* a bakterií z čeledi *Bifidobacteriaceae*. Dalším cílem je fenotypová a genotypová charakterizace izolovaných bifidobakterií a na ní navazující detekce kmenově specifických funkčních vlastností/genů odrážejících jejich možnou adaptaci na hostitelské prostředí a selekce nových prebiotických substrátů vycházejících z přirozených složek diety daných hostitelů se zaměřením na selektivitu pro rod *Bifidobacterium* a další probiotické komenzální mikroorganismy.

4 Publikované práce

Tato disertační práce předkládána formou souboru vědeckých článků vznikla na základě níže uvedených sedmi publikovaných prací v časopisech databáze Web of Knowledge s Impact Factor indexem a jedné publikované práce v časopise databáze Scopus.

4.1 Neuzil-Bunesova V, Lugli GA, **Modrackova N**, Vlkova E, Bolechova P, Burtscher J, Longhi G, Mancabelli L, Killer J, Domig K, Ventura M. 2020b. Five novel bifidobacterial species isolated from faeces of primates in two Czech zoos: *Bifidobacterium erythrocebi* sp. nov., *Bifidobacterium moraviense* sp. nov., *Bifidobacterium oedipodis* sp. nov., *Bifidobacterium olomucense* sp. nov. and *Bifidobacterium panos* sp. nov. International Journal of Systematic and Evolutionary Microbiology **71**.

4.2 Neuzil-Bunesova V, Lugli GA, **Modrackova N**, Makovska M, Mrazek J, Mekadim C, Musilova S, Svobodova I, Spanek R, Ventura M. 2020a. *Bifidobacterium canis* sp. nov., a novel member of the *Bifidobacterium pseudolongum* phylogenetic group isolated from faeces of a dog (*Canis lupus* f. *familiaris*). International Journal of Systematic and Evolutionary Microbiology **70**:5040-5047.

4.3 **Modrackova N**, Stovicek A, Burtscher J, Bolechova P, Killer J, Domig KJ, Neuzil-Bunesova V. 2021b. The bifidobacterial distribution in the microbiome of captive primates reflects parvorder and feed specialization of the host. Scientific Reports **11**.

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4.8 **Modrackova N**, Copova I, Stovicek A, Makovska M, Schierova D, Mrazek J, Sabolova M, Vlkova E, Hradsky O, Bronsky J, Nevoral J, Neuzil-Bunesova V.. 2021a. Microbial shifts of faecal microbiota using enteral nutrition *in vitro*. Journal of Functional Foods **77**:104330.

4.1 Five novel bifidobacterial species isolated from faeces of primates in two Czech zoos: *Bifidobacterium erythrocebi* sp. nov., *Bifidobacterium moraviense* sp. nov., *Bifidobacterium oedipodis* sp. nov., *Bifidobacterium olomucense* sp. nov. and *Bifidobacterium panos* sp. nov.

Neuzil-Bunesova V, Lugli GA, **Modrackova N**, Vlkova E, Bolechova P, Burtscher J, Longhi G, Mancabelli L, Killer J, Domig K, Ventura M. 2020b. Five novel bifidobacterial species isolated from faeces of primates in two Czech zoos: *Bifidobacterium erythrocebi* sp. nov., *Bifidobacterium moraviense* sp. nov., *Bifidobacterium oedipodis* sp. nov., *Bifidobacterium olomucense* sp. nov. and *Bifidobacterium panos* sp. nov. International Journal of Systematic and Evolutionary Microbiology **71**.

INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Neuzil-Bunesova *et al.*, *Int. J. Syst. Evol. Microbiol.*

DOI 10.1099/ijsem.0.004573

MICROBIOLOGY SOCIETY

Five novel bifidobacterial species isolated from faeces of primates in two Czech zoos: *Bifidobacterium erythrocebi* sp. nov., *Bifidobacterium moraviense* sp. nov., *Bifidobacterium oedipodis* sp. nov., *Bifidobacterium olomucense* sp. nov. and *Bifidobacterium panos* sp. nov.

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Abstract

Five *Bifidobacterium* strains, VB23^T, VB24^T, VB25^T, VB26^T and VB31^T, were isolated from chimpanzee (*Pan troglodytes*), cotton-top tamarin (*Saguinus oedipus*), Goeldi's marmoset (*Callimico goeldii*), moustached tamarin (*Saguinus mystax*) and patas monkey (*Erythrocebus patas*), respectively, which were kept in two Czech zoos. These strains were isolated from faecal samples and were Gram-positive, non-motile, non-sporulating, anaerobic and fructose-6-phosphate phosphoketolase-positive. Phylogenetic analyses based on 16S rRNA revealed close relatedness between VB23^T and *Bifidobacterium angulatum* LMG 11039^T (96.0%), VB24^T and *Bifidobacterium pullorum* subsp. *pullorum* DSM 20433^T (96.1%), VB25^T and *Bifidobacterium goeldii* LMG 30939^T (96.5%), VB26^T and *Bifidobacterium imperatoris* LMG 30297^T (98.1%), and VB31^T and *B. angulatum* LMG 11039^T (99.40%). Internal transcribed spacer profiling revealed that VB23^T, VB24^T, VB25^T, VB26^T and VB31^T had highest similarity to *Bifidobacterium breve* LMG 13208^T (77.2%), *Bifidobacterium longum* subsp. *infantis* ATCC 15697^T (85.8%), *Bifidobacterium biavatii* DSM 23969^T (76.9%), *B. breve* LMG 13208^T (81.2%) and *B. angulatum* LMG 11039^T (88.2%), respectively. Average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) analyses with their closest neighbours supported the independent phylogenetic positions of the strains with values between 86.3 and 94.3% for ANI and 25.8 and 54.9% for dDDH. These genomic and phylogenetic analyses suggested that the evaluated strains were novel *Bifidobacterium* species named *Bifidobacterium erythrocebi* sp. nov. (VB31^T=DSM 109960^T=CCUG 73843^T), *Bifidobacterium moraviense* sp. nov. (VB25^T=DSM 109958^T=CCUG 73842^T), *Bifidobacterium oedipodis* sp. nov. (VB24^T=DSM 109957^T=CCUG 73932^T), *Bifidobacterium olomucense* sp. nov. (VB26^T=DSM 109959^T=CCUG 73845^T) and *Bifidobacterium panos* sp. nov. (VB23^T=DSM 109963^T=CCUG 73840^T).

Bifidobacterium (*B.*) is a genus of Gram-positive bacteria belonging to the phylum Actinobacteria, family *Bifidobacteriaceae* [1]. Bifidobacterial colonization is found in the gastrointestinal tracts of mammals, poultry and social insects [2, 3]. Many newly described bifidobacterial species are associated with the gastrointestinal tract of non-human primates and

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Keywords: *Bifidobacterium*; captivity; FAMEs; monkeys; phylogenomics; taxonomy.

Abbreviations: ANI, average nucleotide identity; COG, cluster of orthologous group; dDDH, digital DNA–DNA hybridization; FAME, fatty acid methyl ester; F6PPK, fructose-6-phosphate phosphoketolase; ITS, internal transcribed spacer; ORF, open reading frame; WSP, supplemented Wilkins–Chalgren medium.

DDBJ/ENA/GenBank under accession numbers (in strain order VB23^T, VB24^T, VB25^T, VB26^T and VB31^T, respectively): JAAIIJ000000000, JAAIII000000000, JAAIIH000000000, JAAIIG000000000 and JAAIIF000000000. 16S rRNA gene sequences under accession numbers: MN707963, MN707964, MN707965, MN707966 and MN707967. ITS gene sequences under accession numbers: MT859136, MT859137, MT859138, MT859139 and MT859140. Genome sequencing reads under accession numbers: SAMN13944611, SAMN13944612, SAMN13944613, SAMN13944614 and SAMN13944615.

Two supplementary tables and two supplementary figures are available with the online version of this article.

most of these species have been isolated from New World monkeys, such as marmosets and tamarins [4–12]. Despite efforts to isolate novel species from Old World monkeys, *B. moukalabense* is the only such newly identified bacterial species, isolated from the faeces of wild western lowland gorilla [13]. Multi-host species described in chimpanzees, the closest relatives of humans, include *B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum* and *B. dentium*, which are also found in human stools [14–16]. In addition, *B. pseudolongum* has been described in the faeces of captive chimpanzees [14]. Moreover, *B. angulatum* and *B. pseudocatenulatum* were isolated from faecal samples of patas and Diana monkeys [16].

In the current study, we describe the identification of novel bifidobacterial species by 16S rRNA gene and internal transcribed spacer (ITS) profiling, followed by genomic comparison based on whole-genome sequencing. Genomic and phylogenetic analyses allowed for the identification of two proposed novel strains from Old Word monkeys and three from New World monkeys.

Faecal samples of primates belonging to various New World and Old World species, kept in two Czech zoos, were analysed for the presence of bifidobacterial species on Wilkins–Chalgren anaerobe agar supplemented with GMO-free soya peptone (5 g l^{-1}), L-cysteine hydrochloride (0.5 g l^{-1}), Tween 80 (1 ml l^{-1}), acetic acid (1 ml l^{-1}), mupirocin (100 mg l^{-1}) and norfloxacin (200 mg l^{-1}), following the methodology described by Vlkova *et al.* [17]. Bifidobacterial counts on selective media were determined to be 8.30, 7.58, 8.74, 9.56 and 7.93 log c.f.u. g^{-1} of faeces for the samples from chimpanzee, cotton-top tamarin, Goeldi's marmoset, moustached tamarin and patas monkey, respectively. Colonies of variable cultivation characteristics were isolated. The isolated strains were routinely cultured in Wilkins–Chalgren agar/broth supplemented with GMO-free soya peptone (5 g l^{-1}), L-cysteine hydrochloride (0.5 g l^{-1}) and Tween 80 (1 ml l^{-1}), used as WSP agar/broth, in an oxygen-free carbon dioxide environment at 37 °C for 24 h. Purity and morphology were routinely monitored by phase-contrast microscopy. Stock cultures were stored at −80 °C in 30% glycerol. Isolates were identified to the genus level based on their fructose-6-phosphate phosphoketolase (F6PPK) activity [18]. Genomic DNA of the strains were extracted using the Fast DNA Spin Kit for soil (MP Biomedicals) according to the protocol for pure culture, which includes a bead-beating step following the supplier's instructions. The partial 16S rRNA genes of F6PPK-positive isolates were amplified and sequenced from both directions using the primers Bif285 (5′-GAGGGTTCGATTCTGGCTCAG-3′) and Bif261 (5′-AAGGAGGTGATCCAGCCGCA-3′) [19]. The obtained sequences were entered into the EzBioCloud database [20] to obtain the closest related taxa. Strains with identity lower than 98.65% [21] or interesting multispecies bifidobacterial strains were selected for future identification. The selected strains were used for molecular typing by means of ITS bifidobacterial profiling using previously described primer sequences [22]. Using BLAST, the ITS sequences were compared to a public database containing bifidobacterial ITS sequences (http://probiogenomics.unipr.it/pbi/). This analysis allowed the identification of five isolates with ITS identity values <90% compared to other bifidobacterial type strain sequences, namely VB23^T, VB24^T, VB25^T, VB26^T and VB31^T, representing possibly putative novel *Bifidobacterium* species. The 16S rRNA gene and ITS sequences were deposited in the NCBI database using the Banklt application (www.ncbi.nlm.nih.gov/WebSub/?tool=genbank). Subsequently, genome sequencing was carried out for the selected strains. The chromosome sequences of these strains were decoded using a MiSeq platform (Illumina) at GenProbio srl (Parma, Italy) according to a previously described protocol [3]. In detail, a genomic library was generated using the Nextera XT DNA Library Prep Kit and loaded into a 600-cycle flow cell version 3 (Illumina). The generated reads were subjected to trimming of adapter sequences, then quality filtered and assembled through the MEGAnnotator pipeline [23]. In specific, sequences were filtered and trimmed using the command-line fastq-mcf software with the option -w 5 -q 20 l 100 (https://expressionanalysis.github.io/ea-utils/). Then, the SPAdes program (version 3.13.0) was used for *de novo* assembly of genome sequences with the pipeline option '--careful' and a list of k-mer sizes of 21, 33, 55, 77, 99, 127 [24]. Contigs were then employed by MEGAnnotator for the prediction of protein-encoding open reading frames (ORFs) using Prodigal [25]. Predicted ORFs were functionally annotated by means of RAPSearch2 (Reduced Alphabet based Protein similarity Search; cutoff *E*-value, 1×10^{-5}; minimum alignment length, 20 amino acids) performed against the NCBI nr database [26] coupled with hidden Markov model profile searches (http://hmmer.org/) performed against the manually curated Pfam-A database (cutoff E-value, 1×10^{-10}). Furthermore, tRNA genes were identified using tRNAscan-SE version 1.4 [27], while rRNA genes were detected using RNAmmer version 1.2 [28].

The highest 16S rRNA sequence identity values for VB23^T, VB24^T, VB25^T, VB26^T and VB31^T were 96, 96.1, 96.5, 98.1 and 99.4% with *B. angulatum* LMG 11039^T, *B. pullorum* subsp. *pullorum* DSM 20433^T, *B. goeldii* LMG 30939^T, *B. imperatoris* LMG 30297^T and *B. angulatum* LMG 11039^T, respectively. ITS bifidobacterial profiling (in the same order) revealed highest similarities with *B. breve* LMG 13208^T (77.2%), *B. longum* subsp. *infantis* ATCC 15697^T (85.8%), *B. biavatii* DSM 23969^T (76.9%), *B. breve* LMG 13208^T (81.2%) and *B. angulatum* LMG 11039^T (88.2%), respectively.

In addition, genetic similarities between VB23^T, VB24^T, VB25^T, VB26^T and VB31^T isolates and other currently recognised bifidobacterial (sub)species were evaluated on the basis of average nucleotide identity (ANI) analysis using the program JSpecies version 1.2.1, which is based on MUMmer (ANIm) [29]. This analysis highlighted bifidobacterial type strains having the highest ANI value with respect to genome sequences of the novel isolates, i.e. genome pairs VB23^T/*B. dentium* LMG 11045^T (86.3%), VB24^T/*B. rousetti* DSM 106027^T (86.5%), VB25^T/*B. avesanii* DSM 100685^T (88.6%), VB26^T/*B. scaligerum* DSM 103140^T (94.3%) and VB31^T/*B. angulatum* JCM 7096^T (88.9%) (Tables 1 and S1, available in the online version of this article). In this context, it is worth mentioning that the two (bifido)bacterial strains displaying

Table 1. General genetic features

	VB23^T	VB24^T	VB25^T	VB26^T	VB31^T
Bifidobacterium sp. nov.	*B. panos*	*B. oedipodis*	*B. moraviense*	*B. olomucense*	*B. erythrocebi*
Biological origin	*Pan troglodytes*	*Saguinus oedipus*	*Callimico goeldii*	*Saguinus mystax*	*Erythrocebus patas*
Sequencing coverage (×)	60	79	90	79	203
Number of assembled contigs	44	14	39	38	23
Genome length	1886785	2990182	2563591	2977969	2293450
Average G+C content (mol%)	59.95	57.74	67.49	58.94	61.22
Number of predicted ORFs	1465	2449	1955	2372	1787
tRNA	38	51	58	61	52
rRNA*	1	3	3	4	3
ANI value	86.3% *B. dentium* LMG 11045^T	86.5% *B. rousetti* DSM 106027^T	88.6% *B. avesanii* DSM 100685^T	94.3% *B. scaligerum* DSM 103140^T	88.9% *B. angulatum* JCM 7096^T
dDDH value	25.8%	26.3%	29.4%	54.9%	31.7%
ITS identity	77.2% *B. breve* LMG 13208^T	85.8% *B. longum* subsp. *infantis* ATCC 15697^T	76.9% *B. biavatii* DSM 23969^T	81.2% *B. breve* LMG 13208^T	88.2% *B. angulatum* LMG 11039^T
Similarity of 16S rRNA gene	96% *B. angulatum* LMG 11039^T	96.1% *B. pullorum* subsp. *pullorum* DSM 20433^T	96.5% *B. goeldii* LMG 30939^T	98.1% *B. imperatoris* LMG 30297^T	99.4% *B. angulatum* LMG 11039^T
Accession no. (genome)	JAAIIJ000000000	JAAIII000000000	JAAIIH000000000	JAAIIG000000000	JAAIIF000000000
Accession no. (16S rRNA gene)	MN707963	MN707964	MN707965	MN707966	MN707967
Accession no. (ITS gene)	MT859136	MT859137	MT859138	MT859139	MT859140

*Predicted number of rRNA loci.
ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; ITS, internal transcribed spacer; ORF, open reading frame.

ANI values ≤94% are considered as two distinct species [30, 31]. Remarkably, based on the concatenation of 165 core gene sequences, VB26^T exhibited a close phylogenetic relatedness with *B. scaligerum* DSM 103140^T, and when the whole genome sequences were taken into account, these two strains exhibited ANI values of 94.3% (Table S1). Thus, we decided to employ the Genome-to-Genome Distance Calculator formula two for estimating the digital DNA–DNA hybridization (dDDH) values between these two taxa. The analysis yielded an estimated dDDH value <70% (dDDH estimate GLM-based: 54.9%). Thus, we propose this isolate as a novel bifidobacterial species (Table S2). A complete overview of dDDH values between all bifidobacterial type strains is shown in Table S2.

In order to evaluate the phylogenetic relationships between VB23^T, VB24^T, VB25^T, VB26^T and VB31^T and other type strains, we analysed 16S rRNA genes, ITS sequences and amino acid sequences of genes representing the so-called core genome of the genus *Bifidobacterium*. These genetic data allowed the reconstruction of a 16S rRNA gene-based phylogenetic tree (Fig. 1), an ITS gene-based phylogenetic tree built using the hypervariable ITS region between conserved sequences 5′-YYBTTGKG-3′ and 5′-TGGACGCG-3′ (Fig. S1) and a phylogenomic tree (Fig. 2) based on the amino acid sequences of the *Bifidobacterium* core genome [30, 32]. In order to precisely map bifidobacterial core genome sequences, comparative genome analysis involving the chromosome sequences of all the currently recognised 84 (sub)species belonging to the genus *Bifidobacterium*, as well as the genome sequences of VB23^T, VB24^T, VB25^T, VB26^T and VB31^T, was carried out. The ORF contents of the bifidobacterial genomes were organised in functional gene clusters using BLAST (*E* value cut off of 1×10^{-10} and 50% identity across at least 80% of sequence length) with the gene family method of the Pan-Genome Analysis Pipeline (PGAP) [33]. Sequences were then clustered into protein families using a graph theory-based Markov clustering algorithm [34]. *In silico* analysis identified 191 clusters of orthologous groups (COGs) shared by all genomes analysed in this study, representing the updated core genome of currently sequenced *Bifidobacterium* representatives. Thus, 26 paralogs were excluded from the analysis in order to collect

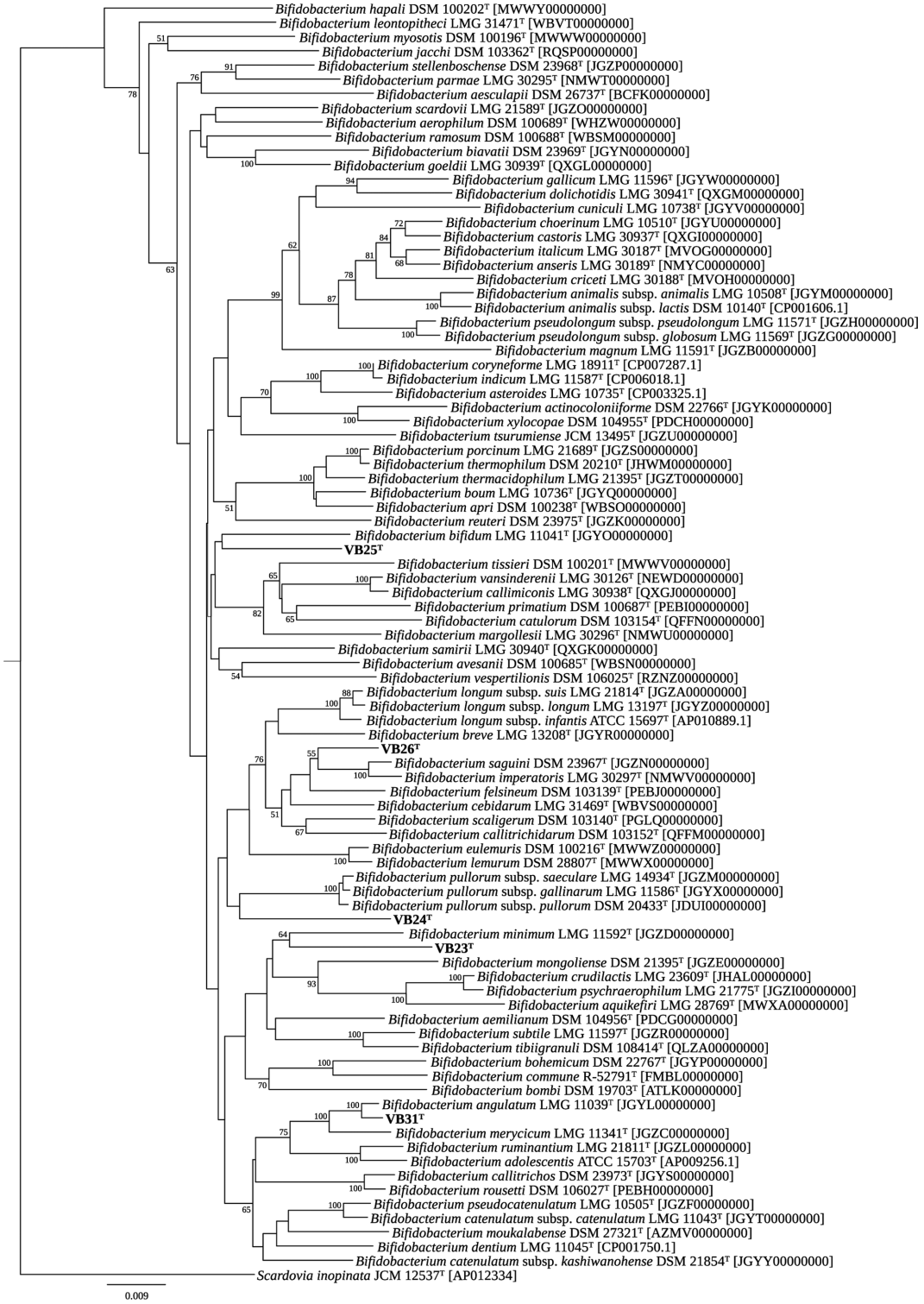

Fig. 1. Phylogenetic relationships between strains VB23^T, VB24^T, VB25^T, VB26^T and VB31^T and members of the genus *Bifidobacterium* based on 16S rRNA gene sequences. The complete nucleic acid sequence of each rRNA gene was extracted from whole-genome sequencing data. The 16S rRNA-based tree was reconstructed by the maximum-likelihood method, and the 16S rRNA gene sequence of *Scardovia inopinata* JCM 12537^T was used as an outgroup. Bootstrap percentages above 50 are shown at node points based on 1000 replicates of the phylogenetic tree.

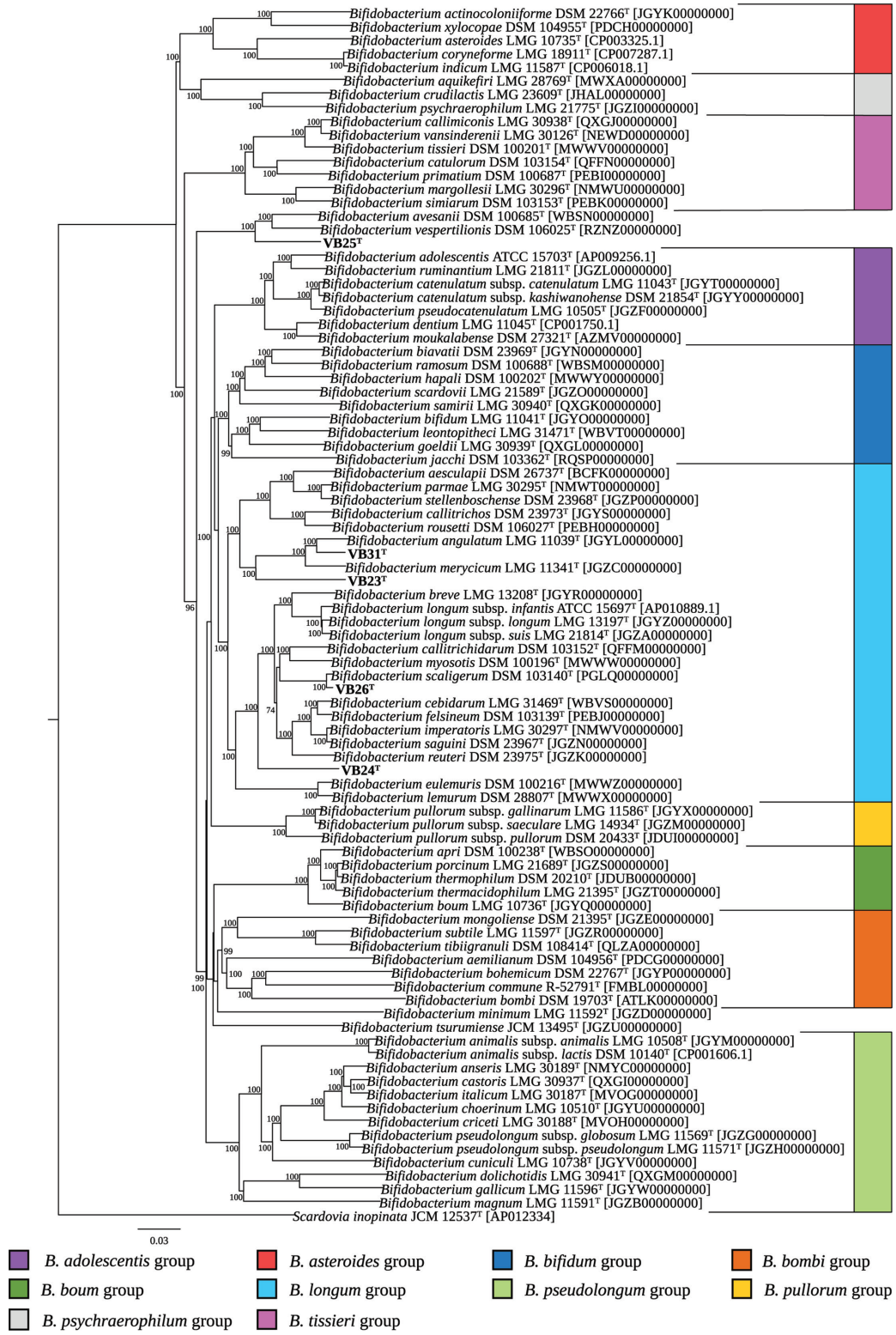

Fig. 2. Phylogenetic tree of the genus *Bifidobacterium* based on the concatenation of 165 amino acid sequences representing the *Bifidobacterium* core genome. The phylogenetic tree was reconstructed by the maximum-likelihood method, and COG sequences of *Scardovia inopinata* JCM 12537^T shared with members of the genus *Bifidobacterium* were used as an outgroup. Bootstrap percentages above 50 are shown at node points based on 1000 replicates of the phylogenetic tree. The amino acid-deduced core gene-based tree shows the division of the genus *Bifidobacterium* into 10 phylogenetic groups as represented by different colours.

COGs identified in a single copy in each bifidobacterial type strain genome. Thus, *Bifidobacterium* core genome sequences based on the concatenation of 165 protein sequences identified in each chromosome were used to build a phylogenomic core tree (Fig. 2). Both the concatenated core genome sequences and the complete 16S rRNA gene sequences were aligned using MAFFT software [35], and corresponding phylogenetic trees (Figs 1 and 2) were reconstructed using the maximum-likelihood method in ClustalW version 2.1 [36]. Accordingly, phylogenetic trees were built using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Bifidobacterial species have previously been subdivided into ten phylogenetic groups [37], and the strains characterized herein indicated that the isolates VB23^T, VB24^T, VB26^T and VB31^T belonged to the *B. longum* group, while VB25^T exhibited close relatedness with *B. avesanii* DSM 100685^T and *B. vespertilionis* DSM 106025^T (Fig. 2).

Fermentation characteristics of the isolated strains were determined using the API 50 CHL kit (bioMérieux) according to manufacturer's instructions; the strains were tested in biologically independent triplicates. The obtained results were evaluated based on colour and pH change. In addition, we evaluated growth on/in WSP agar/broth with different pH values at variable temperatures and oxygen conditions (Table 2).

The growth and cultivation characteristics were similar for all five novel species (Table 2). Only strain VB31^T was able to grow at 46 °C with low density but not at 49 °C. The optimal conditions for the growth of these five novel species were pH of 6.0–6.5, temperature of 37 °C and anaerobic conditions, with the exception of VB31^T, which was able to grow also under microaerophilic conditions. The fermentation profiles of all five novel species were variable, corresponding to taxonomic differences and the different origin and diet of their primate hosts [38, 39].

Cellular fatty acid methyl esters (FAMEs) were obtained from cells grown in WSP broth. Cellular fatty acids were analysed after conversion into FAMEs by saponification, methylation and extraction using minor modifications of the method described by Miller [40] and Kuykendall *et al.* [41] by the DSMZ (Germany). Cellular fatty acid contents of the described isolates representing the novel species are shown in Table 3. The major fatty acids of characterised isolates (VB23^T, VB24^T, VB25^T, VB26^T and VB31^T) were 16:0 FAME, 18:1 CIS 9 FAME and 18:1 CIS 9 DMA, which are also dominant fatty acids in *B. catenulatum*, *B. pseudocatenulatum*, *B. catenulatum* subsp. *kashiwanohense*, *B. dentium* and *B. moukalabense* [42, 43].

On the basis of novel strain characterization, including phylogenetic analyses based on 16S rRNA gene sequences, ITS profiling and whole-genome-based comparisons, all the studied strains were genetically discernible from each other as well as from the currently recognised bifidobacterial species. Thus, they represent five novel taxa for which the names *Bifidobacterium erythrocebi* sp. nov. (VB31^T), *Bifidobacterium moraviense* sp. nov. (VB25^T), *Bifidobacterium oedipodis* sp. nov. (VB24^T), *Bifidobacterium olomucense* sp. nov. (VB26^T) and *Bifidobacterium panos* sp. nov. (VB23^T) are proposed.

Strains VB23^T and VB31^T are the first novel bifidobacterial species identified in chimpanzee and patas monkey, and exhibit close genotypic similarities with bifidobacterial species typically found in humans. Strains VB24^T, VB25^T and VB26^T are another novel bifidobacterial species identified in tamarins.

DESCRIPTION OF *BIFIDOBACTERIUM ERYTHROCEBI* SP. NOV.

Bifidobacterium erythrocebi (e.ry.thro.ce′bi. N.L. gen. n. *erythrocebi* of *Erythrocebus*, the generic name of the patas monkey, *Erythrocebus patas*).

Cells are Gram-positive, non-motile, non-sporulating, F6PPK-positive and grow only under anaerobic conditions. After 48 h of growth on WSP agar under anaerobic conditions at 37 °C, colonies appear as white and circular with a second slimy layer, while embedded colonies are white and elliptical. The diameters of these colonies range from 2 to 3 mm on the surface and 2 mm in the agar. These cells are able to grow under temperatures from 30–37 °C, with limited growth observed at 46 °C; notably, these cells are unable to grow at 10, 15, 20 and 49 °C. Moreover, the cells grow at pH 4–9. Optimal growth occurs under conditions of pH 6 and 37 °C. Cells grown in WSP broth are irregular shorter rods.

Fermentation profiles revealed that strain VB31^T is able to grow well and can produce acids from amygdalin, D-fructose, D-galactose, D-glucose, lactose, maltose, melibiose, raffinose, D-ribose, D-sorbitol, sucrose, turanose, aesculin iron citrate, gentiobiose, glycogen, L-arabinose and starch. Furthermore, weak growth was observed on trehalose, inulin and methyl α-D-glucopyranoside. The cells are not able to grow on arbutin, D-adonitol, D-arabinose, D-arabitol, cellobiose, D-fucose, D-mannitol, D-mannose, melezitose, D-tagatose, D-xylanose, D-xylose, dulcitol, erythritol, glycerol, inositol, L-arabitol, L-fucose, L-rhamnose, L-sorbose, L-xylose, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, *N*-acetylglucosamine, potassium-2-ketogluconate, potassium-5-ketogluconate, potassium gluconate, salicin and xylitol.

Cellular fatty acid composition was dominated by palmitic acid—16:0 FAME (24.46%), stearic acid—18:0 FAME (6.09%), elaidic acid—18:1 CIS 9 FAME (20.90%) and 18:1 CIS 9 DMA (23.48%).

The type strain, VB31^T (=DSM 109960^T=CCUG 73843^T), was isolated from the faecal sample of a patas monkey (*Erythrocebus patas*). The DNA G+C content is 61.22 mol%.

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number JAAIIF000000000, while 16S rRNA gene and ITS sequences were deposited under accession numbers MN707967 and MT859140, respectively. Genome sequencing reads have been deposited under accession number SAMN13944615.

Table 2. Fermentation and cultivation characteristics of tested strains representing the putative novel species

Fermentation characteristics were determined using the API 50 CHL kit (bioMérieux): +, positive reaction (yellow colour, pH <5); +/−, weak reaction (greenish colour, pH 5.0–5.5); −, negative reaction (purple colour, pH 5.6–6.6), basic media pH 6.5, other substrates of the API 50 CHL kit were negative for all tested strains. None of the strains produce acids from D-adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, glycerol, inositol, methyl α-D-mannopyranoside, potassium gluconate, potassium-2-ketogluconate, potassium-5-ketogluconate, L-rhamnose, L-sorbose, D-tagatose, xylitol, D-xylanose or L-xylose. Growth in variable conditions such as at different pH levels, temperatures and in the presence/absence of oxygen were determined based on optical density: +, good growth OD_{600} >0.2; +/−, weak growth 0.1≤ OD_{600} ≤0.2; −, no growth 0.1< OD_{600}. The results were read after 48 h.

Characteristic	VB23^T	VB24^T	VB25^T	VB26^T	VB31^T
Amygdalin	−	+/−	−	+	+
Arbutin	−	−	−	+	−
Cellobiose	−	+/−	−	+	−
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
Lactose (bovine origin)	+	+	+	+	+
Maltose	+	+	+	+	+
D-Mannitol	−	+	−	−	−
D-Mannose	+	+	−	+	−
Melezitose	−	+	−	+	−
Melibiose	+	+	−	+	+
Raffinose	+	+	−	+	+
D-Ribose	+	+	−	−	+
D-Sorbitol	−	−	−	−	+
Sucrose	+	+	+	+	+
Trehalose	−	+/−	−	−	+/−
Turanose	+	+	−	−	+
D-Xylose	+	+	+	+	−
Aesculin iron citrate	+	+	−	+	+
Gentiobiose	−	−	−	+	+
Glycogen	−	−	−	−	+
Inulin	−	−	−	−	+/−
L-Arabinose	+	+	+	+	+
Methyl α-D-glucopyranoside	+/−	−	−	+/−	+/−
N-Acetylglucosamine	−	+/−	−	−	−
Salicin	−	+	−	+	−
Starch	−	−	−	−	+
pH 3.5	−	−	−	−	−
pH 4	−	−	−	−	+
pH 4.5	+	+	+	+	+
pH 5	+	+	+	+	+
pH 6	+	+	+	+	+

Continued

Table 2. Continued

Characteristic	VB23^T	VB24^T	VB25^T	VB26^T	VB31^T
pH 6.5	+	+	+	+	+
pH 7.5	+	+	+	+	+
pH 8.5	+	+	+	+	+
pH 9	+	+	+	+	+
10 °C	–	–	–	–	–
15 °C	–	–	–	–	–
20 °C	–	–	–	–	–
30 °C	+	+	+	+	+
37 °C	+	+	+	+	+
46 °C	–	–	–	–	+/-
49 °C	–	–	–	–	–
Anaerobically	+	+	+	+	+
Microaerophilically	+	+	+	+	–
Aerobically	–	–	–	–	–

DESCRIPTION OF *BIFIDOBACTERIUM MORAVIENSE* SP. NOV.

Bifidobacterium moraviense (mo.ra.vi.en′se. M.L. neut. adj. *moraviense* pertaining to Moravia, the region in the Czechia from which the strain originates).

Cells are Gram-positive, non-motile, non-sporulating and F6PPK-positive. These cells grow under anaerobic and microaerophilic conditions. After 48 h of growth on WSP agar under anaerobic conditions at 37 °C, colonies appear as white and circular, while embedded colonies are white and elliptical. The diameters of these colonies range from 2 to 3 mm on the surface and is 2 mm in the agar. The cells are able to grow at temperatures of 30–37 °C, but are unable to grow at 10, 15, 20, 46 and 49 °C. Moreover, the cells grow at pH 4.5–9. Optimal growth occurs at pH 6.5 and 37 °C. Cells grown in WSP broth are rods of various shapes, forming a branched structure with 'Y' on both sides.

Fermentation profiles revealed that strain VB25^T is able to grow well and produce acids from D-fructose, D-galactose, D-glucose, lactose, maltose, sucrose, D-xylose and L-arabinose.

Cells are not able to grow and produce acids from amygdalin, arbutin, D-adonitol, D-arabinose, D-arabitol, cellobiose, D-fucose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, D-ribose, D-sorbitol, D-tagatose, trehalose, turanose, D-xylanose, dulcitol, erythritol, aesculin iron citrate, gentiobiose, glycerol, glycogen, inositol, inulin, L-arabitol, L-fucose, L-rhamnose, L-sorbose, L-xylose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, potassium-2-ketogluconate, *N*-acetylglucosamine, potassium-5-ketogluconate, potassium gluconate, salicin, starch and xylitol. Cellular fatty acid composition is dominated by saturated palmitic acid—16:0 FAME (33.11%) and myristic acid—14:0 FAME (5.53%), unsaturated elaidic acid—8:1 CIS 9 FAME (16.92%) and 18:1 CIS 9 DMA (11.92%), as well as by the polyunsaturated forms of some acids.

The type strain, VB25^T (=DSM 109958^T=CCUG 73842^T), was isolated from a faecal sample of a Goeldi's marmoset (*Callimico goeldii*). The DNA G+C content is 67.49 mol%.

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number JAAIIH000000000, while 16S rRNA gene and ITS sequences were deposited under accession numbers MN707965 and MT859138. Genome sequencing reads have been deposited under accession number SAMN13944613.

DESCRIPTION OF *BIFIDOBACTERIUM OEDIPODIS* SP. NOV.

Bifidobacterium oedipodis (oe.di.po′dis. N.L. gen. n. *oedipodis* of *oedipus*, referring to the first isolation source, the tamarin *Saguinus oedipus*).

Cells are Gram-positive, non-motile, non-sporulating and F6PPK-positive. Cells grow under anaerobic and microaerophilic conditions. After 48 h of growth on WSP agar under the anaerobic condition at 37 °C, colonies are white and circular, while embedded colonies are white and elliptical. The diameter of each colony ranges from 2 to 3 mm on the surface and is 2 mm in the agar. The cells are able to grow from 30–37 °C, yet are unable to grow at 10, 15, 20, 46 and 49 °C. Moreover, the cells grow at pH 4.5–9. Optimal conditions of

Table 3. Cellular fatty acid content (%) of tested strains representing the putative novel species

FAME, fatty acid methyl ester; DMA, dimethyl acetal; ALDE, aldehyde. Summed features represent groups of two or more fatty acids that could not be separated by GLC with the MIDI system.

Fatty acids	VB23^T	VB24^T	VB25^T	VB26^T	VB31^T
10:0 FAME	0.06	0.39	0.21	0.28	0.26
11:0 DMA	0.52	0.62	0.6	0.55	0.6
12:0 FAME	0.38	0.48	1.2	0.47	0.62
14:0 FAME	0.4	1.09	5.53	1.29	0.86
14:0 DMA	0.22	0.6	2.22	1.07	1.03
16:0 FAME	18.3	21.02	33.11	22.53	24.46
16:0 DMA	1.02	0.94	1.45	1.12	1.16
16:0 ALDE	0.14	–	0.19	0.17	0.14
16:1 CIS 7 FAME	0.26	0.45	0.81	0.37	0.33
16:1 CIS 9 FAME	1.59	1.83	0.7	0.94	0.96
16:1 CIS 9 DMA	1.49	0.62	0.37	0.91	0.94
17:0 FAME	0.26	–	0.35	–	–
18:0 FAME	5.48	4.47	2.63	5.02	6.09
18:0 DMA	–	–	0.52	0.22	0.38
18:1 CIS 9 FAME	45.74	36.23	16.92	20.33	23.48
18:1 CIS 9 DMA	8.63	18.56	11.92	29.22	23.48
18:1 CIS 11 DMA	1.64	1.07	1.01	1.82	1.35
19 CYC 9, 10/:1 FAME	–	0.73	3.73	–	0.37
19:0 CYC 9, 10 DMA	–	–	7.27	–	4.06
Summed feature 1*	–	0.13	0.55	0.21	0.19
Summed feature 4†	0.27	–	–	0.16	0.14
Summed feature 7‡	1.48	2.89	1.69	6.13	4.09
Summed feature 10§	10.69	6.79	6.26	6.44	6.77
Summed feature 12¶	1.44	1.09	0.76	0.75	0.83

*Summed feature 1: 13:1 CIS 12 FAME/14:0 ALDE.
†Summed feature 4: 15:2 FAME.
‡Summed feature 7: 17:1 CIS 8 FAME/ 17:2 FAME at 16.760.
§Summed feature 10: 18:1c11/t9/t6 FAME.
¶Summed feature 12: 19:0 ISO FAME.

growth occur at pH 6.5 and 37 °C. Cells grown in WSP broth are irregular rods without typical bifidobacterial branching.

Fermentation profiles showed that strain VB24^T is able to grow well and produce acids from D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, D-ribose, sucrose, turanose, D-xylose, aesculin iron citrate, L-arabinose and salicin. In addition, weak growth was observed on amygdalin, cellobiose, trehalose and *N*-acetylglucosamine. Cells are not able to grow and produce acids on arbutin, D-adonitol, D-arabinose, D-arabitol, D-fucose, D-sorbitol, D-tagatose, D-xylanose, dulcitol, erythritol, gentiobiose, glycerol, glycogen, inositol, inulin, L-arabitol, L-fucose, L-rhamnose, L-sorbose, L-xylose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, potassium gluconate, potassium-2-ketogluconate, potassium-5-ketogluconate, starch and xylitol.

Cellular fatty acid composition was dominated by palmitic acid—6:0 FAME (21.02%), stearic acid—18:0 FAME (4.47%) and elaidic acid—18:1 CIS 9 FAME (36.23%) and 18:1 CIS 9 DMA (18.56%).

The type strain, VB24^T (=DSM 109957^T=CCUG 73932^T), was isolated from a faecal sample of a cotton-top tamarin (*Saguinus oedipus*). The DNA G+C content is 57.74mol%.

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number JAAIII000000000, while 16S rRNA gene and ITS sequences were deposited under accession numbers MN707964 and MT859137. Genome sequencing reads have been deposited under accession number SAMN13944612.

DESCRIPTION OF *BIFIDOBACTERIUM OLOMUCENSE* SP. NOV.

Bifidobacterium olomucense (o.lo.mu.cen′se. N.L. neut. adj. *olomucense* pertaining to Olomouc; city of Czechia where the type strain was isolated).

Cells are Gram-positive, non-motile, non-sporulating and F6PPK-positive. Cells grow under anaerobic and microaerophilic conditions, and exhibit limited aerobic growth. After 48 h of growth on WSP agar under anaerobic conditions at 37 °C, colonies are white and circular, while embedded colonies are white and elliptical. The diameter of each colony ranges from 1 to 2 mm on the surface and is 2 mm in the agar. The cells are able to grow at 30–37 °C, yet are unable to grow at 10, 15, 20, 46 and 49 °C. Moreover, the cells grow at pH 4.5–9. Optimal conditions of growth occur at pH 6.5 and 37 °C. Cells grown in WSP broth are irregular rods without typical bifidobacterial branching.

Fermentation profiles revealed that strain VB26^T is able to grow well and produce acids from amygdalin, arbutin, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melezitose, melibiose, raffinose, sucrose, D-xylose, aesculin iron citrate, gentiobiose, L-arabinose and salicin. In addition, weak growth was observed on methyl α-D-glucopyranoside.

Cells are not able to grow on D-adonitol, D-arabinose, D-arabitol, D-fucose, D-mannitol, D-ribose, D-sorbitol, D-tagatose, trehalose, turanose, D-xylanose, dulcitol, erythritol, glycerol, glycogen, inositol, inulin, L-arabitol, L-fucose, L-rhamnose, L-sorbose, L-xylose, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, *N*-acetylglucosamine, potassium-2-ketogluconate, potassium-5-ketogluconate, potassium gluconate, starch and xylitol.

The cellular fatty acid composition is dominated by palmitic acid—16:0 FAME (22.53%), stearic acid—18:0 FAME (5.02%) and elaidic acid—18:1 CIS 9 FAME (20.33%) and 18:1 CIS 9 DMA (29.22%).

The type strain, VB26^T (=DSM 109959^T=CCUG 73845^T), was isolated from a faecal sample of a moustached tamarin (*Saguinus mystax*). The DNA G+C content is 58.94mol%.

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number JAAIIG000000000, while 16S rRNA gene and ITS sequences were deposited under accession numbers MN707966 and MT859139. Genome sequencing reads have been deposited under accession number SAMN13944614.

DESCRIPTION OF *BIFIDOBACTERIUM PANOS* SP. NOV.

Bifidobacterium panos (pa′nos. L. gen. n. *panos* of *Pan*, the generic name of the chimpanzee).

Cells are Gram-positive, non-motile, non-sporulating and F6PPK-positive. Cells grow under anaerobic and microaerophilic conditions. After 48 h of growth on WSP agar under anaerobic conditions at 37 °C, colonies are transparent white and slightly irregular. The colonies are very small, and their diameter is around 1 mm. The cells are able to grow at 30–37 °C, yet are unable to grow at 10, 15, 20, 46 and 49 °C. Moreover, the cells grow at pH 4.5–9. Optimal conditions of growth occur at pH 6.5 and 37 °C. Cells grown in WSP broth are rods of various shapes, forming a branched structure with 'Y' on both sides.

Fermentation profiles revealed that strain VB23^T is able to grow well and produce acids from D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melibiose, raffinose, D-ribose, sucrose, turanose, D-xylose, aesculin iron citrate and L-arabinose. Weak growth was observed on methyl α-D-glucopyranoside. Cells are not able to grow and produce acids from amygdalin, arbutin, D-adonitol, D-arabinose, D-arabitol, cellobiose, D-fucose, D-mannitol, melezitose, D-sorbitol, D-tagatose, trehalose, D-xylanose, dulcitol, erythritol, gentiobiose, glycerol, glycogen, inositol, inulin, L-arabitol, L-fucose, L-rhamnose, L-sorbose, L-xylose, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, *N*-acetylglucosamine, potassium-2-ketogluconate, and potassium-5-ketogluconate, potassium gluconate, salicin, starch and xylitol.

The cellular fatty acid composition is dominated by palmitic acid—16:0 FAME (18.3%), stearic acid—18:0 FAME (5.48%), elaidic acid—18:1 CIS 9 FAME (45.74%) and 18:1 CIS 9 DMA (8.63%).

The type strain, VB23^T (=DSM 109963^T=CCUG 73840^T), was isolated from a faecal sample of a chimpanzee (*Pan troglodytes*). The DNA G+C content is 59.95mol%.

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number JAAIIJ000000000, while 16S rRNA gene and ITS sequences were deposited under accession numbers MN707963 and MT859136. Genome sequencing reads have been deposited under accession number SAMN13944611.

Funding information
The work was supported from the European Regional Development Fund—Project 'Centre for the investigation of synthesis and transformation of nutritional substances in the food chain in interaction with potentially harmful substances of anthropogenic origin: comprehensive assessment of soil contamination risks for the quality of agricultural products' (No. CZ.02.1.01/0.0/0.0/16_019/0000845) and by the cooperation project (8J19AT028) between Austria and the Czech Republic.

Acknowledgements
We thank the Zoo Liberec (Lidové sady 425/1, 460 01 Liberec 1, Czechia) and the Zoo Olomouc (Darwinova 29, 779 00 Olomouc – Svatý Kopeček, Czechia). We would also like to thank Jitka Vokurková.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The sampling of primate faeces was performed during routine daily procedures. All procedures involving animals adhered to recommendations of the 'Guide for the Care and Use of Animals' by the Czech University of Life Sciences Prague.

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4.2 *Bifidobacterium canis* sp. nov., a novel member of the *B. pseudolongum* phylogenetic group isolated from faeces of a dog (*Canis lupus* f. *familiaris*)

Neuzil-Bunesova V, Lugli GA, **Modrackova N**, Makovska M, Mrazek J, Mekadim C, Musilova S, Svobodova I, Spanek R, Ventura M. 2020a. *Bifidobacterium canis* sp. nov., a novel member of the *Bifidobacterium pseudolongum* phylogenetic group isolated from faeces of a dog (*Canis lupus* f. *familiaris*). International Journal of Systematic and Evolutionary Microbiology **70**:5040-5047.

INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Neuzil-Bunesova *et al.*, *Int. J. Syst. Evol. Microbiol.*

DOI 10.1099/ijsem.0.004378

MICROBIOLOGY SOCIETY

Bifidobacterium canis sp. nov., a novel member of the *Bifidobacterium pseudolongum* phylogenetic group isolated from faeces of a dog (*Canis lupus* f. *familiaris*)

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Abstract

A fructose-6-phosphate phosphoketolase-positive strain (GSD1FS^T) was isolated from a faecal sample of a 3 weeks old German Shepherd dog. The closest related taxa to isolate GSD1FS^T based on results from the EZBioCloud database were *Bifidobacterium animalis* subsp. *animalis* ATCC 25527^T, *Bifidobacterium animalis* subsp. *lactis* DSM 10140^T and *Bifidobacterium anseris* LMG 30189^T, belonging to the *Bifidobacterium pseudolongum* phylogenetic group. The resulting 16S rRNA gene identities (compared length of 1454 nucleotides) towards these taxa were 97.30, 97.23 and 97.09%, respectively. The pairwise similarities of strain GSD1FS^T using *argS*, *atpA*, *fusA*, *hsp60*, *pyrG*, *rpsC*, *thrS* and *xfp* gene fragments to all valid representatives of the *B. pseudolongum* phylogenetic group were in the concatenated range of 83.08–88.34%. Phylogenomic analysis based on whole-genome methods such as average nucleotide identity revealed that bifidobacterial strain GSD1FS^T exhibits close phylogenetic relatedness (88.17%) to *Bifidobacetrium cuniculi* LMG 10738^T. Genotypic characteristics and phylogenetic analyses based on nine molecular markers, as well as genomic and comparative phenotypic analyses, clearly proved that the evaluated strain should be considered as representing a novel species within the *B. pseudolongum* phylogenetic group named as *Bifidobacterium canis* sp. nov. (GSD1FS^T=DSM 105923^T=LMG 30345^T=CCM 8806^T).

Bifidobacteria are saccharolytic Gram-stain-positive bacteria that colonize different ecological niches connected primarily to the gastrointestinal tract of social mammals, poultry and insects [1, 2]. Their occurrence seems to be common in the gastrointestinal tract of dogs too [3–5]. Over time, the dog diet has changed, starting from carnivorous behaviour with a high protein diet to a carbohydrate-rich diet with the tendency to live an urban lifestyle [6, 7]. Cohabiting dogs and humans share more bacterial operational taxonomic units compared with hosts from separate households [8]. Despite this human–dog co-evolution, multi-host bifidobacterial species such as *Bifidobacterium animalis* and *Bifidobacterium pseudolongum* are frequently detected in dog faeces [3–5]. Moreover, detailed profiling of the bifidobacterial population of dogs based on ITS-based sequencing approaches has identified the occurrence of putative new bifidobacterial taxa [4].

In total, 49 dog faecal samples (Table 1) were analysed for bifidobacterial occurrence on Wilkins–Chalgren anaerobe agar supplemented with GMO-free soya peptone (5 g l^{-1}; both Oxoid), L-cysteine (0.5 g l^{-1}) and Tween 80 (1 m l^{-1}; both Sigma-Aldrich), mupirocin (100 mg l^{-1}) and norfloxacin

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Keywords: *Bifidobacterium*; *Bifidobacterium pseudolongum* phylogenetic group; dogs; MALDI-TOF MS.
Abbreviations: AIC, Akaike information criterion; ANI, average nucleotide identity; BLAST, Basic Local Alignment Search Tool; COGs, Cluster of Orthologous Groups; DDH, DNA–DNA hybridization; F6PPK, fructose-6-phosphate phosphoketolase; GF, gene family; GGDC, Genome-to-Genome Distance Calculator; GMO, genetically modified organism; HCCA, α-cyano-4-hydroxycinnamic acid; ITS, internal transcribed spacer; MCL, Markov clustering algorithm; MUP, mupirocin; NORF, norfloxacin; ORF, open reading frame; PGAP, Pangenome Analysis Pipeline; WSP, Wilkins–Chalgren with soya peptone.
DDBJ/ENA/GenBank accession number: WNLP00000000. The version described in this paper is WNLP01000000. Genome sequencing reads under accession number: SRR12172981. 16S rRNA, *argS*, *atpA*, *fusA*, *hsp60*, *pyrG*, *rpsC*, *thrS* and *xfp* genes under accession numbers: MG028631, MK267100, MK267166, MK267167, MK267170, MK267172, MK267130, MK267174 and MK267175, respectively.
Four supplementary tables and 12 supplementary figures are available with the online version of this article.

Table 1. Strains isolated on bifidobacterial media identified using MALDI-TOF MS and their detected counts

ND, Not detected; NRI, not reliable identification; F, female; M, male; w, weeks; m, month(s); NORF-MUP agar, Wilkins–Chalgren anaerobe agar supplemented with GMO-free soya peptone (5 g l^{-1}; both Oxoid), acetic acid (1 ml l^{-1}), L-cysteine (0.5 g l^{-1}), and Tween 80 (1 ml l^{-1}; all Sigma-Aldrich), mupirocin (100 mg l^{-1}) and norfloxacin (200 mg l^{-1}; both Oxoid).

No.	Dog breed	Age (years)	Sex	Bacterial isolates from NORF-MUP agar identified by MALDI-TOF MS	NORF-MUP agar (log CFU/g±SD)		
1	German Shepherd	3 w	M	***Bifidobacterium animalis***, NRI (GSD1FS^T)	7.56	±	0.32
2	German Shepherd	3 w	M	***Bifidobacterium animalis***, NRI	7.48	±	0.79
3	German Shepherd	3	F	***Bifidobacterium pseudolongum***	6.37	±	0.12
4	Golden Retriever	1.5	F	*Clostridium perfringens*	5.76	±	0.25
5	Samoyed	3	M	*Clostridium perfringens*	5.85	±	0.01
6	German Shepherd	10	M	*Clostridium sordellii*	5.88	±	0.03
7	German Shepherd	4	M	***Bifidobacterium pseudolongum***	5.88	±	0.03
8	German Shepherd	7.5	M	*Clostridium perfringens*, *Clostridium sordellii*	6.66	±	0.06
9	German Shepherd	3	M	*Clostridium perfringens*	4.00	±	0.01
10	Czechoslovakian Wolfdog	4.5	M	*Clostridium perfringens*	3.62	±	0.06
11	Crossbreed	9	M	*Clostridium sordellii*	3.51	±	0.01
12	German Shepherd	3	F	*Escherichia coli*, *Clostridium sordellii*, *Lactobacillus murinus*	4.52	±	0.07
13	Swiss Shepherd	3	M	***Bifidobacterium adolescentis***, ***Bifidobacterium longum***, ***Bifidobacterium animalis***	8.54	±	0.09
14	German Shepherd	5	M	***Bifidobacterium animalis***	7.46	±	0.01
15	Labrador Retriever	3	F	No isolates	<2		
16	Fox Terrier	7	F	***Bifidobacterium catenulatum/pseudocatenulatum***	9.16	±	0.08
17	Belgian Shepherd	4 m	M	***Bifidobacterium catenulatum/pseudocatenulatum***, ***Bifidobacterium pullorum***	5.21	±	0.00
18	Czechoslovakian Wolfdog	7.5	M	***Bifidobacterium pseudolongum***	4.78	±	0.12
19	Swiss Shepherd	4	M	*Clostridium sordellii*	6.88	±	0.27
20	Crossbreed	1.5	M	***Bifidobacterium pseudolongum***	5.51	±	0.02
21	Crossbreed	ND	M	*Clostridium perfringens*	4.31	±	0.00
22	German Shepherd	ND	M	*Clostridium perfringens*	8.79	±	0.00
23	German Shepherd	5	F	No isolates	<2		
24	Belgian Shepherd	6 m	M	No isolates	<2		
25	Havanese	ND	F	***Bifidobacterium catenulatum/pseudocatenulatum***, ***Bifidobacterium pullorum***	2.58	±	3.64
26	Havanese	2 w	ND	*Clostridium perfringens*	1.45	±	2.05
27	Havanese	2 w	ND	*Pediococcus acidilactici*	2.08	±	2.93
28	Crossbreed	ND	F	*Clostridium sordellii*, *Clostridium perfringens*	6.28	±	0.01
29	Crossbreed	ND	M	***Bifidobacterium longum***, *Lactobacillus murinus*, *Clostridium perfringens*	8.17	±	0.02
30	Crossbreed	ND	M	***Bifidobacterium catenulatum/pseudocatenulatum***, ***Bifidobacterium longum***, *Lactobacillus murinus*, *Clostridium perfringens*	7.14	±	0.01
31	Crossbreed	ND	F	*Clostridium perfringens*	6.29	±	0.01

Continued

Table 1. Continued

No.	Dog breed	Age (years)	Sex	Bacterial isolates from NORF-MUP agar identified by MALDI-TOF MS	NORF-MUP agar (log CFU/g±SD)
32	Golden Retriever	6	F	*Propionibacterium acnes*	<2
33	German Shepherd	7	M	*Clostridium perfringens*	4.89 ± 0.01
34	German Shepherd	5	M	*Escherichia coli*	7.60 ± 0.00
35	German Shepherd	1	M	***Bifidobacterium longum***	4.84 ± 0.01
36	German Shepherd	7.5	F	***Bifidobacterium longum***	5.43 ± 0.02
37	German Shepherd	3.5	F	***Bifidobacterium pseudolongum***	6.66 ± 0.00
38	German Shepherd	1	F	***Bifidobacterium pseudolongum***	5.39 ± 0.04
39	German Shepherd	8.5	F	***Bifidobacterium pseudolongum***	9.03 ± 0.01
40	German Shepherd	8.5	F	***Bifidobacterium pseudolongum*****,** *Clostridium perfringens*	7.13 ± 0.01
41	German Shepherd	8.5	F	***Bifidobacterium pseudolongum*****,** *Clostridium perfringens*	5.93 ± 0.02
42	German Shepherd	8.5	F	***Bifidobacterium pseudolongum***	6.81 ± 0.01
43	German Shepherd	7	F	No isolates	1.65 ± 2.33
44	German Shepherd	7	F	*Clostridium perfringens*	3.50 ± 0.01
45	German Shepherd	6	F	*Clostridium sordellii*	5.92 ± 0.02
46	German Shepherd	5	F	***Bifidobacterium pseudolongum*****, *Bifidobacterium animalis***	8.04 ± 0.01
47	German Shepherd	4	F	***Bifidobacterium pseudolongum*****, *Bifidobacterium animalis*****,** *Lactobacillus murinus*	6.32 ± 0.07
48	German Shepherd	ND	F	*Clostridium perfringens*	1.81 ± 2.56
49	German Shepherd	ND	F	*Clostridium perfringens*	3.69 ± 0.02

(200 mg l^{-1}; both Oxoid) according to Vlkova *et al.* [9], which was used as an norfloxacin-mupirocin (NORF-MUP) agar. Isolates with variable cultivation characteristics were subcultivated in Wilkins–Chalgren anaerobe broth/agar (Oxoid) supplemented with GMO-free soya peptone (5 g l^{-1}), acetic acid (1 ml l^{-1}), L-cysteine (0.5 g l^{-1}), and Tween 80 (1 ml l^{-1}) under anaerobic conditions, used as Wilkins–Chalgren with soya peptone (WSP broth)/agar. Obtained cultures were identified using MALDI-TOF MS (ethanol–formic acid extraction procedure with an HCCA matrix according to the manufacturer's instructions; Bruker Daltonik) and by the detection of fructose-6-phosphate phosphoketolase (F6PPK) activity [10]. From 285 bacterial isolates, 155 were identified as *Bifidobacterium* species and other isolates belonged to *Clostridium perfringens*, *Clostridium sordellii*, *Lactobacillus murinus*, *Escherichia coli*, *Pediococcus acidilactici* and *Propionibacterium acnes*. The most-detected bifidobacterial species were *Bifidobacterium animalis* and *Bifidobacterium pseudolongum*, followed by *Bifidobacterium catenulatum/pseudocatenulatum*, *Bifidobacterium longum*, *Bifidobacterium adolescentis* and *Bifidobacterium pullorum* (Table 1). An F6PPK-positive strain, GSD1FS^T, was not reliably identified using MALDI-TOF MS and selected for precise identification and characterization.

The 16S rRNA gene of isolate GSD1FS^T was amplified and sequenced from both directions using the Bif285 (5′-GAGG GTTCGATTCTGGCTCAG-3′) and Bif261 (5′-AAGGAG-GTGATCCAGCCGCA-3′) [11] primers. The obtained sequence (MG028631; 1454 nts long) was inserted into the EZBioCloud database [12] to obtain the closest related taxa. Representatives of the *Bifidobacterium pseudolongum* phylogenetic group [13] including *Bifidobacterium animalis* subsp. *animalis* ATCC 25527^T, *Bifidobacterium animalis* subsp. *lactis* DSM 10140^T, *Bifidobacterium anseris* LMG 30189^T, *Bifidobacterium castoris* LMG 30937^T, *Bifidobacterium italicum* LMG 30187^T, *Bifidobacterium pseudolongum* subsp. *globosum* DSM 20092^T, *Bifidobacterium pseudolongum* subsp. *pseudolongum* LMG 11571^T and *Bifidobacterium choerinum* DSM 20434^T were revealed as the closest relatives with pairwise identities in the range of 96.12–97.30%. These results suggested that the examined strain could represent a novel species within the *B. pseudolongum* phylogenetic group. Multilocus sequence analysis on the basis of eight housekeeping genes was used to confirm the status of a novel species. The partial sequences of the *fusA*, *hsp*60, *pyrG*, *thrS* and *xfp* genes were amplified and sequenced using specific primers and PCR conditions, as described in previous studies [14–18]. New primers and PCR conditions for amplification and sequencing of the *argS* (encoding the arginyl-tRNA synthase), *atpA* (encoding the ATP synthase alpha subunit) and *rpsC* (encoding the 30S ribosomal protein S3) gene regions applicable to the entire

family *Bifidobacteriaceae* were designed and optimized in this study (Table S1, available in the online version of this article). Methods for primer design and optimization of PCR conditions were described in our previous studies [17, 18]. The consensus sequences were obtained using Geneious version 7.1.7 software based on the sequences of 12 complete genome representatives belonging to the family *Bifidobacteriaceae* (Table S2).

Sequences of the 16S rRNA gene and eight housekeeping genes were obtained for strain GSD1FS^T using the methods mentioned above and deposited in the NCBI database using the Banklt application (www.ncbi.nlm.nih.gov/WebSub/?tool=genbank). Sequences of the same genes were retrieved from the complete genomes of the 13 representatives belonging to the *B. pseudolongum* phylogenetic group [13, 19] to provide gene comparative and phylogenetic analyses (Table S3). To ensure better phylogenetic tree topology, two species classified to the *Bifidobacterium boum* phylogenetic group (*B. boum* and *Bifidobacterium porcinum*) and *Aeriscardovia aeriphila* DSM 22365^T were exploited as a root of trees and included in Table S3.

Gene comparative and phylogenetic analyses were performed using individual and concatenated gene alignments. The 16S rRNA (length of 1425 nt), *argS* (741), *atpA* (642), *fusA* (774), *hsp60* (588), *pyrG* (798), *rpsC* (288), *thrS* (726) and *xfp* (477) gene alignments, created using the CLUSTAL_W algorithm in the Geneious version 7.1.7 software package, were used for this purpose. Gene pairwise identities of novel strain GSD1FS^T towards all valid taxa classified into the *B. pseudolongum* group have been automatically computed by the Geneious software package. All phylogenetic trees were reconstructed in MEGA 5.05 software [17] using the maximum-likelihood method, the best fit AIC (Akaike information criterion) ML model and 1000 bootstrap replicates.

The pairwise similarities of strain GSD1FS^T using the *argS*, *atpA*, *fusA*, *hsp*60, *pyrG*, *rpsC*, *thrS* and *xfp* gene fragments to all valid representatives of the *B. pseudolongum* phylogenetic group were at intervals 77.73–87.72, 83.65–90.65, 87.08–90.70, 82.82–86.91, 79.45–85.09, 85.42–94.79, 81.41–89.53 and 83.23–92.24%, respectively (Table S3). These results markedly suggest that strain GSD1FS^T should be considered as representing a novel species [14–18].

A separated position and the closest affinity of strain GSD1FS^T to *B. animalis* subspecies within the *B. pseudolongum* phylogenetic group is obvious from a phylogenetic tree reconstructed using an alignment of concatenated sequences of the *argS*, *atpA*, *fusA*, *hsp60*, *pyrG*, *rpsC, thrS* and *xfp* genes (Fig. 1). An almost identical tree topology was obtained based on

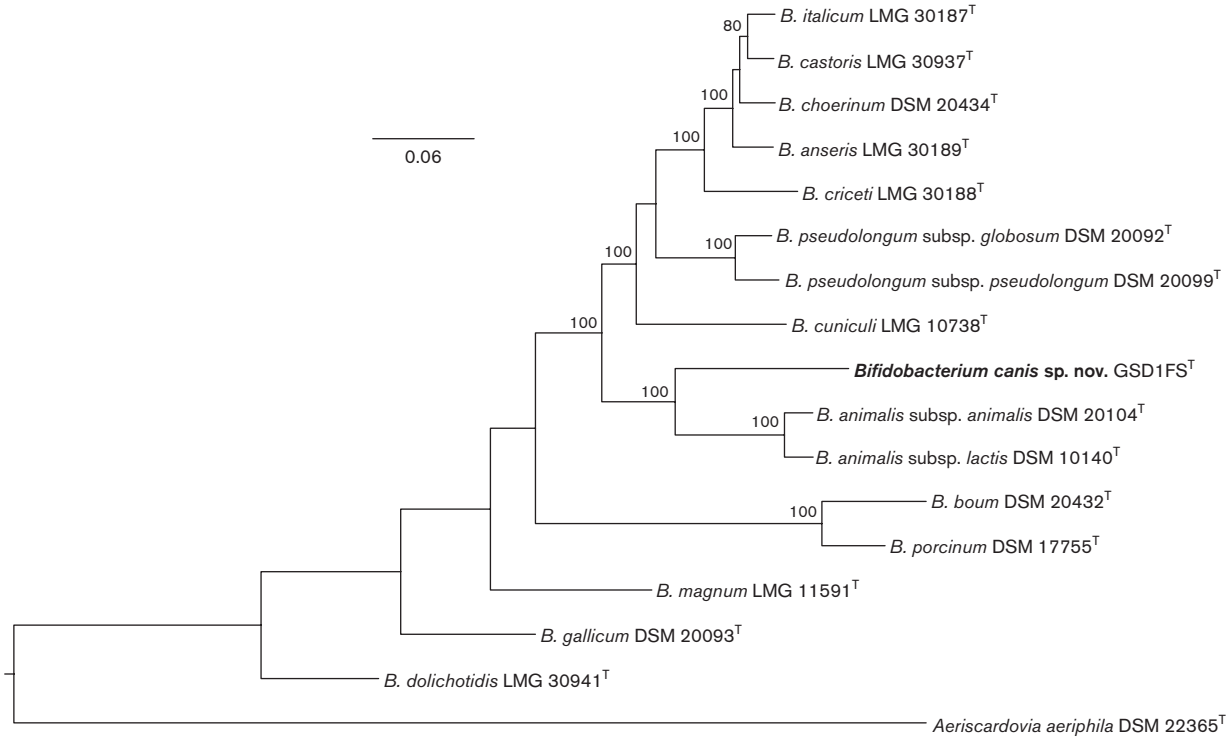

Fig. 1. Maximum-likelihood phylogenetic tree reconstructed using an alignment consisting of concatenated sequences of the *argS* (741 nts), *atpA* (642), *fusA* (774), *hsp60* (588), *pyrG* (798), *rpsC* (288), *thrS* (726) and *xfp* (477) genes, respectively (total 5034 nts). The GTR +G+I best fit AIC (Akaike information criterion) model in the MEGA version 5.05 software package was used for the reconstruction. Bootstrap values (≥70), expressed as percentages of 1000 replicates, are given at nodes. The tree was rooted by *Aeriscardovia aeriphila* DSM 22365^T. Bar, 0.06 substitutions per nucleotide position. The phylogeny clearly documents the close affinity of strain GSD1FS^T to *B. animalis* subspecies within the *B. pseudolongum* phylogenetic group.

Table 2. General genetic features of strain GSD1FS^T

Feature	GSD1FS^T
Biological origin	*Canis lupus* f. *familiaris*
Average coverage	58×
Number of assembled contigs	22
Genome length	2270696
Average G+C content (mol%)	57.5
Number of predicted ORFs	1883
tRNA	56
rRNA*	4
Similarity of 16S rRNA gene	97.3% *Bifidobacterium animalis* subsp. *animalis* ATCC 25527^T
ANI value	88.17% *Bifidobacterium cuniculi* LMG 10738^T

*Predicted number of rRNA loci.

amino-acid (aa) alignment derived from the concatenate (Fig. S1). The close relatedness of strain GSD1FS^T to *B. animalis* subspecies within the *B. pseudolongum* phylogenetic group was revealed in almost all individual phylogenetic trees reconstructed based on 16S rRNA, *argS*, *atpA*, *fusA*, *hsp60*, *pyrG*, *rpsC, thrS* and *xfp* gene alignments (Figs S2–S10).

Genomic DNA of strain GSD1FS^T was isolated using DNeasy UltraClean Microbial DNA kit (Qiagen). The genome was sequenced with Ion Torrent technology on a Proton sequencer at the Seqme.eu company (Czechia). The generated reads were depleted of adapter sequences, quality-filtered and assembled through the MEGAnnotator pipeline [20]. In addition, ORF identification and functional annotation of ORFs were carried out as previously reported [20]. Table 2 shows the basic genome features of strain GSD1FS^T. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number WNLP00000000. Genetic similarity at the genomic level of GSD1FS^T with respect to the other currently recognized bifidobacterial (sub)species was evaluated based on average nucleotide identity (ANI) analysis, and

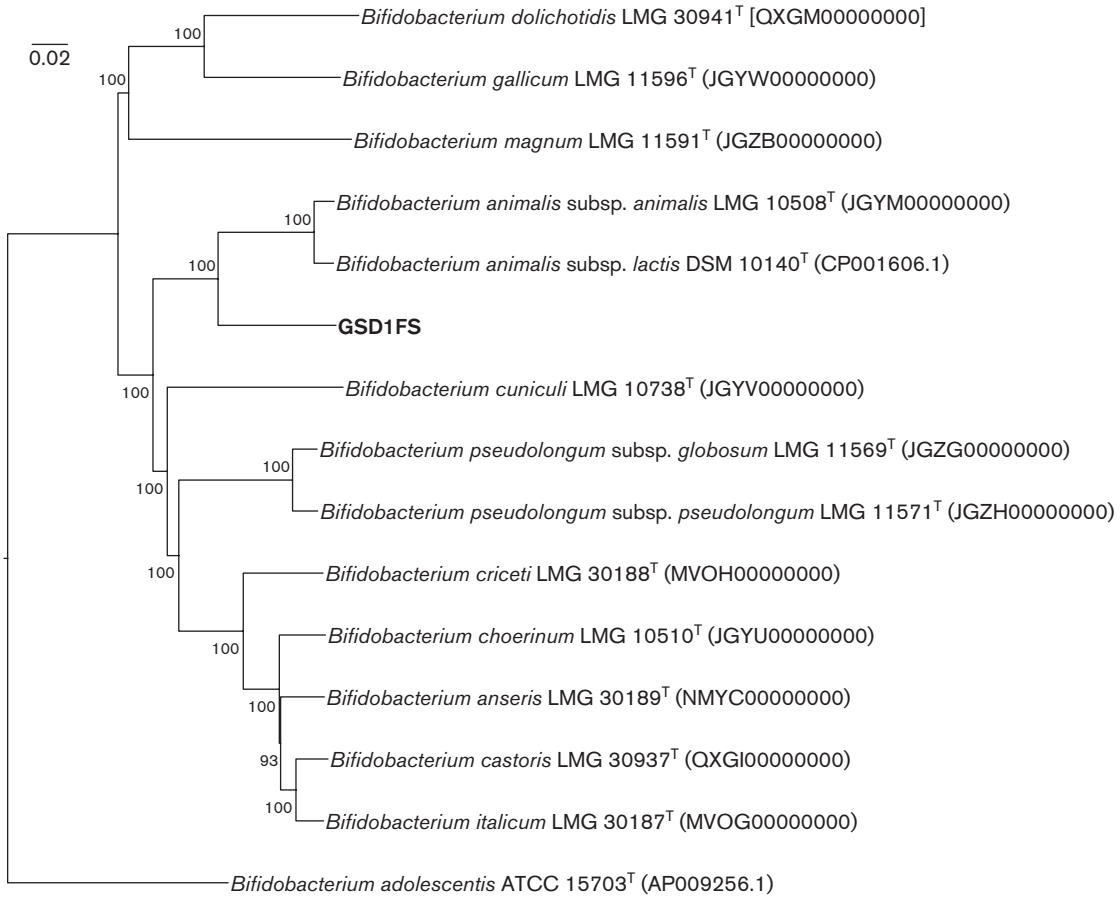

Fig. 2. Phylogenetic tree of bifidobacteria based on the concatenation of 619 protein sequences that represent the *B. pseudolongum* phylogenetic group core genome sequences. The phylogenetic tree was reconstructed by the maximum-likelihood method and the gene sequences of *Bifidobacterium adolescentis* ATCC 15703^T were used as outgroups. Bootstrap percentages above 50% are showed at node points, based on 1000 replicates of the phylogenetic tree.

Table 3. Phenotypic characteristic of GSD1FS^T and species of the closest-related *Bifidobacterium* taxa and species common for dogs

Strain: 1, *B. anseris* LMG 30189^T; 2, *B. animalis* subsp. *lactis* DSM 10140^T; 3, *B. animalis* subsp. *animalis* DSM 20104^T; 4, GSD1FS^T; 5, *B. cuniculi* DSM 20435^T; 6, *B. pseudolongum* subsp. *pseudolongum* DSM 20099^T; 7, *B. pseudolongum* subsp. *globosum* DSM 20092^T. The results were read after 48 h. Fermentation characteristics were determined using API 50 CHL kit (bioMérieux): +, positive reaction (yellow colour, pH <5); +/−, weak reaction (greenish colour, pH 5.0–5.5); −, negative reaction (purple colour, pH 5.6–6.6), v, variable. Basic media pH 6.5. Other substrates in the API 50 CHL kit were negative for all tested strains.

Growth in variable conditions such as pH level, temperature and presence of oxygen were determined based on optical density: +, good growth OD_{600}>0.2; +/−, weak growth 0.1≤ OD_{600}≤0.2; −, no growth 0.1< OD_{600}.

Characteristic	1	2	3	4	5	6	7
Starch	+	−	−	v	+	+	+
Amygdalin	−	+	−	−	−	−	−
Cellobiose	−	−	−	+/−	+/−	+/−	+/−
D-Fructose	−	+/−	+/−	+/−	+/−	+/−	+/−
D-Galactose	−	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+
Lactose (bovine origin)	+	+	+	+/−	+/−	+	+
Maltose	+	+	+	+	+	+	+
D-Mannose	−	−	−	−	+	−	−
Melibiose	+	+	+	+	−	+	+
Raffinose	+	+	+	+	−	+	+
D-Ribose	−	+	+	+	+	+	−
Sucrose	+	+	+	+	+/−	+	+
Turanose	−	−	−	+/−	−	+/−	+/−
D-Xylose	−	−	+	+	+	+	−
Esculin iron citrate	−	+	+	+	+	+	+
Gentibiose	−	−	−	−	+	−	−
Glycogen	+	−	−	v	+	+	+
L-Arabinose	−	−	+	+	+	+	−
L-Fucose	+	−	−	v	−	−	−
Xylitol	−	−	−	−	+/−	−	−
pH 3.5	−	−	−	−	−	−	−
pH 4	−	−	−	−	−	−	−
pH 4.5	+	+	−	+	−	−	−
pH 5	+	+	−	+	−	+	+/-
pH 6	+	+	+	+	+	+	+
pH 6.5	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+
pH 8.5	+	+	+	+	+	+	+
10 °C	−	−	−	−	−	−	−
15 °C	−	−	−	−	−	−	−
20 °C	−	−	−	−	−	−	−
30 °C	+	+	+	+	+	+	+

Continued

Table 3. Continued

Characteristic	1	2	3	4	5	6	7
37 °C	+	+	+	+	+	+	+
46 °C	+	+/−	+/−	+	+/−	+	+
Anaerobic growth	+	+	+	+	+	+	+
Microaerophilic growth	+	+	+	+	+	+	+
Aerobic growth	−	+/−	+/−	−	−	−	−

the Genome-to-Genome Distance Calculator (GGDC) was used to estimate the DNA–DNA hybridization (DDH) values (Table S4). The highest sequence identity value of GSD1FS^T was 88.17% when compared to the chromosome sequences of *Bifidobacterium cuniculi* LMG 10738^T, which belongs to the *B. pseudolongum* phylogenetic group. The estimated DDH value below 70% between these two taxa (DDH estimate generalized linear model-based, 27.8%) led to the proposal of GSD1FS^T as representing a novel bifidobacterial species.

In order to evaluate the phylogenetic relationship of GSD1FS^T with other currently recognized bifidobacterial (sub)species, we investigated the core genome of members belonging to the *B. pseudolongum* phylogenetic group, allowing the reconstruction of a genomic-based tree, i.e. phylogenomic tree (Fig. 2) [19, 21, 22]. Accordingly, a comparative genome analysis involving the chromosome sequences of the currently recognized 13 (sub)species belonging to the *B. pseudolongum* phylogenetic group [23], as well as the genome sequences of GSD1FS^T, was carried out. The ORF content of each genome was organized in functional gene clusters using the gene family method of the PanGenome Analysis Pipeline (PGAP) [24], involving the Basic Local Alignment Search Tool (BLAST; E value cutoff of 1×10–10 and 50% identity across at least 80% of sequence lengths). Sequences were then clustered into protein families, using a graph theory-based Markov clustering algorithm [25]. This *in silico* analysis identified 642 clusters of orthologous groups that are shared by the genomes used in this study. Therefore, the *B. pseudolongum* phylogenetic group core genome sequences, based on the concatenation of 619 protein sequences with the exclusion of paralogs, identified in each chromosome sequence were used to build a phylogenomic core tree (Fig. 2).

The rod-shaped cell morphology of strain GSD1FS^T culture was observed using phase-contrast microscopy (Figs S11 and S12). The cell-wall peptidoglycan composition was examined according to Schumann [26] and was found to comprise L-Orn(Lys)–L-Ala(L-Ser)–L-Ala$_2$.

Fermentation characteristics of GSD1FS^T and the most related species, and at the same time the most common species for dogs (*B. animalis* subsp. *animalis* DSM 20104^T, *B. animalis* subsp. *lactis* DSM 10140^T, *B. anseris* LMG 30189^T, *B. pseudolongum* subsp. *pseudolongum* DSM 20099^T, *B. pseudolongum* subsp. *globosum* DSM 20092^T and *B. cuniculi* DSM 20435^T), were determined using API 50 CHL kit (bioMérieux) according to manufacturer's instructions. Strains were tested in biologically independent triplicates. The obtained results were evaluated based on colour and pH change. The fermentation profile of strain GSD1FS^T corresponded closely to the profiles of the tested strains of *B. pseudolongum* and *B. animalis*; *B. anseris* and *B. cuniculi* differed more (Table 3). Other tested cultivation characteristics were similar between strain GSD1FS^T and the other tested type strains. The cultivation temperature of 37 °C, at pH 6–6.5 and under anaerobic conditions, appeared to be optimal.

Strain GSD1FS^T seemed to be highly genotypically and phenotypically similar to *B. animalis* and *B. pseudolongum*, species which are known to be frequent bifidobacterial species of the dog microbiota.

DESCRIPTION OF *BIFIDOBACTERIUM CANIS* SP. NOV.

Bifidobacterium canis [ca'nis. L. gen. n. *canis* of a dog; common scientific name of a domestic dog (*Canis lupus* f. *familiaris*)].

Cells are Gram-stain-positive, non-motile, non-sporulating and F6PPK-positive. Cells grow under anaerobic and microaerophilic conditions. Colonies grown on the surface of modified Wilkins–Chalgren agar are white and circular, while embedded colonies are white and elliptical. The diameter of each colony ranges from 1.0 to 1.5 mm after 48 h growth on modified Wilkins–Chalgren agar. Cells are able to grow from 30–46 °C, yet are unable to grow at 10, 15, and 20 °C. Moreover, cells grow at pH 4.5–8.5. Optimal conditions of growth occur at pH 6.5 and 37 °C. Cells grown in WSP broth are rods of various shapes, forming a branched structure with 'Y' at both sides.

Fermentation profiles show that strain GSD1FS^T is able to grow well and produce acids on D-galactose, D-glucose, maltose, melibiose, raffinose, D-ribose, sucrose, D-xylose, esculin iron citrate, and L-arabinose. Variable or weak growth was found on cellobiose, D-fructose, lactose, turanose, glycogen, L-fucose and starch. Furthermore, cells are not able to utilize amygdalin, arbutin, D-adonitol, D-arabinose, D-arabitol, D-fucose, D-mannitol, D-mannose, melezitose, D-sorbitol, D-tagatose, trehalose, D-xylanose, dulcitol, erythritol, gentibiose, glycerol, inositol, inulin, L-arabitol, L-rhamnose, L-sorbose, L-xylose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside,

methyl β-D-xylopyranoside, *N*-acetylglucosamine, potassium-2-ketogluconate, potassium-5-ketogluconate, potassium gluconate, salicin and xylitol.

The peptidoglycan type is L-Orn(Lys)–L-Ala(L-Ser)–L-Ala_2.

The type strain, GSD1FS^T (=DSM 105923^T=LMG 30345^T=CCM 8806^T), was isolated from a faecal sample of a 3 weeks old German Shepherd dog (*Canis lupus* f. *familiaris*). The DNA G+C content is 57.5mol%. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number WNLP00000000 and the accession number of the 16S rRNA gene sequence is MG028631.

Funding information
This study was supported by grant 420 MVČR VI20172020088 provided by the Ministry of the Interior of the Czech Republic.

Conflicts of interest
The authors declare that there are no conflicts interest.

Ethical statement
The sampling of the dog faeces was made during routine life situations. All procedures involving animals adhered to recommendations in the 'Guide for the Care and Use of Animals' by the Czech University of Life Sciences Prague. The protocol of the experiment was approved by the Czech Central Committee for the Protection of Animals (Permit number: 63479/2016-MZE-17214).

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4.3 The bifidobacterial distribution in the microbiome of captive primates reflects parvorder and feed specialization of the host

Modrackova N, Stovicek A, Burtscher J, Bolechova P, Killer J, Domig KJ, Neuzil-Bunesova V. 2021b. The bifidobacterial distribution in the microbiome of captive primates reflects parvorder and feed specialization of the host. Scientific Reports **11**.

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The bifidobacterial distribution in the microbiome of captive primates reflects parvorder and feed specialization of the host

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Bifidobacteria, which commonly inhabit the primate gut, are beneficial contributors to host wellbeing. Anatomical differences and natural habitat allow an arrangement of primates into two main parvorders; New World monkeys (NWM) and Old World monkeys (OWM). The number of newly described bifidobacterial species is clearly elevated in NWM. This corresponds to our finding that bifidobacteria were the dominant group of cultivated gut anaerobes in NWM, while their numbers halved in OWM and were often replaced by *Clostridiaceae* with sarcina morphology. We examined an extended MALDI-TOF MS database as a potential identification tool for rapid screening of bifidobacterial distribution in captive primates. Bifidobacterial isolates of NWM were assigned mainly to species of primate origin, while OWM possessed typically multi-host bifidobacteria. Moreover, bifidobacterial counts reflected the feed specialization of captive primates decreasing from frugivore-insectivores, gummivore-insectivores, frugivore-folivores to frugivore-omnivores. Amplicon sequencing analysis supported this trend with regards to the inverse ratio of Actinobacteria and Firmicutes. In addition, a significantly higher diversity of the bacterial population in OWM was found. The evolution specialization of primates seems to be responsible for *Bifidobacterium* abundance and species occurrence. Balanced microbiota of captive primates could be supported by optimized prebiotic and probiotic stimulation based on the primate host.

Primates are a remarkably species-rich order of mammals[1]. Their anatomical differences and natural habitat allow their arrangement into two main parvorders. Platyrrhines, referred as New World monkeys (NWM), naturally occurring in central and southern American tropical and subtropical regions and catarrhines (Cercopithecoidea and Hominoidea), referred as Old World monkeys (OWM), coming from tropical, subtropical, and temperate regions of Asia and Africa[2]. Many primate species are endangered[3] and they must be protected. The conservation of threatened species is a complex and demanding process consisting of elaborated breeding programs and providing of habitat sanctuaries in captive or semi-captive centres, e.g. zoological institutions or forest corridors, which usually aim to reintroduce these species back into their natural habitat[4,5]. Unfortunately, health of captive animals is compromised by emerging recurring infectious diseases mediated through human contact and habitat modifications, and frequent therapeutic doses of antibiotics[6,7]. Furthermore, captive breeding modifies primate microbiome[8,9] and these microbial shifts can substantially affect the host's health[10,11]. Captivity may be also associated with the occurrence of potential pathogens that further increase risk of gut dysbiosis and illnesses[12,13].

Besides exposure to antibiotics, dietary changes and lifestyle seem to be significant modifiers of primate gut microbiome[14]. To provide nutritional needs, primates consume a wide range of plants and animal tissues and possess a variety of dietary specializations based on the proportion of individual dietary components (one type of feed component is dominating only), such as generalist feeders or omnivores, e.g. Cercopithecines[15–18].

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The generalist feeders are adapted to receive a wide variety of feed components, depending on their availability in the environment, and can be split by extension into groups classified by their majority feeds, with seasonal variation in their ratio. Among the generalist feeders, there are highly frugivorous representatives, namely chimpanzees[19–21]. If there is a lack of fruit, these primates consume various feed reaching from plants, nectar, seeds to insects or small vertebrates. Such a feeding type can be described as frugivore-omnivore. If the preferred fruit is less available during the season, primates start to consume more leaves or other parts of plants. Gibbons, for instance pursue this frugivore-folivore feeding strategy[22–25]. Similarly, if the second major component alongside fruit consists of insects, primates are classified as frugivores-insectivores (tamarins)[26–30]. Exudates are another important nutritious feed apart from fruit and animal prey. Some primate species have specially adapted teeth for gum intake[31,32]. This type of feeding behaviour is called gummivory. It is typical for marmosets and can either be dominant or it can be supplemented with insect intake[33–37]. These primates are counted in the gummivore-insectivore feeding category.

Unfortunately, despite all efforts of breeders, composition of diet in captivity does not completely simulate that in the wild, in which primates consume a wider range of natural local plant and animal species[9,38]. In addition, Amato et al.[39] points out the seasonality that is one of the natural phenomena of wild primate diet, which results in a seasonal variation of the gut microbiome.

Deviation from the natural lifestyle in captivity and associated modified diet led to a shift of native gut microbiota and a decrease in diversity and an increased relative abundance of Bacteroidetes[8,9,40,41]. Furthermore, the microbiome of captive primates displays a reduction in Actinobacteria compared to wild groups[14,41]. However, members of the *Bifidobacteriaceae* family (Actinobacteria phylum) are important natural commensals, which possess a large amount of adaptive genes involved in carbohydrate metabolism[42–44]. Moreover, bifidobacteria can utilize a diverse range of dietary carbohydrates that escape degradation in the upper parts of the intestine[45].

Although, bifidobacterial abundance in the gut microbiota usually decreases with host aging[46], bifidobacteria persist throughout the lifespan of primates[42,47]. Moreover, their abundance is confirmed by a recent boom of novel bifidobacterial species isolation and characterization connected to primate gut environment[48–50].

However, data are still scarce about the bifidobacterial microbiota of captive primates and the impact of different diets. We hypothesize that the quantity and species richness of bifidobacteria in captive primates are affected by the host and feed classification. The aim of this study was to compare the quantity and diversity of bifidobacteria in faecal microbiota of captive NWM and OWM by a combination of culture-dependent and culture-independent approaches.

Results

Cultivation analysis. *Quantification of cultivable bifidobacteria in primate faecal samples.* Non-selective and selective media were used for the quantification of anaerobic bacteria and bifidobacteria in primate faecal samples (FS) (Table 1). Cultivation counts significantly varied between the NWM and the OWM in each monitored group of bacteria (Fig. 1A, Suppl. Tab. 1). NWM harboured significantly more anaerobic bacteria (9.52 ± 0.62 log CFU g^{-1}) compared to OWM (8.62 ± 0.71 log CFU g^{-1}) ($t_{(50)} = 4.84$, $p = 1.30e\text{-}05$). A similar statistically significant trend was found in colony forming units cultivated on WPS-MUP medium intended for bifidobacteria that reached 8.91 ± 1.38 log CFU g^{-1} in the NWM compared to 7.02 ± 0.93 log CFU g^{-1} in the OWM ($t_{(50)} = 5.87$, $p = 3.50e\text{-}07$). In case of FS with lower numbers of bifidobacteria and the presence of clostridia, this medium was not sufficiently selective also allowing the growth of clostridia[51,52]. Consequently, a notably greater statistically significant difference was detected between primate parvorders on more selective WSP-NORF medium with bifidobacterial counts of 8.57 ± 2.13 log CFU g^{-1} for the NWM and 4.32 ± 2.04 log CFU g^{-1} for the OWM ($Z = 5.17$, $p = 2.38e\text{-}07$). Cultivation differences between parvorders were also reflected within the primate sub-division based on feed specialization (Fig. 1B). Specifically, gummivore-insectivores (9.63 ± 0.71 log CFU g^{-1}) and frugivore-insectivores (9.46 ± 0.57 log CFU g^{-1}) exhibited significantly higher numbers of anaerobic bacteria including bifidobacteria than frugivore-folivores (8.72 ± 0.49 log CFU g^{-1}) and frugivore-omnivores (8.60 ± 0.78 log CFU g^{-1}). The same statistically significant trend was found on WPS-MUP in gummivore-insectivores (8.99 ± 1.19 log CFU g^{-1}) and frugivore-insectivores (9.19 ± 0.96 log CFU g^{-1}) in comparison with frugivore-folivores (6.58 ± 1.05 log CFU g^{-1}) and frugivore-omnivores (7.07 ± 1.01 log CFU g^{-1}), as well as on WSP-NORF in gummivore-frugivores (8.46 ± 2.34 log CFU g^{-1}) and frugivore-insectivores (9.15 ± 0.76 log CFU g^{-1}) compared to frugivore-folivores (4.29 ± 1.95 log CFU g^{-1}) and frugivore-omnivores (4.22 ± 2.13 log CFU g^{-1}) (Supplementary S5).

Bifidobacterial species detected by MALDI-TOF MS. Bacterial colonies with variable cultivation characteristics from bifidobacterial selective media were isolated for further identifications (Suppl. Tab. 1). From a total of 326 isolates, 210 were F6PPK-positive bifidobacteria and the remaining 116 isolates (isolated mainly from WSP-MUP) were F6PPK-negative gas producing clostridial rods or cells with sarcina morphology. All F6PPK-positive strains were also identified with MALDI-TOF MS using an expanded custom database for bifidobacterial identification. 54% of the strains ($n = 112$) were assigned to 18 different bifidobacterial species, 36% ($n = 76$) were assigned only to the *Bifidobacterium* genus, and 11% ($n = 22$) were not identified reliably (Fig. 2A, C).

B. parmae, B. imperatoris/saguini, and *B. ramosum* were the most frequently identified species in the NWM, whereas *B. dentium* and *B. catenulatum/pseudocatenulatum* were most common in the OWM. Interestingly, *B. adolescentis* was equally represented in both primate parvorders. A more diverse species representation of bifidobacteria was found in the NWM (14 spp.) compared to the OWM (5 spp.). Genus-level assignment and the presence of not reliable identifications (NRI) was mainly detected in the NWM. Related presumed species compliance and the closest match of *Bifidobacterium* spp. strains was found predominantly with *B. parmae* and *B. stellenboschense* in the NWM, and *B. angulatum/merycicum* in the OWM (Fig. 2B).

ID	Primate host species	Family	Parvorder	Zoo	Feed category
PR1	Common Marmoset (*Callithrix jacchus*)	*Calitrichidae*	NWM	Pilsen, CZ	Gummivore-insectivore
PR2	Common Marmoset (*Callithrix jacchus*)	*Calitrichidae*	NWM	Pilsen, CZ	Gummivore-insectivore
PR3	White-faced Saki (*Pithecia pithecia*)	*Pitheciidae*	NWM	Pilsen, CZ	Frugivore-omnivore
PR4	Emperor Tamarin (*Saguinus imperator*)	*Calitrichidae*	NWM	Pilsen, CZ	Frugivore-insectivore
PR5	Moustached Tamarin (*Saguinus mystax*)	*Calitrichidae*	NWM	Pilsen, CZ	Frugivore-insectivore
PR6	Brown-mantled Tamarin (*Saguinus fuscicollis*)	*Calitrichidae*	NWM	Pilsen, CZ	Frugivore-insectivore
PR7	Red-handed Tamarin (*Saguinus midas*)	*Calitrichidae*	NWM	Pilsen, CZ	Frugivore-insectivore
PR8	Red-handed Tamarin (*Saguinus midas*)	*Calitrichidae*	NWM	Pilsen, CZ	Frugivore-insectivore
PR9	Emperor Tamarin (*Saguinus imperator*)	*Calitrichidae*	NWM	Pilsen, CZ	Frugivore-insectivore
PR10	Silvery Marmoset (*Mico argentatus*)	*Calitrichidae*	NWM	Pilsen, CZ	Gummivore-insectivore
PR11	Silvery Marmoset (*Mico argentatus*)	*Calitrichidae*	NWM	Pilsen, CZ	Gummivore-insectivore
PR15	Silvery Marmoset (*Mico argentatus*)	*Calitrichidae*	NWM	Pilsen, CZ	Gummivore-insectivore
PR16	Emperor Tamarin (*Saguinus imperator*)	*Calitrichidae*	NWM	Pilsen, CZ	Frugivore-insectivore
PR17	Emperor Tamarin (*Saguinus imperator*)	*Calitrichidae*	NWM	Pilsen, CZ	Frugivore-insectivore
PR18	Chimpanzee (*Pan troglodytes*)	*Hominidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR19	Northern White-cheeked Gibbon (*Nomascus leucogenys*)	*Hylobatidae*	OWM	Liberec, CZ	Frugivore-folivore
PR20	Golden-bellied Mangabey (*Cercocebus chrysogaster*)	*Cercopithecidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR21	Diana Monkey (*Cercopithecus diana*)	*Cercopithecidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR22	Lion-tailed Macaque (*Macaca silenus*)	*Cercopithecidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR23	Hamadryas Baboon (*Papio hamadryas*)	*Cercopithecidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR24	Pygmy Marmoset (*Cebuella pygmaea*)	*Calitrichidae*	NWM	Liberec, CZ	Gummivore-insectivore
PR26	Cotton-top Tamarin (*Saguinus oedipus*)	*Calitrichidae*	NWM	Liberec, CZ	Frugivore-insectivore
PR27	Golden Lion Tamarin (*Leontopithecus rosalia*)	*Calitrichidae*	NWM	Olomouc, CZ	Frugivore-insectivore
PR28	Common Marmoset (*Callithrix jacchus*)	*Calitrichidae*	NWM	Olomouc, CZ	Gummivore-insectivore
PR29	Patas Monkey (*Erythrocebus patas*)	*Cercopithecidae*	OWM	Olomouc, CZ	Frugivore-omnivore
PR30	Goeldi's Marmoset (*Callimico goeldii*)	*Calitrichidae*	NWM	Olomouc, CZ	Frugivore-insectivore
PR31	White-headed Marmoset (*Callithrix geoffroyi*)	*Calitrichidae*	NWM	Olomouc, CZ	Gummivore-insectivore
PR32	White-headed Marmoset (*Callithrix geoffroyi*)	*Calitrichidae*	NWM	Olomouc, CZ	Gummivore-insectivore
PR33	Moustached Tamarin (*Saguinus mystax*)	*Calitrichidae*	NWM	Olomouc, CZ	Frugivore-insectivore
PR34	Patas Monkey (*Erythrocebus patas*)	*Cercopithecidae*	OWM	Olomouc, CZ	Frugivore-omnivore
PR35	Silvery Marmoset (*Mico argentatus*)	*Calitrichidae*	NWM	Olomouc, CZ	Gummivore-insectivore
PR36	Campbell's Mona Monkey (*Cercopithecus campbelli*)	*Cercopithecidae*	OWM	Dvur Kralove, CZ	Frugivore-omnivore
PR37	Putty-nosed Monkey (*Cercopithecus nictitans*)	*Cercopithecidae*	OWM	Dvur Kralove, CZ	Frugivore-omnivore
PR38	Northern Talapoin Monkey (*Miopithecus oguensis*)	*Cercopithecidae*	OWM	Dvur Kralove, CZ	Frugivore-omnivore
PR39	De Brazza´s Monkey (*Cercopithecus neglectus*)	*Cercopithecidae*	OWM	Pilsen, CZ	Frugivore-omnivore
PR40	Northern White-cheeked Gibbon (*Nomascus leucogenys*)	*Hylobatidae*	OWM	Liberec, CZ	Frugivore-folivore
PR41	Chimpanzee (*Pan troglodytes*)	*Hominidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR42	Chimpanzee (*Pan troglodytes*)	*Hominidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR43	Chimpanzee (*Pan troglodytes*)	*Hominidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR44	Chimpanzee (*Pan troglodytes*)	*Hominidae*	OWM	Liberec, CZ	Frugivore-omnivore
PR45	Patas Monkey (*Erythrocebus patas*)	*Cercopithecidae*	OWM	Olomouc, CZ	Frugivore-omnivore
PR46	Southern Yellow-cheeked Gibbon (*Nomascus gabriellae*)	*Hylobatidae*	OWM	Olomouc, CZ	Frugivore -folivore
PR47	Southern Yellow-cheeked Gibbon (*Nomascus gabriellae*)	*Hylobatidae*	OWM	Olomouc, CZ	Frugivore -folivore
PR51	Southern Yellow-cheeked Gibbon (*Nomascus gabriellae*)	*Hylobatidae*	OWM	Bratislava, SK	Frugivore -folivore
PR52	Green Monkey (*Chlorocebus sabaeus*)	*Cercopithecidae*	OWM	Hodonin, CZ	Frugivore-omnivore
PR55	Hamlyn's Monkey (*Cercopithecus hamlyni*)	*Cercopithecidae*	OWM	Bojnice, SK	Frugivore-omnivore
PR56	Roloway Monkey (*Cercopithecus roloway*)	*Cercopithecidae*	OWM	Bojnice, SK	Frugivore-omnivore
PR57	Lesser Spot-nosed Monkey (*Cercopithecus petaurista*)	*Cercopithecidae*	OWM	Bojnice, SK	Frugivore-omnivore
PR58	Southern Yellow-cheeked Gibbon (*Nomascus gabriellae*)	*Hylobatidae*	OWM	Bojnice, SK	Frugivore-folivore

Continued

ID	Primate host species	Family	Parvorder	Zoo	Feed category
PR59	Northern White-cheeked Gibbon (*Nomascus leucogenys*)	*Hylobatidae*	OWM	Liberec, CZ	Frugivore-folivore
PR60	Northern White-cheeked Gibbon (*Nomascus leucogenys*)	*Hylobatidae*	OWM	Liberec, CZ	Frugivore-folivore
PR61	Golden Lion Tamarin (*Leontopithecus rosalia*)	*Calitrichidae*	NWM	Olomouc, CZ	Frugivore-insectivore

Table 1. List of monkey hosts kept in zoological gardens**.** General information about primate taxonomy, parvorder and feed classification. Primate general feeders (n = 52) were grouped to 4 individual feed categories based on proportion of dominating feed components – frugivore-omnivore, frugivore-folivore, frugivore-insectivore, and gummivore-insectivore. Zoo, zoological garden; CZ, Czechia; SK, Slovakia; NWM, New World monkey; OWM, Old World monkey.

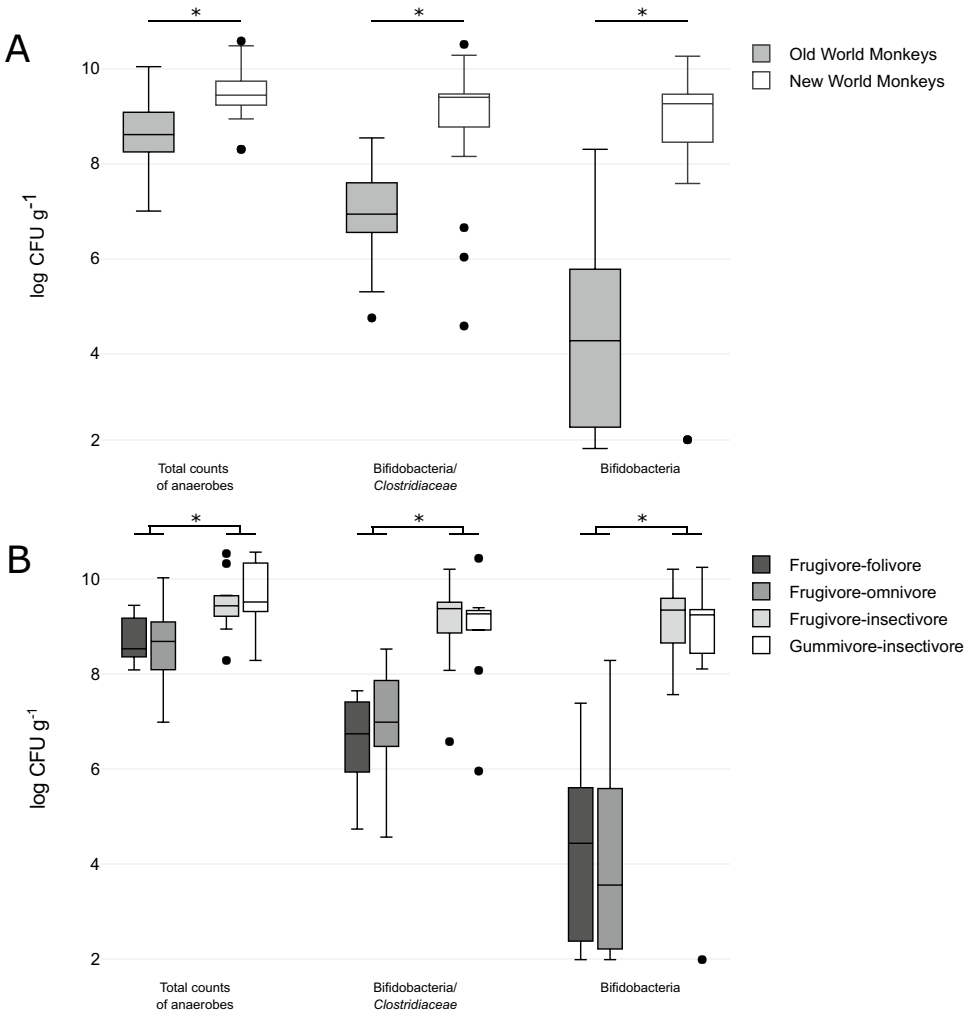

Figure 1. Quantification of cultivable anaerobic bacteria (log CFU g^{-1}) in primate faecal samples. (**A**) Cultivation counts of bacteria per parvorder: New World monkeys (n = 24) and Old World monkeys (n = 28). (**B**) Cultivation counts of bacteria per feed category: frugivore-folivore (n = 8), frugivore-omnivore (n = 21), frugivore-insectivore (n = 13), gummivore-insectivore (n = 10). Asterisks (*) denote statistically significant differences as determined by t-test and ANOVA ($p < 0.05$).

Species assignment verification by 16S rRNA gene sequencing. The MALDI-TOF MS identification was verified by 16S rRNA gene Sanger sequencing of 46 strains, whose selection was randomly executed based on determined species frequency and identification scores (Suppl. Tab. 2). Due to similar MALDI-TOF MS spectra, some bifidobacterial species could not be distinguished. However, the results consistently suggest an assignment to either of the two indistinguishable species. These indistinguishable groups were merged to produce consistent

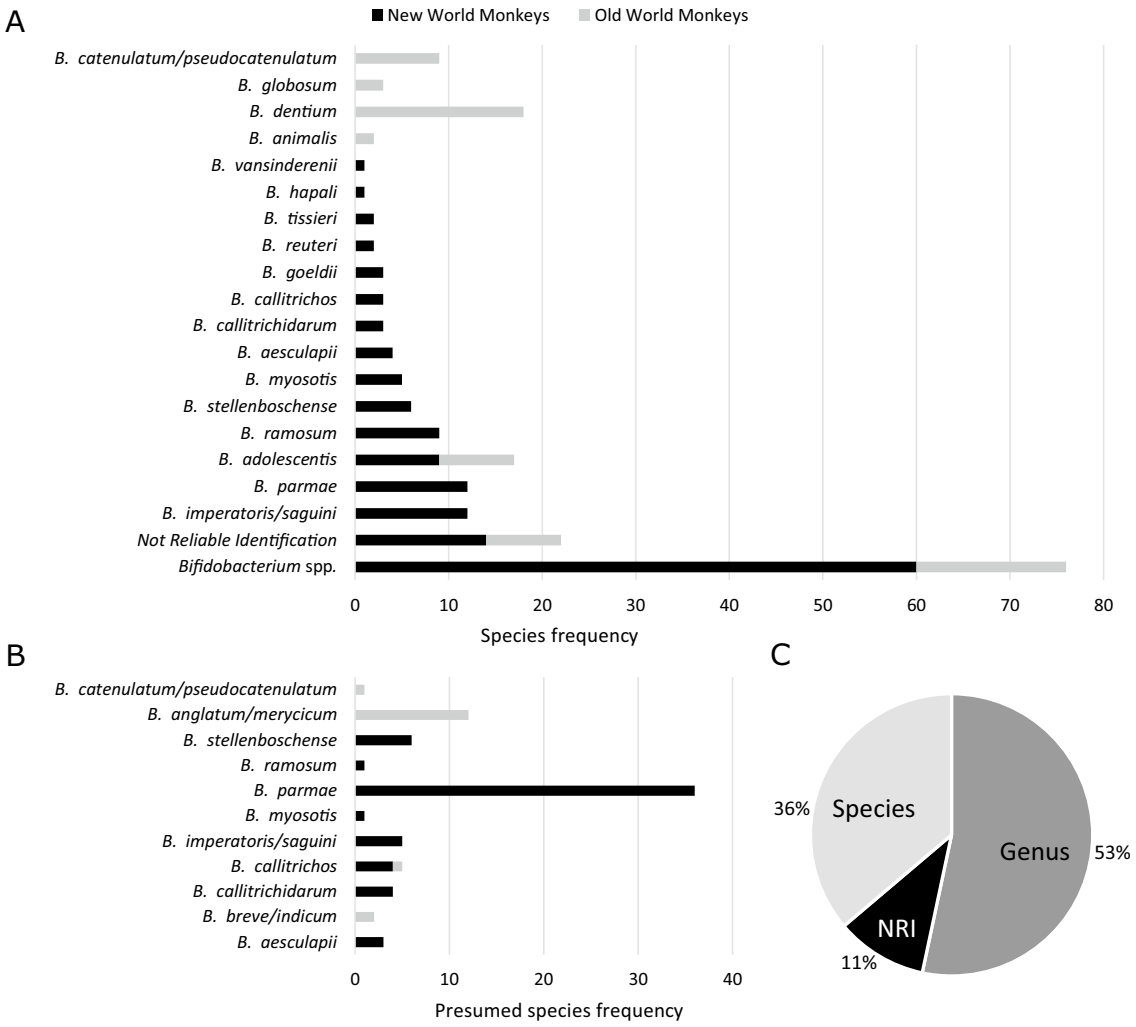

Figure 2. MALDI-TOF MS identification of primate bifidobacterial isolates. (**A**) MALDI-TOF MS identification of 210 bifidobacterial strains. (**B**) The closest probable species match of isolates with unambiguous genus MALDI-TOF MS identification (*Bifidobacterium* spp). (**C**) Proportion of species assignment, genus assignment and not reliable identification (NRI) of bifidobacterial isolates. Bruker criteria (scores) for assignment: 0.000–1.699 not reliable identification, 1.700–1.999 probable genus identification, 2.000–3.000 genus and species identification.

MALDI-TOF MS assignment and are presented together in the following groups: *B. angulatum/merycicum*, *B. breve/indicum*, *B. catenulatum/pseudocatenulatum*, and *B. imperatoris/saguini*.

An agreement between the MALDI-TOF MS species assignment and the sequencing of 16S rRNA gene was confirmed for 38 strains. Only 3 strains were identified differently by the two methods. Namely, strain N127 identified as *B. faecale* by 16S rRNA gene sequencing was mistaken for *B. adolescentis* by the MALDI-TOF MS, *B. imperatoris* for NRI (N40), and PEBJ_s for *B. imperatoris/saguini* (N50). Interestingly, mentioned strain N50 together with N74, N94, N97, and N115, exhibiting MALDI-TOF MS NRI score (< 1.69), were considered potential novel species of bifidobacteria. In addition, this sample set also contained 5 problematic strains (N16, N70, N81, N119, and N125), whose 16S rRNA gene sequencing failed repeatedly and thus their MALDI-TOF MS identity was not confirmed.

Amplicon sequencing analysis. Amplicon sequencing profiles of the FS collected from captive primates were determined by sequencing the V4 region of the 16S rRNA gene. The bacterial α-diversity was expressed as an ASV count, Shannon diversity, and Pielou evenness. Each diversity parameter between the primate parvorders was significantly higher in the OWM (ASV count: $F_{(1,50)} = 30.47$, $p = 1.21 \times 10^{-6}$, $\eta^2 = 0.379$, Shannon: $F_{(1,50)} = 38.01$, $p = 1.21e\text{-}07$, $\eta^2 = 0.432$, Pielou: $F_{(1,50)} = 38.41$, $p = 1.08e\text{-}07$, $\eta^2 = 0.434$) (Fig. 3A). Similarly, there was a significantly higher diversity, evenness, and richness of the bacterial population in the frugivore-folivores and frugivore-omnivores compared to the frugivore-insectivores and gummivore-insectivores (Fig. 3B, Supplementary S1).

Microbial community shifts were found between the NWM and OWM parvorders. The relative abundance of phylum Actinobacteriota (W = 13) and Campylobacterota (W = 12) was significantly higher in the NWM

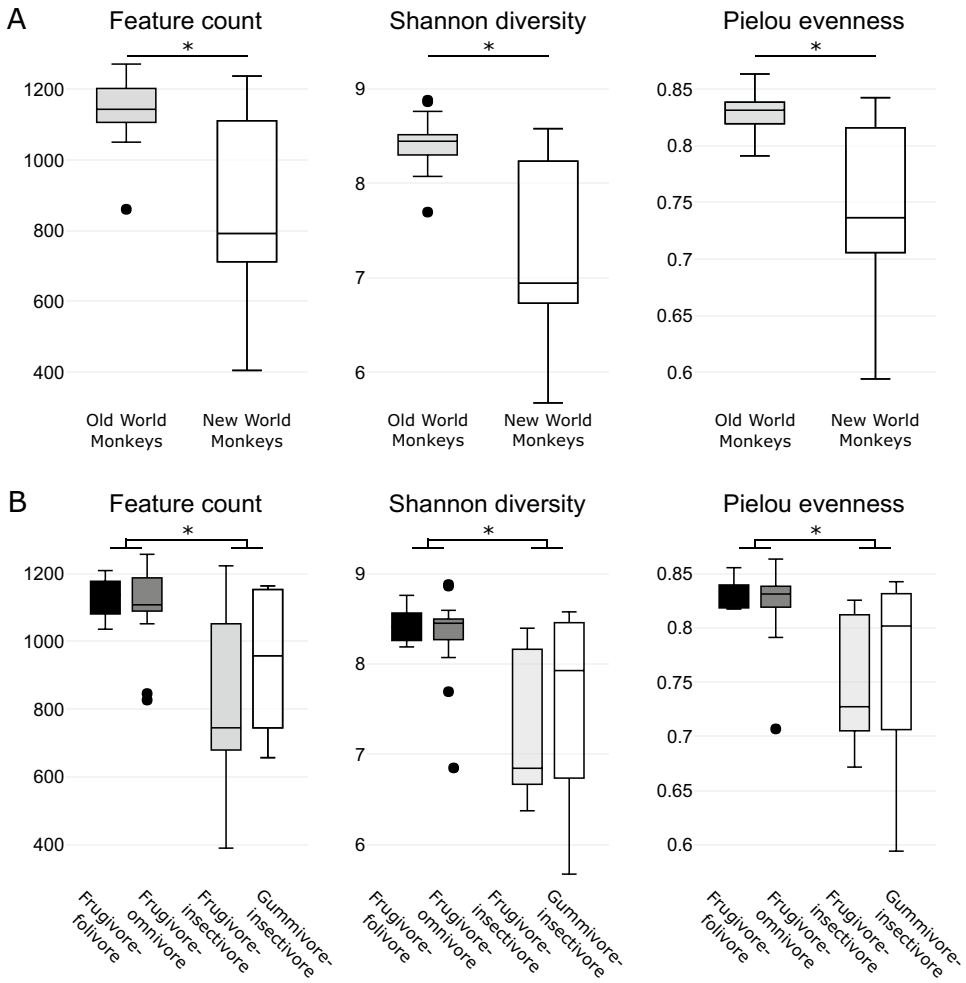

Figure 3. Alfa-diversity of primate gut microbiota. (**A**) Bacterial α-diversity per parvorder: New World monkeys and Old World monkeys. (**B**) Bacterial α-diversity per feed category: frugivore-folivore, frugivore-omnivore, frugivore-insectivore, gummivore-insectivore. Asterisks (*) denote adjusted statistically significant differences (adj. $p < 0.05$).

compared to the OWM as confirmed by the ANCOM statistics. Meanwhile, the phylum Firmicutes showed an opposite trend, which was however not statistically significant (Fig. 4A). The difference in the Actinobacteriota can be attributed specifically to the family *Bifidobacteriaceae* which was significantly higher in the NWM (16%) compared to the OWM (3%) (W = 139) (Fig. 4B, Supplementary S2). These findings corroborate the cultivation results.

The proportion of the phylum Actinobacteriota was statistically significantly different among the primate feed categories (W = 12) and it was the highest in the frugivore-insectivores followed by gummivore-insectivores, frugivore-omnivores, and frugivore-folivores. Furthermore, the phyla Proteobacteria and Campylobacterota were statistically significantly different among the categories (W = 8, W = 7 respectively) with a notable enrichment of both in the frugivore-insectivores followed by gummivore-insectivores compared to frugivore-omnivores and frugivore-folivores. Moreover, although not statistically significant, the opposite ratio of Firmicutes was also detected (Fig. 4C). The relative abundance of *Bifidobacteriaceae* was significantly different across the categories (W = 140); the most abundant in the frugivore-insectivores (19%), followed by the gummivore-insectivores (12%), the frugivore-omnivores (4%), and the frugivore-folivores (2%) (Fig. 4D).

By comparing 16S rRNA gene sequencing data of cultured bifidobacterial isolates with the results of 16S rRNA gene amplicon sequencing of the FS, we retrospectively confirmed the presence of 18 species within this sample set. *B. callitrichos* and *B. parmae* were significantly enriched in the NWM (W = 38, W = 38 respectively), followed by *B. saguini* (W = 34), *B. biavatii* (W = 34), *B. vansinderenii* (W = 34), *B. aerophilum* (W = 34), unclassified II ASV (W = 33) and sp. I ASV (W = 30). The distribution of bifidobacteria corresponds to the proportion of *Bifidobacteriaceae* among the total relative bacteria in samples normalized to 42 134 sequences/sample in the primate feed categories as determined by amplicon sequencing.

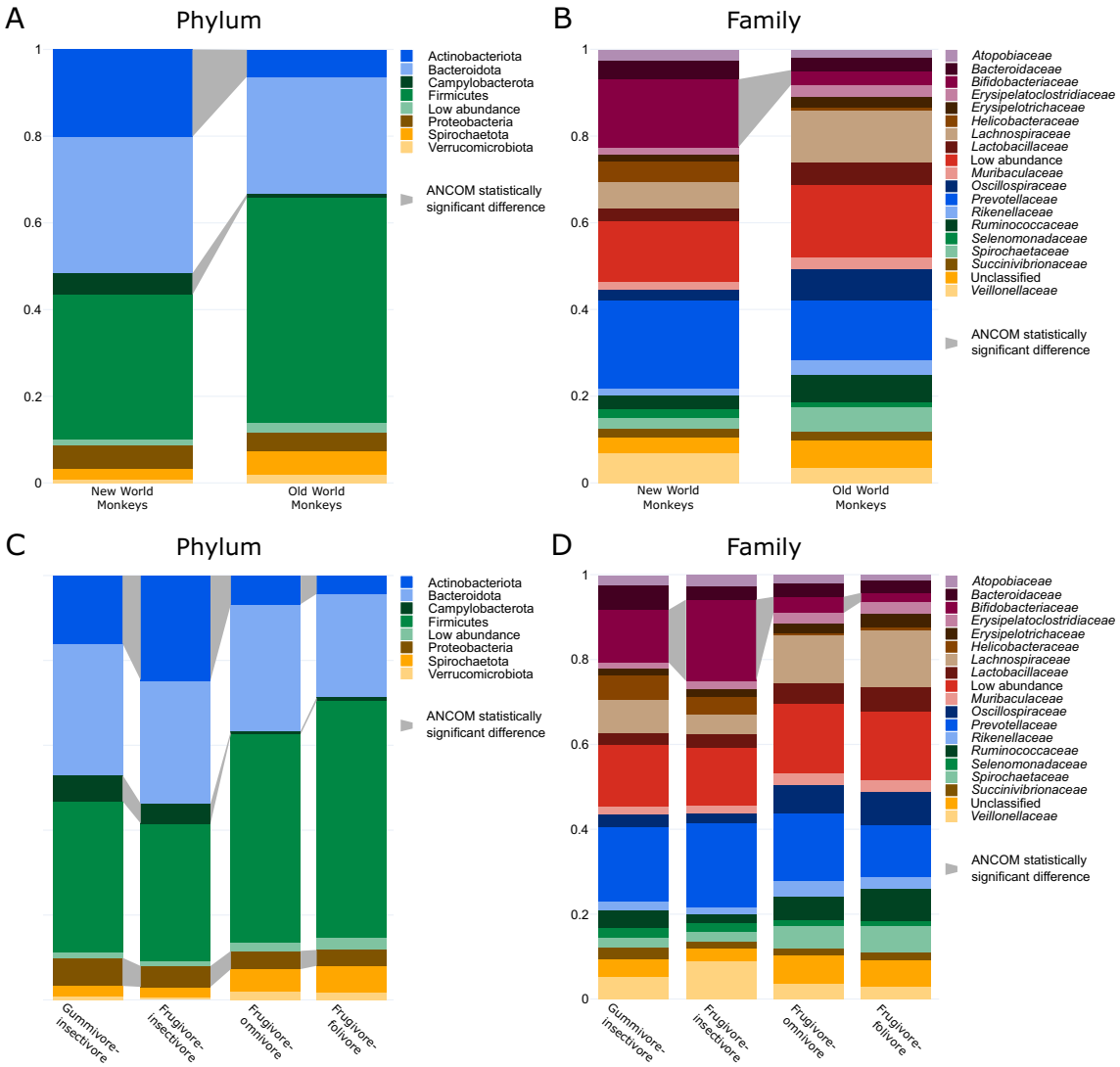

Figure 4. Relative abundance of primate gut microbiota. (**A**) Relative abundance of bacteria within parvorders on phylum level. (**B**) Relative abundance of bacteria within parvorders on family level. (**C**) Relative abundance of bacteria within feed categories on phylum level. (**D**) Relative abundance of bacteria within feed categories on family level. ANCOM statistically significant differences are denoted with grey links.

Discussion

Dynamic microbial communities aid the living and surviving of animals in changing environmental conditions, including habitat degradation, captive breeding, and diet. If microbial balance of the host is disturbed and dysbiosis occurs, there is a presumption of disease development[5,53,54]. Among others, commensal microorganisms, such as bifidobacteria, play a crucial role in maintaining the gut homeostasis[55–57]. Bifidobacterial diversity and adaptation are connected to their hosts and environments with possession of specific genomic traits[58–60] which includes primates[42].

Two independent approaches, cultivation with subsequent MALDI-TOF MS identification and amplicon sequencing of the V4 region of the 16S rRNA gene, were used to analyse the microbiome composition and the prevalence of bifidobacterial species in primate gut microbiota. NWM are a significant source of cultivable bifidobacteria with average counts of 10^8 CFU g^{-1} of faeces compared to the OWM with four orders of magnitude lower counts. Interestingly, although no health complications were evident, FS of primate individuals with reduced or undetectable cultivation counts of bifidobacteria contained *Clostridiaceae*, mainly displaying sarcina morphology. This was mainly observed in individuals belonging to the OWM parvorder (Suppl. Tab. 1). Spore-forming bacteria identified as *Sarcina ventriculi* (syn. *Clostridium ventriculi*) were previously isolated also from primates without apparent health problems[61–63]. Although they are considered pathogens[64], this may indicate sarcina as common bacteria of the primate gut microbiota. In the gut of NWM, the abundance of sarcina is probably decreased by the presence of bifidobacteria, which exhibit potential to hamper growth of clostridia[65–67]. The inverse ratio and balancing of the bifidobacteria and clostridia are typically described in the gut microbiome of infants[68–70].

Timperio et al.[71] showed that the screening of bacterial isolates from environmental samples can be performed efficiently, quickly, and inexpensively using MALDI-TOF MS and should be refined by implementation of environmental strains into the database. Within our study, the use of an extended custom database for MALDI-TOF MS allowed reliable species differentiation and identification of wild bifidobacterial isolates. Higher species diversity was observed in NWM. Interestingly, the multi-host species *B. adolescentis* was present among most screened captive primates. In OWM *B. dentium* and *B. catenulum/pseudocatenulatum*, that are common species of the human gut microbiota, as well as *B. adolescentis,* were found[72]. Lugli et al.[42] detected *B. adolescentis* and *B. dentium* in OWM as well, and indicated possible joint development and evolutionary relatedness. In contrast, NWM exhibited the presence of cultivable bifidobacteria mainly with primate origin. Interestingly, Brown et al.[73] pointed out that marmoset bifidobacteria are closely related to those in tamarins. Furthermore, we found that bifidobacterial species variability in NWM significantly exceeds that in OWM. Furthermore, we hereby confirmed that we can re-isolate recently described primate *Bifidobacterium* spp. also from primate species with various captive locations other than those from which bifidobacteria were originally isolated.

Moreover, MALDI-TOF MS screening allowed us to identify 5 potential novel species of bifidobacteria isolated from tamarins that were confirmed by 16S rRNA gene sequencing. That indicates primate gut as a promising environment for the discovery of novel species of bifidobacteria[42,48,50]. To achieve an accurate identification of potential novel species, a combination with other methods, such as sequencing of phylogenetic markers[74–76], multi-locus sequence typing[77], and genome sequencing[78], should be included.

The significantly lower species richness and high relative abundance of bifidobacteria in NWM compared to OWM was confirmed by sequencing of the V4 region of 16S rRNA gene. The relative abundance of *Bifidobacteriaceae* reached 16% in the NWM and only 3% in the OWM. The same trend was also detected for *Prevotellaceae* and *Veillonellaceae*. In particular, marmosets and tamarins exhibited 32% bifidobacterial abundance compared to 0.03% in the OWM[42]. This high relative bifidobacterial proportion in adult marmosets could be a consequence of their housing as family groups and their constant subjection to the gut microbiota of other individuals[73]. Conversely, *Lachnospiraceae*, *Oscillospiraceae*, *Ruminococcaceae*, and *Spirochaetaceae* showed an opposite trend with high abundances in OWM. Interestingly, we showed that the captive NWM have high relative levels of bifidobacteria, which is similar to what they display in the wild[47,79–81]. It indicates that NWM gut is a rich bifidobacterial environment that is also supported by other studies[42,82,83]. In contrast to our results in captive individuals, some microbiome studies point to a slightly increased bifidobacterial relative proportions in wild OWM as well[84,85]. Although the captivity was previously described as a factor influencing the presence of Actinobacteria in the primate gut microbiome[14,41], our results suggest that it is probably not as strong as the affiliation to the primate parvorder, which seems to be considerably more significant.

Primate gut microbiome seems to be significantly modified by dietary changes of the host species and geography[14]. Frugivore-insectivores and gummivore-insectivores possessed significantly more abundant *Bifidobacteriaceae* compared to frugivore-omnivores and frugivore-folivores. Interestingly, if insects constitute an important component of the diet, bifidobacteria are highly abundant. Ecologically beneficial symbionts leading to host evolutionary dependence have been previously described in other animal taxa, such as sap-feeding insects, which generate essential amino acids exclusively for their microbial symbionts[86]. Bifidobacteria are known as a commensal bacterial group of insects with social life[87], whereas the importance of insects in the diet of primates in relation to bifidobacterial occurrence remains unclear.

Although captive feeding inevitably modifies primate gut microbiome to decreased diversity, the feed optimization could improve the animals health condition[40]. In contrast to Amato et al.[88], who state that the host phylogeny is stronger driver in shifts of microbial composition than the diet and geographic location, our results suggest that both diet and the host itself affect the microbiome composition, especially the relative abundance of *Bifidobacteriaceae*. Moreover, it is important to mention, that the diet of captive animals usually includes fruits, vegetables, and leaves that may not completely match the available components present in the wild. In addition, the natural microbiota reflects diet seasonality and location that may affect trophic interactions in the gastrointestinal tract of the host[89,90].

Clayton et al.[91] confirms that modified diet in captive primates is related to the alteration of microbiome composition and host health. Captive primate individuals susceptible to health disorders may show clinical signs including chronic diarrhoea, weight loss, lethargy, cardiac disease, and poor reproductive success[9,12,92,93]. Therefore, it is necessary to further monitor the relationship between the microbiome, diet, and the health of captive primates[40]. Microbiota modulation is an effective and affordable strategy for host health support of threatened animals[5]. Therefore, applicable mitigation strategies such as optimized dietary[40] and prebiotic interventions[94] could be pursued towards supporting balanced microbiota in captive primates. Moreover, probiotic supplementation with focus on bifidobacteria, that naturally colonize primate guts, can be a further promising approach[42,43,95]. Furthermore, this may provide a potential approach in human probiotic intervention. Due to the ever-decreasing diversity of the human microbiome through diet and antimicrobial intake, the microbiome of originally living evolutionarily close relatives has the potential to design a probiotic that is no longer part of the human microbiota and could have the potential to strengthen health[96]. Probiotic intervention should be optimized according to the gut microbiota composition and should be supported by appropriately selected prebiotic stimulation in synbiotic mixtures for long-term maintenance of balanced microbiome and host health.

Materials and methods

Sampling and cultivation analysis. Faecal samples of primate hosts (n = 52) belonging to two parvorders, NWM (n = 24) and OWM (n = 28), were preliminary screened for quantitative content of cultivable bifidobacteria. The list of primate hosts and classification into parvorders and feed category is shown in Table 1. Sampling was performed in zoological gardens in Dvur Kralove, Hodonin, Liberec, Olomouc, Pilsen

(all Czechia), Bojnice, and Bratislava (both Slovakia) between 2017–2019. FS were collected in tubes containing dilution buffer (5 g L^{-1} tryptone, 5 g L^{-1} nutrient broth No. 2, 2.5 g L^{-1} yeast extract (all Oxoid, Basingstoke, UK), 0.5 g L^{-1} L-cysteine, 1 mL L^{-1} Tween 80 (both Sigma-Aldrich, St. Louis, Missouri, USA), 30% glycerol (VWR, Radnor, Pennsylvania, USA), and glass pearls for homogenization. Media were prepared in an oxygen-free carbon dioxide environment[97] and then sterilized. After sampling, the tubes were stored at –20 °C and within the 14 days transported into the laboratory for analysis. Then, decimal serial dilutions of FS were spread on the following media.

Wilkins-Chalgren Anaerobe Agar was supplemented with 5 g L^{-1} GMO-Free Soya Peptone (both Oxoid), 0.5 g L^{-1} L-cysteine, and 1 mL L^{-1} Tween 80 to determine total counts of anaerobic bacteria (WSP medium). Moreover, two selective media were used for bifidobacterial quantification and isolation: WSP-NORF (WSP agar supplemented with 100 mg L^{-1} of mupirocin, 200 mg L^{-1} of norfloxacin (both Oxoid), and 1 mL L^{-1} of acetic acid (Sigma-Aldrich)[52]) and WSP-MUP (WSP agar supplemented with 100 mg L^{-1} of mupirocin and 1 mL L^{-1} of acetic acid[98]). All plates were incubated anaerobically using GENbag anaer (bioMérieux, Craponne, France) at 37 °C for 2 days.

Isolation and culture identifications. Based on variable cultivation characteristics, the isolation of colonies from selective media and consecutive sub-cultivation was performed in tubes containing WSP broth under anaerobic conditions[97] at 37 °C for 1 day. Whether a culture belonged to *Bifidobacterium* spp. was verified by fructose-6-phosphate phosphoketolase (F6PPK) test with cetrimonium bromide for cell disruption according to Orban and Patterson (2000)[99]. Subsequently, bifidobacterial isolates were identified to the species level using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF MS) with ethanol-formic acid extraction procedure with HCCA matrix solution according to the manufacturer's instructions (Bruker Daltonik GmbH, Bremen, Germany). An extended custom database (based on Bruker Biotyper software tools), which included 50 additional bifidobacterial species in addition to the already available entries, was used for identification. An overview about the database entries is provided in Suppl. Tab. 3. Stock cultures of bifidobacteria were stored at –80 °C in 30% glycerol.

Selected isolates (n = 46) were further identified by 16S rRNA gene amplicon sequencing. DNA was isolated from freshly grown bifidobacterial cultures in WSP broth using PrepMan Ultra™ (Applied Biosystems, Waltham, Massachusetts, USA) according to manufacturer's instructions and stored at –20 °C. Primers 285F (5′-GAGGGTTCGATTCTGGCTCAG-3′) and 261R (5′-AAGGAGGTGATCCAGCCGCA-3′) were used for PCR amplification of nearly the full 16S rRNA gene according to Kim et al.[100] enabling longer reads and thus more precise taxonomic identification. PCR products were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Norcross, Georgia, USA) and sequenced by Eurofins Genomics (Ebersberg, Germany). The obtained sequences were processed in Chromas Lite 2.5.1 (Technelysium Pty Ltd., Tewantin, Australia), BioEdit[101] with ClustalW algorithm[102], and compared with 16S rRNA gene sequences in BLAST rRNA/ITS (https://blast.ncbi.nlm.nih.gov/) and EZBioCloud databases (https://www.ezbiocloud.net/). The sequences of the 16S rRNA gene are available in the GenBank database under accession numbers MN736337–341, 342, 344–346, 348, 350–355, 357–360, 363–365, 367, 369, 372–378, 381, 387–388, 390–392, and MW678772–74.

Amplicon sequencing analysis. Total genomic DNA was extracted from 200 mg of FS using the Fast DNA SPIN kit for soil (MP Biomedicals, Illkirch-Graffenstaden, France) according to the manufacturer's instructions. The DNA concentration of each sample was determined using the Qubit 1X dsDNA HS Assay Kit (Invitrogen, Paisley, UK) and a Qubit fluorometer. Subsequent library preparation and sequencing were performed by NovoGene (Cambridge, UK). As amplicon sequencing method supports only shorter fragments, the V4 region of the 16S rRNA gene (300 bp fragments) was amplified using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and a Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, Massachusetts, USA). The library was prepared using the NEB Next® UltraTM DNA Library Prep Kit for Illumina and paired-end 250 bp sequencing was performed using the NovaSeq machine (Illumina, San Diego, California, USA). The resulting sequences were submitted to the NCBI database with the accession number ERP128111. Amplicon sequence variants (ASV) were obtained using the DADA2 pipeline (bioconductor-dada2 v1.16.0)[103] and Silva non redundant database v138[104] (Supplementary S3) with custom manual species assignment. The depth of sequencing of the resulting data was normalized by rarefaction to the lowest sequencing depth (42 134 sequences/sample) and a relative abundance on several taxonomic levels in different variable groups were explored (Supplementary S4). Total bacterial diversity was expressed as Shannon entropy[105], the population richness was expressed as simple feature or ASV counts and the evenness was expressed as Pielou's index[106].

Statistical analyses. Counts of bacterial colonies in log CFU g^{-1} within the parvorders and feed categories are shown as boxplots. The normality of data was evaluated by Shapiro–Wilk W test ($\alpha = 0.05$). Differences in bacterial counts were assessed using a Mann–Whitney U Test ($\alpha = 0.05$) within the parvorders, and a one-way ANOVA within the feed categories ($\alpha = 0.05$) using STATISTICA software (StatSoft, Prague, Czechia) and Microsoft Office Professional Plus 2016.

To detect differentially abundant taxa between the sample categories, the ANCOM statistical test[107] was used from the package skbio v0.5.2 (scikit-bio.org). The one-way F statistics from the scipy package v1.4.1[108] was used to determine that statistical significance with $\alpha = 0.05$. Several categories of the data were explored on both the Phylum and Family level. Furthermore, the bifidobacterial sub-population was extracted for each sample and the differentially abundant species were calculated. Statistically significant results are presented in form of boxplots (Supplementary S2).

The statistical significance of difference in means of the diversity metrics (Shannon, Pielou, and ASV counts) was assessed using the ordinary least squares method coupled with a pairwise T-test. The data was Box-Cox transformed and the resulting residuals were normally distributed (Jarque-Berra and Omnibus probability > 0.05), however, the groups were highly heteroskedastic. To mitigate this, we have used the ordinary least square method from the package statsmodels v0.11.0[109] with MacKinnon and White's heteroscedasticity robust standard errors[110] (Supplementary S1).

Ethical approval. The sampling of primate faeces was performed during routine daily procedures. All procedures involving animals adhered to recommendations of the "Guide for the Care and Use of Animals" by the Czech University of Life Sciences Prague. The research conducted herein was approved by Ethic and Animal Care Committee of the Czech University of Life Sciences Prague (protocol number: CZU/17/19) and was performed in accordance with the relevant guidelines and regulations. All zoological institutions have rigorous standards for animal welfare and are accredited by the European Association of Zoos and Aquaria. The research adhered to the legal requirements of the Czech Republic for the ethical treatment of nonhuman primates as well as in accordance with European Directive 2010/63/EU.

Received: 12 April 2021; Accepted: 9 July 2021
Published online: 27 July 2021

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Acknowledgements

Research was funded by the European Regional Development Fund-Project, "Centre for the investigation of synthesis and transformation of nutritional substances in the food chain in interaction with potentially harmful substances of anthropogenic origin: comprehensive assessment of soil contamination risks for the quality of agricultural products" [No: CZ.02.1.01/0.0/0.0/16_019/0000845], by the cooperation project (8J19AT028)

between Austria and the Czech Republic – BMBWF and Ministry of Education, Youth and Sports of the Czech Republic, and by METROFOOD-CZ research infrastructure project [MEYS Grant No: LM2018100] including access to its facilities. The authors thank Tamara Rudavsky and Cristina Ukowitz for their valuable assistance in laboratory experiments. We also thank the zoos in Dvur Kralove, Hodonin, Liberec, Olomouc, Pilsen, Bojnice, and Bratislava for their kind cooperation. The MALDI-TOF-MS (Bruker Biotyper) was kindly provided by the EQ-BOKU VIBT GmbH and the BOKU Core Facility Food & Bio Processing.

Author contributions

N.-B.V. designed experiments. M.N. and N.-B.V. carried out experiments. M.N. and S.A. participated at data analysis, interpretation, and visualization of results. M.N., N.-B.V., and S.A. drafted the manuscript. B.P. and K.J. were involved in the writing up of the manuscript. B.J. and D.K.J. participated at data evaluation and revised the manuscript critically. The study was supervised by N.-B.V. and D.K.J. All authors reviewed the manuscript and approved the version to be published.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-94824-y.

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4.4 Prebiotic potential of natural gums and starch for bifidobacteria of variable origins

Modrackova N, Makovska M, Mekadim C, Vlkova E, Tejnecky V, Bolechova P, Bunesova V. 2019b. Prebiotic potential of natural gums and starch for bifidobacteria of variable origins. Bioactive Carbohydrates and Dietary Fibre **20**:100199.

Bioactive Carbohydrates and Dietary Fibre 20 (2019) 100199

Contents lists available at ScienceDirect

Bioactive Carbohydrates and Dietary Fibre

journal homepage: http://www.elsevier.com/locate/bcdf

Prebiotic potential of natural gums and starch for bifidobacteria of variable origins

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ARTICLE INFO

Keywords:
Bifidobacterium
Origin
Prebiotic
Substrate
Natural gums
Starch
SCFAs

ABSTRACT

Complex polysaccharide polymers of natural origin are widely used as natural food thickeners. They are useful for their technological properties, and at the same time they are biodegradable and safe for consumers. In addition, natural food thickeners, such as natural gums (NGs) and starch, may also represent suitable potentially prebiotic substrates for probiotic genera such as the genus *Bifidobacterium*. Therefore, 204 bifidobacterial strains of 60 species and subspecies were tested for their ability to utilize 6 NGs (locust bean, guar, tragacanth, arabic, xanthan, and karaya gums) and starch.

Here we observed that the ability to utilize these substrates as a single carbon source is species and strain specific trait reflecting the host origin and diet. The utilization was evaluated based on the pH change, metabolite formation, and detection of viable bifidobacterial counts. In conclusion, 114 strains of human and animal origin (37 bifidobacterial species and subspecies) were able to utilize starch. Compared to that, mostly bifidobacteria of the animal origin were able to utilize a wider range of available natural substrates compared to the human bifidobacteria. In total, 29 strains were able to use NGs (10 species and subspecies). Most often used locust bean, guar, tragacanth, and arabic gums represent possible prebiotic sources for bifidobacteria in animal nutrition, ideally in synbiotic applications. Natural food thickeners were found to be useful potential prebiotics. However, a suitable combination with probiotic *Bifidobacterium* strains is required.

1. Introduction

Natural food thickeners are predominantly complex polysaccharide polymers with a hydrocolloid nature and the capability of gel formation and emulsion stabilization. These are mainly natural gums (NGs) and starches. Their application can affect the binding of water and structural properties of modified products. For this reason, the NGs, such as locust bean (E 410), guar (E 412), tragacanth (E413), arabic (E 414), xanthan (E 415), and karaya (E 416) gums, are widely used also as emulsifiers, stabilizers, foaming agents, and edible coatings and films (Bashir, Warsi, & Sharma, 2016; FAO/WHO, 2017; Mirhosseini & Amid, 2012; Saha, Tyagi, Gupta, & Tyagi, 2017). NGs have advantages over the synthetic additives, since they are chemically inert, nontoxic, less expensive, biodegradable, and widely available. They can also be modified in different ways to obtain tailor-made materials for drug delivery systems and thus can compete with the available synthetic excipients (Choudhary & Pawar, 2014). These features are used in the encapsulation and administration of probiotic bacteria (Eratte et al., 2015; Weinbreck, Bodnár, & Marco, 2010). For supporting of these probiotic bacteria can be used prebiotics, which are defined as substrates that are selectively used by host's microorganisms at the site of action with health benefit of the host; without previous target host enzyme degradation (Gibson et al., 2017). NGs are considered to be potential substrates for probiotic bacteria such as bifidobacteria, lactobacilli and lactococci (Mudgil, Barak, Patel, & Shah, 2018; Salavati Schmitz & Allenspach, 2017), their fermentation has impact on the faecal microbiota and short chain fatty acid production (Alarifi, Bell, & Walton, 2018), and their prebiotic properties are documented (Calame, Weseler, Viebke, Flynn, &

Abbreviations: NGs, natural gums; SCFAs, short chain fatty acids.

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https://doi.org/10.1016/j.bcdf.2019.100199
Received 18 February 2019; Received in revised form 9 September 2019; Accepted 11 September 2019
Available online 12 September 2019

Siemensma, 2008; Slavin, 2013).

Bifidobacteria are commensal microorganisms with probiotic nature, which are strictly anaerobic or facultative anaerobic pleomorphic rods. They occur in many ecological habitats and belong to phylum *Actinobacteria* and family *Bifidobacteriaceae* (Biavati & Mattarelli, 2015, pp. 1–57; Bottacini, van Sinderen, & Ventura, 2017). A variety of bifidobacterial species have been isolated from warm-blooded mammals including human and various animals; presently, the novel described species are mainly from primates (Michelini et al., 2018; Modesto et al., 2018). Nevertheless, their occurrence also includes insects living in social life (Killer et al., 2011; Praet et al., 2015), fermented milk products (Delcenserie et al., 2013; Laureys, Cnockaert, De Vuyst, & Vandamme, 2016), and sewage (Scardovi & Trovatelli, 1974). Bifidobacteria have a long history of safe use (Abe et al., 2010; Doron & Snydman, 2015), and their presence is associated with the health benefits of the host (Awasti et al., 2016; Hidalgo-Cantabrana et al., 2017). For instance, strain *B. animalis* subsp. *lactis* BB12 (Merenstein et al., 2015) and species *B. adolescentis, B. bifidum, B. breve* and *B. longum* are considered safe as addition to food and feed (Ricci et al., 2017). Bifidobacterial genome encodes enzymes that are required for the metabolism of complex carbohydrates, and the ability to utilize these specific polysaccharides depends on the species and origin of bifidobacteria (Bunešová, Joch, Musilová, & Rada, 2017; Milani et al., 2016).

We hypothesized that natural food thickeners could be possible sources of potential prebiotics for bifidobacteria of different species and origins. The aim of this study was to test the utilization of NGs and starch by bifidobacterial strains of human, animal, insect, sewage, fermented milk, probiotic and infant formula product origin.

2. Materials and methods

2.1. Bacterial strains

A total of 60 bifidobacterial species and subspecies with 204 individual strains were selected for testing. The selected strains were comprised of type and collection strains from DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*, Germany), CCM (*Czech Collection of Microorganisms*, Czechia), BCCM/LMG (*Belgian Coordinated Collections of Microorganisms*, Belgium), and wild strains originating from various ecological niches stored in collection of the Department of Microbiology, Nutrition and Dietetics (CULS, Czechia) are listed in Table 1. Most strains have been identified and characterized previously (references in Table 1) and other new strains were identified using MALDI-TOF MS (ethanol-formic acid extraction procedure and mixed with HCCA matrix, both according to the manufacturer's instructions; Bruker Daltonik GmbH, Germany) and sequencing 16S rRNA (B. J. Kim, Kim, Yun, & Kook, 2010). Stock cultures of bifidobacteria were maintained at −80 °C in 30% glycerol. The strains were cultivated in Wilkins-Chalgren broth (Oxoid, UK) supplemented with soya peptone (5 g/L, Oxoid), L-cysteine (0.5 g/L, Sigma-Aldrich, USA), and tween 80 (1 mL/L, Sigma-Aldrich) in an oxygen-free carbon dioxide environment at 37 °C for 48 h. To prepare working cultures, the freshly grown cultures were sub-cultivated overnight and used as an inoculum for future testing.

2.2. Testing of ability to utilize prebiotic substrates by bifidobacteria

Bifidobacterial strains were investigated for their ability to utilize different sources of potentially prebiotic substrates. Locust bean gum from *Ceratonia siliqua* seeds, guar gum, tragacanth gum, gum arabic, xanthan gum from *Xanthomonas campestris*, karaya gum, and starch from wheat (all, Sigma-Aldrich) were used as a single carbon source in the final concentration at 2 g/L in API 50 CHL medium (API medium) prepared according to manufacturer's instructions (BioMérieux, France). The pH of the medium was adjusted to 7.5 before subsequent sterilization by autoclaving at 121 °C for 1 h. The pH after sterilization was between 6.57 depending on the used substrate.

2.2.1. 96-Well microtiter plate assay

Bifidobacterial ability to utilize given substrates was performed in 96-well microtiter plates in triplicates. Overnight bifidobacterial cultures were washed and resuspended in bifidobacterial phosphate buffer; K_2HPO_4 1.2 g/L, KH_2PO_4 0.333 g/L (both Lachner, Czechia). Bifidobacteria (20 µl) were inoculated into 180 µl API medium supplied with the tested substrate. Thus prepared microtiter plates were incubated under anaerobic conditions created by the GENbag anaer (bioMérieux, France) at 37 °C for 48 h. Glucose (Penta, Czechia) was used as a positive control for bifidobacteria that were able to grow under given assay. A negative control without substrate addition was also used. The growth and utilization of the carbohydrate source was judged by colour change of the medium from purple to yellow. In addition, the purity and morphology of positive cultures were checked by phase-contrast microscopy.

2.2.2. Hungate tube assay

Based on the same principle, the second test was repeated in larger volumes. API media supplied with the carbohydrate substrates (mentioned above in Section 2.2.) were prepared and filled with 10 mL into Hungate tubes using the roll-tube technique in an oxygen-free carbon dioxide environment and were then sterilized. The overnight cultures of bifidobacteria were inoculated in volume of 0.5 mL (5% w/v) and cultivated at 37 °C for 48 h. After that, the colour changes and morphology of the cultures were observed and pH values were measured using pH tester Checker Plus (Hanna Instruments, Czechia). For the selected strains, metabolite formation was assessed via ion-exchange chromatography (IC) with suppressed conductivity. Moreover, the growth ability of bifidobacterial strains on the tested substrates was detected by desk plate method, both as outlined below.

2.3. Microbiological plate assay

After cultivation on tested substrates in Hungate tubes, the bifidobacterial counts of the selected strains were evaluated using the plate method according to Musilova, Rada, Vlkova, Bunesova, and Nevoral (2015) on Wilkins-Chalgren Anaerobe Agar supplemented with soya peptone (5 g/L), L-cysteine (0.5 g/L), and tween 80 (1 mL/L). The plates were cultivated in anaerobic conditions created by the GENbag anaer (bioMérieux, France) at 37 °C for 48 h.

2.4. Measurement of short chain fatty acid and lactate levels

After testing the bifidobacterial ability to utilize the provided substrates *in vitro*, primary metabolites such as lactate, acetate and formate, in the chosen samples were determined by IC with suppressed conductivity using a ion chromatograph ICS 1600 (Dionex, Sunnyvale, CA, USA) equipped with an IonPac AS11-HC (Dionex) guard and analytical columns. The eluent comprised 1–35.5 mM KOH (Erba Lachema) with a gradient of 1–65 min and a flow rate of 1 mL/min. The ASRS 300 (−4 mm) suppressor (Dionex) was used to suppress the eluent conductivity. Chromatograms were processed and evaluated using the software Chromeleon 6.80 (Dionex). Standards were prepared from 1 g/L anion or cation concentrates (Analytika, Czechia; Inorganic Ventures, USA) and deionised water (conductivity < 0.055 µS/cm; Adrona, Latvia) in the range of 0.1–40 mg/L. Limits of detection were calculated from a 3:1 signal-to-noise ratio (Shabir, 2003) and for determined anions and cations the limits of detection.

3. Results

3.1. Bifidobacterial utilization of natural substrates

In total, 204 strains of 60 species and subspecies of bifidobacteria

Table 1
Utilization of natural substrates by bifidobacteria.

Species	Strain code	Origin	n	Substrates							
				GLU	STA	LOC	GUAR	TRAG	ARAB	XANT	KARA
B. actinocoloniiforme	DSM 22766	Bumblebee digestive tract	1	+	–	–	–	–	–	–	–
B. adolescentis	DSM 20083	Intestine of adult	1	+	+	–	–	–	–	–	–
	DSM 24849	Human faeces	1	+	+	–	–	–	–	–	–
	DSM 20087	Bovine rumen	1	+	+	–	–	–	–	–	–
	CCM 4987	Human intestine	1	+	–	–	–	–	–	–	–
	(B3/8, 1MBif, MB9/3)[1]	Human faeces	3	+	–	–	–	–	–	–	–
	(B1/1, B2/7, B9/11)[1]	Human faeces	3	+	+	–	–	–	–	–	–
	JK7	Infant faeces	1	+	+	–	–	–	–	–	–
	(1/3A, 1/5)[2]	Elephant faeces	2	+	+	–	–	–	–	–	–
B. aesculapii	**DSM 26737**	**Marmoset faeces**	1	+	+	-	-	+	+	-	-
B. angulatum	DSM 20098	Human faeces	1	+	+	–	–	–	–	–	–
	PR21/6E	Diana Monkey	1	+	–	–	–	–	–	–	–
	PR34/7A	Patas monkey	1	+	+	–	–	–	–	–	–
	PR23/5D	**Hamadryas baboon**	1	+	+	+	+	-	-	-	-
B. animalis subsp. *animalis*	DSM 20104	Rat faeces	1	+	–	–	–	–	–	–	–
	DSM 26074	Wildtype mouse	1	+	–	–	–	–	–	–	–
	(L3, L4, L5, L7)[3]	Lamb faeces	4	+	+	+	+	–	–	–	–
	(023II, 3/10, 012II1, 1/11, 805III2, 813P2)[4]	Calf faeces	6	+	+	+	+	–	+	–	–
	L1[3]	Lamb faeces	1	+	+	+	+	–	+	–	–
	805P4[4]	**Calf faeces**	1	+	+	+	+	+	+	-	-
B. animalis subsp. *lactis*	DSM 10140	Yoghurt	1	+	–	–	–	–	–	–	–
	Danone – BLAC[5]	Milk product	1	+	–	–	–	–	–	–	–
	Nestlé – BLAC[5]	Infant formula	1	+	–	–	–	–	–	–	–
	(ZDK7, ZDK8)[5]	Okapi faeces	2	+	–	–	–	–	–	–	–
	PR36/2NA	Campbell's mona monkey	1	+	–	–	–	–	–	–	–
	MUF1[5], MUF2	Mouflon faeces	2	+	–	–	–	–	–	–	–
	ZDK1[5]	Cameroon sheep faeces	1	+	–	–	–	–	–	–	–
	ZDK4[5]	Barbary sheep faeces	1	+	–	–	–	–	–	–	–
	S7[5,7]	Ovine cheese	1	+	–	–	–	–	–	–	–
	P2N1[5], 3N/1, 10/7C, 11/6A	German shephard dog	4	+	–	–	–	–	–	–	–
	MB4/1[1]	Human faeces	1	+	–	–	–	–	–	–	–
B. asteroides	DSM 20089	Hindgut of honeybee	1	+	–	–	–	–	–	–	–
B. avesanii	DSM 100685	Tamarin faeces	1	+	–	–	–	–	–	–	–
B. biavati	DSM 23969	Tamarin faeces	1	+	–	–	–	–	–	–	–
B. bifidum	DSM 20456	Infant faeces	1	+	–	–	–	–	–	–	–
	DSM 29521	Stool of breastfed infant	1	+	–	–	–	–	–	–	–
	DSM 20082	Intestine of adult	1	+	–	–	–	–	–	–	–
	DSM 20239	Infant faeces	1	+	–	–	–	–	–	–	–
	DSM 20215	Human intestine	1	+	–	–	–	–	–	–	–
	CCM 3762	Human intestine	1	+	–	–	–	–	–	–	–
	(B10/1, BifiX)[1]	Human faeces	2	+	–	–	–	–	–	–	–
	MA1, V33N, MA5, KI300, KI8M	Infant faeces	5	+	–	–	–	–	–	–	–
	NUTRA BONA-BBIF	Probiotic product	1	+	–	–	–	–	–	–	–
B. bohemicum	DSM 22767	Bumblebee digestive tract	1	+	+	–	–	–	–	–	–
B. bombi	DSM 19703	Bumblebee digestive tract	1	+	+	–	–	–	–	–	–
B. boum	DSM 20432	Bovine rumen	1	+	+	–	–	–	–	–	–
	2/3A[2]	Elephant faeces	1	+	+	–	–	–	–	–	–
	P6[6]	Pig faeces	1	+	+	–	–	–	–	–	–
	P3, **P7**	**Pig faeces**	2	+	+	+	+	-	-	-	-
B. breve	DSM 15700	Infant faeces	1	+	+	–	–	–	–	–	–
	VB-TA1[6]	Infant faeces	1	+	+	–	–	–	–	–	–
	BR03, Yakult	Probiotic product	2	+	+	–	–	–	–	–	–
B. callitrichos	DSM 23973	Common marmoset faeces	1	+	–	–	–	–	–	–	–
B. catenulatum	DSM 16992	Human faeces	1	+	–	–	–	–	–	–	–
	CCM 4989	Human faeces, sewage	1	+	–	–	–	–	–	–	–
	(B8/2, B9/1)[1]	Human faeces	2	+	–	–	–	–	–	–	–
	20ToBifN	Infant faeces	1	+	–	–	–	–	–	–	–
	(MB6/2, B3/10)[1]	Human faeces	2	+	+	–	–	–	–	–	–

(*continued on next page*)

Table 1 (*continued*)

Species	Strain code	Origin	n	Substrates							
				GLU	STA	LOC	GUAR	TRAG	ARAB	XANT	KARA
	MA3	Infant faeces	1	+	+	–	–	–	–	–	–
B. choerinum	DSM 20434	Piglet faeces	1	+	+	–	–	–	–	–	–
	(L6, L7, L9, L12, L13)[3], L24	Lamb faeces	6	+	+	–	–	–	–	–	–
	23I2[4]	Calf faeces	1	+	+	–	–	–	–	–	–
B. coryneforme	DSM 20216	Hindgut of honeybee	1	+	–	–	–	–	–	–	–
B. crudilactis	LGM 23609	Raw milk, raw milk cheese	1	+	–	–	–	–	–	–	–
	(S13, S17)[7]	Ovine cheese	2	+	–	–	–	–	–	–	–
B. cuniculi	DSM 20435	Rabbit faeces	1	+	+	–	–	–	–	–	–
B. dentium	DSM 20436	Dental caries	1	+	+	+	+	–	–	–	–
	J36	Infant faeces	1	+	+	+	+	–	–	–	–
	PR18/8A, PR42/7C, PR44/7D	**Chimpanzee**	3	+	+	+	+	-	-	-	-
	PR22/6B	Lion-tailed macaque	1	+	+	+	+	+	–	–	–
B. eulemuris	DSM 100216	Lemur faeces	1	+	–	–	–	–	–	–	–
B. faecale	PR30/8C	Goeldi's marmoset	1	+	+	–	–	–	–	–	–
B. gallicum	DSM 20093	Human faeces	1	+	+	–	–	–	–	–	–
B. gallinarum	DSM 20670	Chicken caecum	1	+	+	–	–	–	–	–	–
B. hapali	DSM 100202	Faeces of baby common marmosets	1	+	–	–	–	–	–	–	–
B. indicum	DSM 20214	Hindgut of honeybee	1	+	+	–	–	–	–	–	–
B. kashiwanohense	DSM 21854	Infant faeces	1	+	–	–	–	–	–	–	–
	BCK-INF	Infant faeces	1	+	+	–	–	–	–	–	–
B. lemurum	DSM 28807	Ring-tailed lemur faeces	1	+	–	–	–	–	–	–	–
B. longum subsp. *infantis*	DSM 20088	Infant faeces	1	+	–	–	–	–	–	–	–
	DSM 20218	Intestine of infant	1	+	–	–	–	–	–	–	–
	DSM 20090	Intestine of infant	1	+	–	–	–	–	–	–	–
	CCM 4990	Human intestine	1	+	–	–	–	–	–	–	–
	KI13a, KI13b, KI81a, KI82	Infant faeces	4	+	–	–	–	–	–	–	–
	Nestlé-BINF	Nestlé infant formula	1	+	–	–	–	–	–	–	–
	NUTRA BONA-BINF	Probiotic product	1	+	–	–	–	–	–	–	–
B. longum subsp. *longum*	DSM 20219	Intestine of adult	1	+	–	–	–	–	–	–	–
	(B1/7, B10/4, B3/5, MB2/1, B10/5, MB6/3, B6/1)[1], KH36, KH37	Human faeces	9	+	–	–	–	–	–	–	–
	B5/8[1]	Human faeces	1	+	+	–	–	–	–	–	–
	KI47	Infant faeces	1	+	–	–	–	–	–	–	–
	MA2	Infant faeces	1	+	+	–	–	–	–	–	–
	T49	Calf faeces	1	+	+	–	–	–	–	–	–
B. longum subsp. *suillum*	DSM 28597	Pig faeces	1	+	–	–	–	–	–	–	–
B. longum subsp. *suis*	DSM 20211	Pig faeces	1	+	+	–	–	–	–	–	–
	(T5/9, 022II)[4]	Calf faeces	2	+	–	–	–	–	–	–	–
B. magnum	DSM 20222	Rabbit faeces	1	+	–	–	–	–	–	–	–
	MOR1/2, MOR2/4	Guinea pig faeces	2	+	–	–	–	–	–	–	–
B. merycicum	CCM 6492	Rumen of cattle	1	+	–	–	–	–	–	–	–
B. minimum	DSM 20102	Sewage	1	+	+	–	–	–	–	–	–
B. mongoliense	DSM 21395	Airag, Mongolian traditional beverage	1	+	+	–	–	–	–	–	–
B. moukalabense	**DSM 27321**	**Wild western lowland gorilla faeces**	1	+	+	+	+	-	-	-	-
B. myosotis	DSM 100196	Baby common marmoset faeces	1	+	+	–	–	–	–	–	–
B. pseudocatenulatum	DSM 20438	Infant faeces	1	+	+	–	–	–	–	–	–
	MA7	Infant faeces	1	+	+	–	–	–	–	–	–
	T63	Calf faeces	1	+	+	–	–	–	–	–	–
	(L15, L17)[3]	Lamb faeces	2	+	+	–	–	–	–	–	–
	PR21/5NC	Diana Monkey	1	+	+	–	–	–	–	–	–
	PR23/4NA	Hamadryas baboon	1	+	+	–	–	–	–	–	–
	PR25/6NB	Black lemur	1	+	+	–	–	–	–	–	–
	PR19/6A	Chinese white-cheeked gibbon	1	+	+	–	–	–	–	–	–
*B. pseudolongum**	D15/4A, **W1**, W11	**Dog faeces**	3	+	+	+	+	-	-	-	-
	T46, T71, T113	Calf faeces	3	+	+	–	–	–	–	–	–

(*continued on next page*)

Table 1 (*continued*)

Species	Strain code	Origin	n	Substrates							
				GLU	STA	LOC	GUAR	TRAG	ARAB	XANT	KARA
	D17/4H	Dog faeces	1	+	+	–	–	–	–	–	–
	P9, P11	Pig faeces	2	+	+	–	–	–	–	–	–
	JU4/3C, JU4/4E, JU4/3B	Spalax faeces	3	+	+	–	–	–	–	–	–
	PR40/5A	Chinese white-cheeked gibbon	1	+	+	–	–	–	–	–	–
	KI9, KI81bB	Infant faeces	2	+	+	–	–	–	–	–	–
B. pseudolongum subsp. *globosum*	DSM 20092	Rumen of cattle	1	+	+	–	–	–	–	–	–
	(L10, L11)[3]	Lamb faeces	2	+	+	–	–	–	–	–	–
	T19	Calf faeces	1	+	+	–	–	–	–	–	–
	BPG-INF	Infant faeces	1	+	+	–	–	–	–	–	–
B. pseudolongum subsp. *pseudolongum*	DSM 20099	Pig faeces	1	+	+	–	–	–	–	–	–
B. psychraerophilum	DSM 22366	Pig caecum	1	+	–	–	–	–	–	–	–
B. pullorum	CCM 20433	Chicken faeces	1	+	+	–	–	–	–	–	–
	CCM 4987	Chicken faeces	1	+	+	–	–	–	–	–	–
B. ramosum	DSM 100688	Adult cotton-top tamarin faeces	1	+	+	–	–	–	–	–	–
B. reuteri	DSM 23975	Common marmoset faeces	1	+	+	–	–	–	–	–	–
B. ruminantium	CCM 6489	Rumen of cattle	1	+	+	–	–	–	–	–	–
B. saeculare	CCM 6531	Rabbit faeces	1	+	–	–	–	–	–	–	–
B. saguini	DSM 23967	Tamarin faeces	1	+	–	–	–	–	–	–	–
B. scardovii	**DSM 13734**	**Human blood**	1	+	-	+	-	-	-	-	-
B. stellenboschense	DSM 23968	Tamarin faeces	1	+	–	–	–	–	–	–	–
B. stercoris	DSM 19555	Human faeces	1	+	+	–	–	–	–	–	–
B. subtile	DSM 20096	Sewage	1	+	+	–	–	–	–	–	–
B. thermoacidophillum subsp. *porcinum*	DSM 17755	Piglet faeces	1	+	+	–	–	–	–	–	–
	MK60	Calf faeces	1	+	+	–	–	–	–	–	–
B. thermoacidophilum subsp. *thermoacidophilum*	DSM 15837	Sewage (tofu)	1	+	+	–	–	–	–	–	–
B. thermophilum	DSM 20210	Piglet faeces	1	+	+	–	–	–	–	–	–
	DSM 20212	Rumen of cattle	1	+	+	–	–	–	–	–	–
	(017III1, 017III2, 025II, 12II1)[4]	Calf faeces	4	+	+	–	–	–	–	–	–
	J7	Wild pig faeces	1	+	–	–	–	–	–	–	–
B. tissieri	**DSM 100201**	**Baby common marmoset faeces**	1	+	-	+	-	-	-	-	-
B. tsurumiense	**DSM 17777**	**Hamster dental plaque**	1	+	+	+	+	-	-	-	-

Bifidobacterial ability to utilize the provided natural substrates was tested in modified API CHL medium supplied with 5 g/L of a prebiotic substrate. The positive reaction (+) was evaluated as a colour change detection of API 50 CHL medium after 48 h of incubation at 37 °C under anaerobic conditions; and is highlighted in the table. Utilization of substrates was verified by measurement of primary metabolites for the selected samples (Table 2). n, number of strains; GLU, glucose; STA, starch; LOC, locust bean gum; GUAR, guar gum; TRAG, tragacanth gum; ARAB, gum arabic, XANT, xanthan gum; KARA, karaya gum; *, the strains reliably identified to the species level. Previously identified and characterized strains: human faeces[1] (Bunešová et al., 2017), elephant faeces[2] (Bunesova, Vlkova, Rada, Killer, & Kmet, 2013), lamb faeces[3] (Bunesova, Vlkova, Killer, Rada, & Rockova, 2012b), calf faeces[4] (Bunesova, Domig, et al., 2012a), *Bifidobacterium animalis* subsp. *lactis* strains[5] (Bunešová et al., 2017), variable bifidobacterial strains characterized by ThrS gene[6] (J. Killer et al., 2018), ovine cheese[7] (Bunesova et al., 2014).

were tested for their ability to utilize 7 different natural substrates, in particular, 6 NGs (locust bean gum, guar gum, tragacanth gum, gum arabic, xanthan gum, and karaya gum), and starch. Utilization ability of the tested strains with positive (+) or negative (−) result is shown in Table 1. NG utilization was found to be species and strain dependent. All bifidobacterial strains were able to use glucose as a control substrate that confirms the given assay; as well as, they were unable to change colour based on the pH indicator and produce metabolites in control API medium without added substrates. Of the 204 tested strains, 29 strains belonging to 10 species and subspecies, were able to utilize a NG as a single carbon source in the media. In contrast, the tested starch was utilized by 114 strains belonging to 37 species and subspecies. The results indicate that starch, locust bean gum, and guar gum were the most frequently fermented substrates in case of tested bifidobacteria. Namely, *B. angulatum* PR23/5D, twelve strains of *B. animalis* subsp. *animalis* (L1, L3, L4, L5, L7, 023II, 3/10, 012II1, 1/11, 805III2, 813P2, 805P4), two strains of *B. boum* (P3, P7), six strains of *B. dentium* (DSM 20436, J36, PR18/8A, PR42/7C, PR44/7D, PR22/6B), *B. moukalabense* (DSM 27321), three strains of *B. pseudolongum* (D15/4A, W1, W11), and *B. tsurumiense* (DSM 17777) utilized locust bean and guar gums, and starch as well. The ability to utilize gum arabic was found in seven strains of *B. animalis* subsp. *animalis* (023II, 3/10, 012II1, 1/11, 805III2, 813P2, 805P4), and to use tragacanth gum in one strain of *B. dentium* PR22/6B. Ultimately, only *B. aesculapii* (DSM 26737), and the strain 805P4 of *B. animalis* subsp. *animalis* were able to utilize both mentioned gums, tragacanth and arabic. Most strains using starch were able to utilize locust bean gum. However, *B. scardovii* (DSM 13734) and *B. tissieri* (DSM 100201) were able to utilize only locust bean gum and control glucose without starch. Comparatively, xanthan and karaya gums were not suitable as a potentially prebiotic source for any of the

tested bifidobacterial strains.

3.2. Bifidobacterial growth and metabolite formation after cultivation

The visual detection of colour changes after cultivation indicated the utilization of the tested substrate. The utilization was confirmed by the detected amounts of monitored metabolites such as lactate, acetate and formate in the selected samples. The short chain fatty acid and lactate production by bifidobacteria, pH values and bifidobacterial counts after cultivation are demonstrated in Table 2. The measured acetate levels corresponded to the pH values and colour changes. The pH values of the samples with the positive reaction varied between 4.5 and 5.5. In that case, the acetate levels were measured around 7–8 mM, mostly two orders of magnitude higher than those at negative controls and samples. The intermediate products, lactate and formate, were detected in lower amounts and varied depending on the tested strain and substrate.

The detected counts of bifidobacteria in the samples capable of growth and utilization of tested substrates attained values 10^7–10^8 CFU/mL. An increase by 3–4 orders was observed compared to the inoculation dose of 10^4 CFU/mL. The counts of bifidobacteria on the negative controls and samples were lesser by two orders (Table 2).

4. Discussion

Bifidobacteria possess sacharolytic type of metabolism with production of acetic and lactic acid in a ratio 3:2. By fermentation of some substrates, ethanol and formic acid can be produced in small amounts (Amaretti et al., 2007; Bottacini et al., 2017; Falony et al., 2009; Palframan, Gibson, & Rastall, 2003). In our study, the biochemical tests with bromcresol purple, as pH indicator, were used for evaluation of the ability of bifidobacteria to utilize natural food thickeners as potentially prebiotic sources. These tests were sufficient to detect the colour changes in the media with starch, which were utilized by 112 bifidobacterial strains, and media with NGs, which were used only by 28 strains. The growth in the medium detected by cultivation was not found fully decisive. The basal API medium without carbon sources such as NGs and starch provided the growth of tested bifidobacterial cultures in decreased counts (Table 2). The content of peptones and other nutrients in media, which can be probably used by bifidobacteria to growth with a limitation, specifically, in two orders of magnitude lesser cultivation counts than those at positive controls. Therefore, it is necessary to confirm the results with further analyses. Production of SCFAs and decrease of pH values were related to the bifidobacterial growth and substrate utilization that was confirmed just by colour change. This was linked to two orders of magnitude higher levels of acetate compared to the negative control. However, the ratio is presumably influenced by the provided carbohydrate substrate and bifidobacterial species and strain character (Palframan et al., 2003). In addition, the polymeric structure of the provided carbohydrate substrate with glucan backbones can terminate with diverse residues, e.g. galactose in locust bean gum, which could be utilized by some strains of bifidobacteria (Bunešová et al., 2012, 2017). This probable mechanism may be used for explanation of partially substrate utilization, although without positive colour interpretation of the reaction. Therefore, the changes in the tested parameters, such as increased numbers of cultivated bacteria and the related decreased pH values and increased acetate levels, may be detected. For example, xanthan gum contains mannose side chains that were probably used by *B. aesculapi* and strain *B. animalis* subsp. *animalis* 805P4 with slight increase in the acetate levels. According to Milani et al. (2015), the genes encoding mannose cleavage are not common within most genomes of bifidobacterial species; however, the number of analyzed genomes is still limited.

Based on our results, there can be a correlation between the ability of bifidobacterial strains to metabolize substrates and the diet of the bacterial host. Bifidobacterial strains isolated from faeces of omnivores, ruminants, and monkeys possessed a high ability to utilize various substrates, such as locust bean, guar, and arabic gums. Strains isolated from dental carries, *B. tsurumiense* and *B. dentium*, were able to metabolize only locust bean and guar gums, and also starch. Interestingly, one of these *B. dentium* strains was isolated from infant faeces. In contrary, the other bifidobacteria of human origin had shown low utilization ability that was not influenced by the stage of human life, and only few strains of those were capable to use starch. Cronin, Ventura, Fitzgerald, and Van Sinderen (2011) reported, that in case of bifidobacteria, there are species and strain specific differences in presence of glycosyl hydrolases and connected ability to ferment and use the metabolic substrates. Moreover, according to McLaughlin et al. (2015), the ability to utilize various substrates is usually strain specific within the bifidobacterial species. Milani et al. (2015) also confirmed that carbohydrate metabolism is species dependent. Finally, strain variability of tested multi-host species, e.g. *B. angulatum*, *B. animalis* subsp. *animalis*, *B. boum*, *B. dentium*, and *B. pseudolongum,* indicated strain and host variability in utilizing the tested natural food thickeners. In contrast, the subspecies *B. animalis* subsp. *lactis,* represented by 16 strains with the variable origin, were able to grow and utilize only control glucose. Crociani, Alessandrini, Mucci, and Biavati (1994) stated that bifidobacterial strains with animal origin revealed reduced complex carbohydrate fermentation ability compared to the strains with human origin. In our case, conversely, the results indicate the better NG and starch utilization by bifidobacteria of animal origins. However, since their testing of complex saccharides, the identification and taxonomy of bifidobacteria has been significantly improved (Killer, Mekadim, Pechar, Bunešová, & Vlková, 2018) and the genus *Bifidobacterium* has considerably expanded (Lugli et al., 2018); therefore, further testing and verification is desirable.

In our study, there were used six NGs, such as locust bean, guar, tragacanth, arabic, xanthan, and karaya gums, which are the final products of a process called gummosis. The mechanism of gum creation is not clearly understood, but it is obviously considered that plant-derived gums are formed by the decaying of carbohydrate plant tissues, which are then transformed into the form of exudates excreted from specialized bodies (Goswami & Naik, 2014; Williams, 2016). For instance, arabic, karaya, and tragacanth gums are tree exudates; whereas guar and locust bean gums are seed-derived (Choudhary & Pawar, 2014). Thanks to the technically beneficial character of starch and NGs, some of them could be usable as potentially prebiotic packaging materials for bifidobacteria (Lian, Hsiao, & Chou, 2002; Sun & Griffiths, 2000). Moreover, prebiotic substrates could be also used in synbiotic mixture with probiotics for desirable synergistic effect (Cencic & Chingwaru, 2010; de Vrese & Schrezenmeir, 2008).

Based on this study, some NGs exhibited prebiotic potential for the growth of several strains of bifidobacteria, in particular, locust bean and guar gums were the most used substrates; tragacanth and arabic gums were also utilized, but a little less than mentioned previous. On the other hand, the most strains of bifidobacteria were able to use starch, which is a common part of human and animal diet. In case of bifidobacterial metabolism, starch is an universal source of carbon, which is degradable by bifidobacteria (Liu et al., 2015) with following production of SCFAs (Haenen et al., 2013). For example, the genomes of *B. adolescentis* (Duranti et al., 2014) and *B. choerinum* (Jung et al., 2018) contain genes encoding utilization of starch. As well as, *B. pseudolongum* revealed significant ability in hydrolysis of resistant starch (Centanni et al., 2018). Furthermore, our study indicates that most strains of *B. adolescentis, B. boum, B. choerinum, B. dentium, B. pseudolongum*, and *B. thermophilum* had showed frequent starch utilization ability. Nevertheless, starch as prebiotic substrate is not sufficiently selective and is utilized also by potentially pathogenic bacteria, such as clostridia (Luo et al., 2018). The non-selectivity of some prebiotics has been emphasized previously (Bunešová et al., 2012; Rada et al., 2008). However, resistant and modified starch, e.g. Nutriose, could be potentially prebiotic source for bifidobacteria (Kim, Shin, Lee, Moon, & Lee, 2018) with the ability to reduce *Clostridium perfringens* (Lefranc-Millot et al., 2012).

Table 2
Growth and metabolite formation of selected bifidobacterial species.

Species	Strain code	Medium	pH	log CFU/mL	Metabolite production (mM)		
					Lactate	Acetate	Formate
B. aesculapii	DSM 26737	API	5.89	5.38	0.30	5.28	0.27
		GLU	**4.55**	**6.30**	**2.12**	**8.41**	**0.60**
		STARCH	**4.92**	**7.65**	**1.56**	**7.35**	**0.29**
		LOCUST	6.38	5.39	0.10	6.29	0.03
		GUAR	6.30	6.64	0.03	6.51	0.16
		TRAG	**5.44**	**7.70**	**0.48**	**6.66**	**0.42**
		ARAB	**5.32**	**7.87**	**0.20**	**7.20**	**0.03**
		XANT	6.11	5.04	0.09	6.93	0.04
		KARAYA	5.76	5.23	0.03	6.70	0.03
B. angulatum	PR23/5D	API	6.04	6.53	0.04	5.33	0.04
		GLU	**4.57**	**6.44**	**2.29**	**8.54**	**0.57**
		STARCH	**4.82**	**7.54**	**1.90**	**7.62**	**0.30**
		LOCUST	**4.63**	**6.66**	**1.99**	**7.97**	**0.54**
		GUAR	**4.65**	**6.53**	**1.72**	**7.85**	**0.71**
		TRAG	5.71	7.16	0.42	5.98	0.39
		ARAB	5.69	5.28	0.52	6.04	0.29
		XANT	6.02	6.61	0.38	5.81	0.21
		KARAYA	5.86	7.04	0.37	6.15	0.18
B. animalis subsp. *animalis*	805P4	API	5.97	6.48	0.31	5.42	0.16
		GLU	**4.53**	**8.22**	**1.45**	**8.42**	**1.05**
		STARCH	**5.28**	**8.44**	**0.51**	**6.68**	**0.61**
		LOCUST	**4.62**	**8.22**	**1.81**	**7.75**	**0.53**
		GUAR	**4.57**	**8.35**	**1.76**	**8.79**	**0.82**
		TRAG	**6.12**	**7.35**	**0.24**	**6.44**	**0.03**
		ARAB	**5.26**	**8.18**	**0.53**	**6.57**	**0.17**
		XANT	6.06	6.92	0.24	7.38	0.06
		KARAYA	5.89	7.24	0.19	6.42	0.04
B. boum	P7	API	6.04	6.98	0.10	6.11	0.03
		GLU	**4.70**	**6.78**	**0.77**	**8.54**	**1.38**
		STARCH	**4.81**	**7.64**	**1.89**	**7.42**	**0.20**
		LOCUST	**4.73**	**6.54**	**2.07**	**7.80**	**0.33**
		GUAR	**4.75**	**5.62**	**1.52**	**7.61**	**0.59**
		TRAG	6.00	7.74	0.45	5.63	0.16
		ARAB	6.25	7.61	0.31	5.36	0.12
		XANT	6.20	6.34	0.30	5.52	0.13
		KARAYA	5.96	7.42	0.25	5.55	0.10
B. dentium	PR18/8A	API	5.97	5.65	0.33	5.31	0.13
		GLU	**4.57**	**7.56**	**1.72**	**8.37**	**0.81**
		STARCH	**4.86**	**7.59**	**1.70**	**7.61**	**0.22**
		LOCUST	**4.63**	**8.24**	**2.24**	**7.97**	**0.27**
		GUAR	**4.64**	**8.06**	**1.86**	**8.53**	**0.79**
		TRAG	5.88	7.59	0.55	6.82	0.05
		ARAB	5.94	7.38	0.40	6.52	0.04
		XANT	6.14	5.93	0.35	5.81	0.05
		KARAYA	5.92	7.89	0.31	6.56	0.04
B. moukalabense	DSM 27321	API	5.92	7.49	0.44	5.25	0.21
		GLU	**4.56**	**6.78**	**2.22**	**8.30**	**0.57**
		STARCH	**4.89**	**7.63**	**1.55**	**7.07**	**0.32**
		LOCUST	**4.50**	**7.02**	**2.82**	**8.58**	**0.39**
		GUAR	**4.85**	**4.84**	**1.60**	**7.26**	**0.19**
		TRAG	5.64	6.27	0.42	5.62	0.29
		ARAB	6.17	6.44	0.36	5.32	0.12
		XANT	6.14	6.54	0.38	5.50	0.10
		KARAYA	5.96	7.57	0.51	6.02	0.13
B. pseudolongum	W1	API	6.05	6.55	0.73	5.13	0.10
		GLU	**4.66**	**8.22**	**1.56**	**8.26**	**0.81**
		STARCH	**4.85**	**8.02**	**1.75**	**7.58**	**0.20**
		LOCUST	**4.67**	**8.57**	**2.10**	**7.99**	**0.35**
		GUAR	**4.74**	**8.80**	**1.56**	**7.87**	**0.54**
		TRAG	5.85	7.63	0.66	5.78	0.04
		ARAB	6.28	7.50	0.48	4.41	0.03
		XANT	6.06	7.19	0.53	5.20	0.04
		KARAYA	5.95	7.27	0.64	5.71	0.10
B. scardovii	DSM 13734	API	5.93	6.94	0.32	5.43	0.23
		GLU	**4.62**	**7.67**	**2.13**	**8.22**	**0.51**
		STARCH	6.23	7.03	0.31	5.52	0.24
		LOCUST	**4.66**	**8.26**	**1.77**	**7.83**	**0.45**
		GUAR	5.26	8.62	0.43	6.42	0.63
		TRAG	6.06	7.48	0.33	5.35	0.14

(*continued on next page*)

Table 2 (*continued*)

Species	Strain code	Medium	pH	log CFU/mL	Metabolite production (mM)		
					Lactate	Acetate	Formate
		ARAB	6.15	7.31	0.19	5.85	0.11
		XANT	5.92	7.55	0.31	5.50	0.20
		KARAYA	5.83	6.72	0.39	5.78	0.05
B. tissieri	DSM 100201	API	5.85	6.30	0.17	5.13	0.07
		GLU	**4.49**	**6.78**	**2.17**	**8.31**	**0.59**
		STARCH	5.73	6.40	0.29	5.25	0.34
		LOCUST	**4.78**	**7.41**	**0.62**	**7.38**	**1.16**
		GUAR	5.57	7.32	0.27	5.97	0.50
		TRAG	5.81	7.36	0.73	5.74	0.36
		ARAB	5.82	7.34	0.46	5.59	0.40
		XANT	5.92	6.93	0.31	5.52	0.31
		KARAYA	5.72	6.57	0.30	6.10	0.30
B. tsurumiense	DSM 17777	API	6.01	5.89	0.30	4.96	0.04
		GLU	**4.52**	**8.39**	**2.04**	**8.34**	**0.91**
		STARCH	**5.09**	**7.97**	**1.11**	**6.29**	**0.21**
		LOCUST	**4.69**	**7.82**	**1.47**	**7.79**	**0.69**
		GUAR	**4.83**	**8.14**	**0.92**	**7.41**	**0.80**
		TRAG	5.77	7.74	0.53	5.34	0.04
		ARAB	6.17	6.30	0.38	5.33	0.04
		XANT	6.09	6.47	0.28	3.76	0.03
		KARAYA	5.89	6.51	0.41	5.04	0.04

Formation of lactate, acetate, and propionate of selected bifidobacterial species in API 50 CHL medium supplied with 5 g/L of a prebiotic substrate was measured after 48 h of incubation by ion-exchange chromatography with suppressed conductivity. The analysis was further enhanced by measuring the pH values and determining the cultivation numbers of bifidobacteria. The positive reaction is highlighted in the table.

Moreover, some bifidobacteria are specialized human/infant gut commensals, for example *B. bifidum* and *B. longum* subsp. *infantis* that is reflected in their metabolism. These bifidobacterial species are able to utilize human milk oligosaccharides (LoCascio, Desai, Sela, Weimer, & Mills, 2010; Rockova et al., 2012; Sela et al., 2008; Turroni et al., 2014) and only *B. bifidum* can degrade and grow also in presence of mucin (Duranti et al., 2015; Ruas-Madiedo, Gueimonde, Fernandez-Garcia, Reyes-Gavilan, & Margolles, 2008).

The environment of host gastrointestinal tract, in which bifidobacteria live, is associated with their metabolic adaptation. The gut is an important source of a wide range of complex carbohydrates, which are not used during the passage through the upper parts of the intestines, and which bifidobacteria can metabolize due to the content of genes encoding enzymes for carbohydrate cleavage in the bifidobacterial genomes (Milani et al., 2016; Pokusaeva, Fitzgerald, & van Sinderen, 2011). Our study also indicates that bifidobacteria may presumably adapt to the environment in which their hosts live. For example, *B. animalis* subsp. *animalis* of ruminant origin revealed increased ability to utilize the natural plant substrates in contrast to the strains of omnivore origin. The diet changes are directly connected with the species representation in the intestinal microbiota (Flint, Duncan, Scott, & Louis, 2015). The presence of these microbial representatives is associated with coevolution of the host and is closely related to the host's microbiome (Albert, Rani, & Sela, 2018). Thus, NG induction into the host diet could positively influence the composition of microbial representatives with desirable properties for following host life.

5. Conclusion

The ability of bifidobacteria to utilize natural food thickeners was host, species and strain dependent. Bifidobacterial strains with animal origin were able to utilize a wider range of available substrates, whereas strains of human origin were not. Locust bean, guar, tragacanth, and arabic gums represent possible potentially prebiotic sources in animal nutrition and can ensure the protection of administered bifidobacteria, as well. Frequently detected bifidobacterial starch utilization reflects its high content in the diet of human and various animals; however, for the application of starch as a prebiotic substrate, it is advisable to use its modified and resistant forms to avoid the promotion of undesirable bacteria.

Funding

This work was supported by the University Internal Grant Agency of the Czech University of Life Sciences Prague [grant number CIGA 20182018] and by METROFOOD-CZ research infrastructure project [MEYS Grant No: LM2018100] including access to its facilities.

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4.5 *Bifidobacterium* β-glucosidase activity and fermentation of dietary plant glucosides is species and strain specific

Modrackova N, Vlkova E, Tejnecky V, Schwab C, Neuzil-Bunesova V. 2020. *Bifidobacterium* β-glucosidase activity and fermentation of dietary plant glucosides is species and strain specific. Microorganisms **8**:839.

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Article

Bifidobacterium β-Glucosidase Activity and Fermentation of Dietary Plant Glucosides Is Species and Strain Specific

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Received: 13 May 2020; Accepted: 1 June 2020; Published: 3 June 2020

Abstract: Dietary plant glucosides are phytochemicals whose bioactivity and bioavailability can be modified by glucoside hydrolase activity of intestinal microbiota through the release of acylglycones. Bifidobacteria are gut commensals whose genomic potential indicates host-adaption as they possess a diverse set of glycosyl hydrolases giving access to a variety of dietary glycans. We hypothesized bifidobacteria with β-glucosidase activity could use plant glucosides as fermentation substrate and tested 115 strains assigned to eight different species and from different hosts for their potential to express β-glucosidases and ability to grow in the presence of esculin, amygdalin, and arbutin. Concurrently, the antibacterial activity of arbutin and its acylglycone hydroquinone was investigated. Beta-glucosidase activity of bifidobacteria was species specific and most prevalent in species occurring in human adults and animal hosts. Utilization and fermentation profiles of plant glucosides differed between strains and might provide a competitive benefit enabling the intestinal use of dietary plant glucosides as energy sources. Bifidobacterial β-glucosidase activity can increase the bioactivity of plant glucosides through the release of acylglycone.

Keywords: amygdalin; arbutin; esculin; bifidobacteria; β-glucosidase; hydroquinone; antibacterial activity

1. Introduction

Phytochemicals are found in leaves, fruits, vegetables, grains, and beans. Some glycosidic phytochemicals have been used in traditional medicine for centuries [1]. For example, the phenolic β-glucoside arbutin is a component of *Arctostaphylos uva-ursi* (bearberry leaf), which has been used in urinary tract infections [2]. Other plant derived β-glucosides include amygdalin (naturally occurring in almonds), esculin (dandelion coffee), fraxin (kiwi), polydatin (grapes), sinigrin (broccoli), and vanillin (vanilla) [3]. The biological effects of many glycosides are not attributed to their glycoside forms but to the corresponding aglycones (Figure 1). Aglycones are bioactive compounds that have lower molecular weight and hydrophilicity. After consumption, plant glucosides can be either taken up in the small intestine and undergo enterohepatic circulation, or they can be hydrolyzed by glycosidic activity of the gut microbiota [2]. Bacterial β-glucosidases, which have been classified within glycoside hydrolases (GH) families GH1, GH3, GH5, GH9, GH30, and

GH116, cleave β-D-glucosidic linkages liberating glucose and the corresponding acylglycones. Some acylglycones have been shown to be antimicrobially active [4].

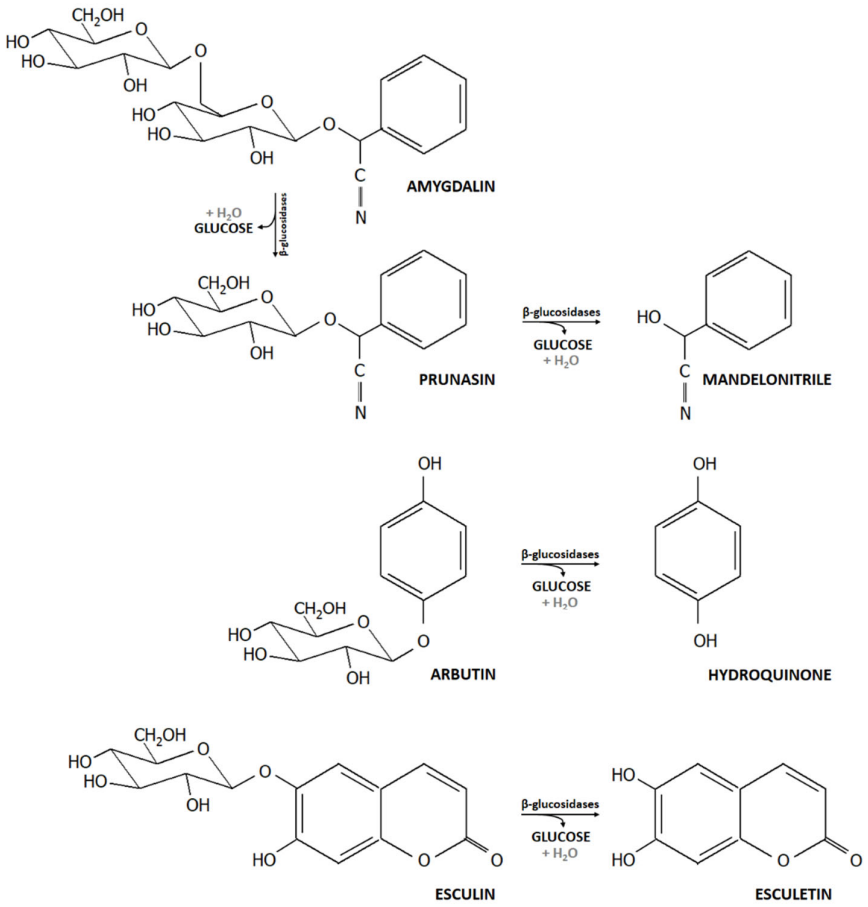

Figure 1. Structures of dietary plant glucosides (amygdalin, arbutin, and esculin) and their products after β-glucosidase hydrolysis.

Several taxa of the major gut colonizers Firmicutes, Bacteroidetes and Actinobacteria possess β-glucosidase activity [5]. *Bifidobacterium* spp. (Actinobacteria) represent an important group of human commensals, being among the first microbial colonizers with considerable relevance for health in later life [6,7]. Intestinal competitiveness of bifidobacteria is attributed to their ability to degrade and metabolize a diversity of carbohydrates, and to carbohydrate resource sharing and cross-feeding [8,9]. Host adaptation seems to be linked to the ability to use dietary or host-derived glycans in a glycan-rich gut environment and differs between species and strains [10,11]. *Bifidobacterium* spp. frequently possess the potential to encode a wide array of glycosyl hydrolases, including β-glucosidases [12].

In humans, prevalence and diversity of members of the genus *Bifidobacterium* change in succession during life, with the species *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum* representing about 80% of the intestinal microbiota of infants, while *Bifidobacterium adolescentis*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, and *Bifidobacterium pseudocatenulatum* prevail in adults representing about 1% of gut microbes [13–18]. Colonization of *B. bifidum* and *B. breve* seems to be limited to the human intestinal tract [12,19], whereas *B. adolescentis*, *B. longum* subsp. *longum*, *Bifidobacterium animalis* subsp. *animalis* and *lactis*, *B. catenulatum*, and *B. pseudocatenulatum* are considered multi-host species that were isolated from other mammals such as dogs, primates, and young ruminants on the milk diet [11,20]. *Bifidobacterium dentium* likely colonizes the oral cavity, and might only transiently pass the intestine [21,22].

Bifidobacterium spp. are common inhabitants of the human intestinal tract throughout life, and intestinal bifidobacterial β-glucosidase activity might modify the bioactivity and bioavailability of dietary plant glucosides. However, as there has been no systematic investigation, we tested 115 *Bifidobacterium* strains belonging to eight species that were associated with different hosts for β-glucosidase activity, the ability to grow in the presence of the coumarin glucoside esculin, the cyanogenic diglucoside amygdalin and the phenolic β-glucoside arbutin (Figure 1), and investigated the antibacterial activity of arbutin and its acylglycone hydroquinone. We additionally used genomic data of representative strains to screen for the presence of β-glucosidases encoding genes.

2. Materials and Methods

2.1. Bacterial Strains

Bifidobacterial strains (n=115) of the species *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. dentium*, *B. longum*, and *B. catenutalum*, and *B. pseudocatenulatum* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) or the strain collection of the Department of Microbiology, Nutrition, and Dietetics (CZU, Czechia) (Table 1). Strain identity was confirmed with MALDI-TOF MS (Bruker Daltonik GmbH, Germany) according to Modrackova et al. (2019) [23]. MALDI-TOF MS failed to distinguish *B. catenulatum* and *B. pseudocatenulatum*, and to identify subspecies of *B. longum*. Subspecies of *B. animalis* were classified in previous studies [11,24,25]. Strains were routinely cultured in Wilkins–Chalgren broth (Oxoid, UK) supplemented with soya peptone (5 g L^{-1}, Oxoid), L-cysteine (0.5 g L^{-1}), and Tween 80 (1 mL L^{-1}, both Sigma-Aldrich, USA) (WSP broth) in an oxygen-free carbon dioxide environment at 37 °C for 24 h. Stock cultures were stored at –80 °C in 30% glycerol and were reactivated in WSP broth for 24 h to obtain working cultures. Purity was routinely confirmed by phase-contrast microscopy.

Table 1. Bifidobacterial utilization of β-glucosylated substrates during growth, and β-glucosidase activity. The ability to utilize plant glucosides was tested in API 50 CHL media. The color change detection of cultivation media after 72 h of incubation at 37 °C under anaerobic conditions was determined spectrophotometrically (434 nm/588 nm). A ratio of <2 was considered no growth (-); 2.1–2.4: poor growth (+); 2.5–3.4: growth (++); and >3.5: very good growth (+++). Beta-glucosidase activity was tested by enzymatic assay with 4-nitrophenyl β-D-glucopyranoside (β-GLU) as substrate with spectrophotometric measurement at 405 nm; the difference of >0.1 was considered positive (+). The release of the acylglycone from esculin was determined visually using ammonium iron citrate as scavenger (ESN rel.).

Species/Subspecies	Strain	Origin	GLU	ESC	AMYG	ARB	API	β-GLU	ESN rel.
B. adolescentis	DSM 20083	Intestine of adult	+++	-	-	-	-	+	+
	B34	Stool of infant	+++	-	+++	-	-	+	+
	B35	Stool of infant	+++	++	+++	+	-	+	+
	B36	Stool of infant	+++	-	-	-	-	+[R]	+
	B38	Stool of infant	+++	+	-	-	-	+	+
	B2	Stool of adult	++	-	++	-	-	+[R]	+
	B9	Stool of adult	++	-	++	-	-	+[R]	+
	B30	Stool of adult	+++	-	+++	-	-	+[R]	+
	B39	Stool of adult	+++	++	+++	+++	-	+	+
	B41	Stool of adult	+++	-	+++	-	-	+	+
	B56	Stool of adult	++	-	-	-	-	+	+
	PEG038	Stool of adult	+++	-	+++	-	-	+	+
	10/6d	Dog feces	+++	-	+++	-	-	+	+
B. animalis subsp. *animalis*	DSM 20104	Rat feces	+++	-	-	-	-	+	+
	805P4	Calf feces	+++	+++	-	-	-	+[R]	+
	012II1	Calf feces	+++	+	-	-	-	+[R]	+
	023II	Calf feces	+++	-	-	-	-	+[R]	+
	J1 (L1)	Lamb feces	++	-	-	-	-	+[R]	+
	J5 (L4)	Lamb feces	+++	+	-	-	-	+[R]	+
	J6 (L3)	Lamb feces	+++	++	-	-	-	+	+
B. animalis subsp. *lactis*	DSM 10140	Yoghurt	+++	-	+++	-	-	+	+
	BB12	Probiotic product	++	++	++	-	-	+	+

	Dan	Probiotic product	++	+	+++	-	-	+	+
	Nestlé	Infant nutrition	+++	++	++	-	-	+	+
	S7	Ovine cheese	++	++	++	-	-	+	+
	B22	Stool of infant	+++	++	+++	-	-	+	+
	B25	Stool of infant	+++	+	+++	-	-	+	+
	PEG042	Stool of adult	+++	-	+++	-	-	+	+
	PEG084	Stool of adult	++	+	++	+++	-	+	+
	P2N1	Dog feces	+++	-	-	-	-	+	+
	43/7nb	Dog feces	+++	-	-	-	-	+	+
	11/6a	Dog feces	++	-	-	-	-	+	+
	ZDK1	Cameroon sheep feces	+++	++	+++	-	-	+	+
	ZDK4	Barbary sheep feces	++	++	++	-	-	+	+
	ZDK7	Okapi feces	+++	++	+++	-	-	+	+
B. bifidum	DSM 20456	Stool of infant	+++	-	-	-	-	-	-
	DSM 20239	Stool of infant	+++	-	-	-	-	-	-
	B6	Stool of infant	+++	-	-	-	-	-[R]	-
	B33	Stool of infant	+++	-	-	-	-	-[R]	-
	B10	Stool of adult	+++	-	-	-	-	-[R]	-
	B29	Stool of adult	+++	-	-	-	-	-[R]	-
	B40	Stool of adult	+++	-	-	-	-	-	-
	B55	Stool of adult	+++	-	-	-	-	-	-
B. breve	DSM 20213	Intestine of infant	+++	++	+++	+++	-	+	+
	BR03	Probiotic product	++	++	+++	-	-	+	+
	B13	Stool of infant	+++	+++	+++	++	-	+[R]	+
	B14	Stool of infant	+++	++	+++	+++	-	+[R]	+
	B37	Stool of infant	+++	+++	+++	-	-	+	+
	B42	Stool of infant	++	++	++	+++	-	+	+
	B43	Stool of infant	+++	-	++	-	-	+	+
	B50	Stool of infant	+++	+++	+++	+++	-	+	+
	B57	Stool of infant	+++	+++	+++	-	-	+	+
	PEG010	Stool of adult	++	++	+++	-	-	+	+
	PEG064	Stool of adult	+++	+++	+++	-	-	+	+
	PEG071	Stool of adult	+++	++	+++	-	-	+	+
	PEG074	Stool of adult	+++	+++	+++	++	-	+	+
B. catenulatum	DSM 16992	Human feces	+++	+++	+++	+++	-	+	+
B. catenulatum subsp. *kashiwanohense*	DSM 21854	Stool of infant	+++	-	+++	+	-	+	+
B. pseudocatenulatum	DSM 20438	Stool of infant	+++	+++	+++	+++	-	+	+
B. catenulatum/ *pseudocatenulatum*	B12	Stool of infant	+++	-	+++	-	-	+[R]	+
	B46	Stool of infant	+++	++	+++	+++	-	+	+
	B48	Stool of infant	+++	+++	-	-	-	+	+
	B23	Stool of adult	+++	++	+++	-	-	+[R]	+
	B32	Stool of adult	+++	++	+++	-	-	+[R]	+
	B51	Stool of adult	++	-	+++	-	-	+	+
	B52	Stool of adult	+++	+	-	+++	-	+	+
	B53	Stool of adult	+++	-	+++	-	-	+	+
	22/4nb	Dog feces	+++	++	+++	-	-	+	+
B. dentium	DSM 20436	Dental caries	+++	+++	+++	-	-	+	+
	FD1	Stool of infant	+++	++	+++	+++	-	+[A]	+
	TH1	Stool of infant	+++	+++	+++	-	-	+[A]	+
	VOK II	Stool of infant	+++	++	+++	-	-	+[A]	+
	PEG020	Stool of adult	+++	++	+++	+++	-	+	+
	A1/5A	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N12	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N21	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N23	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N26	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N77	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N79	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N105	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N109	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N110	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N111	Monkey feces	+++	+++	+++	+++	-	+[A]	+
	N112	Monkey feces	+++	+++	+++	+++	-	+[A]	+

B. longum subsp. *infantis*	DSM 20088	Stool of infant	+++	-	-	-	-	-	-
B. longum subsp. *longum*	DSM 20219	Intestine of adult	+++	-	-	-	-	-	-
B. longum subsp. *suillum*	DSM 28597	Feces of piglets	+++	-	-	-	-	-[A]	-
B. longum subsp. *suis*	DSM 20211	Pig feces	+++	-	+++	-	-	+	-
	5/9	Calf feces	+++	-	-	-	-	-	-
B. longum	INFNUT	Probiotic product	++	-	-	-	-	-	-
	B3	Stool of infant	+++	-	-	-	-	-[R]	-
	B4	Stool of infant	++	-	-	-	-	-[R]	-
	B7	Stool of infant	+++	-	-	-	-	-	-
	B8	Stool of infant	+++	-	-	-	-	-	-
	B11	Stool of infant	+++	-	-	-	-	-	-
	B16	Stool of infant	+++	-	-	-	-	-	-
	B17	Stool of infant	+++	-	-	-	-	-[R]	-
	B19	Stool of infant	+++	-	-	-	-	-[R]	-
	B20	Stool of infant	++	-	-	-	-	-[R]	-
	B27	Stool of infant	++	-	-	-	-	-	-
	B28	Stool of infant	+++	-	-	-	-	-	-
	B44	Stool of infant	+++	-	-	-	-	-[A]	-
	B49	Stool of infant	+++	-	-	-	-	-	-
	B1	Stool of adult	+++	-	-	-	-	+	-
	B26	Stool of adult	+++	-	-	-	-	-[R]	-
	PEG057	Stool of adult	+++	-	-	-	-	-	-
	PEG059	Stool of adult	+++	-	-	-	-	-	-
	PEG080	Stool of adult	++	-	++	-	-	+	+
	PEG104	Stool of adult	+++	-	-	-	-	-	-
	022II	Calf feces	+++	-	-	-	-	-[R]	-
	10/6b	Dog feces	+++	-	-	-	-	+	+
	32/3na	Dog feces	+++	-	-	-	-	+	-
	33/5nb	Dog feces	++	-	-	-	-	+	-
	33/4nc	Dog feces	++	-	-	-	-	+	-

GLU, glucose; ESC, esculin; AMYG, amygdalin; ARB, arbutin; API, negative control; β-GLU, β-glucosidase activity; ESN rel., esculetin release; superscript letter A, the shown reaction (positive/negative) of β-glucosidase activity is confirmed by ANAEROtest 23; and superscript letter R, the shown reaction of β-glucosidase activity is confirmed by RAPID ID 32 A.

2.2. Utilization of Selected Dietary Plant Glucosides

The ability of bifidobacteria to utilize glucosylated substrates was investigated in sterile 96-well microtiter plates (VWR, USA). Stock solutions of esculin (7-hydroxycoumarin-6-glucoside; Sigma-Aldrich), amygdalin (D-mandelonitrile-β-gentiobioside; Sigma-Aldrich), and arbutin (hydroquinone-β-D-glucopyranoside; Alfa Aesar, USA) were prepared in concentrations corresponding to 28 mM (5 g L^{-1}) glucose (Penta, Czechia) in API 50 CHL medium (BioMérieux, France) with bromocresol purple as a pH indicator, and were filter sterilized. Glucose served as a growth positive control, API medium without added substrate was used to determine background growth.

Overnight cultures were centrifuged and re-suspended in the same volume of API medium. Strains were added (20 µL) to 180 µL API medium with or without added substrate. Plates were incubated under anaerobic conditions (GENbag anaer, bioMérieux, France) at 37 °C for 72 h. The color change from purple to yellow indicated a positive reaction. We measured absorbance at 434 nm and 588 nm using a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland) and calculated the ration 434/588 [26], which was categorized as: no growth (<2); poor growth (2.1–2.4); growth (2.5–3.4); and very good growth (>3.5). Every strain was tested two or three times.

2.3. Metabolite Formation Analysis Using Ion Chromatography with Suppressed Conductivity Detection

Concentration of main fermentation metabolites lactate, acetate, and formate, was determined for selected strains using capillary high-pressure ion-exchange chromatography with suppressed conductivity detection. A Dionex ICS 4000 system equipped with IonPac AS11-HC 4 µm (Thermo Scientific, USA) guard and analytical columns. Eluent composition was as follows: 0–10 min isocratic: 1 mM KOH; 10–20 min linear gradient: 1–60 mM KOH; and 20–25 min again isocratic: 60 mM KOH.

The flow rate was set to 0.012 mL min^{-1}. An ACES 300 suppressor (Thermo Scientific, USA) was used to suppress eluent conductivity, while a carbonate Removal Device 200 (Thermo Scientific) was implemented to suppress carbon dioxide baseline shift. Chromatograms were processed with Chromeleon 7.20 (Dionex, USA). Standards were prepared from 1 g L^{-1} stock solutions (Analytika, Czechia; Inorganic Ventures, USA). Deionized water (conductivity <0.055 μS cm^{-1}; Adrona, Latvia) was used for eluent and standard preparation (0.1–40 mg L^{-1}).

2.4. Determination of Whole Cell β-Glucosidase Activity, and Release of the Acylglycone Esculetin

Beta-glucosidase activity of whole cells was assessed by enzymatic assay with 4-nitrophenyl β-D-glucopyranoside (PNP-G; Sigma-Aldrich, USA) as substrate. All samples were tested at least twice. Overnight cultures (1 mL) were centrifuged, supernatant was discarded and cell pellets were frozen at –20 °C. Frozen cells were re-suspended in 20 μL BifiBuffer (1.2 g L^{-1} K_2HPO_4, 0.333 g L^{-1} KH_2PO_4; Lachner, Czechia), 1 μL of this suspension was added to 99 μL of PNP-G solution (20 mM in Bifibuffer). Absorbance at 405 nm was measured before and after 4 h of incubation at 37 °C using a Tecan Infinite M200 spectrometer: reactions with a difference of absorbance >0.1 units were considered positive.

For selected strains, β-glucosidase activity was additionally tested using kits RAPID ID 32 A (bioMerieux, France), or ANAERO test 23 (Erba Lachema, Czechia) which employ PNP-G.

The release of esculetin from esculin was tested using ammonium iron citrate (Sigma Aldrich, USA) as scavenger, the reaction of esculetin with ferric ions changes the color from purple to opaque black. Bifidobacteria were inoculated in API medium supplied with 10.2 g L^{-1} esculin (corresponding to 28 mM solution of glucose) and 1 g L^{-1} ammonium iron citrate in microtiter plates as described above; and were incubated under anaerobic conditions (GENbag anaer) at 37 °C for 72 h. The color change was assessed visually. Every strain was tested at least twice.

2.5. Antibacterial Activity of Arbutin and Hydroquinone against Selected Bifidobacterium Strains

The antibacterial activity of arbutin and its acylglycone hydroquinone (Sigma-Aldrich, St. Louis, MI, USA) was tested using two-fold broth dilution assay in 96-well sterile microtiter plates. Overnight cultures of selected strains (DSM 20083, DSM 20104, DSM 10140, DSM 20456, DSM 20213, DSM 20211, DSM 20219, and DSM 20088), which represented five of the species tested (Table 2), grown in WSP broth, were centrifuged, and the cell pellet was resuspended in the same volume of API medium supplied with 14 mM glucose. A two-fold dilution series was prepared in microtiter plates using API stock solutions containing 14 mM glucose, and 28 mM arbutin or hydroquinone. Cultures (10%) were added, and plates were incubated under anaerobic conditions (GENbag anaer) at 37 °C for 24 h. Absorbance at 434 and 588 nm was determined using a spectrophotometer and the ratio of 434 nm/588 nm was calculated as described above. The minimal inhibitory concentration (MIC) was defined as the concentration that prevented growth, metabolite formation and thereby color change of the API medium. Every strain was analyzed at least three times.

Table 2. Minimal inhibitory concentrations of hydroquinone and arbutin. Minimal inhibitory concentrations (MIC) were determined using a two-fold dilution assay in microtiter plates and API medium supplied with glucose (14 mM), and hydroquinone or arbutin. The MIC was defined as the concentration that completely inhibited growth of strains determined using the absorbance ration 434 nm/588 nm. MIC were tested in 3–5 independent replicates.

Species or Subspecies	Strain	Minimal Inhibitory Concentration (mM)	
		Hydroquinone	Arbutin
B. adolescentis	DSM 20083	0.05–0.10	>25.5
B. animalis subsp. *animalis*	DSM 20104	≤0.05	>25.5
B. animalis subsp. *lactis*	DSM 10140	0.10–0.20	>25.5
B. bifidum	DSM 20456	0.10–0.20	>25.5
B. breve	DSM 20213	0.10–0.20	>25.5
B. longum subsp. *suis*	DSM 20211	≤0.05	>25.5

B. longum subsp. *longum*	DSM 20219	≤0.05	>25.5
B. longum subsp. *infantis*	DSM 20088	≤0.05	>25.5

To test whether the presence of arbutin in API medium impacted growth, we additionally conducted growth kinetics of selected strains that were able or lacked the ability to grow with arbutin in API medium supplied with 28 mM glucose, 14 mM glucose and 14 mM arbutin, or 28 mM arbutin to ensure the availability of the same concentration of glucose. Strains were grown in microtiter plates as described before, and absorbance was measured at 0, 3, 6, 9, 12, 24, 30, 36, and 48 h at 434 and 588 nm, to calculate the ratio of 434 nm/588 nm as described above.

2.6. Identification and Comparison of β-Glucosidases Encoded by Representative Bifidobacterium spp. of the Species Investigated

Homologues of previously characterized β-glucosidases of *B. animalis* subsp. *lactis* [27] and *B. pseudocatenulatum* IPLA 36007 [28] were identified in genomes of *B. adolescentis* DSM 20083 (AP009256), *B. animalis* subsp. *lactis* BB12 (CP001853.1), DSM 10140 (CP001606.1), *B. animalis* subsp. *animalis* DSM 20104 (CP002567.1), *B. bifidum* DSM 20456 (AP012323.1), *B. breve* DSM 20213 (ACCG02000000), *B. longum* subsp. *longum* DSM 20219 (AP010888.1), *B. longum* subsp. *infantis* DSM 20088 (CP001095.1), *B. longum* subsp. *suis* 20211 (JGZA01000002.1), *B. catenulatum* DSM 16992 (ABXY01000009.1), *B. catenulatum* subsp. *kashiwanohense* DSM 21854 (JGYY01000015.1), *B. pseudocatenulatum* DSM 20438 (ABXX02000004.1) and *B. dentium* DSM 20436 (FNSE01000001.1) using blastP. To identify additional β-glucosidases, genomic data were obtained from NCBI and were annotated with RAST using default settings [29]. Glycosyl hydrolases of family 1 and 3 were identified using the dbCAN database based on a search for signature domains of every CAZyme family [30].

3. Results

3.1. Distribution of (Putative) β-Glucosidases Encoding Genes in Genomes of Representative Bifidobacterium spp.

We screened the genomes of selected strains for the presence of GH1 and GH3 encoding genes (Table 3). Homologous proteins related to the four GH3 β-glucosidases characterized in *B. pseudocatenulatum* IPLA 36007 were present in *B. adolescentis* DSM 20083, *B. breve* DSM 20213, *B. catenulatum* subsp. *kashiwanohense* DSM 21854, *B. catenulatum* DSM 16992, and *B. pseudocatenulatum* DSM 20438. Multiple β-glucosidases (n=1 GH1, and n=11 GH3) were encoded by the genome of *B. dentium* DSM 20438 including homologues to the four β-glucosidases of *B. pseudocatenulatum* IPLA 36007. Strains of *B. animalis* harbored a homologue of Bbg572 (GH1) of *B. animalis* subsp. *lactis* SH5, and in addition a homologue of r-β-gluE of *B. pseudocatenulatum* IPLA 36007. The distribution of β-glucosidases in *B. longum* differed between subspecies, *B. longum* subsp. *longum* DSM 20219 and *B. longum* subsp. *suis* DSM 20211 possessed homologues of r-β-gluE, which were also highly similar (>96%) to a characterized β-glucosidase of *B. longum* H1 [31], while r-β-gluD was present in all three subspecies but was truncated in *B. longum* subsp. *longum* DSM 20219. *B. longum* subsp. *infantis* DSM 20288 additionally possessed a homologue of Bbg572. *B. bifidum* DSM 20456 harbored only one putative GH1 β-glucosidase with low homology to Bbg572.

Table 3. Distribution of β-glucosidases in representative strains of *Bifidobacterium* species. Beta-glucosidases putatively encoded by the genomes were compared to characterized β-glucosidases of *B. animalis* subsp. *lactis* SH5 (Bbg572, GH1) or to four GH3 β-glucosidases r-β-gluA, r-β-gluB, r-β-gluD, and r-β-gluE of *B. pseudocatenulatum* IPLA36007.

Species	Strain	Characterized Beta-Glucosidase					Not Yet Characterized β-Glucosidases
		GH Family 1	GH family 3				
		Bbg572 (JX274651) 461 AA	**r-β-gluE** (AW18_08090, KEF28001.1) 787 AA	**r-β-gluB** (AW18_09810, KEF27912.1) 809 AA	**r-β-gluD** (AW18_08145, KEF28010.1) 748 AA	**r-β-gluA** (AW18_01575, KEF29323.1) 964 AA	
B. breve	DSM 20213	-	EFE88733.1A 93% I, 90% P in 774 AA	EFE90113.1 80% I, 97% P in 833 AA	EFE88739.1 82% I, 90% P in 757 AA	EFE90117.1 70% I, 81% P in 811 AA	
B. adolescentis	DSM 20083	-	BAF39978.1 96% I, 98% P in 780 AA	BAF40379.1 90% I, 94% P in 811 AA	BAF39975.1 [B] 85% I, 92% P in 748 AA	BAF40392.1 97% I, 93% P in 962 AA	BAF40391.1
B. longum subsp. *longum*	DSM 20019	-	BAJ67169.1[C] 82% I, 89% P in 776 AA	-	BAJ67164.1 78% I, 87% P in 507 AA[D]	-	
B. longum subsp. *suis*	DSM 20211	-	KFI73778.1[E] 82% I, 90% P in 775 AA	-	KFI73782.1 83% I, 91% P in 752 AA	-	KFI73422.1
B. longum subsp. *infantis*	DSM 20088	ACJ52977.1 69% I, 81% P in 417 AA	-	-	ACJ51732.1 82% I, 90% P in 756 AA	-	
B. animalis subsp. *animalis*	DSM 20104	AFI62379.1 96% I, 98% P in 460 AA	AFI63691.1 73% I, 84% P in 776 AA	-	-	-	
B. animalis subsp. *lactis*	DSM 10140	ACS47112.1 100% I, 100% P in 476 AA	ACS48458.1 73% I, 84% P in 771 AA	-	-	-	
B. animalis subsp. *lactis*	BB12	ADC85172.1 100% I, 100% P in 460 AA	ADC84934.1 73% I, 84% P in 771 AA	-	-	-	ADC84934.1
B. bifidum	DSM 20456	BAQ97280.1 47% I, 63% P in 437 AA	-	-	-	-	
B. catenulatum subsp. *kashiwanohense*	DSM 21854	KFI63440.1 71% I, 82% P in 458 AA	KFI67404.1 97% I, 98% P in 780 AA	KFI63941.1 95% I, 97% P in 728 AA	KFI67400.1 98% I, 99% P in 748 AA	KFI63834.1 92% I, 99% P in 299 AA*	

B. catenulatum	DSM 16992	-	EEB22212.1 96% I, 98% P in 780 AA	EEB21148.1 95% I, 97% P in 809 AA	EEB22216.1 98% I, 99% P in 748 AA	EEB22373.1 93% I, 96% P in 696 AA	EEB22212.1
B. pseudocatenulatum	DSM 20438	-	EEG71163.1 96% I, 98% P in 780 AA	EEG71238.1 99% I, 99% P in 809 AA	EEG71159.1 98% I, 99% P in 748 AA	EEG70226.1 99% I, 99% P in 964 AA	
B. dentium	DSM 20436	SEC02936.1 69% I, 80% P in 457 AA	SEC47920.1 99% I, 95% P in 774 AA	SEC11609.1 87% I, 93% P in 809 AA	SEC48734.1 94% I, 98% P in 748 AA	SEC11364.1 90% I, 95% P in 962 AA SEC11543.1 61% I, 76% P in 962 AA	SEC18208.1 SEB97687.1 SEB79266.1 SEC47658.1 SEC14043.1 SEB96905.1

[A] I = Identities, P = Positives; [B] BaBgl3 was characterized by Florindo et al. (2018) [32]; [C] 96% I, 98% P in 783 AA to β-glucosidase of *B. longum* H1 [31]; [D] truncated protein; [E] 96% I, 98% P in 787 AA to β-glucosidase of *B. longum* H1 [31].

3.2. Beta-Glucosidase Activity of Bifidobacterium spp.

Whole cell β-glucosidase activity was investigated by enzymatic assay using PNP-G as substrate. *B. adolescentis*, *B. animalis*, *B. breve*, *B. catenulatum/pseudocatenulatum*, and *B. dentium* were consistently β-glucosidase positive (Table 1). In contrast, all tested strains of *B. bifidum*, with a few exceptions, and most strains of *B. longum,* were β-glucosidase negative. From *B. longum*, only four strains that originated from dog feces (10/6b, 32/3na, 33/5nb, and 33/4nc), two isolates from stool of adults (B1, PEG080) and one from pig feces (DSM 20211) showed β-glucosidase activity. For selected strains, β-glucosidase activity was confirmed using RAPID ID 32 A and ANAEROtest 23 (Table 1).

3.3. Growth in the Presence of β-Glucosides

We observed that β-glucosidase activity is a common yet species dependent feature of *Bifidobacterium* spp. To investigate whether β-glucosidase activity relates to the ability to use plant glucosides, we grew strains with esculin, amygdalin, and arbutin as a sole carbohydrate source in API 50 CHL medium (Table 1). All strains were able to grow in the presence of glucose, verifying the suitability of the assay.

In general, amygdalin was the preferred β-glucosylated substrate used by 54% of strains, followed by esculin (47%), and arbutin (24%).

Strains belonging to *B. dentium* were most versatile in the utilization of the provided β-glucosides as all strains grew in the presence of amygdalin and esculin. Only three *B. dentium* strains (DSM 20436, TH1, and VOK II) were not able to use arbutin. All strains of *B. breve* used amygdalin and esculin (with one exception, B43), while the utilization of arbutin was less frequent (46%). Within *B. catenulatum/pseudocatenulatum*, 83% strains grew in the presence of amygdalin, while 67% utilized esculin and 38% arbutin. The majority of the *B. adolescentis* strains (69%) was capable of using amygdalin, strains B35 and B39 grew in presence of arbutin and esculin. For *B. animalis*, we observed subspecies dependent differences in substrate utilization. The majority of *B. animalis* subsp. *lactis* strains, except three isolates from dog feces (P2N1, 43/7nb, and 11/6a), utilized amygdalin (80%), 67% used esculin, and only PEG084 grew in the presence of arbutin. In contrast, *B. animalis* subsp. *animalis* strains were not capable to grow with amygdalin and arbutin, while 57% utilized esculin. None of the *B. longum* strains was able to use esculin and arbutin, amygdalin utilization was detected for *B. longum* DSM 20211 and PEG080, while *B. bifidum* strains were not able to utilize any of the provided plant β-glucosides.

3.4. Release of the Acylglycone Esculetin

We qualitatively determined whether β-glucoside hydrolysis of esculin would lead to the release of the acylglycone esculetin using ammonium iron citrate as a scavenger (Table 1). The majority (94%) of the strains that were positive in the PNP-G enzymatic assay released esculetin confirming β-glucosidase activity. Few strains (namely DSM 20211, B1, 32/3na, 33/5nb, and 33/4nc), all from *B. longum*, were PNP-G positive, while esculetin release was not detected. More than half (66%) of the strains that were able to release esculetin grew when esculin was present as substrate.

3.5. Metabolite Formation during Growth of Bifidobacteria in the Presence of Plant Glucosides

For representative strains from different species, we investigated metabolite formation as an indicator of fermentation activity. Lactate, acetate, and formate concentrations were measured by capillary ion-exchange chromatography with suppressed conductivity detection (Figure 2). All strains grew in the presence of glucose, producing mainly acetate metabolite (44%–83% of metabolites formed), followed by lactate (3%–51%) and formate (0%–22%). In negative controls (API medium without added glycan source), and samples without visible growth, acetate was formed as the major metabolite, otherwise the acetate:lactate:formate ratios differed between strains, and compared to growth in glucose.

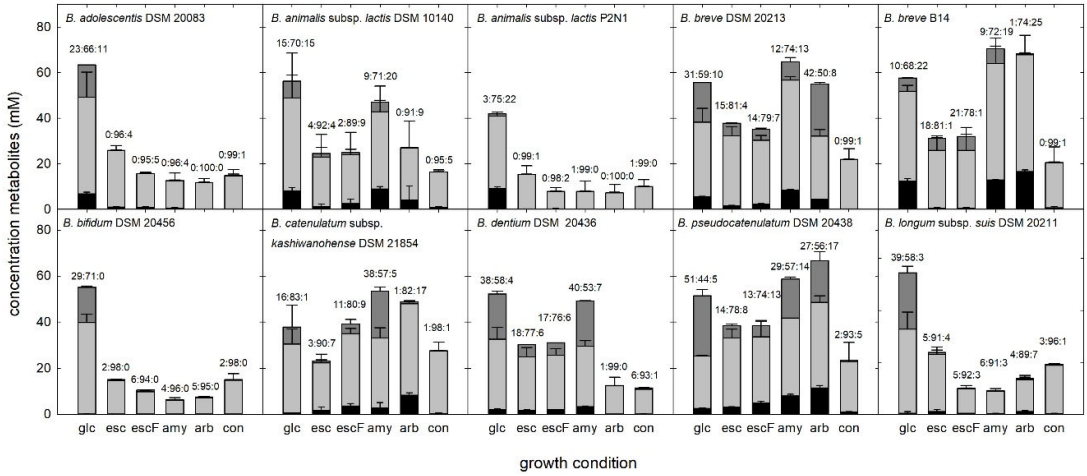

Figure 2. Metabolites formed during growth in the presence of glucose or plant glucosides. Formation of fermentation metabolites, lactate (dark grey), acetate (light grey), and formate (black), by selected strains grown in API 50 CHL medium supplied with plant glucosides (equivalent to 28 mM glucose) was analyzed after 72 h incubation by capillary ion-exchange chromatography with suppressed conductivity. The proportion of major metabolites lactate, acetate, and formate for each growth condition are shown above the bar. Strains were tested two or three times. glc, glucose; esc, esculin; escF, esculin and ammonium iron citrate; amy, amygdalin; arb, arbutin; and con, negative control.

3.6. Antibacterial Activity of Hydroquinone and Arbutin and Impact on Growth Kinetics

We tested the antibacterial and growth affecting activity of arbutin and its acylglycone hydroquinone on representative strains using two-fold dilution assay in microtiter plates and growth kinetics, respectively. Glucose (14 mM) was added to API medium to avoid growth inhibition due to substrate limitation. None of the strains were affected by the presence of arbutin even at the maximum concentration tested (25.5 mM) (Table 2). *B. animalis* subsp. *animalis* DSM 20104, *B. longum* subsp. *longum* DSM 20019, *B. longum* subsp. *suis* DSM 20211, and *B. longum* subsp. *infantis* DSM 20088 were most sensitive towards hydroquinone with MIC≤0.05 mM while the MIC of the other strains was 0.1–0.2 mM (Table 2).

Strains *B. animalis* subsp. *animalis* DSM 20104 and *B. animalis* subsp. *lactis* DSM 10140, *B. breve* DSM 20213, and *B. longum* subsp. *infantis* DSM 20088 were additionally grown in the presence of glucose, glucose and arbutin, or arbutin (Figure 3). The presence of arbutin did not impact the growth of *B. breve* DSM 20213, while the lag phase of the other strains was delayed in the presence of glucose and arbutin and the final absorbance ratio reached was approximately 50% compared to glucose only.

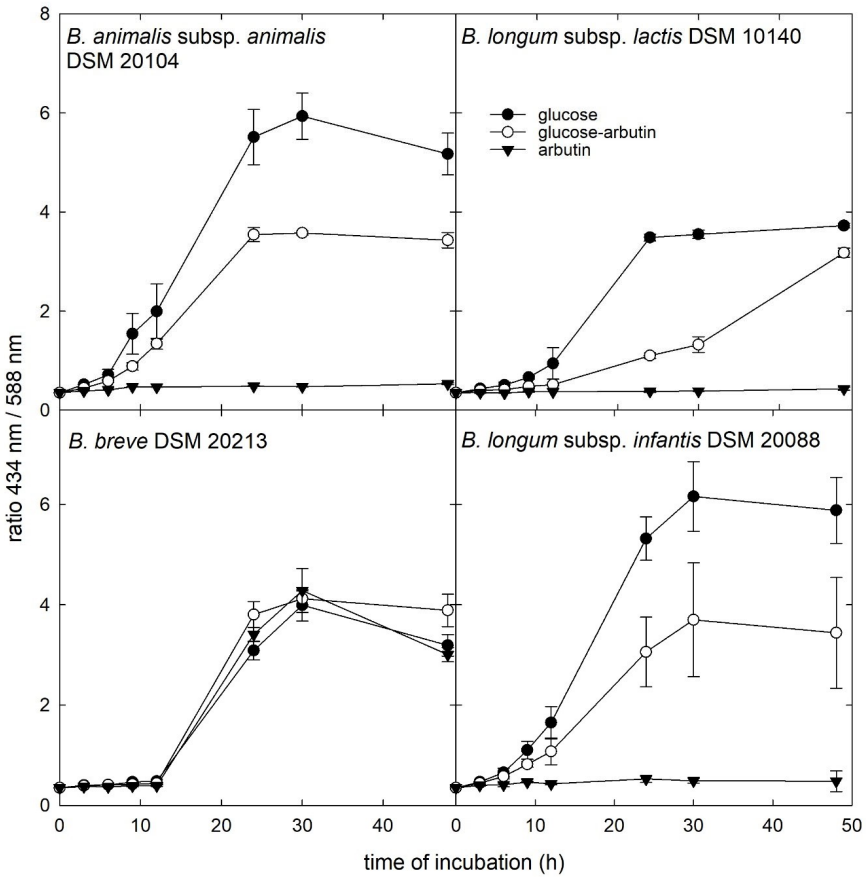

Figure 3. Impact of arbutin addition on growth. Growth kinetics of selected strains were tested in microtiter plates with API medium supplied with 28 mM glucose, 14 mM glucose and arbutin, or 28 mM arbutin. The plates were spectrophotometrically measured at 0, 3, 6, 9, 12, 24, 30, 36, and 48 h at 434 and 588 nm, to calculate the ratio of 434 nm/588 nm.

4. Discussion

Plant derived β-glucosides encompass structurally diverse compounds, which, when ingested, can reach the colon and be enzymatically modified by gut microbes. Beta-glucosidases are widely distributed in gut microbes and play important roles in biological processes. Here, we demonstrate species and strain dependent variability of *Bifidobacterium* spp. in the utilization of dietary plant glucosides linked to aromatic aglycones.

4.1. Genomic Potential for β-Glucosidase Activity Partly Predicts Activity

Based on genomic data, strains of the phylogenetically closely related species *B. adolescentis*, *B. catenulatum*, *B. pseudocatenulatum*, *B. dentium*, and the more distant *B. breve* [10], harbored a core of four β-glucosidases. In agreement, all strains of these species possessed β-glucosidase activity but showed preference for different substrates. Indeed, the purified β-glucosidases of *B. pseudocatenulatum* IPLA 36007 varied in their ability to release of the aglycones daidzein and genistein from isoflavone glycosides daidzin and genistin, indicating enzyme dependent substrate preference [28].

Strains of *B. longum* did not show β-glucosidase activity despite the presence of genes encoding β-glucosidases with high homology to a purified β-glucosidases of *B. longum* H1, which hydrolyzed arbutin. Lack of β-glucosidase activity of whole cells might suggest that enzymes were either not expressed or expressed at concentrations not sufficient to confer activity at test conditions.

B. animalis subsp. *lactis* and *B. animalis* subsp. *animalis* harbored highly similar GH1 and GH3 β-glucosidases and possessed β-glucosidase activity. The similar GH1 β-glucosidase Bgl572 hydrolyzed PNP-G and arbutin when purified [31]. However, growth in the presence of plant glucosides differed between *B. animalis* subspecies, possibly due different transport mechanism or sensitive towards the released acylglycones. Indeed, *B. animalis* subsp. *animalis* DSM 20104 was more sensitive towards hydroquinone than *B. animalis* subsp. *lactis* DSM 10140.

4.2. Bifidobacterium β-Glucosidase Increases Bioactivity and Bioavailability of Plant Glucosidases and Acylglycones

Plants are used for antibacterial properties in medical applications, and bacterial β-glucosidase activity leads to the release of bioactive acylglycones. Indeed, we confirmed the release of esculetin from esculin, by almost all strains with β-glucosidase activity, modifying bioactivity and bioavailability. Despite the ability to hydrolyze esculin, not all strains were able to grow when esculin was supplied as substrate, which might be due to the antimicrobial activity of esculetin [33].

Bioactivity of plant glucosides is likely lower than of acylglycones due to larger molecular weight and higher hydrophobicity. In confirmation, arbutin at the concentrations tested did not show inhibition while hydroquinone conferred strong antibacterial activity against the *Bifidobacterium* strains tested. Hydroquinone MIC values of bifidobacteria ranging from ≤0.05–0.2 mM were lower than reported for *Staphylococcus aureus* (1–11 mM) [34,35] and various aerobic Gram-positive and -negative strains [4] including *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* (1.5–6 mM).

MIC were up to 10-fold lower than the levels of hydroquinone theoretically released during growth. However, in the MIC assay, a low concentration of bacterial cells is exposed to high concentrations of the antibacterial at the beginning of the growth phase, while in the growth assay, increasing amounts of hydroquinone face an increasing number of bacterial cells. Sensitivity of bifidobacteria to hydroquinone, which, if released in the intestinal tract, could reduce the growth potential of β-glucosidase active strains, but also of neighboring cells.

In addition, β-glucosidase activity of *Bifidobacterium* spp. increased the bioavailability of acylglycones. Hydroquinone has been linked to anticancer activity in vitro and in vivo [36] and has been shown to confer antimycobacterial and antileishmanial activity in vitro [37].

4.3. Niche Adaption of Bifidobacteria Seems Related to β-Glucosidase Activity

Host adaptation of *Bifidobacterium* spp. was suggested to be linked to the ability to use dietary or host-derived glycans and differs between species and strains [10,11]. Indeed, strains of *B. bifidum* and *B. longum* subsp. *infantis*, which are among the most abundant microbes during the first months of life, lacked β-glucosidase activity in agreement to previous observations [5], and with the absence of β-glucosidase encoding genes in the genomes. Both species occur in the infant gut when glycan substrates are limited to human breast milk, endogenous mucin, or infant formula. Indeed, a previous cohort of studies observed that fecal β-glucosidase activity was low after birth and gradually increased with the introduction of a more diverse diet [38]. Interestingly, *B. animalis* subsp. *animalis* showed only little growth in the presence of plant glucosides despite possessing β-glucosidase activity. Most isolates were obtained from lamb and calf feces early when animals received a milk diet.

B. breve colonizes infant and adults, and similar to *B. adolescentis* and other adult or multi-host species, possesses multiple β-glucosidases. In the adult gut, *Bifidobacterium* spp. contribute a minor share of the population, and compete with other β-glucosidase positive taxa for substrate [5]. Beta-glucosidase activity of *Bifidobacterium* strains colonizing adults might enhance ecological competitiveness.

For the multi-host subspecies of *B. animalis* subsp. *lactis*, plant glucoside utilization profiles likewise suggested host adaption in agreement with genetic and phenotypic host-specific differences previously observed [11]. Strains from adult ruminant hosts (Cameroon sheep, Barbary sheep, and

an okapi), that naturally receive a plant-based diet, used plant glucosides in contrast to strains isolated from omnivorous dogs.

4.4. Substrate Source Impacted Fermentation Profiles

Bifidobacteria metabolize hexoses via the "bifid shunt" with fructose-6-phosphoketolase as the key enzyme. Glucose (1 mol) theoretically yields 1.5 mol acetate, 1 mol lactate, and 2.5 ATP [39]. Whether the intermediate pyruvate is cleaved to acetyl phosphate and formate, or reduced to lactate, depends on type and supply of the substrate carbohydrate [40], possibly in the presence of oxygen [8] and on different rates in substrate consumption. A previous study reported that with a decrease of consumption rate, relatively more acetic and formic acid and less lactic acid was produced by *Bifidobacterium* spp. [41]. Indeed, the proportion of lactate was reduced for most strains grown in the presence of esculetin, and to a lesser extent with arbutin, which might indicate that hydrolysis activity reduced the consumption rate.

5. Conclusions

Our data shows that bifidobacterial β-glucosidase activity is preserved among species which might be related to niche adaption. Structural homology of a core set of β-glucosidases of species associated with adult humans could suggest that these enzymes evolved together. Beta-glucosidase activity may provide a competitive advantage in the mammalian gut proving access to energy sources, but they might also have environmental impact due to the release of bioactive antibacterial acylglycones; thus, increasing bioavailability due to the formation of fermentation metabolites.

Author Contributions: Conceptualization, V.N.-B. and C.S..; methodology, V.N.-B. and C.S.; validation, N.M., V.N.-B., and C.S.; formal analysis, N.M.; investigation, N.M., V.T., C.S., and V.N.-B.; resources, V.N.-B.; data curation, C.S.; writing—original draft preparation, N.M., C.S., and V.N.-B.; writing—review and editing, V.N-B., C.S., and N.M.; visualization, N.M. and C.S.; supervision, V.N.-B. and C.S.; project administration, E.V.; and funding acquisition, E.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by European Regional Development Fund-Project, "Centre for the investigation of synthesis and transformation of nutritional substances in the food chain in interaction with potentially harmful substances of anthropogenic origin: comprehensive assessment of soil contamination risks for the quality of agricultural products" [No: CZ.02.1.01/0.0/0.0/16_019/0000845], and by METROFOOD-CZ research infrastructure project [MEYS Grant No: LM2018100] including access to its facilities.

Acknowledgments: The authors thank Katerina Kindlova for her valuable assistance in laboratory experiments.

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

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4.6 Antibiotic susceptibility screening of primate-associated *Clostridium ventriculi*

Makovska M, **Modrackova N**, Bolechova P, Drnkova B, Neuzil-Bunesova V. 2021. Antibiotic susceptibility screening of primate-associated *Clostridium ventriculi*. Anaerobe **69**:102347.

Anaerobe 69 (2021) 102347

Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/anaerobe

Antimicrobial susceptibility of anaerobic bacteria

Antibiotic susceptibility screening of primate-associated *Clostridium ventriculi*

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ARTICLE INFO

Article history:
Received 30 November 2020
Received in revised form
7 February 2021
Accepted 8 February 2021
Available online 16 February 2021

Handling Editor: Dr. Jamal Wafaa

Keywords:
Sarcina ventriculi
Monkeys
Antibiotics
Disk diffusion
Susceptibility
Resistance

ABSTRACT

Clostridium ventriculi (syn. *Sarcina ventriculi*) is a Gram-positive opportunistic pathogen with sarcina morphology. In the case of gastrointestinal disorders, the treatment is often empirical. Due to the common occurrence in primates and the potential risk of dysbiosis; the antibiotic susceptibility screening of *C. ventriculi* strains isolated from guenon monkeys and crested gibbons to 58 antibiotics was performed to reduce potentially ineffective antibiotic use in case of disease. Isolates were found to be susceptible to the majority of the tested antibiotics, mainly to (fluoro)quinolones, macrolides, penicillins, and tetracyclines. The susceptibility profiles were similar despite the hosts. Tested strains showed also natural resistance to a few antibiotics on the genus level. Detected *in vitro* antibiotic efficiency is consistent with documented human treatment cases.

1. Introduction

Clostridium ventriculi (syn. *Sarcina ventriculi*) and *Clostridium maximum* (syn. *Sarcina maxima*) are morphologically very atypical almost spherical cells forming packets, usually of eight or more cells. These clostridia have properties rarely observed in other bacteria, such as the ability to grow at very low pH values and to form up to extremely large bundles [1]. They were formerly a separated genus *Sarcina,* comprising of two species: *S. ventriculi* and *S. maxima*. In 2016, they were reclassified based on the 16S rRNA gene and included in the genus *Clostridium* [2]. *C. ventriculi* strains naturally occur in soil, mud, and cereal grains [1]. Their presence is also associated with various pathologies of the human and animal digestive tract. The documented occurrence of *C. ventriculi* in the human body is causally related to stomach disorders, nausea, vomiting, ulcers, and chronic dyspepsia [3–5]. In animals, these strains are generally associated with excessive salivation and ulcers. Bleeding from the spleen of sheep, goats, and calves, and also cases of flatulence in cats and horses have been also reported [6–10]. Remarkably, these clostridia were found in healthy hosts, especially in people consuming a vegetarian diet [11] and in the gastrointestinal tract of animals without apparent health problems [12]. Disorders of humans caused by *C. ventriculi* have been often empirically treated with antibiotics (Table 1). Interestingly, the cultivation of these clostridia is very difficult, and they have limited viability and lifetime *in vitro* [1]. This may also be a reason for the limited number of results of their susceptibility testing to antimicrobial agents. The aim of this work was to determine the susceptibility or possible resistance of *C. ventriculi* primate strains to

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https://doi.org/10.1016/j.anaerobe.2021.102347
1075-9964/

Table 1
Documented human cases of *Clostridium ventriculi* (syn. *Sarcina ventriculi)* infections treated by antibiotics.

Age (years)	Sex	Histologic findings	Treatment including ATB	Reference
65	M	Large ulcerations of the cecal mucosa filled with fibrin and leukocyte aggregates, compatible with infectious colitis, one positive anaerobic blood culture with *C. ventriculi*	Laparotomic ileocecal resection and creation of a double-barrelled ileocolostomy; piperacillin-tazobactam, gentamicin, fluconazole	[22]
52	M	*C. ventriculi* in the upper respiratory tract and in the sputum cytology in a pulmonary tuberculosis patient	Rifampicin, isoniazid, pyrazinamide, ethambutol	[31]
54	M	A slight amount of retained gastric contents in the fundus	Proton pump inhibitor treatment; after identification of *C. ventriculi* ciprofloxacin and metronidazole	[24]
70	M	Barret's esophagus, ulcers, *C. ventriculi* in biopsies	Metronidazole, ciprofloxacin - this dual antibiotic therapy had failed; surgical revision of hiatus hernia and partial fundoplication	[32]
8	M	Ulceration in mid-esophagus with fibro-inflammatory debris and *C. ventriculi*; rare *C. ventriculi* and chronic inflammation in biopsies of the gastric body	Ciprofloxacin, metronidazole, proton pump inhibitor	[33]
14	F	Sarcina morphology microorganisms in biopsies (samples were taken during gastroscopy)	Ciprofloxacin, metronidazole	[26]
86	F	Emphysematous gastritis in the presence of bacterial overgrowth by *C. ventriculi*	Vancomycin, piperacillin-tazobactam, pantoprazole	[26]
69	M	Biopsy from pylorus and pancreas showed acute and chronic inflammation and bacteria morphologically consistent with *C. ventriculi*; biopsy sections of pyloric mucosa – acute and chronic gastritis, *C. ventriculi* marked increase in eosinophils	Ciprofloxacin, metronidazole, omeprazole	[5]
45	F	Diffuse circumferential thickening of the stomach wall affecting the pylorus and causing excessive distraction of the stomach, indicating obstruction of the gastric discharge	Oral proton pump inhibitors, metronidazole, and ciprofloxacin	[34]
12	F	Severe erosive esophagitis of the distal 15 cm esophagus; in particular, a large amount of gastric retention has been observed, indicating delayed gastric emptying	Ciprofloxacin and metronidazole	[23]
15	F	Active gastritis with the presence of microorganisms arranged in tetrads, characteristic of *C. ventriculi*	Ciprofloxacin, metronidazole, and omeprazole	[23]
10 m	M	Urethral narrowing and inflammation after transurethral fulguration	Ciprofloxacin and metronidazole	[35]
68	F	Gastric perforation complicated by gastrohepatic ligament abscess and mediastinal cyst; there were markers of acute inflammation, necrosis, granulation tissue and bacterial organisms identified as *C. ventriculi*	Ceftriaxone, metronidazole, piperacillin-tazobactam	[27]
32	F	Ulcer biopsy tissue with *C. ventriculi*	Fluoroquinolone and metronidazole, a proton pump inhibitor	[36]
50	M	Chronic gastritis with intestinal metaplasia with the presence of *C. ventriculi*	Metronidazole and ciprofloxacin with sucralfate	[25]
73	M	Inflammation with the formation of an ulcer bed and the presence of *C. ventriculi*	Ciprofloxacin and metronidazole	[4]
48	F	Fever, stomach cramps, vomiting and watery diarrhoea (congenital chloride diarrhoea – diagnosed in childhood)	Amoxycillin	[29]
3	F	Polymorphic inflammatory infiltrate with *C. ventriculi* and gas bubbles	Imipenem, fluconazole, and omeprazole	[30]
14	M	Diffuse acute haemorrhagic gastritis and *C. ventriculi*	Gentamicin and metronidazole	[28]

Footnotes: M – male; F – female.

available antibiotics and determine effective antimicrobial agents for their reduction in the case of some disorders.

2. Material and methods

2.1. Strain isolation and identification

Sarcina morphology microorganisms isolated from Wilkins-Chalgren agar supplemented with soya peptone (5 g L^{-1}, both Oxoid, UK), L-cysteine (0.5 g L^{-1}), and Tween 80 (1 mL L^{-1}, both Sigma-Aldrich, USA), primarily used to isolate bifidobacteria from faecal samples of primates, were used in this study. Visually distinguishable irregular yellow colonies with sarcina morphology were isolated and routinely cultivated in Wilkins-Chalgren broth (Oxoid) supplemented with soya peptone (5 g L^{-1}), L-cysteine (0.5 g L^{-1}), and Tween 80 (1 mL L^{-1}) used as WSP, or in Reinforced clostridial (RC) broth (Oxoid), or Brain heart infusion (BHI) broth (Oxoid), all filled with carbon dioxide and kept under oxygen-free conditions at 37 °C for 24 h. The grown cultures were stored at room temperature to keep bacterial cells viable for an extended period. Also, the growth in the agar form of media mentioned above (WSP, BHI, RC) and other media recommended for anaerobes such as Fastidious anaerobe agar (FAA) with horse blood (Oxoid) were verified to find a suitable solution for future antibiotic susceptibility testing. The RC agar was found to be the most suitable for antimicrobial susceptibility testing of *C. ventriculi*.

The origin of selected cultures is shown in Table 2. Genomic DNA from isolates was extracted from 1 mL of culture using PrepMan® Ultra™ Sample Preparation Reagent protocol (Thermo Fisher Scientific, USA). DNA was stored at −20 °C. The 16S rRNA of isolates was amplified and sequenced (Eurofins Genomics, Germany) using the universal primers fd1 and rp2 [13]. The obtained sequences have been inserted into the EZBioCloud [14] to obtain the closest related taxa. The type strain of *C. ventriculi* DSM 286^T was used as a control, respectively.

2.2. Antimicrobial susceptibility testing

Totally, 8 primate strains and one type strain DSM 286^T (Table 2) of *C. ventriculi* were used for disk diffusion antimicrobial susceptibility testing method (6 antibiotic discs per plate) with RC medium supplemented with 10 g L^{-1} of technical agar (No. 3, Oxoid). Strains were exposed to 58 antibiotics (Table 3; all Oxoid) with chosen concentration according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing) and/or CLSI (The Clinical & Laboratory Standards Institute). An aliquot (1 ml) of freshly grown clostridial culture was transferred into the Petri dish and suffused by 20 ml of RC agar. All antibiotics were tested in duplicates. Plates were incubated anaerobically at 37 °C for 48 h.

3. Results and discussion

Colonies with sarcina morphology were routinely detected on modified Wilkins-Chalgren agar during cultivation analyses of

Table 2
Clostridium ventriculi (syn. Sarcina ventriculi) isolates used for antibiotic suspensibility testing.

Strain	Isolated from the faecal sample of		Place of sampling
SV1	De Brazza's monkey	*Cercopithecus neglectus*	Zoo Plzeň, Czechia
SV2	De Brazza's monkey	*Cercopithecus neglectus*	Zoo Plzeň, Czechia
SV3	Norhren white-cheeked gibbon	*Nomascus leucogenys*	Zoo Liberec, Czechia
SV4	Norhren white-cheeked gibbon	*Nomascus leucogenys*	Zoo Liberec, Czechia
SV5	Roloway monkey	*Cercopithecus roloway*	Zoo Bojnice, Slovakia
SV6	Roloway monkey	*Cercopithecus roloway*	Zoo Bojnice, Slovakia
SV7	Lesser white-nosed Monkey	*Cercopithecus petaurista*	Zoo Bojnice, Slovakia
SV8	Lesser white-nosed Monkey	*Cercopithecus petaurista*	Zoo Bojnice, Slovakia
DSM 286^T	Soil	–	DSMZ - type strain

Footnotes: T – type strain; DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH/German Collection of Microorganisms and Cell Cultures GmbH.

faecal samples of guenon monkeys and crested gibbons. However, to isolate and keep these strains in both pure and viable culture for repeated testing is very demanding. Therefore, only the results of a limited number of isolates are presented. Finally, 8 isolates from 8 hosts (Table 2) originated from faecal samples of the De Brazza's monkeys, Northern white-cheeked gibbons, Roloway monkeys, and Lesser white-nosed monkeys kept in Zoo Liberec (Czechia) and Zoo Bojnice (Slovakia) were tested in this study. The type strain *C. ventriculi* DSM 286^T originated from soil was included as a control. The mentioned primate hosts were free of obvious health and gastrointestinal problems. *C. ventriculi* counts on modified Wilkins-Chalgren agar were determined to be ordinarily from 10^5 to 10^7 CFU g^{-1} of faecal sample. The identity of tested gas-producing aggregating isolates with typical sarcina morphology (Fig. 1) was confirmed by the 16S rRNA gene sequencing. The obtained sequences were inserted into the EZBioCloud and NCBI database and *C. ventriculi* (syn. *S. ventriculi*) was found as the closest related taxa. Because the clostridia have been detected repeatedly in faecal samples of guenon monkeys and crested gibbons, *C. ventriculi* seems to be a common part of their faecal microbiota. *C. ventriculi* strains were also isolated from faeces of wild Yakushima macaques [12], wild gorilla [15], and chimpanzees with damaged intestinal mucosa due to the simian immunodeficiency virus [16].

All 8 selected primate isolates of *C. ventriculi* were tested simultaneously with 58 different antibiotics using the disc diffusion method together with the type strain. As no recommendations are available for minimal inhibitory concentration (MIC) and clinical breakpoints for *C. ventriculi*, most concentrations of tested antibiotics were selected according to the EUCAST and/or CLSI. In absence of recommendations for clinical categorization of strains we decided as follows; detected inhibition zones were arbitrarily divided into three categories based on their size. The diameter of the inhibition zones (including the disc diameter of 6 mm) was measured in millimeters. Results <8 mm were expressed as resistant, 8–21 mm as moderately susceptible, and >21 mm as susceptible. The limit <8 mm for resistance was chosen because *C. ventriculi* cultures do not form homogenous growth visible as cell density, but form aggregates on the agar, which affects the reading of the inhibition zone. All tested isolates of *C. ventriculi* were found to be resistant to 13 tested antibiotics (Table 3). Based on test results, the tested strains seem to be resistant to most antibiotics from the group of cephalosporins (cefixime, cefadroxil, cefotaxime, cefpodoxime, ceftriaxone, cefuroxime sodium, cephalexin, cephazolin). Cephalosporins are generally less active against clostridia [17]. An intrinsic resistance was also observed to aztreonam (monobactams), sulfamethoxazole/trimethoprim, trimethoprim (sulfonamides), fluconazole (azoles), and mupirocin (pseudomonic acids). In summary, the detected results indicate some natural resistance on the genus level. Above that, some of these tested antibiotics are commonly used for the treatment of primates kept in zoos; therefore, the potential of the resistance should be considered in order to reduce its spread [18].

The highest inhibition zones (>21 mm) were observed for all antibiotics from the group tetracyclines (doxycycline, tetracycline, tigecycline) and for all of the penicillins. Further for rifampicin (ansamycins), ciprofloxacin, levofloxacin, moxifloxacin, and norfloxacin ((fluoro)quinolones), chloramphenicol (chloramphenicols), metronidazole (imidazoles), clarithromycin, clindamycin, and quinupristin/dalfopristin (macrolides), and linezolid (oxazolidinons). *C. ventriculi* strains were moderately susceptible to all other tested antibiotics (Table 3) and the susceptibility profiles were alike. Also, the type strain of soil origin showed very similar susceptibility. However, lower susceptibility of the type strain DSM 286^T compared to primate isolates was detected with amoxycillin-clavulanic acid, ampicillin-sulbactam, clarithromycin, and ticarcillin. For glycopeptides (30 μg) some diameters (Table 3) may appear small due to poor diffusion around the disk of these drugs. EUCAST does not recommend diffusion method on the grounds of poor diffusion.

Although the diffusion test is not recommended for anaerobes, it has been demonstrated that disk diffusion can predict antibiotic susceptibility with few very major errors [19–21]. Only Bortolotti et al. [22] determined *in vitro* antimicrobial susceptibility of *C. ventriculi* isolate of human origin as it is recommended by the CA-SFM 2019 (Comité de l'Antibiogramme de la Société Française de Microbiologie) guidelines. This *C. ventriculi* isolate was susceptible to penicillin (MIC = 0.25 mg L^{-1}), amoxycillin (MIC = 0.50 mg L^{-1}), amoxycillin-clavulanic acid, piperacillin-tazobactam, imipenem, clindamycin, levofloxacin, rifampicin, vancomycin, metronidazole, and linezolid, which corresponds with our results.

The detected *in vitro* susceptibility of *C. ventriculi* isolates to most of the tested antibiotics is in correlation with their mostly empirical application (Table 1). Based on documented records, ciprofloxacin together with metronidazole are the mostly used antibiotics [5,23–27], followed by piperacillin-tazobactam [22,26,27]. Less frequently used antibiotics were gentamicin [28], vancomycin [26], amoxycillin [29], imipenem [30], and others [31–36]. *C. ventriculi* isolates were found susceptible to these antibiotics also according to our and Bortolotti et al. [22] *in vitro* results. Therefore, early well-chosen antibiotic therapy appears to be effective and would not cause possible antibiotic resistance.

4. Conclusions

Despite of the fact that *C. ventriculi* presence is often connected with different pathologies of the gastrointestinal tract, this opportunistic pathogen seems to be a common bacterial taxon of primates without apparent health problems. The susceptibility profiles of tested primate isolates were very similar despite the different hosts and their locations. Primate-associated *C. ventriculi* isolates seem to be susceptible to most of the common antibiotics. Some presence of natural resistance on the genus level was also

Table 3
Susceptibility of *Clostridium ventriculi* isolates to antibiotics determined using disk diffusion method. The diameter of the inhibition zones was measured in millimeters (including the disc diameter of 6 mm) and were arbitrarily divided into three categories based on size. Results <8 mm were expressed as resistant, 8–21 mm as moderately susceptible, and >21 mm as susceptible.

Antibiotic	concentration	SV1	SV2	SV3	SV4	SV5	SV6	SV7	SV8	DSM 286^T
Aminoglycosides										
Amikacin	30 μg*,^{E}	9	9	9	10	9	8	10	12	13
Gentamicin	10 μg*,^{E}	14	13	14	12	17	15	13	17	13
Kanamycin	30 μg*	17	14	17	15	14	15	16	18	12
Neomycin	30 μg*	12	11	14	13	12	15	15	11	10
Netilmicin	10 μg^E	9	9	9	9	9	8	9	9	8
Streptomycin	10 μg*	12	12	15	10	18	11	12	12	13
Tobramycin	10 μg*,^{E}	8	8	8	9	9	8	8	8	9
Ansamycins										
Rifampicin	5 μg*,^{E}	28	30	30	30	35	32	30	32	28
Azoles										
Fluconazole	25 μg*	6	6	6	7	7	6	6	6	6
Carbapenems										
Ertapenem	10 μg*,^{E}	21	20	20	19	20	19	20	19	22
Imipenem	10 μg*,^{E}	15	14	15	15	18	15	18	16	18
Meropenem	10 μg*,^{E}	14	13	12	15	14	15	16	14	17
Cephalosporins										
Cefadroxil	30 μg^E	6	6	6	6	6	6	6	6	6
Cefepime	30 μg*,^{E}	20	20	21	21	20	20	21	21	21
Cefixime	5 μg*,^{E}	6	6	6	6	6	6	6	6	6
Cefotaxime	5 μg*	6	6	6	6	6	6	7	6	6
Cefpodoxime	10 μg*,^{E}	6	6	6	6	6	6	7	6	6
Ceftazidime	10 μg^E	11	11	11	11	13	10	11	11	13
Ceftriaxone	30 μg*,^{E}	6	6	7	6	7	6	6	6	6
Cefuroxime sodium	30 μg*,^{E}	6	6	7	6	6	6	7	6	6
Cephalexin	30 μg^E	6	6	7	6	6	6	7	6	6
Cephazolin	30 μg*	6	6	6	6	6	6	6	6	6
Cefamycins										
Cefotetan	30 μg*	19	19	19	14	18	14	18	16	16
Cefoxitin	30 μg*,^{E}	11	10	12	10	10	10	13	12	13
Fluoro (quinolones)										
Ciprofloxacin	5 μg*,^{E}	30	26	28	23	30	25	25	22	22
Levofloxacin	5 μg*,^{E}	27	27	28	24	28	27	28	28	22
Moxifloxacin	5 μg*,^{E}	28	26	28	28	28	28	27	22	22
Nalidixic acid	30 μg*,^{E}	14	13	13	15	15	10	13	13	15
Norfloxacin	10 μg*,^{E}	23	26	25	29	25	22	25	22	21
Ofloxacin	5 μg*,^{E}	22	21	20	15	19	12	20	21	21
Pefloxacin	5 μg^E	14	17	14	14	14	13	14	12	16
Glycopeptides										
Teicoplanin	30 μg*,^{E}	15	13	15	15	15	13	12	16	18
Vancomycin	30 μg*	20	23	21	23	21	20	21	21	16
Chloramphenicols										
Chloramphenicol	30 μg*,^{E}	32	32	32	32	30	30	30	32	26
Imidazoles										
Metronidazole	5 μg	30	30	32	32	32	30	29	30	23
Macrolides										
Azithromycin	15 μg*	15	15	20	16	14	17	18	18	14
Clarithromycin	15 μg*	28	27	29	26	28	27	27	27	21
Clindamycin	2 μg*,^{E}	33	32	33	32	33	32	32	33	28
Erythromycin	15 μg*,^{E}	19	18	19	18	16	16	17	18	15
Quinupristin/Dalfopristin	15 μg*,^{E}	26	26	28	28	28	26	25	28	22
Monobactams										
Aztreonam	30 μg*,^{E}	6	6	6	6	6	6	9	6	6
Nitrofurans										
Nitrofurantoin	100 μg^E	22	20	20	18	20	20	20	22	20
Oxazolidinones										
Linezolid	10 μg^E	30	30	30	30	30	28	30	30	30
Penicillins										
Amoxycillin/Clavulanic acid	30 μg*,^{E}	30	30	30	32	30	27	30	32	21
Amoxycillin	25 μg	30	30	30	30	30	30	30	30	26
Ampicilin	10 μg*,^{E}	30	25	26	23	28	27	22	24	22
Ampicillin/Sulbactam	20 μg*,^{E}	28	28	30	30	28	25	27	23	21
Penicillin G	10 μg*	25	28	28	28	26	28	27	27	20
Piperacillin	30 μg^E	28	28	30	28	30	28	30	30	28
Piperacillin/Tazobactam	36 μg^E	33	33	32	33	33	32	30	32	23
Ticarcillin	75 μg*,^{E}	30	30	28	30	30	30	30	30	20
Pseudomonic acids										
Mupirocin	200 μg^E	6	6	6	6	6	6	6	6	6
Sulfonamides										
Sulfamethoxazole/Trimethoprim	25 μg	7	7	6	6	6	6	6	7	6
Trimethoprim	5 μg*,^{E}	6	7	6	6	6	6	6	7	6
Steroid antibiotics										

Table 3 (*continued*)

Antibiotic	concentration	SV1	SV2	SV3	SV4	SV5	SV6	SV7	SV8	DSM 286^T
Fusidic acid	10 μg*,^{E}	20	18	17	18	20	15	18	18	19
Tetracyclines										
Doxycycline	30 μg*	33	33	33	33	33	35	32	33	34
Tetracycline	30 μg*,^{E}	33	33	33	33	32	33	33	35	31
Tigecycline	15 μg*,^{E}	33	33	33	30	32	33	32	33	25

Footnotes: The used antibiotics are classified in the classes according to "Categorization of antibiotics in the European Union" published by European Medicines Agency, 2020. ^{E}standard concentration of antibiotic disks according to the EUCAST; *standard concentration of antibiotic disks according to the CLSI.

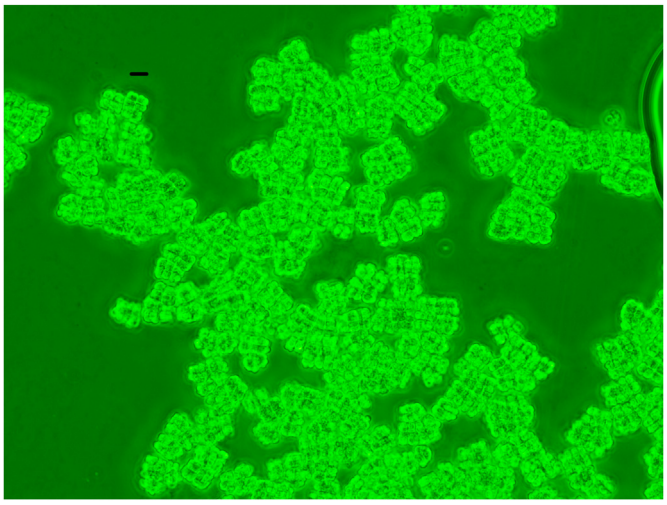

Fig. 1. Cell morphology of *Clostridium ventriculi* isolate (SV5) using phase contrast microscopy (bar, 10 μm).

detected. However, none antibiotic resistance on the strain- or host-level was found.

Funding

The work was supported by the European Regional Development Fund-Project "Centre for the investigation of synthesis and transformation of nutritional substances in the food chain in interaction with potentially harmful substances of anthropogenic origin: comprehensive assessment of soil contamination risks for the quality of agricultural products" (No. CZ.02.1.01/0.0/0.0/16_019/0000845).

Ethical approval

The sampling of the primate faeces was performed during zookeepers' routine activities. All procedures involving animals adhered to recommendations outlined in the "Guide for the Care and Use of Animals" by the Czech University of Life Sciences Prague.

Declaration of competing interest

There are no conflicts of interest associated with this publication.

Acknowledgements

This research was performed within the Centre for Infectious Animal Diseases (CINeZ) initiative.

We thank Zoo Liberec (Lidové sady 425/1, 460 01 Liberec 1, Czechia), Zoo Plzeň (Pod Vinicemi, 301 00 Plzeň, Czechia), and Zoo Bojnice (Zámok a okolie 939/6, 972 01 Bojnice, Slovakia).

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4.7 Enteral nutrition as a growth medium for cultivable commensal bacteria and its effect on their quantity in the stool of children with Crohn’s disease

Modrackova N, Bunesova V, Vlkova E, Musilova S, Mrvikova I, Bronsky J, Copova I, Hradsky O, Nevoral J. 2019a. Enteral nutrition as a growth medium for cultivable commensal bacteria and its effect on their quantity in the stool of children with crohn's disease. Journal of Medicinal Food **22**:810-816.

JOURNAL OF MEDICINAL FOOD
J Med Food 22 (8) 2019, 810–816

DOI: 10.1089/jmf.2019.0021

Enteral Nutrition as a Growth Medium for Cultivable Commensal Bacteria and Its Effect on Their Quantity in the Stool of Children with Crohn's Disease

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ABSTRACT Current studies indicate a link between the intake of exclusive enteral nutrition (EEN) and the induction of complex changes in the intestinal microbiota, as well as the clinical improvement of Crohn's disease (CD). The first aim of this study was to test the ability of various commensal bacterial strains ($n = 19$) such as bifidobacteria, lactobacilli, and *Escherichia coli* to grow on three different polymeric EN *in vitro*. Tested EN formulas were found to be suitable growth media for tested commensals. Furthermore, the counts of these bacteria and total counts of anaerobic bacteria in the fecal samples of children with CD ($n = 15$) before and after 6 weeks of EEN diet administration were determined using cultivation on selective media. The counts of cultivable commensal bacteria in the fecal samples of CD children were not significantly affected by EEN. However, tested bacteria showed some individual shifts in counts before and after EEN therapy. Moreover, cultured bifidobacteria were found to be in reduced counts in CD children. Therefore, the application of bifidogenic prebiotic compounds to EN for CD patients might be considered.

KEYWORDS: • ***bifidobacteria*** • **Escherichia coli** • ***exclusive enteral nutrition*** • ***inflammatory bowel disease*** • ***lactobacilli*** • ***probiotics***

INTRODUCTION

Based on ECCO/ESPGHAN guidelines, exclusive enteral nutrition (EEN) is recommended as first-line therapy to induce remission in children with active luminal Crohn's disease (CD).[1] Administering EEN is considered important for preserving the structural and functional integrity of the gut and also to maintain intestinal microbial diversity.[2] The outbreak of inflammatory bowel disease (IBD), and especially in CD, is associated with microbial imbalance[3] and was originally considered to occur in developed countries with Westernized lifestyles. However, the incidence of CD has also recently increased in other parts of the world with a significantly different lifestyle.[4,5]

CD is chronic inflammation of the gastrointestinal tract with alternating phases of relapse and remission, and known symptoms include abdominal pain, bloody or mucous diarrhea, and weight loss, which can result in patient malabsorption. Relapse is the acute phase of that disease, whereas remission is the resting phase that occurs as symptoms disappear.[6–8] The disruption of mucosal intestinal homeostasis, and also the interaction between environmental and genetic factors are associated with the outbreak of the disease.[9] Unfortunately, no permanent treatment options have been discovered to date.[4,10,11] There are many benefits of EEN, such as prolonging remission, improving growth, promoting mucosal healing, and restoring bone mineral density.[1,7,12] Furthermore, it can be used to treat relapse,[13] and is closely associated with changes to the patient microbiota and the suppression of intestinal dysbiosis.[14]

The microbial composition of the human intestine and the occurrence of commensal bacteria are associated with living in symbiosis with the host and providing important protective, trophic, and metabolic functions.[15] Clearly, it is desirable to promote the growth of potentially probiotic commensal bacteria in the host microbiota, such as bifidobacteria, lactobacilli, and nonpathogenic *Escherichia coli*.[16,17] The aims of this work were thus to verify the growth ability of selected commensal bacteria in three different EN formulas *in vitro* and then to evaluate the effects of EEN on the abundance of cultivable bifidobacteria, lactobacilli, and *E. coli* in the stool microbiota of children with CD.

Manuscript received 1 February 2019. Revision accepted 26 February 2019.

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MATERIALS AND METHODS

In vitro *growth of commensal bacteria in EN*

The first part of this study was focused on verifying of bacterial ability to grow in three different EN formulas *in vitro* using the microtiter plate assays. The compositions of tested polymeric EN are described in Table 1. EN Modulen IBD (Nestlé Netherland BV, Netherlands), Fortini Neutral (N. V. Nutricia, Zoetermeer, Netherlands), and Fresubin Energy Fiber Strawberry (Fresenius Kabi Deutschland GmbH, Germany) were used as the only source of nutrients for the 19 tested probiotic strains of bacteria (Table 2).

Overnight cultures of bacterial strains from the genera *Bifidobacterium*, *Lactobacillus*, and *Escherichia* were used for this study after isolation and identification using MALDI-TOF MS according to Bunesova *et al.*[18] The strains had different origins, including probiotic milk products (BIF1 and LB2), probiotic supplements (BIF2, BIF3, BIF7, and EC2), infant feces (BIF4, BIF5, BIF6, LB1, LB3, LB4, LB5, LB6, and LB7), and adult human feces (EC1, EC3, EC4, and EC5). The strains were cultivated in an oxygen-free carbon dioxide environment at 37°C for 24 h in Wilkins-Chalgren anaerobe broth (Oxoid, UK) supplemented with GMO-Free Soya Peptone (5 g/L; Oxoid), L-cysteine (0.5 g/L; Sigma-Aldrich, USA), and tween 80 (1 mL/L; Sigma-Aldrich), which was used as WSP broth.

Table 1. Composition of Tested Enteral Nutrition Formulas

Nutrients per 100 mL	*Modulen IBD*	*Fortini Neutral*	*Fresubin Energy Fiber Strawberry*
Energy value (kJ/kcal)	414/100	640/153	630/150
Fat (g)	4.6	6.8	5.8
Saturated	2.6	0.7	0.4
Monounsaturated	0.78		3.8
Polyunsaturated	0.48		1.6
Carbohydrate (g)	11.0	18.8	17.8
Sugars	4.2	4.6	5.6
Lactose	<0.100	<0.025	<0.260
Fiber (g)	0	1.5	2.0
Protein (g)	3.6	3.4	5.6
Salt (g)	0.085	0.17	
Other nutrients			
Carotenoids (mg)		0.15	3
Choline (mg)	7	30	26.7
α-linolenic acid (mg)	0.04		
Linoleic acid (g)	0.42		
L-carnitine (mg)		3	
Taurine (mg)		11	
Medium-chain triglycerides (g)	1.2		
Osmolality (mOsm/L)	290	380	410

Representation of macronutrients and other nutrients in tested EN formulas. Complete content of the products, including vitamins and minerals, can be obtained from the manufacturers' package leaflets.

EN, enteral nutrition; IBD, inflammatory bowel disease.

Tested EN formulas (90 μL) were inoculated into the microtiter plates, and then the volume was adjusted to 100 μL with pure fresh overnight cultures of tested bacterial strains. The amount of the inoculated cells was $\sim 10^4$ CFU per 1 mL of EN before cultivation. WSP broth was used as a positive control for growth, and nutrient-poor dilution medium consisting of Tryptone (5 g/L; Oxoid), Nutrient Broth No. 2 (5 g/L; Oxoid), yeast extract (2.5 g/L; Oxoid), tween 80 (0.5 mL/L), and L-cysteine hydrochloride monohydrate (0.25 g/L) was used as a negative control. The microtiter plates were incubated under anaerobic conditions in CO_2/H_2 (20/80%) created by the GENbag anaer (bioMérieux, France) at 37°C for 24 h. After cultivation, the bacterial counts were evaluated using the plate method on Wilkins-Chalgren Anaerobe Agar supplemented with GMO-Free Soya Peptone (5 g/L), L-cysteine (0.5 g/L), and tween 80 (1 mL/L), which was used as WSP agar. Plates were cultivated again in anaerobic conditions and maintained for 48 h.

Enumeration of commensal bacteria in fecal samples

The study was approved by the Ethics Committee of the authors' institution. Fecal samples of 15 children diagnosed with CD (13.60 ± 3.50 years of age) on a 6-week polymeric EEN regimen were collected at week 0 and 6 at the Department of Pediatrics, University Hospital Motol, Charles University in Prague. The taste and brand choice of the aforementioned EN were influenced by personal patient preferences. Patients could choose a single or combinations of any polymeric EN. However, throughout the therapy time, the mentioned formulas were only used (Table 1). Microbiological analyses of stool samples were performed using the plate technique with media for total counts of anaerobic bacteria, bifidobacteria, lactobacilli, and *E. coli* according to Musilova *et al.*[19] Briefly, WSP agar was used for the determination of total counts of anaerobic bacteria, WSP agar with mupirocin (100 mg/L; Oxoid) and acetic acid (1 mL/L; Sigma-Aldrich) for bifidobacteria, Rogosa Agar (Oxoid) with acetic acid (1.32 mL/L) for lactobacilli, and chromogenic T.B.X. medium (Oxoid) for *E. coli*. Cultivation was performed under anaerobic conditions for bifidobacteria and total anaerobes at 37°C for 48 h, whereas microaerophilic conditions were used for lactobacilli at 37°C for 48 h and aerobic conditions were used for *E. coli* at 37°C for 24 h.

Statistical analysis

Counts of bacterial colonies were expressed as the mean with standard deviation. Analysis of variance using one-way ANOVA with Scheffe's method was applied to determine statistical significance between tested groups of commensal bacteria and EN based on *in vitro* testing with a 95% confidence level. The normality of the data was evaluated by the Shapiro-Wilk W test. The effect of 6-week EEN therapy on the quantity of commensal bacteria in stool samples from children with CD was evaluated by performing a Mann–Whitney U Test ($P < .05$). All statistical analyses were processed using STATISTICA software (Version 12.0, 2013).

Table 2. Average Counts of Tested Commensal Bacteria After *In Vitro* Cultivation on Different Enteral Nutrition Formulas (log CFU/mL)

		Enteral nutrition		
		Modulen	*Fortini*	*Fresubin*
Average counts of bifidobacteria		6.71 ± 1.15^{1a}	7.56 ± 1.59^{1a}	7.63 ± 1.91^{1a}
BIF1	*Bifidobacterium animalis* ssp. *lactis*	5.18 ± 0.01	5.42 ± 0.05	5.46 ± 0.15
BIF2	*Bifidobacterium animalis* ssp. *lactis*	5.01 ± 0.04	5.30 ± 0.00	4.30 ± 0.00
BIF3	*Bifidobacterium bifidum*	7.81 ± 0.22	8.38 ± 0.01	8.63 ± 0.07
BIF4	*Bifidobacterium bifidum*	7.40 ± 0.02	7.47 ± 0.09	9.00 ± 0.10
BIF5	*Bifidobacterium bifidum*	7.37 ± 0.01	8.26 ± 0.01	8.57 ± 0.06
BIF6	*Bifidobacterium longum*	6.75 ± 0.02	8.93 ± 0.01	8.65 ± 0.05
BIF7	*Bifidobacterium longum*	7.47 ± 0.00	9.15 ± 0.15	8.79 ± 0.03
Average counts of lactobacilli		9.38 ± 0.11^{2a}	8.44 ± 0.69^{1b}	8.96 ± 0.28^{1ab}
LB1	*Lactobacillus gasseri*	9.33 ± 0.03	9.35 ± 0.07	8.77 ± 0.10
LB2	*Lactobacillus paracasei*	9.36 ± 0.00	9.41 ± 0.03	9.29 ± 0.03
LB3	*Lactobacillus paracasei*	9.17 ± 0.03	8.62 ± 0.02	9.11 ± 0.04
LB4	*Lactobacillus paracasei*	9.38 ± 0.05	7.84 ± 0.06	9.26 ± 0.04
LB5	*Lactobacillus rhamnosus*	9.49 ± 0.01	7.90 ± 0.28	8.57 ± 0.01
LB6	*Lactobacillus paracasei*	9.42 ± 0.01	7.93 ± 0.03	9.02 ± 0.05
LB7	*Lactobacillus rhamnosus*	9.49 ± 0.00	8.06 ± 0.02	8.72 ± 0.02
Average counts of *Escherichia coli*		9.15 ± 0.25^{2a}	8.81 ± 0.36^{1a}	9.10 ± 0.20^{1a}
EC1	*Escherichia coli*	9.20 ± 0.03	8.65 ± 0.08	9.24 ± 0.04
EC2	*Escherichia coli*	9.43 ± 0.05	8.91 ± 0.04	9.03 ± 0.04
EC3	*Escherichia coli*	9.05 ± 0.05	8.39 ± 0.07	9.00 ± 0.04
EC4	*Escherichia coli*	8.78 ± 0.06	8.76 ± 0.03	8.86 ± 0.00
EC5	*Escherichia coli*	9.28 ± 0.00	9.35 ± 0.01	9.37 ± 0.06

Counts of commensal bacteria are averages in log CFU/mL ± standard deviation. Data of individual bacterial strains were obtained from three independent measurements. Superscript letters represent differences within the rows regarding the ability of one group of bacteria to grow in three different EN; superscript numbers represent differences within the columns in terms of the ability of three bacterial groups to grow in tested EN (significance level $P < .05$). Differences among counts of the three groups of commensal bacteria in one medium, and also within one bacterial group for three media were evaluated by analysis of variance with one-way ANOVA (Scheffe's test) using STATISTICA software (Statistica 12.0, Tulsa, USA).

RESULTS

Growth of commensal bacteria in EN

In total, 19 strains of bifidobacteria, lactobacilli, and *E. coli* were tested for their ability to grow *in vitro* using three different EN formulas (Modulen IBD, Fortini, and Fresubin). Bacterial counts are shown in Table 2. All strains of bacteria were able to utilize components of tested EN and grow in each formula, achieving relatively high numbers, with the exception of commercially available strains of *Bifidobacterium animalis* subsp. *lactis* (BIF1 and BIF2), for which counts were only ~5 log CFU/mL. These strains increased the standard deviation of the entire bifidobacterial group due to their poor growth potential, compared to that of other tested bifidobacterial strains isolated from several hosts.

In the control WSP media, all tested strains ($n = 19$) grew to counts greater than 10^9 CFU/mL. Specifically, bifidobacterial counts were in the range of 6.71–7.63 log CFU/mL, lactobacilli counts were 8.44–9.38 log CFU/mL, and *E. coli* counts were 8.81–9.15 log CFU/mL. Comparing the growth of pure commensal bacterial strains on different formulas, no statistically significant differences in bifidobacteria and *E. coli* were detected, whereas the counts of lactobacilli were significantly higher on Modulen IBD (9.38 ± 0.11 log CFU/mL), compared to those on Fortini (8.44 ± 0.69log CFU/mL). However, counts on Fresubin (8.96 ± 0.28 log CFU/mL) were similar to those in both aforementioned EN formulas. In terms of the ability of particular EN formulas to promote the growth of commensal bacteria, on Modulen, bifidobacterial counts (6.71 ± 1.15 log CFU/mL) were significantly lower than *E. coli* (9.15 ± 0.25 log CFU/mL) and lactobacilli counts (9.38 ± 0.11 log CFU/mL). In terms of growth, statistically significant differences were not detected among pure strains on the other two formulas, namely Fortini and Fresubin.

The counts of cultivable commensal bacteria in the fecal microbiota of CD patients after completion of EEN treatment

The counts of bifidobacteria, lactobacilli, and *E. coli* in stool samples of 15 children with CD were determined and compared before (week 0) and at week 6 of EEN. The results are shown by box plots in Figure 1. The examined stool samples contained similar amounts of bacteria at week 0 and 6, and no statistically significant differences were identified within each group. Bifidobacterial counts in the examined stool samples ranged from 7.86 ± 1.58 log CFU/g before the diet to 7.49 ± 1.76 log CFU/g after 6 weeks of EEN therapy, and were approximately two orders of magnitude lower than total numbers of anaerobic bacteria, for which counts ranged from 9.22 ± 0.86 log CFU/g at week 0 to 9.38 ± 0.79 log CFU/g at week 6. Nevertheless, bifidobacteria exhibited the

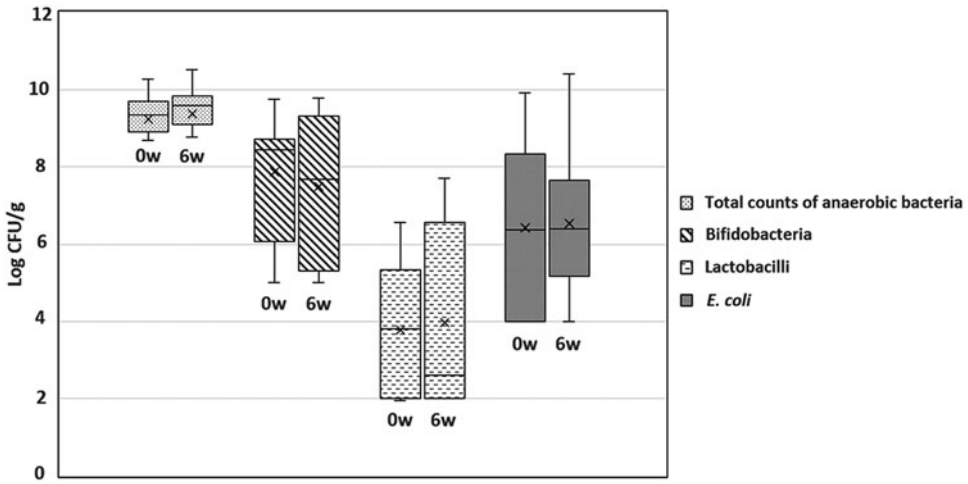

FIG. 1. Counts of cultivated commensal bacteria from stool samples of Crohn's disease patients ($n = 15$) before and after a 6-week exclusive enteral nutrition diet. Values are averages in log CFU/g ± standard deviation of 15 measurements. The data were compared within the same bacterial group at different times (0w, week 0; 6w, week 6) using the Mann–Whitney U test ($P < .05$) for data without normal distribution. The normality of data was evaluated by the Shapiro–Wilk W test. STATISTICA software (Statistica 12.0, Tulsa, USA) was used to perform analyses. The box plots were created using Microsoft Office Professional Plus 2016.

highest occurrence compared to the other two tested groups of bacteria. Lactobacilli were also detected in both groups of stool samples; however, their numbers reached counts of almost 4 log CFU/g (from 3.79 ± 1.70 to 3.96 ± 2.31 log CFU/g). *E. coli* exhibited better growth ability compared to lactobacilli as counts went from 6.42 ± 2.07 log CFU/g at week 0 to 6.54 ± 1.96 log CFU/g at week 6. It was thus confirmed that EEN did not selectively support any of the tested groups of cultivable commensal bacteria after 6 weeks of treatment. In contrast, for bifidobacteria and lactobacilli, as well as *E. coli*, variability in bacterial counts before and after EEN therapy was observed on an individual patient level.

DISCUSSION

EEN is currently considered an effective modality for achieving remission in pediatric patients with CD, and should be used as the first preferable treatment of choice.[20] There is evidence that EEN can induce remission in up to 80% of CD patients, although its mechanism of action in terms of its effects on the composition of the intestinal microbiota is still unclear[21]; moreover, there is no single conclusion regarding how the intestinal microbiota of patients is affected by EEN therapy. Therefore, this study was conducted to obtain further information by testing the growth of pure strains of bifidobacteria, lactobacilli, and *E. coli* on EN. For basic *in vitro* testing, EN would be a suitable source of nutrients for commensal bacteria that are able to utilize compounds found in each of the tested formulas. The absence of bifidogenic components such as fiber, as well as lower carbohydrate levels, in the Modulen IBD formula could result in the inability of bifidobacteria to grow, as well as lactobacilli (9.38 ± 0.11 log CFU/g) and *E. coli* (9.15 ± 0.25 log CFU/g), which was statistically confirmed. Bifidobacteria reached counts that were approximately two orders of magnitude lower than those of the other groups. For example, recently, a bifidogenic effect was found for a mixture of galactooligosaccharides and maltodextrins, which improved the bifidobacterial growth potential,[22] as well as isomaltooligosaccharides,[23] inulin-type fructans, and arabinoxylanoligosaccharides.[24] Moreover, bifidobacteria are anaerobic microorganisms that are sensitive to oxygen exposure[25]; thus, manipulation during testing might result in a lower incidence of these bacteria after cultivation. In addition, the worse growth potential of commercially available bifidobacterial strains could be caused by growth degeneration due to the long cultivation passages in laboratory conditions.[26–28]

Currently, the presence of tested commensal bacteria with probiotic potential, as health-promoting beneficial bacteria, is monitored in the microbiota of patients suffering from CD, which is closely related to EEN therapy. Likewise, the quantitative occurrence of cultivable commensal bacteria in the intestinal microbiota of CD patients before and after EEN therapy was tested in our study. The administration of EEN is associated with decreased variation in the patient microbiota, especially with respect to representation by the genera *Bifidobacterium*, *Faecalibacterium*, *Ruminococcus*, and *Eubacterium*, and with dysbiosis of the microbiota.[29] Moreover, Schwerd *et al.*[30] claimed that a 2-week course of EEN could reduce the numbers of *Bacteroides* and increase *Firmicutes*. However, Shiga *et al.*[31] suggested that EEN therapy does not cause any change to the bacterial diversity in the intestine. Similarly, in our study, there were no statistically significant differences in the counts of cultivable commensal bifidobacteria, lactobacilli, and *E. coli* in the fecal microbiota, which were compared before and after 6 weeks of EEN therapy. The counts of cultivable bifidobacteria are usually found to be $\sim 10^9$ CFU/g in stool

samples of healthy human donors.[22,32] In this study, bifidobacteria were detected in stool samples of children with CD using the same conditions; however, significantly lower counts (10^7 CFU/g) were found. Moreover, Sheehan *et al.*[33] suggested that the genus *Bifidobacterium* is reduced in the microbiota of CD patients. However, based on our results, the counts of other cultivable commensals such as *Lactobacillus* spp. and *E. coli* were similar compared to those in the stool samples of humans without CD.[22,32]

The unsupported growth promotion of commensal bacteria could probably be caused by the nonselective properties of the provided substrates found in EN formulas. However, there are some possible ways through which the representation of aforementioned bacteria could be affected. Bifidobacterial growth can be supported by the addition of human milk oligosaccharides[34] and short-chain fructooligosaccharides[35] to the EN. However, there is a risk that commercially available prebiotics are not sufficiently selective and will support the growth of potential pathogens.[36,37] However, fructans and galactooligosaccharides are among the most studied prebiotics that have a bifidogenic effect,[38] whereas inulin was suggested to be a prebiotic for lactobacilli growth.[39] These bacteria are commonly found in the human intestine[40] and could be also acquired from common diet.[41]

In contrast, interactions between coexisting bacterial species in the gut could also be desirable for CD patient health. The ability of bifidobacteria to utilize various substrates is associated with the different ability of coexisting bifidobacterial strains to utilize their metabolites in the intestinal microbiota, and is called cross-feeding.[42] This mechanism has also been described for bacteria of various genera, such as between bifidobacteria and *Eubacterium hallii*, wherein bifidobacteria utilize the provided substrates and produce short-chain fatty acids, from which acetate and lactate are utilized by *E. hallii* to produce butyrate.[43] A similar effect is observed during the triculture of *Lactobacillus paracasei* and *Bifidobacterium longum* with *E. hallii* or with *Anaerostipes caccae*.[44]

The final metabolites such as butyrate can be used as a source of energy for epithelial cells in the gut and contribute to the maintenance of intestinal homeostasis.[45] It has also been shown that treatment with n-butyrate leads to the regulation of proinflammatory mediators.[46] Based on this information, it might be desirable to promote the growth of not only aforementioned commensal bacteria but also butyrate-producing bacteria, which have an immediate effect on the inflamed intestine. It is also important to realize that to evaluate the effect of EEN on the composition of the intestinal microbiota, it is necessary to choose appropriate studies with relevant methods. Gatti *et al.*[47] stated that many studies are relatively inconsistent, and thus further investigations are necessary to clarify the mechanisms associated with *in vivo* interactions between EEN and intestinal inflammation.[48] As mentioned previously herein, further testing of the selective growth of commensal bacteria in enriched EN formulas with prebiotic effects is necessary in the future.

CONCLUSION

EN formulas seem to be a suitable source of nutrients for commensal bifidobacteria, lactobacilli, and *E. coli*, which were able to utilize components of all provided formulas and grow to relatively high numbers, with the exception of slightly lower counts for bifidobacteria. Furthermore, the quantitative representation of these commensal cultivable bacteria in the microbiota of CD patients was not significantly affected by EEN administration for 6 weeks, as no differences were detected with respect to counts at week 0. To ensure the selectivity of EN formulas for the growth of commensal bacteria, further testing of various antimicrobial and prebiotic components is necessary.

ACKNOWLEDGMENTS

This work was supported by the Grant Agency of Charles University in Prague (grant nos. 364617, 246216); and by the Ministry of Health, Czech Republic—Conceptual Development of Research Organization, Motol University Hospital, Prague, Czech Republic (grant no. 00064203). The authors thank the study participants. We also thank Monika Stemberkova and Martina Geigerova for their valuable assistance in laboratory experiments.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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4.8 Microbial shifts of faecal microbiota using enteral nutrition *in vitro*

Modrackova N, Copova I, Stovicek A, Makovska M, Schierova D, Mrazek J, Sabolova M, Vlkova E, Hradsky O, Bronsky J, Nevoral J, Neuzil-Bunesova V. 2021a. Microbial shifts of faecal microbiota using enteral nutrition *in vitro*. Journal of Functional Foods **77**:104330.

Journal of Functional Foods 77 (2021) 104330

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Short communications

Microbial shifts of faecal microbiota using enteral nutrition *in vitro*

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ARTICLE INFO

Keywords:
Polymeric enteral formulas
Prebiotics
Cultivation
SCFAs
Microbiome
Therapy

ABSTRACT

Enteral nutrition (EN) formulas of polymeric type ordinarily have similar content of intact macronutrients but may vary in prebiotic saccharides and micronutrients. These components can play an important role in the intestinal microbiota modulation. The aim of this study was to investigate microbial changes of faecal samples after their *in vitro* anaerobic cultivation in four polymeric EN formulas using plate technique method, metabolite analysis, and microbiota profiling using 16S rRNA sequencing. Detected cultivable commensal groups (bifidobacteria, lactobacilli, *Escherichia coli*) in faecal samples of donors were able to grow in EN formulas. However, their counts varied depending on the individual donor and the type of EN formula. Similar trend was found in detected metabolites such as acetate, lactate, and butyrate. Also, taxonomic composition and diversity of original and cultivated faecal microbiota of one individual on different EN formula indicate a possible effect of the prebiotics and micronutrients to modulate gut microbiota.

1. Introduction

Nutrients in diet are the key factor of the microbiota configuration, through modulation of the abundance of specific species and their functions. Moreover, the effects of a diet on individuals in the population differ from person to person and may be influenced by a combination of host and microbiome features (David et al., 2014; Kolodziejczyk, Zheng, & Elinav, 2019; Shanahan, Van Sinderen, O'Toole, & Stanton, 2017; Yang et al., 2020). Enteral nutrition (EN) is a common artificial nutritional support for patients who are unable to achieve their nutritional requirements through oral diet. Exclusive EN represents the use of a complete liquid diet, with the exclusion of normal dietary components for a defined time, except water. In addition, exclusive EN providing a complete diet and simultaneously a therapeutic measure to induce remission of Crohn's disease (CD) in up to 80% of cases (Ashton et al., 2018; Forbes et al., 2017; MacLellan et al., 2017), especially in children and adolescents newly diagnosed with active CD (Cameron et al., 2013; Hradsky, Copova, Zarubova, Nevoral, & Bronsky, 2016). Therefore, exclusive EN is used as the first line therapy for CD patients (Ruemmele et al., 2014). There is significant evidence of this therapy efficacy in the microbiota changes such as specific species appearance, broad taxonomic shifts, and functional changes (Ashton et al., 2017; Ashton, Gavin, & Beattie, 2018; D'Argenio et al., 2013; Kaakoush, Day, Leach, Lemberg, & Mitchell, 2016; Quince et al., 2015). The effect on microbiota varies among patients, as well as the methods used to estimate bacterial distribution and diversity are heterogeneous (Gatti et al., 2017). It ought to be mentioned, that the gut microbiome composition of each individual is unique (Johnson, 2020) and the EN formulas used in the studies differ in type and composition (Cámara-Martos & Iturbide-Casas, 2019), especially in the presence of prebiotics, vitamins, and other nutrients and parameters, which can affect the colon microbiota (Klingbeil & de La Serre, 2018; Walker & Lawley, 2013; Yang et al., 2020). Above that, the time of exclusive EN therapy is usually at least 6 weeks and patients may change brands of formulas and at thus the composition of the EN during the therapy. The aim of this study was to investigate the microbial changes of faecal microbiota after their *in vitro* cultivation on EN formulas of polymeric type with variable prebiotic and nutrient composition.

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https://doi.org/10.1016/j.jff.2020.104330
Received 15 October 2020; Received in revised form 27 November 2020; Accepted 3 December 2020
Available online 30 December 2020

2. Materials and methods

2.1. Experimental design

Microbial composition of faecal samples (FS; n = 10) before and after their *in vitro* anaerobic cultivation in polymeric EN formulas (n = 4) were determined using plate technique method, metabolite analysis, and microbiota profiling of selected samples (n = 3, resp. 3 + 12) by 16S rRNA sequencing (Fig. 1**).**

2.2. Faecal inoculum

One g of fresh FS from human healthy donors (n = 10), aged from 5 to 18 years, with diversified adult-like gut microbiota, without recent intake of antibiotics for the previous 3 months, was collected into sterile anaerobically prepared tubes with 10 mL of dilution buffer consisting of tryptone (5 g L^{-1}), nutrient broth No. 2 (5 g L^{-1}), yeast extract (2.5 g L^{-1}; all Oxoid, UK), L-cysteine (0.5 g L^{-1}), and Tween® 80 (1 mL L^{-1}, both Sigma-Aldrich, USA). Tubes also contained glass pearls for homogenization and were prepared in an oxygen-free carbon dioxide environment according to Hungate (1969). The samples were tested within 3 h of defecation.

2.3. Enteral nutrition formulas as cultivation media

Four polymeric enteral nutrition (EN) formulas of different brands were used as media for FS cultivation: Fortini Multi Fibre (Nutricia, Zoetermeer, Netherlands) – labelled as EN_A, Fresubin Energy Fibre (Fresenius Kabi Deutschland GmbH, Germany) – EN_B, Renutryl Booster (Nestlé Clinical Nutrition, France) – EN_C, and Ensure Plus Advance (Abbott Laboratories, Czech Republic) – EN_D (Table 1**)**. The sterile tubes were filled with 10 mL of tested EN formulas and treated with CO_2 to establish an oxygen-free environment.

2.4. In vitro cultivation of FS in EN formulas

FS (0.1 g per 10 mL of tested EN formula) was anaerobically inoculated into four prepared tubes with different EN formulas and incubated under anaerobic conditions (GENbag anaer, bioMérieux, France) at 37 °C for 24 h. FS inoculum and cultivated FS in EN formulas (marked as FS + EN_A, FS + EN_B, FS + EN_C, FS + EN_D) were further analysed.

2.5. Quantification of selected cultivable bacterial group

Microbiological analysis of FS before and after cultivation in the EN formulas was performed using the plate technique with media for total counts of anaerobic bacteria, bifidobacteria, lactobacilli, and *E. coli* according to Modrackova et al. (2019) with serial dilution of samples using above mentioned dilution buffer. Wilkins-Chalgren agar supplemented with soya peptone (WSP; 5 g L^{-1}, Oxoid), L-cysteine (0.5 g L^{-1}), and Tween® 80 (1 mL L^{-1}, both Sigma-Aldrich, USA) was used for the determination of total counts of anaerobic bacteria, WSP agar with mupirocin (100 mg L^{-1}, Oxoid) and acetic acid (1 mL L^{-1}, Sigma-Aldrich) for bifidobacteria, Rogosa Agar (Oxoid) with acetic acid (1.32 mL L^{-1}) for lactobacilli, and chromogenic T.B.X. medium (Oxoid) for *Escherichia coli*. Cultivation was performed under anaerobic conditions (GENbag anaer) for bifidobacteria and total anaerobes at 37 °C for 48 h, whereas microaerophilic conditions were used for lactobacilli at 37 °C for 48 h, and aerobic conditions for *Escherichia coli* at 37 °C for 24 h.

2.6. Short-chain fatty acid analysis by ion chromatography with suppressed conductivity detection

The main short chain fatty acids (SCFAs) acetate, propionate, butyrate, and two intermediate products, lactate and formate, were measured in fermentation supernatants of batch fermentation samples by capillary high-pressure ion-exchange chromatography with suppressed conductivity detection. Samples were centrifuged at 13 000 × g for 5 min. Supernatants were diluted (500×) and filtered through a 0.45 µm nylon membrane, and analysed using a Dionex ICS 4000 system equipped with IonPac AS11-HC 4 µm guard and analytical columns (Thermo Scientific, USA). Eluent composition was as follows: 0–10 min isocratic: 1 mM KOH; 10–20 min linear gradient: 1–60 mM KOH; and 20–25 min again isocratic: 60 mM KOH. The flow rate was set to 0.012 mL min^{-1}. An ACES 300 suppressor (Thermo Scientific, USA) was used to suppress eluent conductivity, while a carbonate Removal Device 200 (Thermo Scientific) was implemented to suppress carbon dioxide baseline shift. Chromatograms were processed with Chromeleon 7.20 (Thermo Fisher). Standards were prepared from 1 g L^{-1} stock solutions (Analytika, Czech Republic; Inorganic Ventures, USA). Deionised water (conductivity < 0.055 µS cm^{-1}) was used for eluent and standard preparation (0.1–40 mg L^{-1}).

2.7. Microbiota profiling with 16S rRNA sequencing

To investigate microbiota composition, three FS were randomly

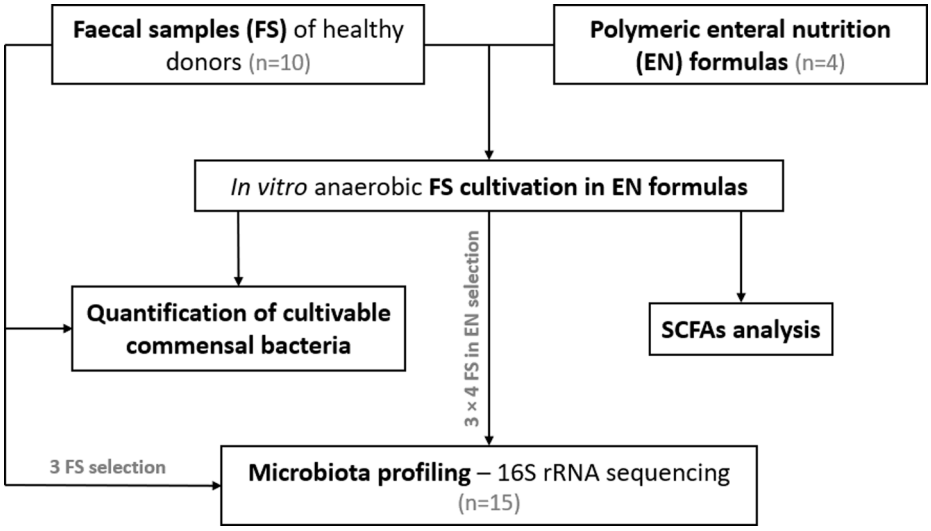

Fig. 1. Experimental design.

Table 1
The composition (per100 mL) of used enteral nutrition formulas for *in vitro* testing.

Enteral Nutrition		Fortini Multi Fibre	Fresubin Energy Fibre	Renutryl Booster	Ensure Plus Advance
Energy value (kJ/kcal)		**640/153**	**630/150**	**840/200**	**631/150**
Osmolarity (mOsm/L)		380	410	580	557
Nutrients	**Fat (g)**	6.8	5.8	7	4.8
	of which saturates (g)	0.7	0.4	0.9	0.42
	monounsaturates (g)		3.8	4	
	polyunsaturates (g)		1.6	1.3	
	Carbohydrate (g)	18.8	17.8	24	16.8
	of which sugars (g)	4.6	5.6	7	6.8
	lactose (g)	<0.025	< 0.26	< 0.05	
	Fibre* (g)	1.5	2	0	0
	Protein (g)	3.4	5.6	10	9.1
	Salt (g)	0.17	0.43		0.43
Minerals	Sodium (mg)	67	80	95	160
	Chloride (mg)	100	100	85	139
	Potassium (mg)	140	135	240	270
	Calcium (mg)	84	135	229	227
	Phosphorus (mg)	75	80	153	120
	Magnesium (mg)	17	21	50	25
	Iron (mg)	1.5	2	1.7	2.1
	Zinc (mg)	1.5	1.5	2.5	1.75
	Copper (mg)	0.135	300	250	0.45
	Iodine (μg)	15	30	25	22
	Selenium (μg)	4.5	10	13	8.5
	Manganese (mg)	0.23	0.4	0.16	0.45
	Chromium (μg)	0.23	10	21	10
	Molybdenum (μg)	6	15	7	15
	Fluorine (mg)	0.11	0.2	0.4	
Vitamins	Vitamin A (μg)	61	120	117	60
	Vitamin D (μg)	1.5	2	1.7	5.7
	Vitamin E (mg α-TE)	1.9	3	3.4	2.5
	Vitamin K (μg)	6	16.7	12	15
	Vitamin C (mg)	15	15	20	16
	Vitamin B1 (mg)	0.23	0.23	0.2	0.26
	Vitamin B2 (mg)	0.24	0.32	0.27	0.34
	Vitamin B6 (mg)	0.18	0.33	0.37	0.734
	Niacin (mg)	0.88	3	4.5	3
	Folic acid (μg)	23	50	67	35
	Vitamin B12 (μg)	0.26	0.6	0.8	0.65
	Pantothenic acid (mg)	0.5	1.2	0.83	1.1
	Biotin (μg)	6	7.5	10	6
Other nutrients	Carotenoids (mg)	0.15			
	L-carnitine (mg)	3			18
	Cholin (mg)	30	26.7		70
	Taurine (mg)	11			
	Betacarotene (μg)		300		60
	Linoleic acid (g)			1.1	
	α-linolenic acid (mg)			210	
	Fructooligosaccharides (g)				0.75
	Hydroxymethylbutyrate (g)				0.55

Footnotes: *Fibre content in Fortini Multi Fibre –soy polysaccharides, inulin, oligofructose, resistant starch, gum arabic, and cellulose; in Fresubin inulin – chicory

selected before and after cultivation in four different EN formulas. Total genomic DNA was extracted from 500 μl of 10 × diluted FS or FS in EN using the Fast DNA SPIN kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA concentration and quality were accessed by absorbance measurements at 260 nm on a NanoDrop® ND-1000 Spectrophotometer (Witec AG, Littau, Switzerland), and samples were stored at − 20 °C prior to the molecular analyses.

The bacterial V4–V5 16S rRNA region was amplified with the primers BactBF (GGATTAGATACCCTGGTAGT) and BactBR (CACGACACGAGCTGACG) according to Fliegerova et al. (2014). The obtained PCR products were purified using the QIAquick® PCR purification kit (Qiagen, Germany).

Purified amplicons were used for library preparation using the NEBNext® Fast DNA Library Prep Set for Ion Torrent (NEB, USA). The Ion Xpress Barcode Adapters were used to label each sample specifically. The sequencing template was prepared on the Ion OneTouch 2 system using the Ion PGM OT2 HiQ View Kit and sequenced on the PGM platform (both, Thermo Fisher Scientific) using the Ion PGM TM Hi-Q TM Sequencing Kit and the Ion 316 TM v2 chip, both according to manufacturer's protocols.

A quality control of the resulting sequences was performed using FastQC package v0.11.8. The resulting sequences were analysed using the Qiime2 (Bolyen et al., 2019) software package with the DADA2 v2019.10.0 (Callahan et al., 2016) pipeline for IonTorrent error prediction (see ***SuplementaryFile1***). The resulting set of ASVs was normalized by sub-sampling to even depth of the lowest sample. Relative bacterial abundance was subsequently plotted on the phylum and family level. Taxonomic groups that accounted for<0.1% of total sequences in each sample were pulled together into a low abundance category for increased legibility. Furthermore, the community diversity was expressed as Shannon's entropy. These procedures are detailed in the ***SupplementaryFile2***.

2.8. Statistics

Counts of bacterial colonies (log CFU g^{-1}) within the groups FS, FS + EN_A, FS + EN_B, FS + EN_C, and FS + EN_D are shown as boxplots

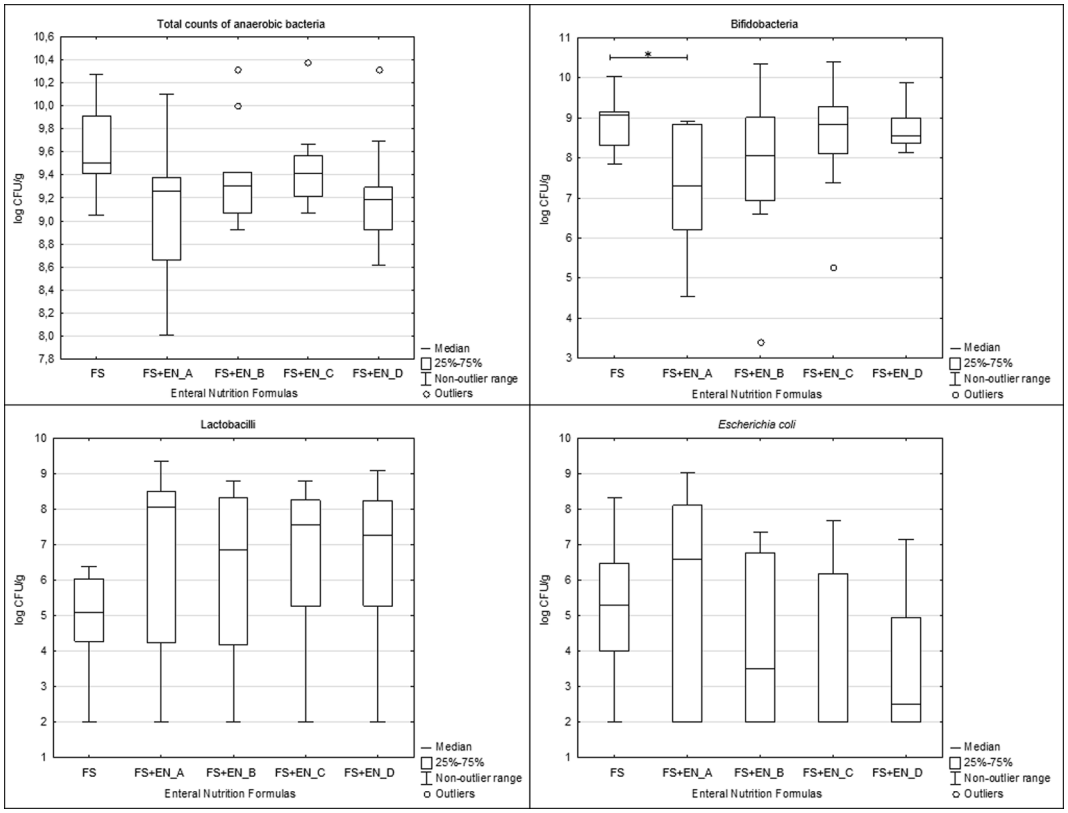

Fig. 2. Enumeration of bacterial groups after FS incubation in four EN formulas. Counts of cultivable commensal groups of bacteria are shown as averages in log CFU g^{-1}. Four different media were used for quantification of total counts of anaerobic bacteria, bifidobacteria, lactobacilli, and *Escherichia coli* within the incubated faecal sample (FS) obtained from ten individual donors (n = 10) in four different polymeric enteral nutrition (EN) formulas; EN_A (Fortini Multi Fibre), EN_B (Fresubin Energy Fibre), EN_C (Renutryl Booster), and EN_D (Ensure Plus Advance). Scheffe's test of one-way ANOVA and Kruskal-Wallis test were used for assessment of statistically significant differences ($P < 0.05$), shown as s horizontal bar, between FS and EN formulas using STATISTICA software (StatSoft, Czech Republic).

(Fig. 2). SCFA levels (mM) of lactate, acetate, propionate, formate, and butyrate are expressed as averages with standard deviations (Fig. 3). The normality of data was evaluated by Shapiro-Wilk W test ($P < 0.05$). Depending on the fulfilment of the testing conditions, Scheffe's method for multiple hypothesis testing adjustment of P-values of One-Way ANOVA or Kruskal-Wallis test were used ($P < 0.05$). All statistical analyses were processed by STATISTICA software (StatSoft, Czech Republic).

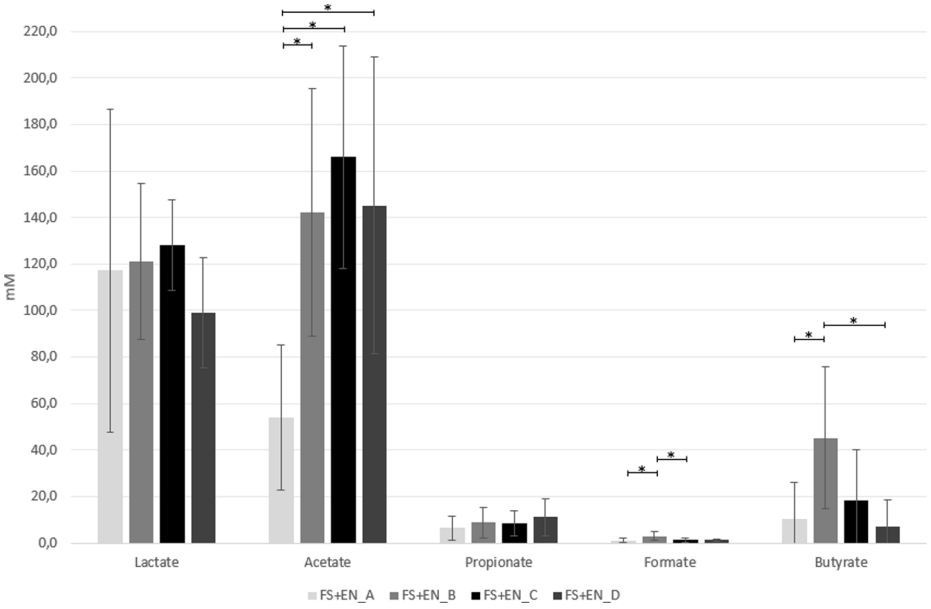

Fig. 3. Short chain fatty acid analysis. Formation of lactate, acetate, propionate, formate, and butyrate was measured in fermentation supernatants of batch fermentation samples within the incubated faecal sample (FS) obtained from individual donors (n = 10) in four different polymeric enteral nutrition (EN) formulas; EN_A (Fortini Multi Fibre), EN_B (Fresubin Energy Fibre), EN_C (Renutryl Booster), and EN_D (Ensure Plus Advance). The measurement was performed after 24 h of *in vitro* cultivation by ion-exchange chromatography with suppressed conductivity. Scheffe's test of one-way ANOVA and Kruskal-Wallis test were used for assessment of statistically significant differences ($P < 0.05$), shown as a horizontal bar, between the concentrations of fermentation metabolites of FS in EN formulas. The statistics was performed using STATISTICA software (StatSoft, Czech Republic) and Microsoft Office Professional Plus 2016.

3. Results

3.1. Detected counts of cultivable bacterial commensal groups

The counts of cultivable commensal groups of bacteria naturally present in FS (n = 10), which were inoculated in four different EN formulas, were quantified by desk-plate method using selective media and factors for their cultivation. The resulting bacterial counts display of a wide spread that was caused by inter-individual differences among the FS, and due the detection limit 10^2 (especially for *E. coli*). Total counts of anaerobic bacteria reached more than 10^9 CFU g^{-1} and were similar among each variant of EN formula as growth media, and as well in comparison to the FS itself (Fig. 2). The same trend was also detected in the other two monitored groups. Lactobacilli exhibited average numbers of (6.21 ± 2.43)–(6.71 ± 2.59) log CFU g^{-1} that is almost 1.5 order of magnitudes higher than in FS (5 log CFU g^{-1}), and *E. coli* of (3.50 ± 1.86)–(6.21 ± 2.74) log CFU g^{-1}. Bifidobacteria were detected in significantly decreased numbers 7.14 ± 1.56 log CFU g^{-1} in Fortini Multi Fibre in comparison with FS 8.86 ± 0.68 log CFU g^{-1}. Other EN formulas do not appear to have enhanced their growth and enabled bifidobacteria to grow in range 10^7–10^9 CFU g^{-1}.

3.2. Metabolic profile of fermentation supernatants

The production of main detected metabolites, such as acetate, propionate, formate, and butyrate, differed among used EN formula as the fermentation substrates (Fig. 3). In general, acetate and lactate reached the highest levels, as main metabolites of bifidobacteria, as well as lactobacilli. The acetate levels after FS incubation in Fortini Multi Fibre were significantly decreased (53.94 ± 31.33 mM) in comparison with other EN formulas where the concentrations were almost triplicated (142.32 ± 53.31 mM in Fresubin Energy Fibre, 165.98 ± 47.90 mM in Renutryl Booster, and 145.22 ± 63.82 mM in Ensure Plus Advance). The low amount of acetate correlates with lower abundance of bifidobacteria in FS incubated in Fortini Multi Fibre. In addition, fermentation supernatant of FS in Fortini Multi Fibre showed the inverse acetate: lactate ratio in favour of lactate compared to other EN formulas. Formate levels were significantly lower in FS in Fortini Multi Fibre (1.14 ± 0.85 mM) and Renutryl Booster (1.41 ± 0.94 mM) compared to FS in Fresubin Energy Fibre (3.05 ± 1.82 mM). Similarly, the butyrate levels were significantly lower in FS in Fortini Multi Fibre (10.45 ± 15.70 mM) and Ensure Plus Advance (7.01 ± 11.49 mM) compared to the FS in Fresubin Energy Fibre (45.26 ± 30.57 mM). In contrast to that, the propionate levels were similar among all tested EN.

3.3. Bacterial composition of the original and cultivated faecal microbiota

Microbiota profile of the analysed FS (n = 3) and FS incubated in four different EN formulas Fortini Multi Fibre, Fresubin Energy Fibre, Renutryl Booster, and Ensure Plus Advance was determined by sequencing the V4–V5 regions of the 16S rRNA gene amplicons. The bacterial diversity in each sample was expressed as the Shannon's entropy. The cultivation samples displayed a considerable spread of values, however the median diversity remained similar to the FS sample (Fig. 4).

The relative abundance of the microbiota of the collective FS was similar in phyla Actinobacteria and Firmicutes (both ≈ 40.53%), followed by Proteobacteria (5.98%), and Bacteroidetes (2.30%). The taxonomic families *Bifidobacteriaceae* (33.73%), *Enterobacteriaceae* (5.52%), and *Lactobacillaceae* (0.16%) were further analysed for comparison with the cultivation data and the SCFAs profiles (Fig. 5). Fresubin Energy Fibre displayed the highest increase of *Bifidobacteriaceae* from 34 to 53%, followed by the Renutryl Booster (42%). In contrast to that, bifidobacteria were decreased in Fortini Multi Fibre to 10%. The *Lactobacillaceae* was nearly undetectable in the FS samples and Fresubin Energy Fibre and Ensure Plus Advance, whereas they sharply increased in Fortini Multi Fibre and Renutryl Booster to 34 and 33%, respectively. The relative abundance of *Enterobacteriaceae* was not strongly affected by any of the EN formulas as fermentation substrates. Further microbial shifts were detected. *Streptococcaceae* extensively multiplied in Fortini

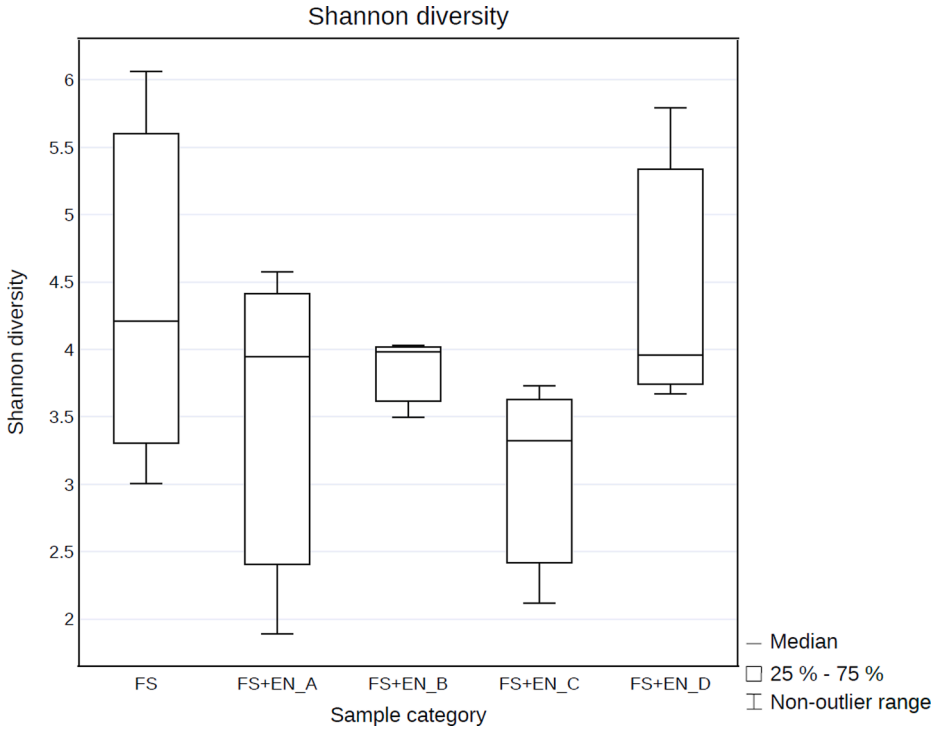

Fig. 4. Shannon's entropy. The bacterial diversity in each sample, where the faecal sample (FS) obtained from three individual donors (n = 3) was incubated in four different polymeric enteral nutrition (EN) formulas; EN_A (Fortini Multi Fibre), EN_B (Fresubin Energy Fibre), EN_C (Renutryl Booster), and EN_D (Ensure Plus Advance); was expressed as the Shannon's entropy.

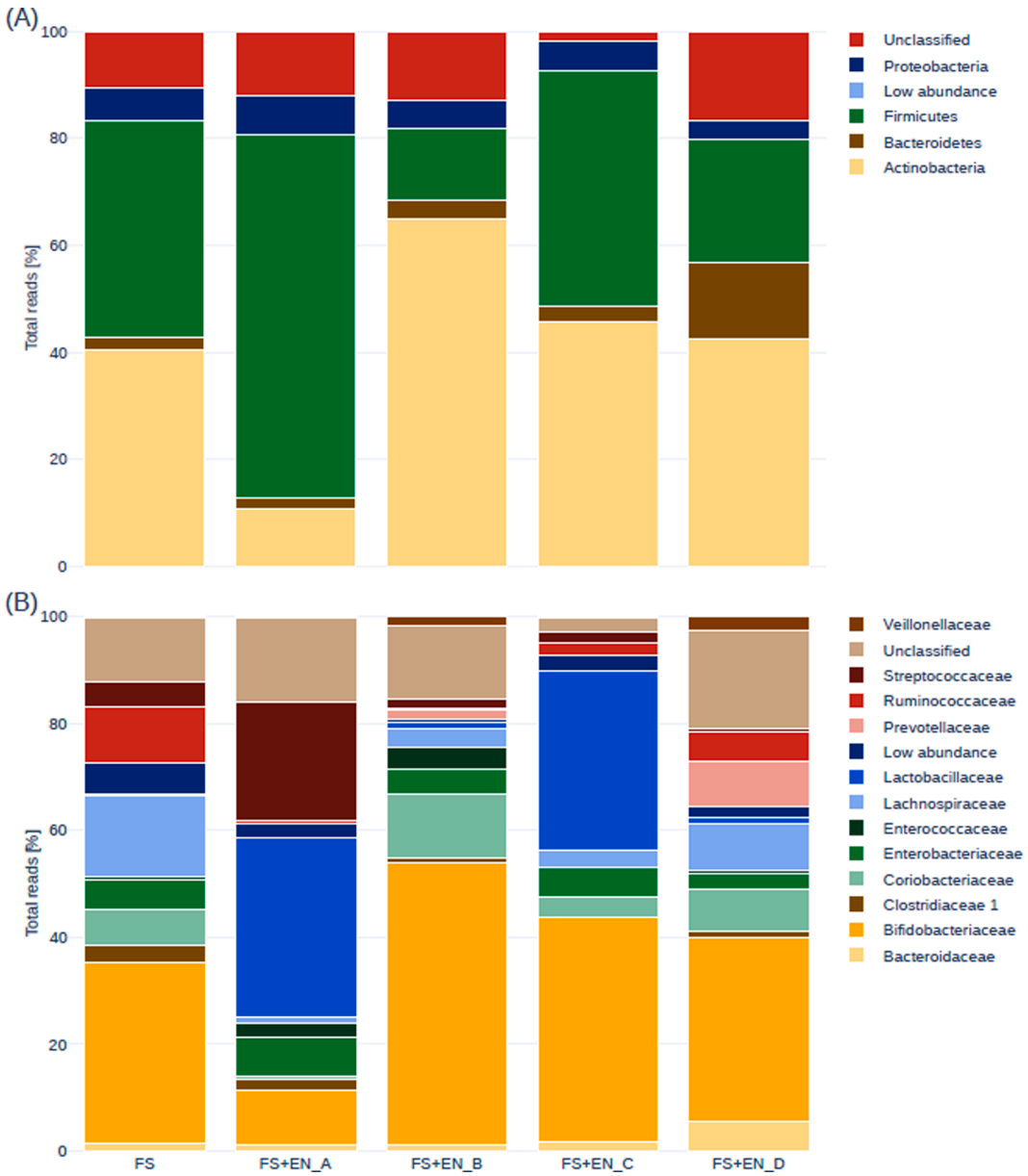

Fig. 5. Microbiota profiling with 16S rRNA sequencing. Microbial phyla (A) and families (B) in the faecal samples (FS) and FS incubated in four different EN formulas; EN_A (Fortini Multi Fibre), EN_B (Fresubin Energy Fibre), EN_C (Renutryl Booster), and EN_D (Ensure Plus Advance). Values presented are averages of the biological replicates (n = 3).

Multi Fibre up to 22% of reads in comparison with 5% in FS and<2% in other EN formulas. Likewise, *Coriobacteriaceae* thrived in Fresubin Energy Fibre (12%) and *Prevotellaceae* in the Ensure Plus Advance (8%). A notable decrease of *Lachnospiraceae*, when compared to the FS (15%), was detected in Ensure Plus Advance with 9%, Fresubin Energy Fibre and Renutryl Booster with 3%, and in Fortini Multi Fibre with tenths of percent reads. Microbial shift of gut representatives was significantly influenced by the brand of the used EN formulas as fermentation substrate and their diverse content.

4. Discussion

Modulation of the human microbiota is an evolving strategy to improve human health. The ability to shift the composition and metabolic outcomes of the gut microbial population is achieved via dietary or non-dietary interventions (David et al., 2014; Gibson et al., 2017; Walker & Lawley, 2013). Commercial enteral formulas usually differ in composition of prebiotic substrates and another specific nutrients, which can modulate gut microbiota profile (Klingbeil & de La Serre, 2018; Walker & Lawley, 2013; Yang et al., 2020). In this study, the prebiotic fibre content of the tested EN formulas varied. Fortini Multi Fibre contained soy polysaccharides, inulin, oligofructose, resistant starch, gum arabic, and cellulose, Fresubin Energy Fibre chicory inulin, and Ensure Plus Advance fructooligosaccharides. Renutryl Booster did not contain any prebiotic fibre, but linoleic and α-linolenic acids unlike the others. Knowledge of the content of these substances should be considered when choosing exclusive EN as the therapy. The effects of EN formulas on specific bacterial species occurrence and metabolite formation are variable and inconsistent between studies (D'Argenio et al., 2013; Guinet-Charpentier, Lepage, Morali, Chamaillard, & Peyrin-Biroulet, 2017; Tjellström et al., 2012), and EN components can play an important role in the intestinal microbiota modulation. Moreover, the presence of soluble dietary fibre can have effect on stool form and short-chain fatty acid production (Mizuno, Bamba, Abe, & Sasaki, 2020).

Dietary changes lead to significant shifts in the human gut microbiota, which can occur in a rapid and reproducible manner (David et al., 2014; Lang et al., 2018; Seo, Lee, Kim, & Park, 2020). The changes are highly variable among individuals, without strong population level trends (Lang et al., 2018). Heterogeneous and highly personalized microbial shifts have also been detected in response to carbohydrates, including dietary fibre with resistant starches and prebiotic carbohydrates (Lockyer & Nugent, 2017; Walker et al., 2011). Fortifying enteral formulas with prebiotics have been proposed as a method to increase beneficial species such as bifidobacteria to assist in colonisation resistance and to increase SCFAs production (Whelan, 2007; Whelan, Gibson, Judd, & Taylor, 2001). However, some results are not so convincing to support bifidobacterial counts (Majid, Emery, & Whelan, 2011; Schneider et al., 2006). According to Modrackova et al. (2019) EN formulas were found to be suitable growth media for commensal groups such as bifidobacteria, lactobacilli, and *E. coli* tested in single culture assay *in vitro*. On the other hand, the counts of cultivable commensal bacteria in the FS of CD children were not significantly affected by 6 weeks exclusive EN therapy *in vivo*. It is known that added prebiotic carbohydrates, which are also part of the EN formulas can also support the growth of other groups with a pathogenic potential (e.g. clostridia and Gram-negative bacteria) present in the intestinal microbiota (Bunesova et al., 2012; Rada et al., 2008). Differences in the prebiotic substrates available in the individual commercial EN formulas (Table 1) may be responsible for the different microbial and metabolic profiles obtained after culturing an identical FS on the tested polymeric EN formulas in this study. Bifidobacteria and lactobacilli detected in FS of donors were able to grow in the tested EN formulas *in vitro* with respect to the other present members of microbiota. In addition to that, microbiota profiling using 16S rRNA sequencing points to the growth also of other taxa than *Bifidobacteriaceae* and *Lactobacillaceae*, such as *Streptococcaceae, Coriobacteriaceae, Prevotellaceae,* and *Lachnospiraceae.*

Batch fermentation represents an opportunity to test the effect of prebiotics and other nutrients on microbial populations from single cell culture to complex faecal microbiota (Bunesova et al., 2012; Modrackova et al., 2019). Whereas, *in vitro* continuous intestinal fermentation technology with immobilized faecal microbiota, mimicking planktonic as well as sessile growth, can be used to produce controlled and stable "artificial" large intestinal microbiota with high cell densities and quantities, moreover is a potential alternative to faecal microbiota transplantation (Bircher, Schwab, Geirnaert, & Lacroix, 2018; Lacroix, de Wouters, & Chassard, 2015; Payne, Zihler, Chassard, & Lacroix, 2012). Both can be used for further research of EN components to modulate homeostatic and dysbiotic microbiota of CD patients.

5. Conclusion

Our results indicate that even slight composition differences of EN formulas can shift microbial profile. The efficacy of prebiotics and other nutrients in modulation of the gut microbiota in health and disease needs further investigation, and an individualized approach is merited, given the great (inter)individual variation in microbiota configurations.

6. Funding sources

Author Ivana Copova has received research grants from the Grant Agency of Charles University in Prague, project No. 136,215 and No. 246216. Project was supported by the Ministry of Health, Czech Republic, for the conceptual development of research organizations 00,064,203 (University Hospital Motol, Prague, Czech Republic).

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the authors' institution (the Ethics Committee Reference number is 1491/16).

CRediT authorship contribution statement

Nikol Modrackova: Software, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Ivana Copova:** Conceptualization, Methodology, Resources, Writing - review & editing. **Adam Stovicek:** Software, Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization. **Marie Makovska:** Investigation. **Dagmar Schierova:** Investigation, Writing - review & editing. **Jakub Mrazek:** Investigation, Funding acquisition. **Monika Sabolova:** Investigation, Writing - review & editing. **Eva Vlkova:** Funding acquisition. **Ondrej Hradsky:** Conceptualization, Writing - review & editing. **Jiri Bronsky:** Conceptualization. **Jiri Nevoral:** Conceptualization, Writing - review & editing. **Vera Neuzil-Bunesova:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank Romina Vezo and Anna Jelinkova for their valuable assistance in laboratory experiments.

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5 Souhrnná diskuze

V této kapitole jsou souhrnně diskutovány výsledky publikovaných prací, jejichž citace jsou v textu označeny tučně. Detailní metodika s podrobnými výsledky jsou uvedeny v předložených publikovaných článcích v kapitolách 4.1–4.8. S přihlédnutím k publikačním výstupům je tato souhrnná diskuze rozdělena do třech dílčích podkapitol.

5.1 Monitoring výskytu bifidobakterií a popis nových druhů

Bifidobakterie běžně kolonizují gastrointestinální trakt teplokrevných živočichů, drůbeže, hmyzu a řadu dalších stanovišť, která jsou ale většinou v přímé souvislosti s těmito hostiteli (Bunesova et al. 2014; Milani et al. 2017b). Právě jejich sacharolytický typ metabolismu je předurčuje k osídlování těchto míst bohatých na sacharidové substráty (Turroni et al. 2018a). Přestože první bifidobakterie byla popsána již na počátku 20. století (Tissier 1900; Orla-Jensen 1924), i nyní o více než dekádu později je stále objevováno velké množství nových druhů s pozoruhodně rostoucím trendem jejich izolace zejména z novosvětských opic, jako jsou kosmani a tamaríni. Konkrétně druhy *B. callitrichos*, *B. saguini*, *B. jacchi*, *B. catulorum*, *B. myosotis*, *B. tissieri*, *B. hapali* byly izolovány z kosmana bělovousého (*Callithrix jacchus* L.) (Endo et al. 2012; Michelini et al. 2016c; Modesto et al. 2018a; Modesto et al. 2019), *B. parmae*, *B. margollesii* z kosmana zakrslého (*Cebuella pygmaea*) (Lugli et al. 2018), *B. primatium*, *B. scaligerum*, *B. felsineum*, *B. aerophilum*, *B. avesanii*, *B. ramosum* z tamarína pinčího (*Saguinus oedipus* L.) (Michelini et al. 2016a; Modesto et al. 2018c), *B. imperatoris*, *B. vansinderenii*, *B. callitrichidarum*, *B. simiarum* z tamarína vousatého (*Saguinus imperator*) (Duranti et al. 2017; Lugli et al. 2018; Modesto et al. 2018b; Modesto et al. 2018c), *B. reuteri*, *B. stellenboschense*, *B. biavatii* z tamarína žlutorukého (*Saguinus midas*) (Endo et al. 2012), *B. cebidarum* z tamarína skákavého (*Callimico goeldii*) (Duranti et al. 2020) a *B. leontopitheci* z lvíčka zlatohlavého (*Leontopithecus chrysomelas*) (Duranti et al. 2020).

Tento trend byl potvrzen v rámci naší studie zabývající se monitoringem výskytu bifidobakterií u primátů chovaných v zajetí v českých zoologických zahradách. Bylo detekováno hojné zastoupení bifidobakterií s jejich významnou převahou u novosvětských opic, v rámci jejichž mikrobioty byly zároveň popsány tři nové druhy bifidobakterií, a to konkrétně *B. moraviense* DSM 109958^{T} z tamarína skákavého (*Callimico goeldii*), *B. oedipodis* DSM 109957^{T} z tamarína pinčího (*Saguinus oedipus* L.) a *B. olomucense* DSM 109959^{T} z tamarína bělovousého (*Saguinus mystax*) **(Neuzil-Bunesova et al. 2020b)**. V rámci našeho navazujícího výzkumu zabývajícího se analýzou mikrobiomu primátu bylo dekováno dalších pět potenciálně

nových druhů bifidobakterií izolovaných v tomto případě výhradně z tamarínů, konkrétně tamarína sedlového, tamarína vousatého, tamarína pinčího, tamarína bělovousého a tamarína žlutorukého **(Modrackova et al. 2021b)**, které budou dále popisovány. Ve střevní mikrobiotě novosvětských opic byla dále detekována také většina druhů bifidobakterií, které byly původně z tohoto prostředí izolovány a v posledních letech popsány jako nové druhy. Konkrétně se nejčastěji jednalo o druhy *B. parmae*, *B. imperatoris/saguini* a *B. ramosum.* U novosvětských opic byla také celkově detekována větší variabilita bifidobakteriálních druhů ve srovnání s opicemi starosvětskými **(Modrackova et al. 2021b)**.

Přestože v minulosti byla snaha izolovat nové druhy i z opic starosvětských, pouze *B. moukalabense* je jedním z druhů izolovaných z gorily nížinné (*Gorilla gorilla gorilla*) (Tsuchida et al. 2014). Druhy bifidobakterií detekovaných u skupiny starosvětských opic většinou zahrnují ty multi-hostitelské, jako je například *B. pseudolongum* (Nomoto et al. 2017), *B. angulatum* (Ushida et al. 2010), *B. adolescentis* a *B. dentium* (D'Aimmo et al. 2014). V rámci naší mikrobiomové studie byla hojná přítomnost těchto multi-hostitelských druhů bifidobakterií u starosvětských opic potvrzena. Konkrétně se nejčastěji jednalo o druhy *B. dentium* a *B. catenulatum/pseudocatenulatum* **(Modrackova et al. 2021b)**. Zároveň byla u této skupiny opic také detekována významně vyšší variabilita bakteriální populace ve srovnání s opicemi novosvětskými, která právě u opic starosvětských koresponduje s nižším kvantitativním zastoupením bifidobakterií **(Modrackova et al. 2021b)**. O to větším úspěchem našich experimentů bylo objevení a popis nových druhů bifidobakterií izolovaných z fekálních vzorků šimpanze (*Pan troglotydes*), konkrétně druhu *B. panos* DSM 109963^{T}, a z kočkodana husarského (*Erythrocebus patas*), druhu *B. erythrocebi* DSM 109960^{T} **(Neuzil-Bunesova et al. 2020b)**.

Přestože je v současnosti většina nově popisovaných druhů bifidobakterií izolována zejména ze střevní mikrobioty primátů, podařilo se nám v rámci screeningu střevní mikrobioty psů detekovat bifidobakterie u německých ovčáků se zastoupením potenciálně nového druhu, který byl následně popsán jako *B. canis* DSM 105923^{T} **(Neuzil-Bunesova et al. 2020a)**. V mikrobiotě psů jsou většinou běžně přítomny multi-hostitelské druhy *B. animalis*, *B. choerinum, B. pseudolongum* (Strompfová & Lauková 2014; Sabbioni et al. 2016) a poddruh *B. animalis* subsp. *lactis* (Bunešová et al. 2012b). V rámci našeho testování byly detekovány další typicky multi-hostitelské druhy *B. longum*, *B. adolescentis*, *B. catenulatum/pseudocatenulatum* a *B. pullorum* **(Neuzil-Bunesova et al. 2020a)**. Životem psů v těsné blízkosti člověka a jejich intenzivním zdomácněním totiž došlo k posunu jejich původní přirozené mikrobioty k mikrobiotě podobající se té lidské (Song et al. 2013; Milani et

al. 2017b). S tím úzce souvisí i změna jejich diety, která je teď mnohem bohatší na sacharidy oproti jejímu původně zejména bílkovinnému charakteru (Clauss et al. 2010; Bosch et al. 2015). Při popisu nových druhů je naším přístupem kombinace kultivačně závislých a kultivačně nezávislých metod. V rámci kultivační kvantifikace bifidobakterií s použitím pro ně selektivních médií obohacených o octovou kyselinu s antibiotikem mupirocinem/mupirocinem spolu s norfloxacinem (Rada & Petr 2000; Vlková et al. 2015) a jejich následné identifikace jsme schopni objevit řadu zajímavých izolátů s potenciálem nového druhu či zajímavými potenciálně probiotickými vlastnostmi. Pro identifikaci bifidobakterií v rámci studie analýzy mikrobiomu primátů chovaných v zajetí byla navíc vytvořena rozšířená MALDI-TOF MS databáze hmotnostních spekter, která se osvědčila jako sofistikovaný nástroj pro rychlý screening druhového bifidobakteriálního zastoupení **(Modrackova et al. 2021b)**. Tato databáze má široké použití při každodenních identifikacích divokých kmenů bifidobakterií variabilního původu. Úspěšnost MALDI-TOF MS identifikace je totiž limitována množstvím uložených kmenů v databázi pro srovnání se spektrem analyzovaného vzorku (Shannon et al. 2018). V případě rozšíření databáze těchto spekter o chybějící typové a divoké kmeny bakterií se pak tato metoda může stát vhodným nástrojem pro identifikaci také environmentálních vzorků bakterií (Santos et al. 2016; Timperio et al. 2017). Přes nespornou výhodu v rychlosti, efektivitě a snížené ceně druhové identifikace bifidobakterií pomocí MALDI-TOF MS, i tato metoda má své limity. Při screeningu druhové zastoupení bifidobakterií v mikrobiotě primátů bylo zjištěno, že z důvodu podobnosti spekter některých izolátů je nelze spolehlivě odlišit na druhové úrovni. Konkrétně jde o druhy *B. adolescentis – B. faecale*, *B. angulatum – B. merycicum, B. breve – B. indicum, B. catenulatum – B. pseudocatenulatum* a *B. imperatoris – B. saguini,* které jsme seskupili do uvedených dílčích identifikačních skupin **(Modrackova et al. 2021b)**. Detekovanou vysokou podobnost těchto spekter lze vysvětlit také blízkou příbuzností těchto druhů na základě jejich podobnosti 16S rRNA sekvencí. Pro další odlišení těchto druhů a obecně pro přesnou identifikaci je proto velmi vhodné kombinovat MALDI-TOF MS s více metodami identifikace bifidobakterií, jako je například sekvenování fylogenetických markerů (Milani et al. 2014b; Srinivasan et al. 2015; Killer et al. 2018) a celogenomové sekvenování (Lugli et al. 2018).

Zcela obráceným přístupem k popisu nových druhů poté přistupuje italská skupina vědců profesora Ventury, kteří nejprve analyzují celá mikrobiální společenstva pomocí sekvenování nové generace (NGS). Na základě metagenomického sekvenování získají informace o přítomnosti potenciálně nových druhů bifidobakterií s predikcí jejich metabolických schopností, díky nimž lze identifikovat specifické substráty, které jsou potenciálně nové druhy

schopny metabolizovat. Tyto substráty jsou následně využity při kultivaci pro izolaci cílených druhů bifidobakterií (Milani et al. 2018; Lugli et al. 2019b). Vykultivovaný izolát je následně dále charakterizován pomocí profilování hypervariabilního regionu ITS s použitím specifických primerů pro amplifikaci fragmentů dlouhých 200 bp (Milani et al. 2014b). Získané sekvence jsou poté porovnávány s popsanou ITS bifidobakteriální databází (Milani et al. 2017b). V případě popisu nového druhu klasickým způsobem začínajícím jeho izolací po kultivaci, tato skupina většinou kombinuje více metod pro charakterizaci daného izolátu, jako je amplifikace V3 region genu 16S rRNA (Milani et al. 2013b) a části ITS sekvence (Milani et al. 2014b) s následným sekvenováním celého genomu (Lugli et al. 2016; Lugli et al. 2018). Právě osekvenování a porovnání ITS regionu bylo klíčové v rámci našeho popisu pěti nových druhů bifidobakterií izolovaných z opic, které bylo nezbytné k jejich druhovému odlišení. Dané izoláty bifidobakterií totiž měly identickou sekvenci genu 16S rRNA a až na základě kombinace sekvenace ITS regionu, DDH a ANI bylo potvrzeno, že se jedná o nové druhy **(Neuzil-Bunesova et al. 2020b)**.

5.2 Prebiotika s bifidogenním potenciálem

S rozvojem molekulárních metod bylo zjištěno mnoho zajímavých informací o metabolických schopnostech bifidobakterií. Genom bifidobakterií totiž kóduje velké množství specifických enzymů podílejících se na jejich sacharolytickém způsobu metabolismu. Jedná se zejména o GH, pomocí nichž jsou schopny využívat širokou škálu komplexních substrátů (Cronin et al. 2011; Pokusaeva et al. 2011). Tato schopnost je nicméně často druhově, kmenově a hostitelsky specifická (Milani et al. 2016; Bunešová et al. 2017; Liu et al. 2021). Zejména z důvodu dlouhodobého a bezpečného používání komenzálních bifidobakterií (Abe et al. 2010; Doron & Snydman 2015; Ricci et al. 2017), jejichž přítomnost ve střevě je navíc spojována s mnoha zdravotními přínosy pro hostitele (Awasti et al. 2016; Hidalgo-Cantabrana et al. 2017), je žádoucí hledání možných způsobů jejich podpory. Jednou z možností je nalezení specifických prebiotických sacharidových substrátů, které není sám hostitel schopen využít (Gibson et al. 2017). Trendem současné doby by zároveň mělo být hledání a upřednostňování látek přírodního původu, které jsou/mohou být přirozenou složkou diety hostitele. Právě selekce nových prebiotických substrátů vycházejících z přirozených složek diety daných hostitelů se zaměřením selektivity pro *Bifidobacterium* spp. byl dalším cílem této práce.

Přírodní gumy a některé škroby jsou jedním z příkladů slibných prebiotických substrátů pro bifidobakterie (Salavati Schmitz & Allenspach 2017; Mudgil et al. 2018). Jedná se převážně o komplexní sacharidové polymery s hydrokoloidní povahou, které jsou vzhledem ke svým

vhodným technologickým vlastnostem a bezpečnosti pro konzumenta běžně používány jako emulgátory, stabilizátory a jedlé obaly nebo filmy v potravinářství (Mirhosseini & Amid 2012; Bashir et al. 2016; FAO/WHO 2017; Saha et al. 2017). Oproti syntetickým přídatným látkám jsou navíc chemicky stálé, netoxické, biologicky rozložitelné a snadno dostupné (Choudhary & Pawar 2014). Z tohoto důvodu by přírodní zahušťovadla mohla být vhodným prebiotickým zdrojem pro bifidobakterie.

Schopnost utilizace karubinu (E410), guarové gumy (E412), tragacanthu (E413), arabské gumy (E414), xanthanu (E415), gumy karaya (E416) a škrobu byla testována u 204 kmenů bifidobakterií různých druhů. Tato schopnost byla shledána jako druhově, kmenově a hostitelsky specifická odrážející dietu a původ hostitele. Obecně totiž byly bifidobakterie živočišného původu, izolované zejména z přežvýkavců a opic, schopny využít širší škálu dostupných přírodních substrátu ve srovnání s kmeny původu lidského. Příkladem zcela k utilizaci nekompetentních bifidobakterií je poddruh *B. longum* subsp. *infantis* a druh *B. bifidum*, zatímco 10 dalších testovaných druhů bifidobakterií (29 kmenů) bylo přírodní gumy schopno utilizovat. Karubin, guarová guma, tragacanth a arabská guma byly nejčastěji dostupnými substráty. Naopak zcela nedostupnými substráty pro bifidobakterie byly xanthan a guma karaya **(Modrackova et al. 2019b)**.

Nejčastěji využívaným testovaným substrátem byl škrob, který byl využíván 37 druhy bifidobakterií (114 kmenů) lidského i živočišného původu **(Modrackova et al. 2019b)**. Škrob je totiž běžnou součástí diety mnoha hostitelů a je zároveň univerzálním zdrojem uhlíku, který je dostupný pro řadu bifidobakteriálních druhů (Liu et al. 2015b; Metzler-Zebeli et al. 2019), například pro *B. adolescentis* (Duranti et al. 2014), *B. choerinum* (Jung et al. 2018) a *B. pseudolongum* (Centanni et al. 2018). Kromě výše zmíněných byla tato schopnost v rámci našeho testování potvrzena také u druhů *B. boum, B. dentium* a *B. thermophilum* **(Modrackova et al. 2019b)**. Přestože škrob vykazuje potenciálně prebiotické vlastnosti pro některé druhy bifidobakterií, existuje předpoklad jeho nedostatečné selektivity a s tím související dostupnosti k jeho využití také potenciálně patogenními bakteriemi. Takovýmto příkladem mohou být některé druhy rodu *Clostridium* a *Bacteroides* (El Kaoutari et al. 2013b; Tsolis & Bäumler 2020). Neselektivní charakter byl dokonce prokázán také u řady komerčně dostupných prebiotik (Rada et al. 2008; Bunesova et al. 2012). Naopak slibnou bifidogenní alternativou s vyšší selektivitou by mohla být rezistentní či modifikovaná forma škrobu (Brüssow 2013; Sybille et al. 2013; Kim et al. 2018).

Jednou z možností, jak předejít možné podpoře nežádoucích mikroorganismů ve střevní mikrobiotě hostitele by mohlo být podávání přírodních gum spolu s bifidobakteriemi, které jsou

kompetentní k jejich utilizaci, ve formě synergistických synbiotik **(Modrackova et al. 2019b)**. Dle aktuálně platné definice jsou synbiotika směsí obsahující živé mikroorganismy a substrát(y) selektivně využívané hostitelskými mikroorganismy, které poskytují hostiteli zdravotní přínos. „Hostitelské mikroorganismy" v této souvislosti dané definice zahrnují jak autochtonní (rezidentní a kolonizující hostitele), tak alochtonní mikroorganismy (externě aplikované jako probiotika), které i když jsou transientní, tvoří součást hostitelské mikrobioty. V případě synergistických synbiotik je prebiotický substrát designován k selektivní utilizaci ko-administrovaných probiotik, zatímco komplementární synbiotika jsou cílena na autochtonní mikroorganismy (Swanson et al. 2020). Administrace přírodních gum v kombinaci s bifidobakteriemi by měla největší potenciál zejména ve výživě zvířat, protože většina schopných druhů bifidobakterií utilizovat tyto substráty byla animálního původu **(Modrackova et al. 2019b)**. Již dříve byly dokonce přírodní gumy takto využívány pro enkapsulaci a administraci probiotických bakterií (Weinbreck et al. 2010; Eratte et al. 2015). Jako slibné synbiotikum se například jeví i kombinace rezistentního škrobu ze semen chlebovníku různolistého spolu s *B. pseudolongum* pro redukci hyperlipidémie u myší (Zhang et al. 2021), galaktooligosacharidů s *B. adolescentis* (Krumbeck et al. 2015), nebo inulinu s *B. animalis* subsp. *lactis* (Anzawa et al. 2019) pro zvýšení žádoucích bakterií v lidském střevě. Přestože mají přírodní zahušťovadla slibný prebiotický potenciál pro podporu růstu bifidobakterií, je stále nezbytné další testování jejich selektivity v rámci komplexní střevní mikrobioty. Právě proto je v návaznosti na naši publikaci **Modrackova et al. (2019b)** v současné době prováděn další experiment, ve kterém jsou přírodní gumy podrobeny fermentaci fekální mikrobiotou přežvýkavých krav. Na základě analýzy mikrobiomu a profilu SCFAs a laktátu bude sledován vliv jednotlivých přírodních gum na modifikaci střevní mikrobioty těchto přežvýkavců.

Dalšími potenciálně prebiotickými látkami přírodního původu jsou rostlinné glykosidy. Vyskytují se v listech, plodech a zrnech, kdy řada z nich je využívána v tradiční medicíně již po celá staletí (Biernat et al. 2018). Část dietárních glykosidů, která není vstřebána v tenkém střevě, totiž může být hydrolyzována glykosidickou aktivitou střevní mikrobioty, kterou je modifikována jejich biologická dostupnost uvolněním acylglykonů (de Arriba et al. 2013), které navíc mohou mít i antimikrobiální charakter (Jurica et al. 2017). Konkrétně bifidobakterie disponují širokou škálou enzymů GH umožňujících degradaci řady dietárních sacharidů (Bottacini et al. 2018), včetně přítomnosti β-glukosidáz, které by mohly katalyzovat degradaci těchto látek. Jejich aktivita je většinou druhově specifická (Marotti et al. 2007).

V rámci naší další studie zabývající se β-glukosidázovou aktivitou byla testována schopnost 115 kmenů bifidobakterií různého původu utilizovat rostlinné glykosidy esculin, amygdalin

a arbutin jako fermentační substráty. Naším testováním jsme potvrdili, že β-glukosidázová aktivita bifidobakterií je druhově specifická. Testované druhy *B. adolescentis, B. animalis, B. breve, B. catenulatum/pseudocatenulatum* a *B. dentium* byly β-glukosidáza pozitivní, zatímco *B. bifidum* a většina testovaných kmenů druhu *B. longum* byly negativní. Je zajímavé, že β-glukosidázová aktivita byla nejčastěji detekována u bifidobakterií izolovaných z dospělých lidí a zvířecích hostitelů **(Modrackova et al. 2020)**. Naše výsledky tedy korespondují se zjištěním, že střevní β-glukosidázový potenciál významně roste se zvyšujícím se věkem hostitele a příjmem rozmanité diety (Mykkänen et al. 1997; Dabek et al. 2008).

Schopnost bifidobakterií prospívat či dokonce dominovat v komplexním ekosystému střeva je totiž umožněna jejich kompetencí k degradaci a metabolizaci rozmanitých sacharidů, které zároveň mohou sdílet v rámci cross-feedingu i s dalšími členy střevní mikrobioty (Schwab et al. 2017; Turroni et al. 2018b). Adaptace bifidobakterií k danému hostiteli je poté spojována se schopností využívat dietární a hostitelské sacharidy, což bývá většinou druhově i kmenově variabilní (Milani et al. 2016; Bunesova et al. 2017). Konkrétním příkladem mohou být typicky kojenecké druhy bifidobakterií jako *B. longum* subsp. *infantis, B. breve* a *B. bifidum*, které díky své enzymatické výbavě využívají hostitelem nestravitelné složky mateřského mléka, díky nimž ve střevě dominují (Turroni et al. 2012a; Thomson et al. 2018; Duranti et al. 2019a). Dalším příkladem je multi-hostitelský druh *B. dentium,* který disponuje geny pro utilizaci širokého spektra sacharidů, což mu umožňuje výskyt nejen v dutině ústní, ale i ve střevě člověka a dalších savců (Lugli et al. 2020b).

V rámci testování β-glukosidázové aktivity byla dále detekována také kmenová i hostitelská variabilita u poddruhu *B. animalis* subsp. *lactis*. V případě původu kmenů izolovaných z dospělých přežvýkavců byly bifidobakterie schopny utilizovat testované substráty, zatímco kmeny izolované z všežravých psů nikoliv **(Modrackova et al. 2020)**. Tato genotypová a fenotypová hostitelsky specifická variabilita kmenů v rámci tohoto poddruhu byla již dříve také popsána (Bunesova et al. 2017).

Dále je zajímavé naše další zjištění, že většina β-glukosidáza pozitivních kmenů uvolňovala esculetin z esculinu, nicméně ne všechny tyto kmeny byly schopné na tomto substrátů významně růst **(Modrackova et al. 2020)**. Mohlo by to být způsobeno právě antimikrobiální aktivitou uvolněného acylgylkonu (Yang et al. 2016). Samotný testovaný β-glykosid nepůsobil antimikrobiálně proti bifidobakteriím ani v případě arbutinu, zatímco jeho acylglykon hydrochinon již tuto antimikrobiální aktivitu vykazoval **(Modrackova et al. 2020)**. Tento efekt byl nicméně významně vyšší například proti patogennímu druhu *Staphylococcus aureus* (Rua et al. 2011; Ma et al. 2019). Přestože uvolněný acylglykon při utilizaci substrátu může snížit

růstový potenciál β-glukosidáza aktivních bifidobakterií, je vysoce pravděpodobné, že ovlivní schopnost růstu také okolních mikroorganismů a lze předpokládat jeho environmentální vliv na celý střevní ekosystém **(Modrackova et al. 2020)**. Uvolněný hydrochinon může mít navíc ještě řadu dalších účinku. Konkrétně je například spojován také s antikancerogenní (Byeon et al. 2018), antimykobakteriální a antileishmaniální aktivitou (Horn et al. 2020).

Na základě našich výsledků lze obecně shrnout, že rostlinné β-glykosidy jsou dalšími potenciálními prebiotiky pro bifidobakterie, kdy nejdostupnějším testovaným substrátem byl amygdalin (pro 54 % všech testovaných kmenů), následně esculin (47 % kmenů) a nejméně dostupným byl arbutin (24 % kmenů). Naše výsledky zároveň naznačují, že β-glukosidázová aktivita bifidobakterií je spojena s jejich adaptací k hostitelskému prostředí a s tím související dietou. Navíc je pravděpodobné, že tato aktivita poskytuje konkurenční výhodu v komplexním mikrobiálním ekosystému savčího střeva **(Modrackova et al. 2020)**, ve kterém dochází k neustálým složitým interakcím jak mezi mikroorganismy a hostitelem, tak mezi mikroorganismy navzájem.

5.3 Adaptace bifidobakterií na hostitelské prostředí

Komplexní a dynamická mikrobiální společenstva osídlující střeva savců jsou nezbytná pro přežití zvířat, potažmo člověka, v měnících se environmentálních podmínkách zahrnujících například degradaci habitatu, chov v zajetí a změnu přirozené diety. Případnou mikrobiální dysbiózou je poté většinou iniciován rozvoj řady onemocnění, jako jsou například zánětlivá onemocnění střev, syndrom dráždivého tračníku, celiakie, alergie, astma, metabolický syndrom, kardiovaskulární onemocnění a obezita (Carding et al. 2015; Wagner Mackenzie et al. 2017; West et al. 2019). Zachování homeostáze střeva je neustále probíhající proces interakcí mezi mikrobiotou a hostitelem, na jehož udržení se podílí významné množství komenzálních mikroorganismů. Konkrétním příkladem jsou bifidobakterie (Tojo et al. 2014; Binda et al. 2018), kterým daný hostitel a prostředí přímo definují jejich diverzitu a adaptaci, s čímž přímo souvisí jejich specifická genová výbava (Sun et al. 2015; Sharma et al. 2018; Rodriguez & Martiny 2020). Přirozeným a bohatým zdrojem těchto komenzálů je střevo primátů (Lugli et al. 2020a).

V rámci naší další publikace byl posuzován vliv chovu v zajetí, diety a samotného hostitele na celkové i druhové zastoupení bifidobakterií ve střevním mikrobiomu 52 různých opic z českých a slovenských zoologických zahrad. Bifidobakterie byly dominantní skupinou kultivovatelných anaerobů u novosvětských primátů v množstvích až 10^8 KTJ/g fekálního vzorku, zatímco jejich počty leckdy více než o polovinu klesaly u opic starosvětských. V těchto případech byly

bifidobakterie v jejich střevní mikrobiotě většinou nahrazovány bakteriemi z čeledi *Clostridiaceae* s morfologií sarcin **(Modrackova et al. 2021b)**.

Je zajímavé, že v případě nízkého počtu bifidobakterií nebo jejich zjevné nepřítomnosti dochází k jevu nedostatečné selektivity běžně používaných médií pro detekci bifidobakterií podle Rada & Petr (2000) a Vlkova et al. (2015), na kterých jsou poté běžně detekovány i další rody bakterií, zejména *Clostridium* spp., ať už *C. perfringens* a *C. sordellii* jako nejčastěji detekované druhy klostridií v mikrobiotě psů **(Neuzil-Bunesova et al. 2020a)**, nebo *C. ventriculi* (syn. sarcina) u primátů **(Makovska et al. 2021; Modrackova et al. 2021b)**.

Výskyt sarcin v rámci analýzy mikrobiomu primátů chovaných v zajetí byl překvapivý, protože analyzované fekální vzorky byly odebrány od opic bez zdravotních obtíží **(Modrackova et al. 2021b)**. Sarciny jsou totiž obecně považovány za patogenní mikroorganismy, které jsou spojovány s úmrtností šimpanzů (Owens et al. 2021) a akutní dilatací žaludku u koček (Im et al. 2017), psů a koní (Vatn et al. 2000). Také u lidí byly popsány souvislosti mezi výskytem sarcin v žaludku a chronickou nevolností, dyspepsií, bolestmi břicha, žaludečními vředy (Lam-Himlin et al. 2011) a vzácně také perforací žaludku (Tolentino et al. 2003). Přítomnost sarcin ve střevní mikrobiotě opic bez zjevných zdravotních obtíží je ale pravděpodobně zcela běžná **(Makovska et al. 2021; Modrackova et al. 2021b)**. To potvrzuje jejich detekce také u zdravých goril (Frey et al. 2006), makaků (Ushida et al. 2016) a lidí (Crowther 1971). Patogenita této bakterie je nicméně kontroverzní a její role při vyvolávání zánětlivé reakce sliznice v gastrointestinálním traktu je stále nejasná (Zare et al. 2019). Proto pro optimalizaci efektivní léčby při případné infekci způsobené sarcinami byla v rámci naší další studie testována jejich citlivost na běžně používaná antibiotika. Bylo zjištěno, že sarcina je nejcitlivější k fluorochinolonům, makrolidům, penicilinům a tetracyklinům. Přestože testované kmeny byly izolovány od různých hostitelů z variabilních míst, nebyla u nich detekována žádná kmenová variabilita a je pravděpodobné, že v rámci vztahu k antibiotikům se jedná o konzervativní vlastnost **(Makovska et al. 2021)**.

Trend kultivačních výsledků byl v rámci analýzy mikrobiomu opic potvrzen také amplikonovým sekvenováním V4 regionu genu 16S rRNA. Zjištěný inverzní poměr kmenů Actinobacteria a Firmicutes korespondoval s kultivačně stanoveným poměrem bifidobakterií ke klostridiím **(Modrackova et al. 2021b)**. Již v dřívějších studiích byl tento jev také pozorován. Konkrétním příkladem je střevní mikrobiota kojenců. Je zcela běžné, že v případě vysokého zastoupení bifidobakterií nejsou detekovány klostridie. Přesně naopak je to v případě hojné detekce klostridií, kdy poté obvykle nejsou významně detekovány bifidobakterie (Bunesova et al. 2012; Korpela et al. 2018; Moore & Townsend 2019). Bifidobakterie tedy

pravděpodobně mohou být zodpovědné za omezení růstu klostridií ve střevní mikrobiotě (Vlková et al. 2008; Wei et al. 2018; Wang et al. 2019).

Vzhledem k předpokladu adaptace bifidobakterií k hostitelskému prostředí a dietě byl u primátů chovaných v zajetí pozorován také vliv těchto faktorů na jejich výskyt. Na základě kombinace kultivačních a molekulárních metod bylo zjištěno, že kvantitativní zastoupení bifidobakterií odráží kromě fylogeneze hostitele diskutované výše, také potravní specializaci primátů. Pokud bylo ovoce, hmyz a stromová pryskyřice významnou složkou diety primátů, byly bifidobakterie v mikrobiotě těchto frugivor-insektivorů a gummivor-insektivorů významně zastoupené. Naopak v mikrobiotě opic frugivor-folivorů a frugivor-omnivorů, které byly více všežravé, jejichž významnou složku diety tvořilo ovoce a listy, zastoupení bifidobakterií významně pokleslo. Dále je zajímavé, že v případě vysokého zastoupení hmyzu v krmné dávce opic, také zastoupení bifidobakterií bylo značně signifikantní **(Modrackova et al. 2021b)**. Přestože jsou bifidobakterie také přirozenou součástí mikrobioty hmyzu žijícího sociálním způsobem života (Scardovi & Trovatelli 1969; Killer et al. 2009; Killer et al. 2011), vliv přítomnosti této složky v dietě primátů ve vztahu k výskytu bifidobakterií je stále nejasný.

Přestože Amato et al. (2019) uvádí fylogenezi hostitele jako silnější rozhodující faktor pro složení střevní mikrobioty ve srovnání s dietou a geografií výskytu zvířete, naše výsledky naznačují, že jak dieta, tak hostitel sám významně ovlivňují složení mikrobiomu, zejména relativní zastoupení bifidobakterií **(Modrackova et al. 2021b)**. Z těchto zjištění lze předpokládat, že evoluční specializace primátů zahrnující jak specializaci gastrointestinálního traktu, tak s tím související potravní preference, je pravděpodobně zodpovědná také za výskyt a druhové zastoupení bifidobakterií ve střevní mikrobiotě opic **(Modrackova et al. 2021b)**. S tím koresponduje také distribuce a evoluce bifidobakteriálních genů kódujících GH, u které byla zjištěna silná asociace s dietou hostitele na rozdíl od fylogeneze zvířete (Satti et al. 2021). Z našich dat dále vyplývá, že zmiňovaná dieta a rozdělení opic do skupin novosvětských nebo starosvětských primátů je silnějším determinantem určujícím zastoupení bifidobakterií v jejich mikrobiotě ve srovnání s vlivem samotného chovu v zajetí v zoologických zahradách, který pravděpodobně výskyt bifidobakterií významně neovlivňuje **(Modrackova et al. 2021b)**. Přesto některé studie poukazují na to, že volně žijící primáti bez kontraktu s lidmi mají vyšší bakteriální diverzitu (Clayton et al. 2016) s nižším zastoupením bifidobakterií (McKenzie et al. 2017; Campbell et al. 2020) a že chov v zajetí je obecně spojován se změnou složení střevního mikrobiomu negativně ovlivňující zdraví hostitele. Přes veškeré snahy chovatelů se totiž mnoho institucí potýká s dysbiotickými a zánětlivými stavy střev svých zvířat často spojovanými s chronickými průjmy, letargií, ztrátou hmotnosti a špatnou reprodukční úspěšností (Amato et

al. 2016; Hale et al. 2019; Koo et al. 2020). Je proto velmi důležité hledat způsoby možné podpory přirozené a vyvážené střevní mikrobioty nejlépe optimalizovanou prebiotickou a probiotickou intervencí cílenou na daného hostitele **(Modrackova et al. 2021b)**. Vzhledem k tomu, že složení střevní mikrobioty všežravých primátů je velmi podobné složení lidskému (Ley et al. 2008), mají tato zjištění navíc přesah také do lidské výživy. Právě evolučně blízcí příbuzní stále žijícím původním způsobem života by mohly být vhodným modelem pro design probiotik, které již nejsou běžnou součástí lidské mikrobioty z důvodu stále se snižující její diverzity (Hicks et al. 2018).

Odchýlení od původního způsobu života způsobuje změnu složení střevního mikrobiomu také u lidí (De Filippo et al. 2017; Valle Gottlieb et al. 2018), která může být u citlivých a predisponovaných jedinců spojována s obezitou, vznikem kardiometabolického syndromu (de la Cuesta-Zuluaga et al. 2018) a propuknutím zánětlivých onemocnění střev, která jsou většinou spojována s mikrobiální dysbalancí (Morgan et al. 2012; Schnorr et al. 2014; Mosca et al. 2016). Konkrétně je s dysbiózou střeva například asociována Crohnova choroba způsobující chronický zánět gastrointestinálního traktu (Joossens et al. 2011). Přestože se léčbou Crohnovy choroby dlouhodobě zabývá řada odborníků, přesná etiologie tohoto onemocnění stále není známá a nebyl tedy stanoven ani obecně uznávaný efektivní konsenzus léčby (M'Koma 2013; Gomollón et al. 2017; Ibraheim et al. 2018; Lee et al. 2018). Většinou jsou podávána různá léčiva jako například mesalazin, lokálně aktivní a systémové steroidy, thiopuriny, methotrexát a biologické terapie (Torres et al. 2020). Pro děti a dospívající je jako primární terapie k indukci remise doporučováno podávání exklusivní enterální výživy (Cameron et al. 2013; Ruemmele et al. 2014; Hradsky et al. 2016; Assa & Shamir 2017; Ashton et al. 2019), které je považováno za důležité pro zachování strukturní a funkční integrity střeva a mikrobiální diverzity (Elke et al. 2016). Důležitá je také přítomnost komenzálních bakterií, které žijí v symbióze se svým hostitelem a zajišťují důležité ochranné, trofické a metabolické funkce (Guarner & Malagelada 2003). Právě proto by jejich výskyt ve střevní mikrobiotě měl být podporován (Ozyurt & Ötles 2014; Sonnenborn 2016).

Z výše popsaných zjištění vyplývá předpoklad, že exkluzivní enterální výživa by mohla být vhodným růstovým médiem pro komenzální bakterie a zároveň by mohla ovlivňovat jejich výskyt také ve střevě dětí s Crohnovou chorobou *in vivo*. Z výsledků naší studie nicméně vyplývá, že přestože všechny vybrané exkluzivní enterální výživy byly vhodným růstovým médiem pro testované bifidobakterie, laktobacily a *E. coli in vitro*, jejich kvantitativní zastoupení po šestitýdenním příjmu exkluzivní enterální výživy *in vivo* nebylo ovlivněno a zároveň bylo značně variabilní na úrovni každého pacienta **(Modrackova et al. 2019a)**.

Je zajímavé, že přestože v jiných studiích bylo detekováno významně nižší zastoupení bifidobakterií v dysbiotické mikrobiotě (Quince et al. 2015) a také v mikrobiotě pacientů s Crohnovou chorobou (Sheehan et al. 2015), v našem experimentu průměrné počty bifidobakterií dosahovaly skoro až 10^8 KTJ/g fekálního vzorku **(Modrackova et al. 2019a)**. Tyto počty se navíc přibližují zastoupení bifidobakterií u lidí bez zjevných zdravotních obtíží (Bunešová et al. 2017; Martinello et al. 2017). Pro ověření těchto výsledků bylo provedeno další testování s cílenou simulací podmínek střeva formou uzavřených anaerobních kultivací fekálních vzorků zdravých lidí v různých enterálních výživách. Předpokládali jsme, že výskyt komenzálních bakterií by mohl být ovlivněn typem podávané enterální výživy a jejími variabilními komponenty. Nicméně žádná z testovaných výživ ale významně nepodpořila růst komenzálních bakterií ve srovnání s jejich počty ve fekálních vzorcích a jedna výživa dokonce množství bifidobakterií o 1,5 řádu snížila **(Modrackova et al. 2021a)**.

Podpora výskytu žádoucích komenzálů a s tím související možná celková modulace střevní mikrobioty by pravděpodobně mohla být zajištěna obohacením enterální výživy o další látky, jako jsou prebiotické substráty a specifické nutrienty (Walker & Lawley 2013; Klingbeil & de La Serre 2018; Yang et al. 2020). Přidáním bifidogenních látek do enterálních výživ, například prebiotických galaktooligosacharidů a maltodextrinu (Scott et al. 2014; Musilova et al. 2015), isomaltooligosacharidů (Plongbunjong et al. 2017), frukooligosacharidů a arabino-xylanooligosacharidů (Rivière et al. 2018), by z důvodu enzymatické výbavy mohl být podpořen růstový potenciál komenzálních bifidobakterií. Nicméně, jak již ale bylo diskutováno výše, přidáním prebiotických složek do enterální výživy by mohly být podpořeny také nežádoucí mikroorganismy. Za účelem výběru vhodných prebiotik pro bifidobakterie by proto měly být dále hledány a testovány vhodné látky s pro ně prokázanou selektivitou. Jako potenciálně prebiotické složky s vyšší specifitou by tedy mohlo být zváženo obohacení výživ například o HMOs (Thomson et al. 2017), zmíněné přírodní gumy **(Modrackova et al. 2019b)** a bioaktivní β-glukosidy **(Modrackova et al. 2020)**.

Další z možností, jak podpořit přítomnost bifidobakterií ve střevní mikrobiotě lidí a dalších hostitelů, je jejich podávání ve formě probiotik. Jak bylo ale diskutováno výše, je velmi důležité daný probiotický kmen bifidobakterie cílit hostiteli, pro kterého je jeho přítomnost v mikrobiotě typická, a tedy i možnost kolonizace je více pravděpodobná. Obecně lze shrnout, že bifidobakterie jsou komenzální skupinou mikroorganismů vyskytující se přirozeně v mikrobiotě jak člověka, tak mnoha zvířat, ve které zajišťují řadu žádoucích efektů. Jejich přítomnost by proto měla být podporována, ať už optimalizovanou dietou a prebiotickou intervencí, či administrací probiotik, nebo kombinací těchto preparátů ve formě synbiotik.

6 Závěr

Všechny stanovené cíle disertační práce byly splněny. Byl proveden monitoring výskytu bifidobakterií s jejich charakterizací pomocí fenotypových a genotypových metod u variabilních hostitelů, který umožnil objevení a popis nových druhů. Zároveň byly detekovány druhově a kmenově specifické vlastnosti bifidobakterií, které odrážely jejich adaptaci k hostitelskému prostředí zahrnující zejména dietu a zároveň také jejich specifické schopnosti k utilizaci přírodních potenciálně prebiotických substrátů.

Přestože jsou komenzální bifidobakterie z komplexního pohledu na střevní mikrobiotu minoritně zastoupenou složkou, hrají důležitou a leckdy nenahraditelnou roli v řadě funkcí spojených se zachováním zdraví svého hostitele a zároveň mají také významný probiotický potenciál, jímž lze kromě hostitelova zdraví a mentální pohody ovlivnit a modifikovat také jeho celá mikrobiální společenstva. Pro popis nových druhů a vývoj probiotik je nezbytná podrobná fenotypová a genotypová charakterizace bifidobakterií až na kmenovou úroveň.

Nové informace o mikrobiálních společenstvech střeva jsou v přímé souvislosti s rozvojem mikrobiomových analýz, které daný mikrobiom hodnotí většinou ze širšího a velmi komplexního pohledu. Pokud je ale cílem zájmu zaměřit se na některé mikroorganismy více do hloubky s důrazem na nižší taxonomickou úroveň, jsou často tyto typy analýz nedostatečné. Z tohoto důvodu je žádoucí kombinovat moderní sekvenační analýzy spolu s kultivačně závislými metodami. V případě kultivace lze totiž detekovat i skupiny mikroorganismů, které jsou v rámci komplexního mikrobiomu skryté. Do budoucna je poté velmi zajímavé zaměřit se na tyto izoláty a jejich interakce s bifidobakteriemi, které jsou v danou chvíli méně abundantní, což umožnilo detekci právě i dalších izolátů v podmínkách vhodných pro růst bifidobakterií. Testování interakcí je poté žádoucí i na úrovni celé mikrobioty.

Aktuální trend objevování a popisování nových druhů bifidobakterií ze střevní mikrobioty primátů má stále velký potenciál. Zejména novosvětské opice jsou nevyčerpaným zdrojem velkého množství potenciálně nových druhů. Na základě našich výsledků bude v budoucnu pravděpodobně popsáno minimálně 5 dalších druhů bifidobakterií izolovaných z tamarínů. Nové druhy bifidobakterií lze objevit také v jiných ekologických nikách, jako je například pes, ale s menší pravděpodobností.

Obecně jsou bifidobakterie markerem prosperující střevní mikrobioty. V případě jejich sníženého zastoupení, ať už z důvodu zánětlivých střevních onemocnění, antibiotické léčby, či alergií a dalších vlivů, je žádoucí jejich cílená podpora. Z tohoto důvodu je nezbytné objevování nových specifických prebiotik, která by byla optimálně přírodního původu a mohla

by být i běžnou součástí diety hostitele. Jako takový lze zmínit potenciál přírodních gum a rostlinných β-glukosidů. Při výběru jakýchkoliv substrátů je ale nezbytné využít znalostí o sacharolytických schopnostech bifidobakterií a otestovat selektivitu těchto substrátů i v rámci celkové mikrobioty *in vivo*.

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8 Seznam použitých zkratek

ABC	–	ATP-vázající kazeta (*ATP-Binding Cassette*)
ANI	–	průměrná nukleotidová identita (*Average Nucleotide Identity*)
CAZy	–	sacharidově aktivní enzymy (*Carbohydrate Active Enzymes*)
DDH	–	DNA-DNA hybridizace
DSM	–	Německá sbírka mikroorganismů a buněčných kultur (*Deutsche Sammlung von Mikroorganismen und Zellkulturen*)
FDA	–	Úřad pro kontrolu potravin a léčiv (*Food and Drug Administration*)
EFSA	–	Evropský úřad pro bezpečnost potravin (*European Food Safety Authority*)
F6PPK	–	fruktóza-6-fosfát fosfoketoláza
G+C	–	guanin a cytosin
GH	–	glykosyl hydrolázy
GNB/LNB	–	galakto-*N*-bióza/lakto-*N*-bióza (*galacto-N-biose/lacto-N-biose*)
HMOs	–	oligosacharidy mateřského mléka
ISAPP	–	Mezinárodní vědecká asociace pro probiotika a prebiotika (*International Scientific Association of Probiotics and Prebiotics*)
ITS	–	vnitřní transkribovaný spacer (*Internal Transcribed Spacer*)
LPSN-PNU	–	*List of Prokaryotic Names with Standing in Nomenclature-Prokaryotic Nomenclature Up-to-Date*
MALDI-TOF MS	–	hmotnostní spektrometrie s laserovou desorpcí a ionizací za účasti matrice s průletovým analyzátorem (*Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry*)
MFS	–	hlavní facilitátorová superskupina (*Major Facilitator Superfamily*)
NGS	–	sekvenování nové generace
SCFAs	–	mastné kyseliny s krátkým řetězcem