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A Comparative Analysis of the Fermentation Capabilities of Various Bifidobacterium Strains

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A COMPARATIVE ANALYSIS OF THE FERMENTATION CAPABILITIES OF VARIOUS BIFIDOBACTERIUM STRAINS

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Abstract

Bifidobacterium is a genus of anaerobic bacteria that are commonly found to inhabit the gastrointestinal tract of many members of the animal kingdom. These microorganisms are adapted to obtain their carbon from the breakdown of complex carbohydrates. Marmosets, a mammal whose gut microbiome is inhabited by high levels of Bifidobacteria, consume gum Arabic as a major part of their diet. The purpose of this experiment is to determine whether *Bifidobacterium* strains isolated from the guts of marmosets are able to degrade and ferment this complex carbohydrate or one of its main constituents, arabinose. This was accomplished by inoculating isolates of *Bifidobacterium* species into tubes containing basal MRS medium supplemented with gum Arabic or arabinose and monitoring pH (color change) over time. Each of the 12 marmoset-derived isolates were tested in liquid media containing either 5% arabinose, 3% gum Arabic, or 1% gum Arabic. A positive phenotype indicating fermentation of the substrate was visualized by a shift in the media's color from purple to yellow. The fermentative capabilities of the marmoset strains were then compared to 13 other *Bifidobacterium* strains that were isolated from other mammals such as rats, pigs, and humans. Two strains from each group expressed a negative phenotype for arabinose, while all other strains were positive. In the marmoset group, 6 of the strains expressed positive phenotypes for the 1% gum and 8 were positive for the 3% concentration. The group of strains from non-marmoset origins sported 5 positive phenotypes for the 1% concentration of gum Arabic, while 6 strains tested positive for the 3% concentration.

Keywords: microbiology, gut microbiota, Bifidobacteria, fermentation, marmoset, arabinose, gum Arabic, diet

Introduction

Bacterial species composition of the gut microbiome is individualized, but a relatively small set of about 20 species are typically found in most humans. These include species of *Bacteriodes, Prevotella, Ruminococcus, Coprococcus, Blautia, Eubacterium, Roseburia,* and *Bifidobacterium* (Qin et al., 2010). These species dominate the colonic microbiome and play important roles in directing the flow of carbon from degradation of complex fibers and other food components to different short chain fatty acids in the colon. *Bifidobacterium* is a genus of Gram-positive bacteria that frequently reside within the digestive tracts of insects and mammals (Milani et al., 2015). Species that commonly inhabit the gastrointestinal tracts of humans and other mammals include *B. longum*, *B. bifidum*, *B. animalis*, *B. breve*, *B. adolescentis*, and *B. catenulatum* (Grönlund et al., 2007). As infants, *Bifidobacterium* species make up between 60% and 70% of the human gut microbiome. This number slowly declines until the individual reaches adulthood and the relative abundance remains steady at 2-14% (Arboleya, Watkins, Stanton, & Ross, 2016). While there is very little data available that quantifies their prominence within the microbiota of non-human mammals, it is known that humans, primates, and domesticated mammals exhibit a remarkably high abundance of Bifidobacteria in comparison to other species (Milani et al., 2017). Species within the genus can be quite variable between the different hosts that they inhabit. Unlike humans, the primary *Bifidobacterium* species commonly found within the gut microbiome of the common marmoset (*Callithrix jaccus*) include *B. reuteri*, *B. callitrichos*, *B. saguini, B. stellenboschense, B. biavatti, B.aesculapii, B. myosotis, B. tissieri,* and *B. hapali* (Endo et al., 2012; Modesto et al., 2014; Michelini et al., 2016).

In humans, *Bifidobacterium* populations within the gut microbiota have been shown to play a central role in disease prevention and gut homeostasis. The latter has historically been attributed to competitive exclusion of enteric and other pathogenic organisms, but recent studies have revealed far more intricate interactions between host and microbiota influence a wide range of metabolic and inflammatory diseases (Tojo et al., 2014). In humans, *Bifidobacterium* species play important roles early in life and the early colonizing species can selectively ferment unique human milk oligosaccharides (HMOs) found in breastmilk. These non-digestible sugars act as pre-biotics for the Bifidobacteria, which then provide significant health benefits to their hosts (Lewis et al., 2015). Bifidobacteria are known to ferment carbohydrates, both simple and complex, as their primary source of carbon. Their ability to break down complex polysaccharides that the host organism is unable to digest by itself is another important characteristic of host-Bifidobacteria mutualism (Flint, Scott, Duncan, Louis, & Forano, 2012).

Marmosets are small omnivorous monkeys native to Brazil. Their diet primarily consists of fruits, flowering plants, insects, lizards, and invertebrates, but they also supplement their diets with tree gums from various indigenous tree species (Passamani & Rylands, 2000; Caton, Hill, Hume, & Crook, 1996). When preferred foods (e.g. fruits and flowering plants) become scarce, marmosets will spend a large proportion of their time (up to 70%) obtaining gums and exudates from trees in their local environment (Power, 1996; Ferrari & Ferrari, 1989). These gums comprise large polysaccharide chains, often with ornate branching patterns that are generally believed to be degraded in the colon by members of the colonic microbiota. Recent work on the gut microbiota of captive populations of the common marmoset have found significant levels of *Bifidobacterium* species in adult animals (Albert, Rani, & Sela, 2018). Gum Arabic is a naturally-occurring non-starch complex carbohydrate that is made up of the hardened sap of

acacia trees. Its makeup consists of rhamnose, galactose, glucuronic acid, and arabinose (Butler & Cretcher, 1929; Osman, Williams, Menzies, & Phillips, 1993). The purpose of this experiment is to determine whether *Bifidobacterium* strains isolated from the fecal samples of marmosets are able to ferment gum Arabic or its main component, arabinose. Non-marmoset strains will also be measured for their ability to ferment gum Arabic and arabinose.

Methods

The marmosets used in this experiment were fed ZuPreem® Marmoset Diet, along with fruit like bananas, apples, apple sauce, melons, and oranges. Additionally, they were fed meal worms and scrambled eggs several times per week as a source of supplemental animal protein. Each marmoset received Mazuri® Enrichment Gum Arabic on a daily basis. Each cage had 2 mL of a 0.5 g/mL solution mixed in with their food, for a total of 1 g of the gum per day per cage. Isolates of *Bifidobacterium* species were obtained from the fecal samples of seven adult marmosets and diluted 1:10 with PBS buffer and homogenized. Homogenized fecal samples were serial diluted and plated on MRS and BSIM, or *Bifidobacterium* Selective Iodoacetate Mupirocin (Lewis et al., 2015). The animal designation and isolation medium are indicated in the character string of each strain ID such that each isolate has a unique ID. For example, *Bifidobacterium* MM5-B-2 was isolated from marmoset no. 5, plated on *Bifidobacterium* selective agar, and sampled from colony no. 2. Alternately, strain MM4-M-3 was retrieved from marmoset no. 4, plated on solid MRS, and sampled from colony no. 3 on the plate.

The non-marmoset *Bifidobacterium* strains were obtained from The Agricultural Research Service Culture Collection (NRRL) Online Catalog, each arriving in a dormant state inside sealed glass tubes. The cells were reactivated by first scratching the center of the tube with

a file, then wiping it down with 70% alcohol. After the tube was opened and the end flamed, the contained cell pellet was transferred to 2 mL of Difco™ Lactobacilli MRS Broth. The solution was then vortexed by hand, and 500 µL of the suspension was added to a 1.5 mL tube containing 500 µL of glycerol. This formed the stock solution for each inoculum, which were stored at -80°C. The marmoset inoculum solutions consisted of similar 1.5 mL tubes of glycerol/MRS solution.

Colonies were inoculated into MRS broth and enriched anaerobically at 37ºC for 24 hours. DNA was extracted from all 25 isolates using the method from Martínez, Kim, Duffy, Schlegel, & Walter (2010). Extractions were performed by first warming 10% SDS and precooling the centrifuge to 4°C. Next, the isolates were centrifuged to collect 1 mL of cells at 8,000 x *g* for 5 minutes. The samples were washed once with cold PBS buffer (pH = 7) then vortexed and centrifuged again at 8,000 x *g* for 5 minutes. The 1 mL was discarded after spinning, leaving the pellet undisturbed. Next, the pellet was resuspended with 750 µL lysis buffer. For this step, 20 mg of lysozyme was added per 1 mL of buffer, then filter sterilized. The lysis buffer with the resuspended pellet was then transferred to a bead beating tube containing 300 mg of zirconium beads (0.1 mm, BioSpec products) and incubated at 37°C for 30 minutes. After incubating, 85 µL of 10% SDS solution and 40 µL of proteinase K (15 mg/mL) were added. The solution was then vortexed and incubated for 15 minutes at 60°C, and 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added. Cells that did not lyse with enzymatic lysis were then disrupted using a bead beater (BioSpec products) set on high (homogenize) for 2 minutes. The samples were removed from the bead beater, placed on ice, and transferred to the centrifuge to be spun at 10,000 x *g* for 5 minutes. The top layer was then moved into a 1.5 mL Eppendorf tube. 500 µL of phenol:chloroform:isoamyl alcohol was added to the new tube, which

was then vortexed, spun at 10,000 x *g* for 5 minutes, and had top layer carefully removed into a new 1.5 mL Eppendorf tube. This process was repeated once more with phenol:chloroform:isoamyl alcohol, then twice more with chloroform:ioamyl alcohol. Next, 2 volumes of EtOH (96-100%) were added to the samples and left overnight at –20°C. The samples were then centrifuged at full speed for 20 minutes, and the ethanol was discarded without disturbing the DNA pellet inside. The pellet was washed by adding 500 µL of 70% EtOH and spinning at full speed for 20 minutes. The liquid was carefully discarded, and the DNA pellet was left to dry for 30 minutes or until dry at room temperature. Finally, the DNA was resuspended in DNA in 100 µL of H_2O and left at 4°C overnight.

After DNA extraction, the quality and concentration of DNA was checked using the NanoDrop (Table 3). The PCR protocol recipe and conditions can be found below in Tables 1 and 2. The primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (Turner, Pryer, Miao, & Palmer, 1999) and 16SU2 (5'-ATCGGYTACCTTGTTACGACTT-3') (Benson et al., 2014) were used to amplify the bacterial 16S rRNA gene. After amplifying the 16S rRNA gene through PCR, the PCR products underwent 1% agarose gel electrophoresis to confirm PCR success. Results from the gel indicate that the amplified section DNA from each strain was around 1,500 bp in length. A picture of the gel under UV light is provided in Figure 4. PCR products were then purified using QIAGEN QIAquick® PCR Purification Kit. Purified PCR products were Sanger sequenced in both directions with the 8F and 16SU2 primers at Michigan State University RTSF Genomics Core. Version 7.0 of MEGA (2016) was used to assemble a consensus sequence from each isolate. Consensus sequences were BLASTed against the NCBI database and the top three identities for each isolate were recorded (Table 3).

For PCR amplification, a 1.5 mL tube was used for the PCR mix, into which all ingredients listed in Table 1 (except for the DNA) were added. The Ex Taq was thawed in the fridge, while all other ingredients were left to thaw at room temperature. Into microcentrifuge tubes, 49 µL of the PCR mix was added, as well as 1 µL of the DNA sample being amplified. These tubes were then placed in a thermocycler to undergo PCR amplification. The ingredients of the PCR mix were as follows: 5 µL of 10X buffer, 4 µL of dNTP mix, 1 µL each for the 8F forward and 16SU2 reverse primers, 0.25 µL of Ex Taq polymerase, 37.75 µL of H_2O, and 1 µL of DNA. All ingredients were scaled up as needed for the amount of DNA used. The thermocycler was programmed to run the pre-denaturation step at 95°C for 2 minutes, the denaturation step at 95°C for 30 seconds, the annealing phase at 55°C for 45 seconds, and the extension step at 72°C for 45 seconds. The denaturation, annealing, and extension steps went through a total of 29 cycles before moving on to the final extension step, which was run at 72°C for 5 minutes.

For the fermentations, tubes containing 5 mL of MRS broth were left in an anaerobic chamber overnight to ensure the removal of any oxygen inside. The following day, the pure frozen stock cultures were introduced into the anaerobic chamber. These were inoculated into each tube and incubated at 37ºC for 72 hours. Next, three sets of tubes containing bMRS and either 1% gum Arabic, 3% gum Arabic, or 5% arabinose were placed in the anaerobic chamber overnight. The formula for 500 mL of bMRS is as follows: 5 g protease peptone no. 3, 5 g beef extract, 2.5 g yeast extract, 0.93 g Tween 80, 1 g ammonium citrate dibasic, 2.5 g sodium acetate trihydrate, 0.05 g magnesium sulfate heptahydrate, 1 g dipotassium phosphate, 0.025 g manganese sulfate, and 30 mg bromocresol purple. These ingredients were then added to 450 mL of boiling water, then stirred until dissolved, and autoclaved. Next, a 50 mL of a filter-sterilized

stock solution of the desired carbohydrate was added. To ultimately obtain media containing 5% arabinose by mass, the stock solution of 25% was made by dissolving 12.5 g of the carbohydrate in 50 mL of distilled water. Stock solutions of gum Arabic for the 3% and 1% media were made by dissolving 7.5 g and 2.5 into 50 mL of distilled water, respectively. Next in the fermentation process, 40 µL of the 72-hour culture tubes was used to inoculate each of the fermentation tubes. These new tubes were then placed in the incubator for 72 hours, then removed and analyzed for a color change. Successful fermentation of the carbohydrate within was indicated by shift in the media from purple color to a yellowish hue.

Results

Data on PCR product quality is found in Table 3, which lists DNA concentration of the product and indicates whether phenol contamination was detected in the samples. This information was gathered using a NanoDrop spectrophotometer. Contamination and low DNA concentration are both factors that can negatively impact the accuracy of sequencing results. Three samples were found to be contaminated with phenol, but no samples had problematically low concentrations of DNA. In addition to NanoDrop analysis, the PCR products underwent agarose gel electrophoresis to confirm successful amplification of an expected size product. Results from the gel electrophoresis indicate that the amplified section of DNA from each strain was around 1,500 bp in length, which is the appropriate size for the 16S rRNA gene. An image of the gel stained with ethidium bromide under UV light is provided in Figure 1.

The data in Table 2 contains information about the possible identities of all 25 *Bifidobacterium* strains. This information was obtained by Sanger sequencing of the samples and BLASTing their consensus sequences against the NCBI database. The identities of most non-

marmoset strains were confirmed to be of the correct species, if not the correct strain or subspecies. The exceptions to this are *B. bifidum* ATCC 11617, *B. suis* ATCC 27533, *Bifidobacterium* sp. 12_1_47BFAA, and *Bifidobacterium* sp. 113. *Bifidobacterium* sp. 113 came back as 100% query cover and 100% identity, despite the mistaken identity. Meanwhile, *Bifidobacterium* sp. 12_1_47BFAA came back with some of the lowest scores of all the strains in both categories. Possible explanations for any discrepancies in identity are provided in the Discussion section of this paper.

Of the marmoset strains, MM5-B-8 had the lowest scores in both identity and query cover, and it should be noted that it was one of just three PCR samples that were contaminated with phenol. *B. reuteri* and *Bifidobacterium* sp. strain LMG 30940 each had 3 hits, making them the most frequently observed alignments. *B. myostosis, B. saeculare, B. callitrichidarum, B. areophilum, B. aesculapii, B. scardovii, B. thermophilum, Bifidobacterium* sp. strain TRE_F, *Bifidobacterium* sp. strain TRE_2, *and Bifidobacterium* sp. strain TRE_H all had two hits each. Many of the marmoset strains shared two or three of their top hits. In total, 21 unique strains were listed as possible identities for the marmoset isolates, and 6 of these strains were not from a specific species. MM4-B-6 and MM5-B-8 were the only two isolates with <90% identity scores, while all other marmoset isolates scored above 95% identity. Ten of the marmoset strains had at least one hit with an identity score of over 97%.

For the fermentation tests, a positive phenotype is expressed as a yellow color change in the medium. This is indicative of acidification ($pH < 6$) resulting from the fermentation process. An intermediate phenotype is brought about by mild acidification, and results in a greenish-purple color change. The pH of the medium in these conditions is about 6.5. No color change indicates a negative phenotype and a lack of fermentation. Examples of images from positive,

intermediate, and negative phenotypes for each substrate can be seen in Figures 2, 3, and 4. All strains cultured in arabinose expressed either a positive or negative phenotype, while intermediate phenotypes were common for both concentrations of gum Arabic. Color changes indicating a positive or intermediate phenotype were considerably starker in the fermentation tubes containing 5% arabinose than they were for either concentration of gum Arabic. Intermediate phenotypes appeared more frequently in the non-marmoset strains. Nearly all *Bifidobacterium* strains, marmoset or non-marmoset, indicated some ability to ferment the 5% arabinose, as well as the gum Arabic at both 1% and 3% concentrations. With the exception of MM5-B-9, any strain expressing a negative phenotype for one substrate expressed the same for the others. The results of the fermentation tests are shown in Table 2.

Tables and Figures

Table 1
Ability of Various Bifidobacterium Strains to Ferment Arabinose and Gum Arabic

Strain	Arabinose	1% Gum Arabic	3% Gum Arabic
B. longum ssp. *longum* (ATCC® 15707™)	+	+	+
B. longum longum JDM301	+	+	+
B. longum DJO10A	-	-	-
Bifidobacterium sp. 12_1_47BFAA	+	+	+
Bifidobacterium sp. 113	+	*i*	*i*
B. adolescentis ATCC 15703	+	*i*	*i*
B. adolescentis L2-32	+	+	+
B. adolescentis IVS-1	+	*i*	*i*
B. animalis ssp. *animalis*	+	*i*	*i*
B. bifidum ATCC 11617	+	+	+
B. animalis ssp. *lactis*	+	*i*	*i*
B. suis ATCC 27533	-	-	-
B. breve	+	*i*	+
MM5-B-8	+	*i*	+
MM5-B-9	+	-	-

MM3-M-6	+	*i*	+
MM4-B-6	-	-	-
MM4-M-3	+	+	+
MM5-B-2	-	-	-
MM9-B-2	+	+	+
MM8-B-4	+	*i*	*i*
MM8-B-9	+	+	+
MM8-M-5	+	+	+
MM10-M-9	+	+	+
MM9-B-6	+	+	+

Note. A (+) signifies a positive phenotype and indicates a strong ability to metabolize the substrate as an energy source. Cells marked with a (-) indicate a negative phenotype and an inability to ferment the substrate, while an (*i*) indicates an intermediate phenotype and a low level of metabolic activity.

Table 2

Bifidobacterium Strain Identification Using BLAST®

Strain Name	Possible Strain Identity	Query Cover	Identity
MM5-B-2	*Bifidobacterium reuteri* strain AFB22-1 16S ribosomal RNA gene, partial sequence	100%	98.41%
	Bifidobacterium boum strain LET414 16S ribosomal RNA gene, partial sequence	99%	97.44%
	Bifidobacterium thermophilum strain NB-168 16S ribosomal RNA gene, partial sequence	100%	97.03%
MM4-M-3	*Bifidobacterium* sp. strain LMG 30940 16S ribosomal RNA gene, partial sequence	100%	97.83%
	Bifidobacterium pseudocatenulatum strain CCFM8408 16S ribosomal RNA gene, partial sequence	100%	97.67%
	Bifidobacterium angulatum gene for 16S ribosomal RNA, partial sequence, strain: JCM 7096	100%	97.67%
B. breve	*Bifidobacterium breve* strain NCTC11815 genome assembly, chromosome: 1	100%	99.85%
	Bifidobacterium breve strain FDAARGOS_561 chromosome, complete genome	100%	99.85%
	Bifidobacterium breve strain DRBB30 chromosome, complete genome	100%	99.85%
MM9-B-6	*Bifidobacterium* sp. MRM 9.26 16S ribosomal RNA gene, partial sequence	100%	99.21%
	Bifidobacterium myosotis strain MRM_5.10 16S ribosomal RNA gene, partial sequence	100%	97.64%
	Bifidobacterium sp. strain TRE_F 16S ribosomal RNA gene, partial sequence	96%	98.98%

B. longum ssp. *longum* (ATCC® 15707™)	*Bifidobacterium longum* strain HBUAS54272 16S ribosomal RNA gene, partial sequence	100%	99.66%
	Bifidobacterium longum ssp. *suillum* strain S3 16S ribosomal RNA gene, partial sequence	100%	99.66%
	Bifidobacterium longum strain NCTC11818 genome assembly, chromosome: 1	100%	99.66%
MM8-B-4	*Bifidobacterium hapali* strain MRM_8.14 16S ribosomal RNA, partial sequence	100%	99.49%
	Bifidobacterium sp. MRM 9.16 16S ribosomal RNA gene, partial sequence	100%	99.49%
	Bifidobacterium myosotis strain MRM_5.9 16S ribosomal RNA, partial sequence	100%	96.71%
MM9-B-2	*Bifidobacterium myosotis* strain MRM_5.9 16S ribosomal RNA, partial sequence	100%	99.36%
	Bifidobacterium sp. strain TRE_F 16S ribosomal RNA gene, partial sequence	100%	98.88%
	Bifidobacterium saeculare strain LET 415 16S ribosomal RNA gene, partial sequence	100%	97.92%
B. suis ATCC 27533	*Bifidobacterium longum* ssp. *suis* strain VB-5/9 16S ribosomal RNA gene, partial sequence	100%	99.71%
	Bifidobacterium longum strain Su859 genome assembly, chromosome: I	100%	99.71%
	Bifidobacterium longum strain TPY3-2 16S ribosomal RNA gene, partial sequence	100%	99.71%
B. bifidum ATCC 11617	*Bifidobacterium gallinarum* strain CACC 514 chromosome CACC514, complete sequence	100%	99.71%
	Bifidobacterium pullorum gene for 16S ribosomal RNA, partial sequence, strain: JCM 1214	100%	99.42%
	Bifidobacterium saeculare strain LET 415 16S ribosomal RNA gene, partial sequence	100%	99.13%
MM3-M-6	*Bifidobacterium reuteri* strain AFB22-1 16S ribosomal RNA gene, partial sequence	100%	98.08%
	Bifidobacterium longum ssp. *infantis* strain NCTC11817 genome assembly, chromosome: 1	100%	96.63%
	Bifidobacterium sp. strain TRE_H 16S ribosomal RNA gene, partial sequence	98%	97.56%
B. longum DJO10A	*Bifidobacterium longum* DJO10A, complete genome	100%	99.70%
	Bifidobacterium longum strain NWAFU0001 16S ribosomal RNA gene, partial sequence	100%	99.70%
	Bifidobacterium longum strain HBUAS55017 16S ribosomal RNA gene, partial sequence	100%	99.70%
MM5-B-8	*Bifidobacterium reuteri* strain AFB22-1 16S ribosomal RNA gene, partial sequence	92%	87.46%
	Bifidobacterium boum strain LET414 16S ribosomal RNA gene, partial sequence	90%	84.29%

	Bifidobacterium thermophilum strain NB-168 16S ribosomal RNA gene, partial sequence	90%	84.29%
B. adolescentis ATCC 15703	*Bifidobacterium adolescentis* strain ATCC 15703 16S ribosomal RNA, complete sequence	100%	99.82%
	Bifidobacterium faecale strain CICC6176 16S ribosomal RNA gene, partial sequence	100%	99.82%
	Bifidobacterium ruminantium gene for 16S ribosomal RNA, partial sequence, strain: JCM 8222	100%	99.64%
MM8-B-9	*Bifidobacterium* sp. strain LMG 30940 16S ribosomal RNA gene, partial sequence	100%	98.12%
	Bifidobacterium callitrichidarum strain TRI 5 16S ribosomal RNA, partial sequence	100%	97.65%
	Bifidobacterium sp. strain TRE_2 16S ribosomal RNA gene, partial sequence	100%	97.65%
B. animalis ssp. *lactis*	*Bifidobacterium animalis* ssp. *lactis* strain HN019 chromosome, complete genome	100%	99.82%
	Bifidobacterium sp. strain BZ11 16S ribosomal RNA gene, partial sequence	100%	99.82%
	Bifidobacterium sp. MC_3 partial 16S rRNA gene, strain DSM-20219, isolate MC_3	100%	99.82%
MM4-B-6	*Bifidobacterium callitrichidarum* strain TRI 5 16S ribosomal RNA, partial sequence	99%	87.68%
	Bifidobacterium gallinarum strain CACC 514 chromosome CACC514, complete sequence	97%	87.80%
	Bifidobacterium saeculare strain LET 415 16S ribosomal RNA gene, partial sequence	97%	87.80%
MM8-M-5	*Bifidobacterium aesculapii* strain MRM 4/2 16S ribosomal RNA gene, partial sequence	100%	97.68%
	Bifidobacterium scardovii JCM 12489 = DSM 13734 gene for 16S ribosomal RNA, partial sequence	98%	95.77%
	Bifidobacterium aerophilum strain TRE 26 16S ribosomal RNA gene, partial sequence	98%	95.48%
B. animalis ssp. *animalis*	*Bifidobacterium animalis* ssp. *animalis* strain CNCM I-4602 chromosome, complete genome	100%	100%
	Bifidobacterium animalis strain SJ19 16S ribosomal RNA gene, partial sequence	100%	100%
	Bifidobacterium sp. MC_8 partial 16S rRNA gene, isolate MC_8	100%	99.82%
MM10-M-9	*Bifidobacterium aesculapii* strain MRM 4/2 16S ribosomal RNA gene, partial sequence	99%	97.88%
	Bifidobacterium scardovii JCM 12489 = DSM 13734 gene for 16S ribosomal RNA, partial sequence	98%	95.93%
	Bifidobacterium aerophilum strain TRE 26 16S ribosomal RNA gene, partial sequence	98%	95.33%
MM5-B-9	*Bifidobacterium pseudocatenulatum* strain CCFM8408 16S ribosomal RNA gene, partial sequence	100%	98.51%
	Bifidobacterium sp. strain LMG 30940 16S ribosomal RNA gene, partial sequence	100%	98.35%

	Bifidobacterium sp. strain TRE_2 16S ribosomal RNA gene, partial sequence	100%	98.35%
B. adolescentis IVS-1	*Bifidobacterium adolescentis* strain HBUAS55097 16S ribosomal RNA gene, partial sequence	100%	99.26%
	Bifidobacterium faecale strain HBUAS55087 16S ribosomal RNA gene, partial sequence	100%	99.26%
	Bifidobacterium sp. CCFM8400 16S ribosomal RNA gene, partial sequence	100%	99.26%
B. adolescentis L2-32	*Bifidobacterium adolescentis* strain HBUAS55097 16S ribosomal RNA gene, partial sequence	100%	100%
	Bifidobacterium faecale strain HBUAS55087 16S ribosomal RNA gene, partial sequence	100%	100%
	Bifidobacterium sp. CCFM8400 16S ribosomal RNA gene, partial sequence	100%	100%
Bifidobacterium sp. 12_1_47BFAA	*Bifidobacterium* sp. PG13 16S ribosomal RNA gene, partial sequence	99%	93.33%
	Bifidobacterium longum ssp. *longum* strain CCUG30698, complete genome	93%	92.91%
	Bifidobacterium crudilactis strain C4/12B 16S ribosomal RNA gene, partial sequence	85%	82.94%
Bifidobacterium sp. 113	*Bifidobacterium adolescentis* strain HBUAS55097 16S ribosomal RNA gene, partial sequence	100%	100%
	Bifidobacterium faecale strain HBUAS55087 16S ribosomal RNA gene, partial sequence	100%	100%
	Bifidobacterium sp. CCFM8400 16S ribosomal RNA gene, partial sequence	100%	100%
B. longum longum JDM301	*Bifidobacterium longum* ssp. *infantis* strain NCTC11817 genome assembly, chromosome: 1	100%	100%
	Bifidobacterium longum ssp. *longum* 16S ribosomal RNA gene, partial sequence	100%	100%
	Bifidobacterium longum strain BXY01, complete genome	100%	100%

Table 3

PCR Product Spectrophotometer Results for Checking DNA Quality

Strain	ng/µL	A260/A280	A260/A230
B. longum ssp. *longum* (ATCC® 15707™)	115.0	1.82	1.24
B. longum longum JDM301	131.4	1.77	1.34
B. longum DJO10A	138.1	1.81	1.14
Bifidobacterium sp. 12_1_47BFAA	134.7	2.07	2.13
Bifidobacterium sp. 113	87.4	1.89	1.94
B. adolescentis ATCC 15703	41.3	1.92	1.39
B. adolescentis L2-32	233.3	1.80	1.63
B. adolescentis IVS-1	117.6	1.91	1.63
B. animalis ssp. *animalis*	85.3	1.86	1.73
B. bifidum ATCC 11617	74.5	1.97	1.73
B. animalis ssp. *Lactis*	63.9	1.72	0.90
B. suis ATCC 27533	87.6	1.94	1.43
B. breve	279.3	1.86	1.74
MM5-B-8	500.6	2.01	1.61
MM5-B-9	54.5*	1.54	0.97
MM3-M-6	1492.2	2.03	1.68
MM4-B-6	303.8	1.94	1.49
MM4-M-3	40.4*	1.51	0.94
MM5-B-2	2864.8	2.07	1.78
MM9-B-2	270.1	1.85	1.29
MM8-B-4	1289.6	2.03	2.11
MM8-B-9	42.0	1.67	1.37
MM8-M-5	56.3	1.91	1.29
MM10-M-9	30.5	1.55	0.61
MM9-B-6	249.8*	1.70	1.04

Note. In the measurements marked with an (*) under the ng/µL column, the presence of phenol was detected in the sample.

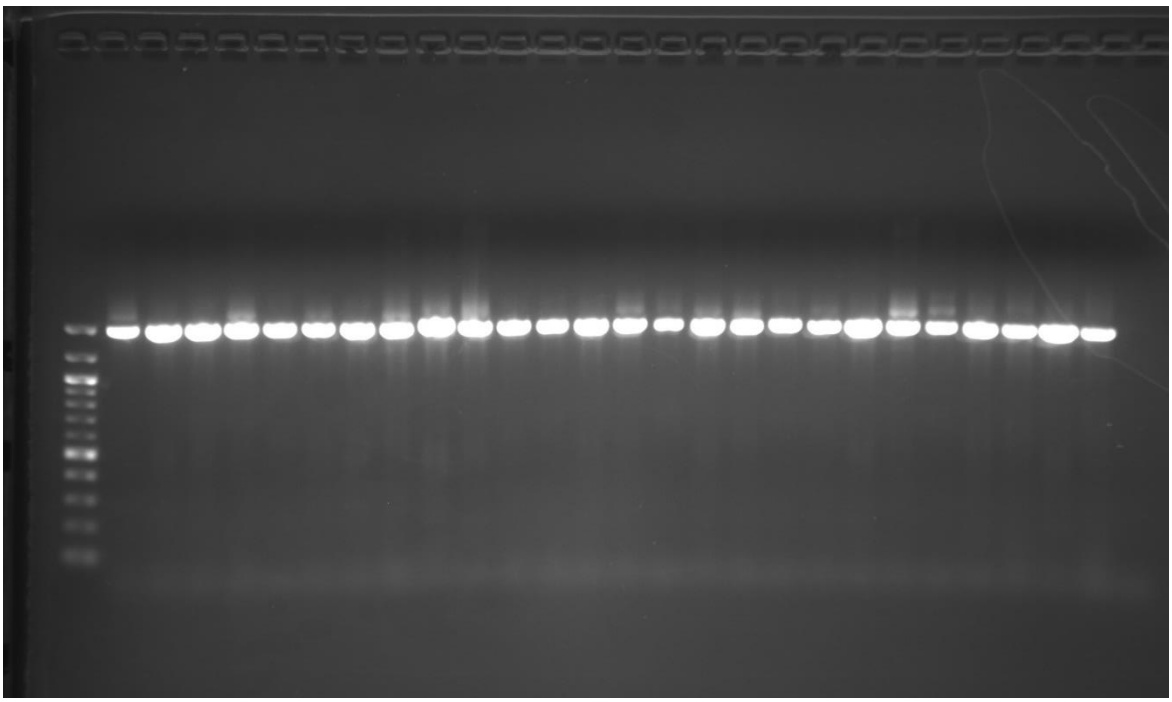

Figure 1. PCR products of amplified 16S rRNA gene sequence of roughly 1,500 bp, viewed under UV light after undergoing agarose gel electrophoresis

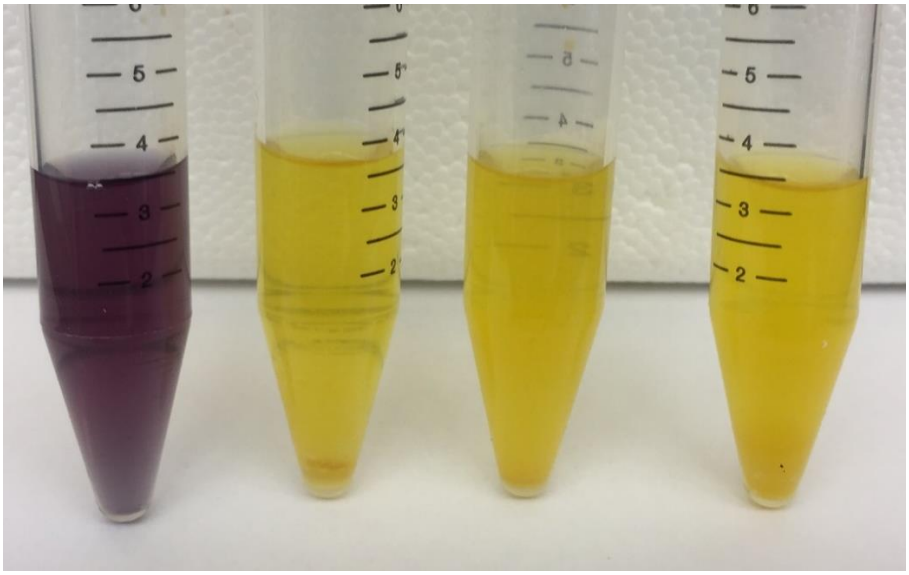

Figure 2. From left to right, one negative and three positive phenotypes for fermentation in media containing 5% arabinose. No intermediate phenotypes are depicted because no strains expressed an intermediate phenotype when incubated with this substrate.

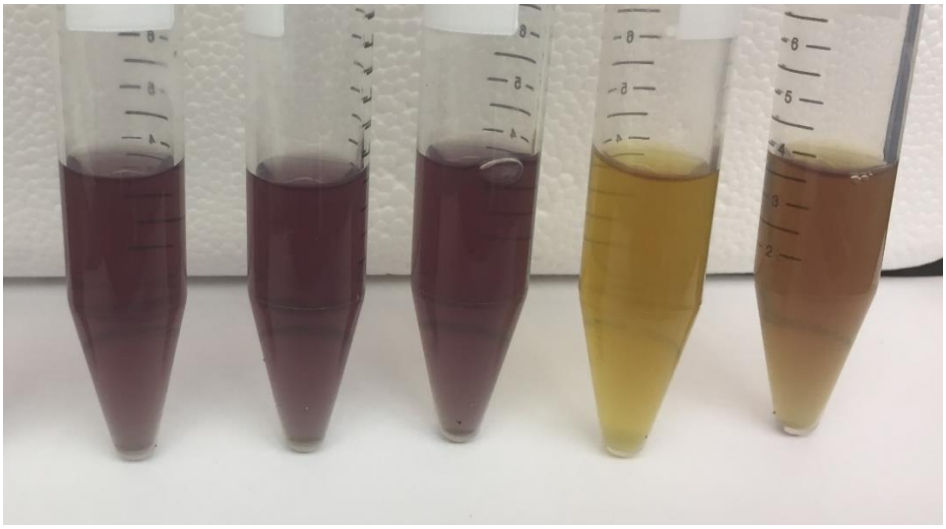

Figure 3. From left to right, three negative phenotypes, one positive phenotype, and one intermediate phenotype for Bifidobacteria incubated in media containing 1% gum Arabic.

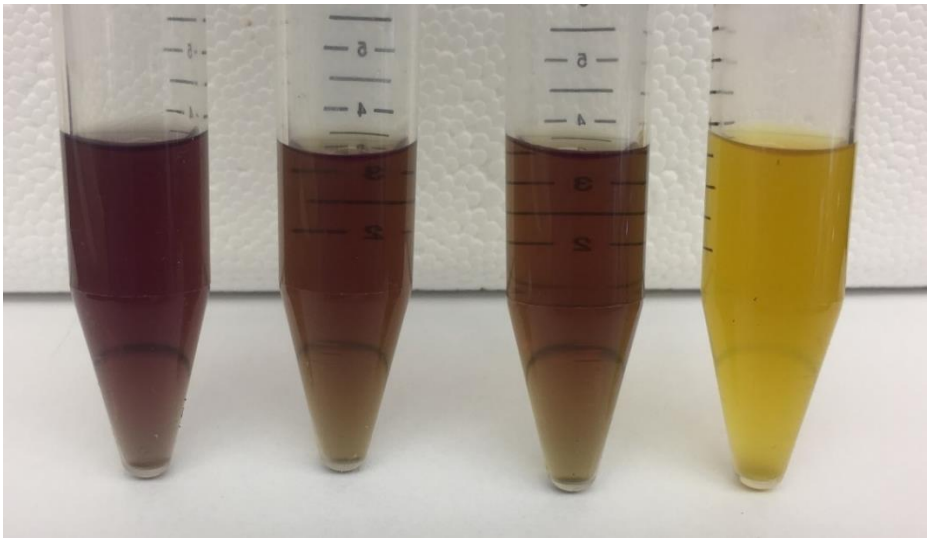

Figure 4. From left to right, one negative phenotype, two intermediate phenotypes, and one positive phenotype for Bifidobacteria incubated in media containing 3% gum Arabic.

Discussion

The results of this experiment have shown that most of the *Bifidobacterium* strains were able to ferment arabinose, regardless of whether or not the strain originated from a marmoset. Only two strains in each group (marmoset vs. non-non-marmoset) tested negative, while all the

others had positive phenotypes. In the marmoset strains, there were more positive phenotypes detected than there were intermediate or negative phenotypes for the gum Arabic. This is true for either concentration; 6 out of 12 strains exhibited positive phenotypes for the 1% concentration, while 8 out of 12 did the same for the 3% concentration. Intermediate phenotypes were more common in the non-marmoset strains, accounting for 6 out of 13 strains for 1% concentration, and 5 out of 13 for the 3%. It is possible that this trend is a result of the marmoset strains being more well-adapted to utilizing gum Arabic as a carbon source.

Some challenges arose during the fermentation testing that prevented obtaining more precise results. Initially, the experimental design called for the use of solid media. Each strain would first be grown in liquid MRS media, then plated on BSIM agar. A single colony would be picked from the plate and used to inoculate a new tube of liquid MRS. After incubation, a loop of inoculum would then be taken from this second tube and plated on solid basal MRS media containing bromocresol purple and the carbohydrate being tested for. Unfortunately, most of the Bifidobacteria would not grow after being plated on the solid media, and it became necessary to exclusively use liquid media for the fermentations.

One of the weaknesses of the tube fermentation experiments is that the phenotype is indirect, observed by acidification of the medium. It is presumed that the acid results from fermentation of the gum Arabic, however it is possible that impurities in the gum are being fermented. Additional studies to detect breakdown of the gum itself during fermentation are needed to confirm actual degradation and fermentation of the gum. Additionally, growth rates should be measured on the gum and other carbohydrate monomers like galactose and rhamnose, which are also constituents of the gum. It may be wise to explore how first treating the gum Arabic enzymatically or with acid hydrolysis that would be expected to occur in the stomach

would affect the ability of Bifidobacterium species to ferment the enzymatic or hydrolysis products.

The results obtained from Sanger sequencing differed in precision between strains, as the size and accuracy of the sequences run through BLAST® were quite variable. Forward and reverse sequences for each strain were compared using MEGA7 software. For several strains, a large portion of the forward sequence would almost perfectly match a section the reverse compliment sequence. Only these closely matched consensus sequences were run through BLAST®, and the shorter queries that resulted from this may have not carried enough information to properly identify the sample when compared to genetically similar strains. For the samples that had significantly mismatched forward and reverse sequences, the entirety of both sequences were run through BLAST®. The top three hits from the sequence that came back with the best identity and query cover scores were recorded, and the information from the other sequence was disregarded. In theory, the compromised accuracy of the gene sequence is largely reconciled by using a much larger portion of the sequenced gene in the query. It should be noted that some of the Bifidobacteria that were isolated from the marmosets may be newly discovered strains, which further contributes to the uncertainty of their identities. Any discrepancies between the forward and reverse sequences may have occurred through contamination, poor DNA quality, or technical errors.

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