Comparative Analysis of Lactulose and Fructooligosaccharide on Growth Kinetics, Fermentation, and Antioxidant Activity of Common Probiotics

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Abstract

Prebiotics are non-digestible oligosaccharides that selectively stimulate the growth of beneficial bacteria in the human gut. Fructooligosaccharide (FOS) is a common prebiotic found in food products and infant formula. Lactulose is primarily used as a pharmaceutical ingredient but also shows potential prebiotic activities. Our objectives were to determine and compare the effects of FOS and lactulose on: 1) growth kinetics of common probiotics in aerobic condition; 2) pH and titratable acidity after fermentation; and 3) antioxidant capacity of the probiotics. Ten probiotic and two non-probiotic strains, representing genera *Lactobacillus*, *Bifidobacterium*, *Bacillus*, and *Escherichia* were assembled. Media used for prebiotics experiment were modified to contain 2% FOS or lactulose as the sole or main carbohydrate source. All experiments were done in triplicate. In aerobic condition, most strains cultured with FOS or lactulose did not grow optimally compared to dextrose (a non-prebiotic), while all four *Bifidobacterium* spp. showed little growth regardless of the carbohydrate source. In anaerobic condition, lactulose and FOS fermentation of *Bifidobacterium* spp. yielded similar pH ($p = 0.2723$), but percent lactic acid, as determined by titratable acidity, was higher after lactulose fermentation ($p = 0.0004$). The non-probiotic strains were able to utilize both FOS and lactulose, but displayed weaker acid production and higher pH ($p < 0.0001$) relative to the probiotic strains. Antioxidant activity of spent medium was measured with Trolox as the reference standard. Overall, the antioxidant activity of probiotics was strain-dependent. FOS enhanced the antioxidant activity of *Bifidobacterium* spp. ($p = 0.0002$) and *Lactobacillus* spp. ($p = 0.0447$), but not probiotic *E. coli* and *Bacillus* spp. ($p = 0.2599$) or non-probiotics ($p = 0.8816$).

In conclusion, lactulose supported growth activities of probiotics to a similar extent as FOS. Lactulose also stimulated higher acid production for *Bifidobacterium* spp. than FOS in anaerobic condition, thus it might be considered for incorporation into functional food products containing bifidobacteria.

Keywords

Prebiotics, Oligosaccharides, Lactulose, FOS, Probiotics

1. Introduction

The gut microbes play an important role in human health. Soon after birth, microbes immediately colonize human skin, oral cavity, and gastrointestinal tract surfaces [1]. A diverse and large amount of bacteria populate the human gastrointestinal tract forming a complex ecosystem of intestinal microbiota [2]. These microbes are an integral part of the gastrointestinal tract due to their role in conserving gut homeostasis, where gut microbes and the human host work together to create a stable ecosystem that provides appropriate responses to microbial "friend" or "foe" [3].

Non-digestible oligosaccharides such as fructooligosaccharide (FOS) and galactooligosaccharide (GOS) are considered prebiotics, which according to Gibson *et al.* (2010), are defined as selectively fermented ingredients that result in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon host health [4]. Some reported benefits include enhanced immune system [5] and defense against pathogens [6], modulation of short-chain fatty acid (SCFA) production [7] and tight junction integrity [8], and mild improvement in iron status [9]. Proliferation of beneficial bacteria, usually lactobacilli and bifidobacteria, in the colon of the host may also be associated with reducing lipid peroxidation in colonic mucosa of intact mammals [10] [11]. The putative antioxidant effect might be due to the ability of the bacteria to scavenge free radicals, and/or an increase in antioxidant capacities of the colon contents. Oxidative stress in the colonic mucosa is presumably involved in the pathogenesis of colon cancer [12].

Lactulose is derived from lactose through an isomerization process in which the glucose moiety in the lactose molecule is converted to fructose, resulting in a disaccharide of galactose and fructose linked together via a $\beta1{\rightarrow}4$ glycosidic linkage [4] [13]. Lactulose has been shown to increase the viability of *Bifidobacterium* and *Lactobacillus* spp. both *in vitro* and *in vivo* [14] [15] [16], and is therefore conventionally classified as a prebiotic [17]. Although available as a functional food ingredient, lactulose is primarily used as a pharmaceutical [18]. On the other hand, FOS is derived from plant sources such as chicory roots [19] [20], and structurally it is a linear chain of β-fructan with the number of fructose unit typically ranging from 2 - 10 [4]. FOS is considered an important commercial prebiotic [21], and studies have examined its use as a functional food ingre-

dient under different food processing environments [22] [23].

The objectives of this study were to determine the prebiotic effects of lactulose on the growth, fermentative ability, and antioxidant activity of common probiotics. The effects of FOS and dextrose were also determined to allow comparisons so as to achieve a better characterization of the prebiotic potential of lactulose as a functional food ingredient.

2. Materials and Methods

2.1. Bacterial Strains

In order to compare the effects of lactulose and FOS on the growth of beneficial bacteria, ten probiotic and two non-probiotic strains, representing genera *Lactobacillus*, *Bifidobacterium*, *Bacillus*, and *Escherichia* were assembled (Table 1). Six species, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Escherichia coli* and *Bacillus pumilus*, were purchased from Hardy Diagnostics (Santa Maria, CA). The *Lactobacillus* and *Bifidobacterium* spp. are considered probiotic-type bacteria, whereas *E. coli* and *B. pumilus* are non-probiotics. In addition, several more probiotics were isolated from commercial products. The species identities according to the product labels were *Lactobacillus rhamnosus*, *L. casei*, *Bifidobacterium infantis*, *Bifidobacterium lactis,* and *Bacillus coagulans*. *Lactobacillus rhamnosus* (LGG) was isolated from the product by incubating a supplement tablet in De Man, Rogosa, Sharpe broth (MRS broth; Hardy Diagnostics, Santa Maria, CA), whereas *L. casei* LcS was isolated by inoculating MRS broth with an aliquot of a probiotic drink obtained from a local grocery store, followed by a 24-hr anaerobic incubation at

Table 1. Bacterial strains used in the study and their sources. Identities of strains that were isolated from commercial products were based on information found on product labels.

Bacterial strain	Source
Lactobacillus acidophilus ATCC 4356	Hardy Diagnostics
Lactobacillus casei ATCC 334	Hardy Diagnostics
Lactobacillus rhamnosus GG (LGG) ATCC 53103	Commercial probiotic supplement
Lactobacillus casei (a proprietary strain, designated as LcS in this study)	Commercial probiotic drink
Bifidobacterium bifidum ATCC 11863	Hardy Diagnostics
Bifidobacterium animalis ssp. *animalis* ATCC 25527	Hardy Diagnostics
Bifidobacterium infantis 35624	Commercial probiotic supplement
Bifidobacterium lactis DN173-010	Commercial yogurt
Escherichia coli Nissle 1917 (ECN)	University of Rhode Island (Dr. Paul S. Cohen)
Bacillus coagulans GBI-30	Commercial probiotic supplement
Escherichia coli (JM 101) ATCC 33876	Hardy Diagnostics
Bacillus pumilus ATCC 14884	Hardy Diagnostics

37°C in a GasPak jar (Becton, Dickinson and Company, Franklin Lakes, NJ). The overnight cultures were streaked for isolation on MRS agar and incubated at 37°C in anaerobic condition for 48 hr. *Bif. infantis* and *Bif. lactis* were isolated by growing the commercial products using Reinforced Clostridium Medium (RCM; Hardy Diagnostics, Santa Maria, CA) for 24 hrs at 37°C in anaerobic condition. Similarly, the overnight cultures were streaked on RCM agar and the plates were incubated anaerobically at 37°C for 48 hr. *B. coagulans* culture was isolated by inoculating the product into Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ) continued with aerobic incubation for 24 hr at 37°C. The broth culture was streaked on a Tryptic Soy Agar (TSA) medium and incubated for 48 hr aerobically at 37°C. One more strain, *E. coli* Nissle 1917 (ECN), was obtained from University of Rhode Island (Dr. Paul S. Cohen). Unlike the aforementioned *E. coli* strain, this ECN strain is considered a probiotic strain [24] [25]. All bacterial strains were checked for purity in their respective agar medium via Gram stain.

2.2. Culture Conditions

All *Lactobacillus* strains were maintained in MRS agar medium supplemented with 0.2% (w/v) sodium thioglycollate (Becton, Dickinson and Company, Franklin Lakes, NJ). *Bifidobacterium* strains were maintained in RCM agar medium supplemented with 0.2% sodium thioglycollate. Both *Lactobacillus* and *Bifidobacterium* strains were cultured at 37°C in an anaerobic GasPak jar with an AnaeroPack® system (Mitsubishi Gas Chemical Company, New York, NY) for 16 - 24 hr prior to experiments. *E. coli* strains and *Bacillus* species were prepared for experiments by growing culture in TSB followed by overnight incubation (16 - 24 hr) at 37°C aerobically. To make frozen stocks, cultures of all bacterial strains were prepared in their corresponding growth medium supplemented with 20% glycerol (Sigma Aldrich, St. Louis, MO) and stored in −80°C freezer.

2.3. Growth Substrates

FOS (Sigma Aldrich, St. Louis, MO), lactulose (Alfa Aesar, Haverhill, MA) and dextrose (Hardy Diagnostics, Santa Maria, CA) were prepared by dissolving the powder in deionized water to yield a 10% (w/v) stock solution. The 10% stock solution was filter sterilized and stored at room temperature. An appropriate amount of either FOS or lactulose stock solution was then added to the tempered MRS basal medium (*i.e.*, MRS without carbohydrate) to yield modified MRS with 2% prebiotics as the sole carbohydrate. The control (non-prebiotic), which was regular MRS medium containing 2% dextrose, was prepared using premixed MRS powder. The RCM medium was prepared by adding 1.5% dextrose to the premixed powder solution that already contained 0.5% basal dextrose to yield a final concentration of 2% dextrose. For the prebiotics experiments on *Bifidobacterium* spp., 1.5% of either FOS or lactulose was added instead of dextrose. For experiments on *E. coli* and *Bacillus* spp., the basal medium of peptone broth (without carbohydrate) was first prepared and autoclaved, fol-

lowed by adding an appropriate amount of the stock solution of dextrose, FOS, or lactulose to the tempered broth to reach a final concentration of 2%.

2.4. Growth Kinetics

Growth curves were determined on bacterial cultures of the ten probiotic and the two non-probiotic strains grown overnight in their respective basal medium with 2% dextrose, FOS, or lactulose as the primary carbohydrate source. *Lactobacillus*, *E. coli* and *Bacillus* spp. were grown in a single carbohydrate source, whereas *Bifidobacterium* spp. were grown in either 2% dextrose, 0.5% dextrose plus 1.5% FOS, or 0.5% dextrose plus 1.5% lactulose. For each bacterial strain, 2 µL of overnight culture was inoculated in duplicate into 200 µL of their respective medium in a 96-well plate. The plate was then incubated at 37°C for 24 hours in SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA) where the start and final OD_{600} of bacterial growth in aerobic condition was measured. OD_{600} of the culture medium alone was used as the blank. The ΔOD_{600} of the sample was calculated with the following equation:

$$\Delta OD_{600} = \left(\text{sample final OD} - \text{sample start OD} \right)$$
$$- \left(\text{blank final OD} - \text{blank start OD} \right)$$

2.5. Fermentation Assay

Similar to the growth curve experiments, overnight cultures of the *Lactobacillus*, *Bifidobacterium*, *E. coli* and *Bacillus* spp. were grown in their respective dextrose-, FOS-, or lactulose-containing medium. Instead of a 96-well plate, broth media were added into sterile glass test tubes containing a Durham tube which collects gas products as a result of fermentation. Overnight cultures were used to inoculate the 10 mL-broth in the test tube at 10% inoculum level. All tubes were incubated overnight at 37°C in anaerobic condition. At the end of the incubation period, the tubes were centrifuged at 2,000 × g for >2 minutes to obtain a cell pellet in the bottom. Supernatant was collected and pH was measured using Orion 3 Star pH meter (Thermo Scientific, Waltham, MA). The blank culture medium was the negative control. In addition to pH, acid production due to fermentation was also measured by titratable acidity (expressed as % lactic acid), in which the supernatant was titrated with 0.1N NaOH.

$$\% \text{ lactic acid} = \frac{\left(0.1N \text{ NaOH} \right)\left(\text{Volume of NaOH used} \right)\left(9 \right)}{\text{Volume of sample}}$$

The final % lactic acid was calculated by subtracting the % lactic acid of the blank medium from that of the sample.

2.6. Antioxidant Activity after Fermentation

Antioxidant activity after fermentation of prebiotics by the bacterial strains was measured using Antioxidant Assay Kit CS0790 (Sigma Aldrich, St. Louis, MO) according to the manufacturer's procedure. Briefly, overnight cultures of the

bacterial strains were prepared in their respective medium with dextrose, FOS, or lactulose. Approximately 10^6 cells were collected and mixed with 0.5 mL of cold 1X Assay Buffer (Sigma Aldrich, St. Louis, MO), then centrifuged at 2,000 × g for 2 minutes (Benchmark Scientific, Edison, NJ). The samples were kept on ice while the cell pellets were sonicated at 22.5 kHz for 10 seconds (Thermo Fisher Scientific, Waltham, MA). The samples were then centrifuged again at 12,000 × g for 15 minutes at 4˚C (GMI Inc., Ramsey, MN). Supernatants were collected and the Trolox equivalent antioxidant capacity (TEAC) was measured. Trolox is a water-soluble vitamin E analog that served as a control antioxidant.

Various Trolox Standards (Sigma Aldrich, St. Louis, MO) were prepared to reach final Trolox concentrations of 0 mM, 0.07 mM, 0.28 mM, 0.50 mM, 0.70 mM, 0.80 mM, 0.90 mM and 1.00 mM. The Trolox Standards were used to construct a Trolox standard curve to calculate the TEAC levels of the samples. In a 96-well plate, 10 μL each of the Trolox Standards and cell-free supernatant samples were added in duplicate, followed by additions of 20 μL of Myoglobin Working Solution (Sigma Aldrich, St. Louis, MO) and 150 μL of ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) Substrate Working Solution (Sigma Aldrich, St. Louis, MO) to each well, continued by a 5-minute incubation at room temperature. Each well was mixed with 100 μL of Stop Solution (Sigma Aldrich, St. Louis, MO) at room temperature to stop the reaction. The endpoint absorbance was read at 405 nm via SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA).

The average absorbance values from the Trolox Standards at different concentrations were used to plot a standard curve to obtain a linear regression equation (and thus the intercept and slope). The TEAC was calculated with the following equation:

$$\text{TEAC}(\text{mM}) = \frac{(\text{Average absorbance of test sample at 405 nm}) - \text{Intercept}}{\text{Slope}}$$

The final TEAC (mM) was calculated by subtracting the TEAC of the blank medium from that of the sample.

2.7. Statistical Analysis

Each experiment was performed independently at least three times. Data sets were checked for normality, and transformed if necessary, before they were analyzed using a general linear model to determine the effects of bacterial species and carbohydrate source. Pairwise comparison among treatments was done by Tukey's HSD with a Bonferroni correction. All statistical analyses were performed with JMP (version 12.1.0, SAS Institute Inc.) with statistical significance indicated by *p*-values of < 0.05.

3. Results and Discussion
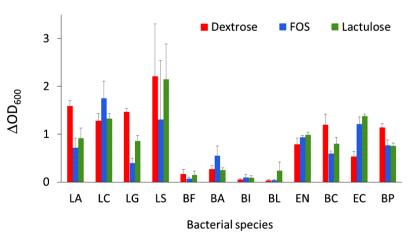
3.1. Bacterial Growth

The aerobic growth activities at 37˚C of the ten probiotics and two non-probiotics

were examined for 24 hours in medium containing dextrose (control), FOS, or lactulose as the main carbohydrate source. The amount of growth was expressed as ΔOD_{600}. When all species were considered, there was a significant decrease in ΔOD_{600} when the medium was supplemented with FOS ($p = 0.0098$) in comparison to either dextrose or lactulose. ΔOD_{600} of *Lactobacillus* spp. was significantly affected by the carbohydrate source (Table 2). LGG grew significantly better in dextrose than in either FOS or lactulose ($p < 0.0001$). A similar trend was observed in *L. acidophilus* ($p = 0.0027$). On the other hand, *L. casei* LcS did not grow as well in medium with FOS than in dextrose or lactulose ($p = 0.0047$). *L. casei* ATCC 334 was the only *Lactobacillus* strain tested that did not exhibit a significantly different aerobic growth pattern in any of the carbohydrate sources provided (Figure 1). Previous studies indicated that *L. acidophilus* ATCC 4356 and LGG performed better with medium supplemented with dextrose than with

Table 2. Mean ± SE of ΔOD_{600} for the four bacterial groups with different carbohydrate sources. Cultures were grown aerobically for 24 hrs at 37°C. Each of the *Lactobacillus* spp. and *Bifidobacterium* spp. groups included four species/strains; the non-lactic probiotics group included *E. coli* Nissle 1917 and *B. coagulans;* the non-probiotics group included *E. coli* and *B. pumilus.*

	ΔOD_{600}			
	Lactobacillus spp.	*Bifidobacterium* spp.	Non-lactic probiotics	Non-probiotics
Dextrose	1.54 ± 0.13^a	0.14 ± 0.04^c	1.00 ± 0.15^{ab}	0.77 ± 0.11^b
FOS	0.94 ± 0.20^b	0.22 ± 0.09^c	0.77 ± 0.08^{ab}	0.99 ± 0.11^{ab}
Lactulose	1.14 ± 0.12^{ab}	0.18 ± 0.03^c	0.90 ± 0.08^{ab}	1.12 ± 0.09^{ab}

[a,b,c]Means with different superscripts are significantly different per Tukey's HSD with a Bonferroni correction ($p < 0.05$).

Figure 1. Mean ± SE of ΔOD_{600} for each bacterial species/strain grown in different carbohydrate sources following aerobic incubation at 37°C for 24 hrs. Error bars indicate standard error of mean. LA = *L. acidophilus*, LC = *L. casei* ATCC 334, LG = *L. rhamnosus* GG, LS = *L. casei* LcS, BF = *Bif. bifidum*, BA = *Bif. animalis*, BI = *Bif. infantis*, BL = *Bif. lactis*, EN = *E. coli* Nissle, BC = *B. coagulans*, EC = *E. coli* (non-probiotic), BP = *B. pumilus* (non-probiotic).

lactulose, FOS, or inulin. Similarly, *L. casei* DN-144-001 did not perform as well when utilizing FOS or inulin as opposed to dextrose [26] [27]. Our results showed that the four *Lactobacillus* strains used in this study generally grew better aerobically when dextrose was the sole carbohydrate source. Dextrose, when available, is the preferred carbohydrate source likely because it is transported into the bacterial cytosol through conservative uptake pathways [28]. Compared to *Lactobacillus*, *Bifidobacterium* spp. grew poorly in aerobic condition regardless of the carbohydrate source (**Table 2**). It is not unexpected because bifidobacteria primarily have been considered obligate anaerobes [29].

While historically most probiotics are Gram-positive non-sporeforming bacteria such as those in the genus *Lactobacillus* or *Bifidobacterium*, some commercially available probiotics are Gram-negative or spore-forming. *E. coli* Nissle 1917 (ECN) could be considered a probiotic because it had been shown to maintain remission in ulcerative colitis patients, reduce relapse occurrence in Crohn's disease patients, and alleviate acute diarrheal symptoms in children [30] [31] [32]. Another non-lactic probiotic, *B. coagulans*, was reported to have beneficial effects on reducing urinary tract infections [33], preventing antibiotic-associated diarrhea in children [33], and relieving symptoms of rheumatoid arthritis [34]. *B. coagulans* started gaining popularity as a probiotic in recent years due to its ability to form endospores, which are resistant to high heat (a common food processing treatment) and acidity of the stomach. As a result, the number of viable cells (spores) reaching the human gut is increased, thereby delivering the putative health benefits [34] [35].

The effects of FOS and lactulose on the growth of ECN and *B. coagulans* were therefore examined. As a group, these non-lactic probiotics did not yield significant changes in ΔOD_{600} among the different types of carbohydrate sources (p = 0.7172). They generally grew sufficiently with any carbohydrate source in aerobic condition, except for the reduced growth observed in *B. coagulans* with FOS. These results are consistent with previous studies that showed some pathogenic and commensal *E. coli* strains could metabolize FOS [36] [37] [38]. Interestingly, while there were no statistically significant differences in ΔOD_{600} among the carbohydrate sources for ECN, the ΔOD_{600} of non-probiotic *E. coli* ATCC 33876 was significantly lower in dextrose than in FOS or lactulose (p < 0.0001, **Figure 1**). *B. pumilus*, another sporeforming *Bacillus* spp yet non-probiotic, exhibited similar growth as *B. coagulans* with all substrates.

3.2. pH and Lactic Acid Production

Fermentative activities of the probiotic and non-probiotic strains in anaerobic condition were evaluated by measuring pH and titratable acidity (expressed as % lactic acid) post-incubation. Except for the group containing *Bifidobacterium* spp., fermentation using dextrose as the sole carbohydrate source resulted in overall lowest pH, followed by lactulose and FOS (p < 0.0001, **Table 3**). A potential benefit of prebiotics and probiotics consumption to the host is their ability to

Table 3. Mean ± SE of pH of the supernatant for the four bacterial groups with different carbohydrate sources after anaerobic incubation for 24 hrs at 37˚C. Each of the *Lactobacillus* spp. and *Bifidobacterium* spp. groups included four species/strains; the non-lactic probiotics group included *E. coli* Nissle 1917 and *B. coagulans;* the non-probiotics group included *E. coli* and *B. pumilus*.
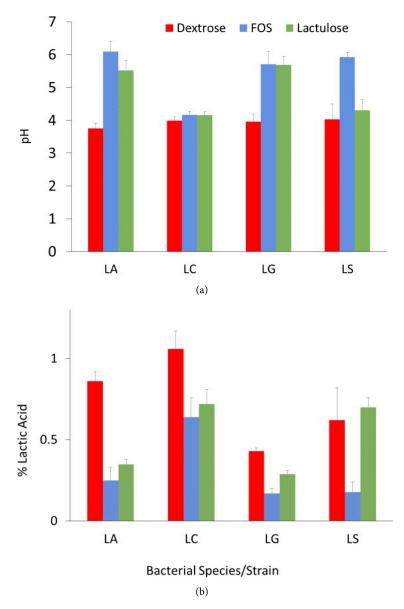
	pH			
	Lactobacillus spp.	*Bifidobacterium* spp.	Non-lactic probiotics	Non-probiotics
Dextrose	3.92 ± 0.10[f]	5.01 ± 0.21[bcde]	3.96 ± 0.05[ef]	5.08 ± 0.14[bcde]
FOS	5.38 ± 0.21[bc]	4.88 ± 0.20[bcdef]	5.64 ± 0.29[abcd]	6.27 ± 0.10[a]
Lactulose	5.02 ± 0.17[cde]	4.37 ± 0.19[def]	4.10 ± 0.15[cdef]	5.91 ± 0.12[ab]

[a,b,c,d,e,f]Means with different superscripts are significantly different per Tukey's HSD with a Bonferroni correction ($p < 0.05$).

decrease gut pH which could suppress pathogens and thereby improve host resistance against intestinal disturbances [39]. As shown in Figure 2, the *Lactobacillus* species/strains produced more acid and lower pH when dextrose was the sole carbohydrate source, when compared to FOS and lactulose ($p < 0.0001$). The only exception was fermentation by *L. casei* ATCC 334 which yielded similar pH regardless of the carbohydrate source (Figure 2(a)). Although there tends to be an inverse relationship between pH and acid concentration, the amount of organic acids produced by the bacteria cannot be directly measured by pH alone. Since lactic acid is the primary organic acid produced by most lactobacilli, % lactic acid in the medium post-fermentation was measured in the present study. As a group, dextrose fermentation by lactobacilli resulted in higher % lactic acid compared to FOS ($p < 0.0001$) or lactulose ($p = 0.0021$, Figure 2(b)) fermentation. Between the two prebiotic supplements, lactulose appeared to increase % lactic acid more than FOS. The most pronounced difference could be seen in *L. casei* LcS ($p < 0.0001$, Figure 2(b)).

In a screening study on acid production by *Lactobacillus* spp., Kaplan and Hutkins (2000) showed that 12 of 16 strains tested (including four *L. acidophilus* strains and two *L. casei* strains) induced a color change in MRS agar medium supplemented with 2% FOS and a pH indicator bromcresol [27], indicating the strains' ability to ferment FOS. Our results corroborated their findings in which all four *Lactobacillus* strains tested in this study could ferment FOS and lactulose, although dextrose fermentation yielded lower pH and higher % lactic acid.
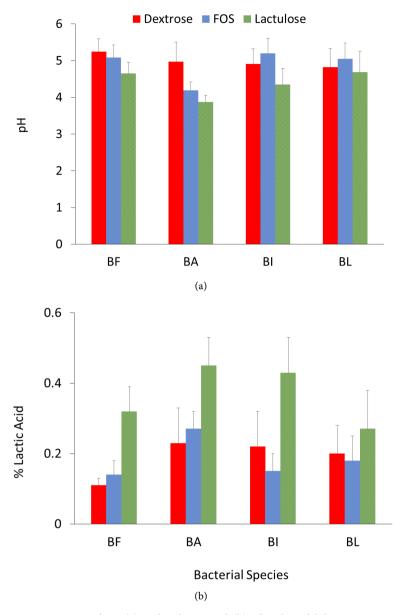
For the group of bifidobacteria containing four species, while all three carbohydrate sources were fermented to produce acid and hence lowering the pH, lactulose supplement in the medium facilitated higher lactic acid production ($p < 0.0001$) and lower pH ($p = 0.0122$) than dextrose alone (Figure 3). Lactulose fermentation by *Bifidobacterium* spp. produced a significantly higher % lactic acid compared to FOS fermentation ($p = 0.0004$; Figure 3(b)). The difference in % lactic acid production between lactulose and FOS could not be derived from pH values which were statistically insignificant ($p = 0.2723$; Figure 3(a)).

(a)

Bacterial Species/Strain

(b)

Figure 2. Mean ± SE of pH (a) and % lactic acid (b) of individual *Lactobacillus* spp. after anaerobic incubation for 24 hrs at 37°C in medium containing dextrose, FOS, or lactulose as the sole carbohydrate source. Error bars indicate standard error of mean. LA = *L. acidophilus*, LC = *L. casei* ATCC 334, LG = *L. rhamnosus* GG, LS = *L. casei* LcS.
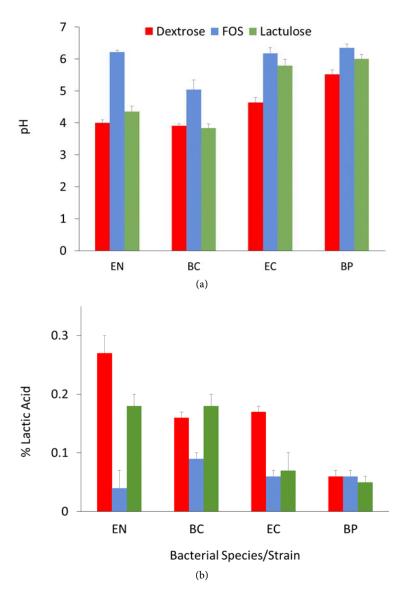
These results further highlight the importance of measuring weak acid content by titration in addition to pH.

Bifidobacteria are important beneficial intestinal microorganisms in the human intestinal environment. They are predominant in the gut of full-term breast-fed infants, presumably offering protection against potential pathogens. Other obligate anaerobes such as *Clostridium* spp. are rarely isolated in such environment [40]. *Bifidobacterium* spp. isolated from infant stools have been shown to exert antimicrobial effects on *Listeria monocytogenes* and *Salmonella* Typhimurium [41]. The environmental condition *in vivo* (*i.e.*, human colon)

Figure 3. Mean ± SE of pH (a) and % lactic acid (b) of each *Bifidobacterium* spp. after anaerobic incubation for 24 hrs at 37°C in medium containing dextrose, FOS, or lactulose as the primary carbohydrate source. Error bars indicate standard error of mean. BF = *Bif. bifidum*, BA = *Bif. animalis*, BI = *Bif. infantis*, BL = *Bif. lactis*.

where bifidobacteria most likely carry out fermentation of carbohydrates is anaerobic. Previous studies showed that more than fifty *Bifidobacterium* strains were found to ferment FOS [42] [43]. Langlands *et al.* (2004), upon examination of biopsy samples taken from the colon and rectum of human subjects during colonoscopy, confirmed that dietary FOS supplementation led to increases in surface counts of *Bifidobacterium* spp. [44]. Our results not only showed that different *Bifidobacterium* spp were able to ferment FOS, but lactulose could be an even more effective prebiotic than FOS in stimulating the fermentative ability of *Bifidobacterium* spp. in anaerobic condition.

Between the non-lactic probiotics ECN and *B. coagulans*, the former species produced higher % lactic acid when dextrose was the sole carbohydrate source ($p = 0.0123$). Statistically, the amount of lactic acid produced by lactulose fermentation was not different from dextrose fermentation ($p = 0.3299$; Figure 4(b)). Between FOS and lactulose, similar to the results obtained from the *Bifidobacterium* group, these two species produced lower pH and higher % lactic acid when lactulose was the carbohydrate source instead of FOS ($p < 0.0001$; Figure 4).

One of the key criteria for prebiotics is that they are *selectively* fermented by beneficial bacteria. In other words, for lactulose to be considered an effective
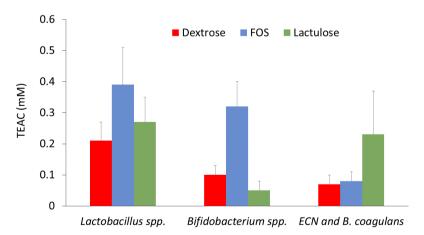

Figure 4. Mean ± SE of pH (a) and % lactic acid (b) of *Escherichia* and *Bacillus* spp. after anaerobic incubation for 24 hrs at 37°C in medium containing dextrose, FOS, or lactulose as the sole carbohydrate source. Error bars indicate standard error of mean. EN = probiotic *E. coli* Nissle 1917, BC = probiotic *B. coagulans*, EC = non-probiotic *E. coli*, BP = non-probiotic *B. pumilus*.

prebiotic, probiotic strains should be able to better utilize lactulose when compared to non-probiotic strains. All probiotic strains in this study (*Lactobacillus* spp., *Bifidobacterium* spp., ECN, *B. coagulans*) yielded lower pH ($p < 0.0001$) and higher % lactic acid ($p < 0.0001$) when given lactulose (or FOS, so far as prebiotic potential is concerned) in comparison to the non-probiotic *E. coli* and *B. pumilus* (Figure 4). These results signify the prebiotic potential of lactulose.

3.3. Antioxidant Activity

Mild statistically difference was observed in TEAC levels among the different carbohydrate sources ($p = 0.0409$, Figure 5). When all strains were considered collectively, FOS as the sole or main carbohydrate source resulted in 0.32 ± 0.14 mM higher TEAC than dextrose. Further examination specifically on the *Lactobacillus* spp. showed that dextrose or lactulose fermentation did not produce significantly different TEAC levels ($p = 0.0958$); however the TEAC from FOS fermentation was significantly higher compared to dextrose ($p = 0.0447$). The *Bifidobacterium* spp. also produced a significantly higher TEAC when supplemented with FOS compared to lactulose ($p = 0.0002$). Lactulose fermentation of probiotic ECN and *B. coagulans* produced higher TEAC than dextrose or FOS fermentation but the difference was not statistically different ($p = 0.2599$). The non-probiotic group showed no differences in TEAC levels regardless of the carbohydrate sources ($p = 0.8816$, data not shown).

Amongst their many putative health benefits, probiotics may neutralize reactive oxygen species (ROS) in the host and repress oxidative stress [45] [46]. Madhu *et al.* (2012) investigated the antioxidant effect of adding 1% (w/v) FOS to the starter cultures, *Streptococcus thermophilus* ATCC 19258 and *L. delbrueckii* ssp. *bulgaricus* CFR2028, supplemented with either *Lactobacillus plantarum* CFR2194 or *Lactobacillus fermentum* CFR2192 in yogurt samples. The total

Figure 5. Mean ± SE of Trolox equivalent antioxidant capacity (TEAC, mM) of probiotics after anaerobic incubation for 24 hrs at 37˚C in medium containing dextrose, FOS, or lactulose as the sole or primary carbohydrate source. Error bars indicate standard error of mean. Each of the *Lactobacillus* spp. and *Bifidobacterium* spp. groups included four species/strains. *E. coli* Nissle (ECN) and *B. coagulans* represented non-lactic probiotics.

antioxidant potential measured via ferric reducing ability (FRAP) assay showed that the synbiotic yogurt samples were able to scavenge 85% and 82% free radicals, when they contained *L. plantarum* and *L. fermentum*, respectively; compared to the 72% from the control samples containing the starter cultures only [47]. Our *Lactobacillus* strains performed comparably in their ability to produce higher TEAC levels with FOS supplementation than with dextrose ($p = 0.0447$).

Our results also show that the antioxidant activity is highly strain-dependent ($p = 0.0026$). Between the two *L. casei* strains, overnight incubation of ATCC 334 with FOS produced 0.74 ± 0.29 mM TEAC, whereas LcS produced 0.07 ± 0.05 mM. Virtanen *et al.* (2007) showed that a combination of *Leuconostoc cremoris* B, *Lactococcus lactis,* and *L. acidophilus* in fermented milk resulted in higher radical scavenging activity than in milk fermented with a single bacterial strain [48], suggesting that a cocktail of carefully chosen probiotic strains might be the best approach to maximize the antioxidant activity of consumer products.

4. Conclusion

The goal of the present study was to compare FOS and lactulose in regard to their effects on the viability and fermentative activity of common probiotic bacteria. Different probiotic strains were tested, along with two non-probiotic strains for additional comparison. Similar to FOS, lactulose supported the growth and fermentative activity of probiotics. In case of *Bifidobacterium* spp., lactulose appeared to stimulate even higher acid production than FOS in anaerobic condition. Food product developers might consider lactulose as the choice of prebiotics when formulating consumer products containing bifidobacteria. Antioxidant seemed to be strain dependent, and while FOS appeared to be more effective than lactulose in stimulating the antioxidant activity of bifidobacteria, further investigations on combinations of probiotics and whether lactulose could be an effective prebiotic to support the activities of probiotic mixtures are warranted.

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