The Effect of Galacto-oligosaccharide Supplementation on Intestinal Microbiota

Madeline Bost

College of Agricultural and Life Sciences, University of Florida

Supplementation with prebiotics, substrates intended to facilitate the growth of beneficial bacteria in the gastrointestinal tract, is anticipated to affect health and reduce disease complications. The purpose of this project was to study an intervention in the diets of University of Florida undergraduate students by providing galacto-oligosaccharide (GOS) fiber packets to consume daily. Of the 427 students participating, a subgroup of 66 individuals provided a total of 262 stool samples. DNA was extracted from fecal samples, and the 16S rRNA gene was amplified non-selectively from the bacterial community and analyzed using denaturing gradient gel electrophoresis (DGGE). Baseline and treatment samples were compared for each subject. According to DGGE analysis using both visual comparison and statistical tests, GOS intake significantly affected the diversity of intestinal microbiota. Ongoing DNA sequencing will provide additional data on which species were affected.

INTRODUCTION

The digestive tract composes a large portion of the immune system. Nearly three quarters of our lymphocytes are housed in the human gastrointestinal (GI) tract (Mueller & Macpherson, 2006). In addition to mucous membranes and stomach acid, the body is protected by a multitude of non-pathogenic bacteria living on the epithelial surface lining the GI tract. These bacteria cover the mucosa and help to prevent pathogenic bacteria from attaching and establishing colonies (Macpherson & Harris, 2004). Additionally, they stimulate an increase in goblet cell proliferation (Mueller & Macpherson, 2006).They are able to do this by stimulating an immune response without attacking the commensals because they adapt to the internal environment without damaging host tissue (Macpherson et al., 2007). Their activities include fermenting indigestible carbohydrates and producing lactate and short chain fatty acids that are absorbed in the colon (Knol et al., 2005).

Most of these bacteria are found in the colon, with the stomach and small intestines having relatively lower quantities (Macpherson & Harris, 2004); the majority of these bacteria include *Bacteroides*, *Clostridium*, *Lactobacillus*, *Eubacterium*, *Faecalibacterium* and *Bifidobacterium* species (Martin et al., 2010). The community composition for each individual is generally stable over time (Tzortis & Vulevic, 2009). Intestinal bacteria have been shown to affect obesity, the onset of diabetes, energy metabolism, inflammatory responses, and many other conditions. Microbial communities display a great range of variability, depending on a number of factors including age, health status, diet, and location in the GI tract.

Bifidobacteria and *lactic acid bacteria* are largely recognized as health-promoting organisms and widely used as probiotics (Tzortis & Vulevic, 2009). This is likely due to their interaction with the host's dendritic cells, which help regulate immune response (Weiss et al., 2010). Administration of probiotic supplementation of certain strains of these bacteria have been shown to beneficially impact the health of infants (Romeo et al., 2010), and breast fed infants tend to have a bifido-dominated gut as opposed to formula-fed infants, which generally have a much more varied microbial community (Khailova et al., 2010).

Significant effort has been put into devising ways to promote the growth specific beneficial bacteria that have been found to improve health and support immune function. One of the ways this is accomplished is through supplementation, namely prebiotics (substrates intended for the microbes), probiotics (live cultures of the beneficial bacteria), and synbiotics, combinations of prebiotics and probiotics (Bosscher, 2009). Prebiotics must be non-digestible to humans so that they can pass through the digestive system intact and selectively stimulate the growth of the preferred bacteria (Gibson & Roberfroid, 1995; Yang et al., 2010). Sources of prebiotics currently investigated include inulin, fructooligosaccharide, lactulose, and galactooligosaccharide (Satokari et al., 2001).

Galacto-oligosaccharide (GOS) is colorless, water soluble, and has low water activity, which increases shelf life by reducing the risk of microbial contamination. Additionally, it is stable within a range of temperatures, which opens wide applications as a supplemental food additive. It also has low caloric value of 1-2 kcal/g (Cummings et al., 1997), which makes it an ideal

supplement because it will not adversely affect certain groups of individuals (e.g., diabetics) or raise the energy value of a food.

Denaturing gradient gel electrophoresis (DGGE) is a technique used to visually determine the population and variety of microbes in a sample and relies on the 16S rRNA gene (Vanhoutte et al., 2004). 16S rDNA sequencing is a particularly useful way to identify bacteria, especially those with unique phenotypes or that cannot be cultured (Woo et al., 2008). In a denaturing polyacrylamide gel, DNA fragments of the same length can be separated based upon base pairs, which affect the mobility of the molecule (Muyzer et al., 1993). As the DNA migrates down the gel, the DNA molecule degenerates from its double-stranded conformation to a melted single-stranded form that has decreased mobility, and its migration stops in specific melting domains based on the number of G-C nucleotide bonds. The bands indicate groups present in the sample (Wanga et al., 2007).

The purpose of this study is to determine the extent to which GOS changes microbial populations in the gut. We hypothesize that beneficial microbes will be enriched with GOS supplementation, specifically, *bifidobacteria* and *lactic acid bacteria*, and changes in gut microbiota populations will be seen using DGGE analysis and proven with sequencing.

METHODS

Participants

Stool samples were obtained from a subgroup of subjects participating in a larger study examining the impact of GOS on overall digestive health and immune strength. Undergraduate students were recruited using fliers, messages through listservs, and announcements in classrooms. 427 students were randomized to three groups: 5 g GOS (provided by PurimmuneTM GTC Nutrition, Golden CO.) treatment, 2.5 g GOS treatment, and 5 g placebo. The procedure dictated a double blind study. Exclusionary criteria included smoking, allergies involving the upper respiratory tract or milk, and refusal to discontinue dietary supplements involved with immune strength (including prebiotics, probiotics, fish oil, Echinacea, other fiber supplements, and vitamin E exceeding 100% of the RDA). Participants were required to mix a 5 g treatment packet with a beverage and answer an online questionnaire daily. These questions pertained to stress level, cold symptoms and their severity, and bowel habits.

A small subset of study participants volunteered to provide stool samples. There were 24, 21, and 22 subjects in groups 0 g GOS, 2.5 g GOS, and 5 g GOS, respectively. Participants dropped off the samples, which were promptly aliquoted within four hours of defecation into tubes for later analysis. The samples were stored at -20°C until extraction to minimize DNA degradation.

DNA Extraction

DNA was extracted from stool samples using the QIAamp Stool Mini Kit (Qiagen) following the manufacturer's protocol. Briefly summarized, the protocol is as follows. A small amount, approximately 0.2 mg stool, was homogenized in a 0.05 M phosphate buffer. Zirconia beads were added to assist in the lysing of cells using the Mini Bead Beater (Biospec). Samples were processed using a series of buffers and washes to elute the DNA.

Because a phenol-chloroform technique is not used, purification of the sample is not necessary to remove impurities. Samples were analyzed using a Nanodrop spectrophotometer to assess nucleic acid concentration and purity.

PCR Amplification

Qiagen reagents 10x reaction buffer, MgCl2 (1 μM), dNTPs, and Taq polymerase (0.625 units/μL) were used to amplify the V6-V8 region of bacterial RNA. The master mix contained 2% formamide and contained 0.2 μM forward and reverse primers. Extracted DNA was diluted 1:10, and the total reaction was 25 μL.

The PCR series consisted of an initial melting temperature of 94°C for 2 min followed by 35 cycles of melting at 94°C for 30 s, annealing at 55°C for 30 s, and polymerase copying at 68°C for 30 s. The final annealing step was at 68°C for 7 min. Reactions were checked on a 1% agarose gel for the presence of a band with length of around 500 base pairs corresponding to the 16S rRNA gene.

DGGE

A 457 base pair fragment from the V6 to V8 region of the bacterial 16S rDNA was amplified with primers U968-GC (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5' GCG TGT GTA CAA GAC CC) as described by Zoetendal et al. (1998). The GC clamp facilitates separation by DGGE, which was performed on an 8% [wt/vol] acrylamide gel with a gradient from 40% at the top to 50% at the bottom at a temperature of 60°C. The 100% denaturing conditions were defined as 7 M urea and 40% formamide. Gels were run for 16 h at 65V and stained with SYBR Gold. Images of the stained gels were scanned in with Quantity One software (Biorad) and analyzed with Diversity database software (Biorad).

DGGE Analysis

The DGGE bands were analyzed using Diversity Database (Biorad) software. There was a total of 262

samples. Marked bands in gels were visually compared to other gels containing the same subjects to determine if there were obvious differences or similarities across treatment groups with regard to band location and intensity.

A phylogenetic tree was derived from all of the gels in the database using the Dice coefficient and Ward's clustering method.

Lane reports for the relative quantity values were exported from each gel and entered into http://www.changbioscience.com/genetics/shannon.html to obtain the Shannon-Weimer diversity index, a measure of the occurrence of species in a sample. The Simpson Diversity index (1/D) was also calculated. These values were utilized in an Excel spreadsheet (Microsoft) using a 2-tailed type 2 Student's T test. The groups compared were as follows: first and second baseline sample within groups; baseline and treatment within groups; baseline (first and second donation) samples between groups; treatment (third and fourth) between groups. P values less than 0.05 were considered significant.

RESULTS

Fecal DNA Extraction and Amplification

The yield of DNA using the Qiagen extraction kit ranged from 75-300 ng/μL. Correct amplification of a 457 base pair fragment from extracted fecal DNA was initially assessed by visualization in an agarose gel (Figure 1).

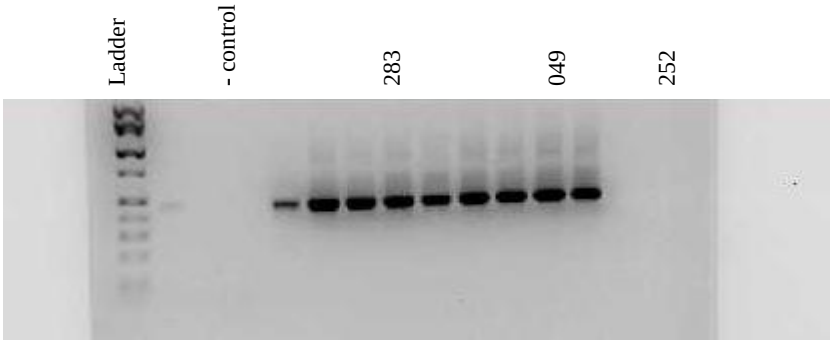

Figure 1. Agarose gel electrophoresis of PCR products from 16S rDNA from fecal samples. For subject 283, the dilutions of the PCR product are 1:10, 1:20, and 1:40 from left to right. The samples 049, 252, 269, and 290 each have two dilutions in the gel, with 1:10 on the left and 1:20 on the right.

Of the three dilutions for sample 283, only the 1:40 dilution is visible in the gel. The negative control shows a faint band but is much lighter than the ladder. Taq Polymerase is the likely cause for this faint band as it is made in E.coli and 16S DNA fragments might be copurified.

DGGE

For each sample lane, the bands were compared to other lanes of the same treatment group on the basis of location and intensity (Figure 2). Rf values between the gels were compared. From this, it was obvious that lanes for a particular subject are more similar to each other than they are to lanes of other subjects, regardless of the group. The changes in band intensities between baseline and treatment samples were compared for each subject in the 5 g GOS group. When a pattern shared between four or more subjects emerged, it was compared to bands in the 0 and 2.5 g GOS groups. No patterns in band intensity after treatment were unique to the 5 g GOS group.

Phylogenetic clustering was observed for baseline and treatment pairs in 0 g GOS, 2.5 g GOS, and 5 g GOS groups using Diversity Database (Figure 3). The expected pattern was as follows: treatment sample pairs would have higher similarity than baseline sample pairs, and treatment pairs would be somewhat similar to baseline pairs. The results from this observation are shown in Table 1. For each group, unexpected or seemingly random clustering was observed in about half of the subjects. The expected pattern was low in 0 g GOS and 5 g GOS, but much higher in 2.5 g GOS; over a third of the subjects followed the expected clustering pattern. Cases where the baseline samples were more similar to each other than treatment samples were more prevalent than the expected pattern in 0 g GOS and 5 g GOS. Some subjects only donated three samples, so a clustering pattern could not be determined.

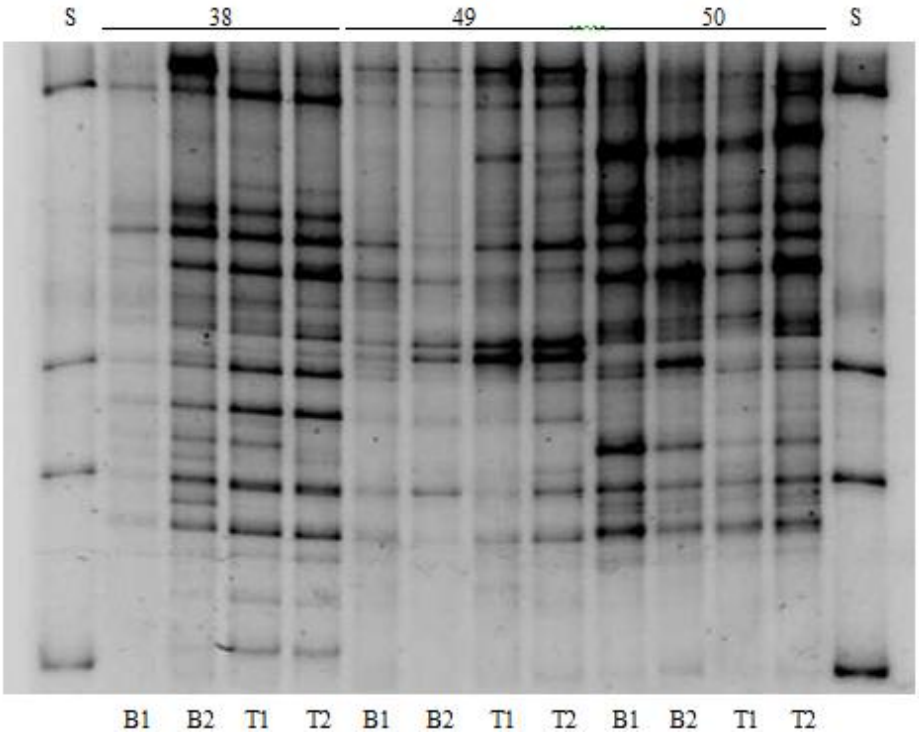

Figure 2. DGGE of PCR products of V6–V8 regions of 16S rDNA from fecal samples. The gel contains three subjects, 038 (5 g GOS), 049 (2.5 g GOS, and 050 (O g GOS), each with two baseline and two treatment samples.

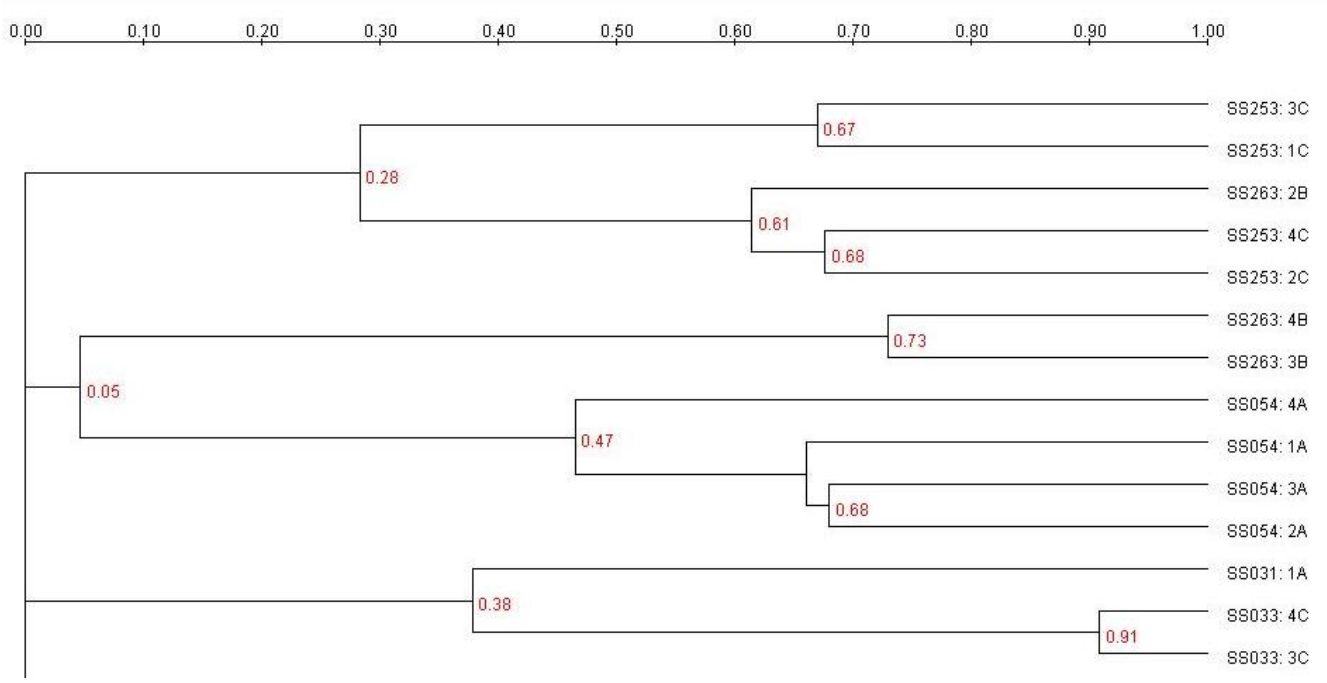

Figure 3. Excerpt of the phylogenetic cluster of PCR products from 16S rDNA of stool samples. A refers to 0 g GOS, B to 2.5 g GOS, and C to 5 g GOS.

Table 1. Patterns Found in the Phylogenetic Clustering

Sample Providing Participants	0 g GOS	2.5 g GOS	5 g GOS
Number of subjects	24	21	22
Unexpected	15	10	14
Expected	2	8	1
Baseline more similar than treatment	3	2	3
Only three samples provided	4	1	4

Statistical Tests

The comparison of the Shannon-Weiner 0 g GOS to 5 g GOS was the only statistically significant value (p-value = 0.0482); the Simpson value for the same data set was not statistically significant (Table 2). From this test, it is evident that there is not a difference between 5 g GOS and 2.5 g GOS or 2.5 g GOS and 0 g GOS, but there is a difference between 0 g GOS and 5 g GOS.

Table 2. Shannon-Weiner and Simpson Diversity Indexes

Parameters	Shannon	Simpson
A1 and A2	0.3529	0.5707
B1 and B2	0.9168	0.8733
C1 and C2	0.9661	0.8062
Ab and At	0.1285	0.0876
Bb and Bt	0.0923	0.0762
Cb and Ct	0.5661	0.2989
Ab and Bb	0.1620	0.2901
Bb and Cb	0.6384	0.8637
Ab and Cb	0.4471	0.2806
At and Bt	0.1978	0.2576
Bt and Ct	0.6059	0.4773
At and Ct	0.0482	0.0638

Note. "1" denotes the first baseline sample and "2" denotes the second baseline sample. "b" and "t" refer to the average of the baseline and treatment samples respectively.

DISCUSSION

When considering the diversity index calculations, there was a statistically significant value for the Shannon-Weiner calculation for the difference between the 0 g GOS and 5 g GOS post treatment groups (p-value = 0.0482). This shows that there is a difference between the 5 g treatment and the placebo that may be due to the fiber consumption. These values were not as apparent when considering the 0 g GOS and 2.5 g GOS groups. It is possible that the amount of fiber administered was not high enough to elicit a response.

On the other hand, when comparing the DGGE gels visually, no unique patterns emerged for 5 g GOS compared to 0 g GOS. This could be due to a number of reasons. Diversity Database is an inexact program, allowing for a large range of subjectivity, specifically with creating the bands. Additionally, there were unexpected results in the phylogenetic tree. Treatment samples, especially for 5 g GOS, were expected to have a greater similarity to each other than the baseline samples, but 5 g GOS was often observed with a subject's baseline sample having a high similarity to a treatment sample. This indicates that either the particular fiber or the amount consumed did not have a strong impact on intestinal microbial populations.

Another factor that affected the expected results is that it is likely that there was a certain level of non-compliance. This is indicated by the number of subjects who provided only three samples. Additional sources of non-compliance include the manner in which the fiber powder was consumed. According to feedback, the GOS did not completely dissolve in 0.5 L of liquid, and so some participants may have disposed of water bottles or washed cups containing residual fiber and consequently not have consumed the whole dose.

From these results, the following conclusions may be drawn. The dosage of fiber was not high enough to yield discernible differences between baseline and treatment samples between groups 0 g GOS, 2.5 g GOS, and 5 g GOS by visually comparing DGGE bands; however, the diversity index calculations indicate there is a difference in the diversity of intestinal microbiota. DNA sequencing can be done to confirm the species present in the groups.

ACKNOWLEDGMENTS

I would like to thank Dr. Bobbi Langkamp-Henken for exposing me to research studies, Dr. Volker Mai for giving me the opportunity to get involved in lab work, and Tyler Culpepper for his guidance and patience at the bench.

REFERENCES

Bosscher D (2009). Food-based strategies to modulate the composition of the intestinal microbiota and their associated health effects. Journal of Physiology and Pharmacology. 60; 5.

Cummings JH, Roberfroid MB, Anderson H, Barth C, Ferro-Luzzi A, Ghoos Y, Gibney M, Hermsonsen K, James WPT, Korver O, Lairon D, Pascal G, Voragen AGS (1997). PASSCLAIM—Gut health and immunity. European Journal of Clinical Nutrition.51; 417–423.

Gibson GR, Roberfroid (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. The Journal of Nutrition.125; 1401–1412.

Khailova L, Mount Patrick SK, Arganbright KM, Halpern MD, Kinouchi T, Dvorak B (2010). *Bifidobacterium bifidum* reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. American Journal of Physiology: GI and liver Physiology.

Knol J, Scholtens P, Kafka C, Steenbakkers J, Gro S, Helm K, Klarczyk M, Scho¨pfer H, Bo¨ckler HM, Wells J (2005). Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed Infants. Journal of Pediatric Gastroenterology and Nutrition. 40; 36–42.

Macpherson AJ, Hapfelmeier S, McCoy KD (2007). The armed truce between the intestinal microflora and host mucosal immunity. Seminars in Immunology.19; 57–58.

Macpherson AJ, Harris NL (2004). Interactions between commensal intestinal bacteria and the immune system. Nature Reviews Immunology.4; 478–485.

Martin FPJ, Sprenger N, Montoliu I, Rezzi S, Kochhar S, Nicholson JK (2010). Dietary Modulation of gut functional ecology studied by fecal metabonomics. Journal of Proteome Research. 9; 5284–5295.

Mueller C, Macpherson AJ (2006). Layers of mutualism with commensal bacteria protect us from intestinal inflammation. Gut. 55; 276–284.

Muyzer G, De Waal EC, Uitierlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology. 59; 695–700.

Romeo J, Nova E, Wärnberg J, Gómez-Martínez S, Díaz Ligia LE, Marcos A (2010). Immunomodulatory effect of fibres, probiotics and synbiotics in different life-stages. Nutricion Hospitalaria. 25; 341–349.

Satokari RM, Vaughan EE, Akkermans ADL, Saarela M, De Vos W (2001). Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal *Bifidobacterium* populations in a prebiotic and probiotic feeding trial. Systemic and Applied Microbiology. 24; 227–231.

Tzortis G, Vulevic J (2009). Galacto-oligosaccharide prebiotics. In Prebiotics and Probiotics Science and Technology. Springer Science+Business Media, LLC. 207–244

Vanhoutte T, Huys G, De Brandt E, Swings J (2004). Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. FEMS Microbiology Ecology. 48; 437–446.

Wanga HF, Zhub WY, Yaob W, Liua JX (2007). DGGE and 16S rDNA sequencing analysis of bacterial communities in colon content and feces of pigs fed whole crop rice. Anaerobe. 13; 127–133.

Weiss G, Rasmussen S, Hjerrild Zeuthen L, Nøhr Nielsen B, Jarmer H, Jespersen L, Frøkiær H (2010). Lactobacillus acidophilus induces virus immune defense genes in murine dendritic cells by a Toll-like receptor-2-dependent mechanism. Immunology. 131; 268–281.

Weiss G, Rasmussen S, Nielson Fink L, Jarmer H, Nøhr Nielsen B, Frøkiær H (2010). *Bifidobacterium bifidum* actively changes the gene expression profile induced by lactobacillus acidophilus in murine dendritic cells. PLoS ONE. 5.

Woo PCY, Lau SKP, Teng JLL, Tse H, and Yuen KY (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clinical Microbiology and Infection. 14; 908–934.

Yang X, Zhao Y, He N, Croft KD (2010). Isolation, characterization, and immunological effects of r-Galacto-oligosaccharides from a new source, the herb lycopus lucidus turcz. Journal of Agriculture and Food Chemistry. 58; 8253–8258.

Zoetendal EG, Akkermans ADL, De Vos WM (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Applied and Environmental Microbiology. 64; 3854–3859.