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Detection Of Beneficial Microbiota In Mouse Colon

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DETECTION OF BENEFICIAL MICROBIOTA IN MOUSE COLON

by

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_____

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DEDICATION

I would like to dedicate this thesis to the women of my family, who show me the meaning of strength.

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TABLE OF CONTENTS

Dedication	ii
Acknowledgments	iii
Table of Contents	iv
List of Tables	v
List of Figures	vi
Chapter 1 “Introduction”	1
Chapter 2 “Materials and Methods”	13
Chapter 3 “Results”	22
Chapter 4 “Conclusion”	44
Chapter 5 “Discussion”	47
References	63
Abstract	73
Autobiographical Statement	75

LIST OF TABLES

Table 1: PCR primers used in this 54

Table 2: The ZR Fecal Kit Extraction Method 55

Table 3: The DNAzol Direct Extraction Method 60

Table 4: Results of PCR for amplification of Lacto, Bifid1, Bifid2, and BiBre 62

LIST OF FIGURES

Figure 1: Adhesins bind to the surface of a host cell 12

Figure 2: The Gram-positive cell wall 12

Figure 3: Gradient PCR results of different annealing temperature 33

Figure 4: Touch-down PCR results on live cell culture, dry cell culture and feces, using Bifid1and BiBre primer 34

Figure 5: Touch-down PCR results on mouse colon mucosa scraping, mouse whole colon and feces, using Bifid2 and Lacto primer 36

Figure 6: Bifid1, Bifid2 and BiBre primer sensitivity test using *B. Breve* live cell colony as a template 38

Figure 7: Touch-down PCR using frozen and fresh collected feces DNA extraction, using Bifid1 and BiBre primer 40

Figure 8: Lacto primer sensitivity test using DNA extracted from feces 42

Figure 9: Touch-down PCR results using Lacto primer, on frozen fresh collected feces DNA 42

Figure 10: DNA Quality and Concentration detected by Nanodrop Spectrophotometer 59

INTRODUCTION

Microbiota

Microbiota or microbial flora is “the microscopic living organisms of a region” (1). It locates in the human gastrointestinal tract and has an effect on our health and well-being. Microbiota has significant influence role in host metabolism and also helps supplies a natural defense mechanism barrier against invading pathogens. Different bacterial groups are found throughout the gastrointestinal tract from mouth to colon in various amounts.

In the human digestive tract, the population of microflora is a very complex but rather stable ecological community (2). The human GI tract is populated by an excess of 10^{10} bacterial cells per gram, at least 10^{12} living bacterial cells in the entire colon (3), that make up from at least 500 different bacterial species (4). In the large intestine, bacteria such as *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Fusobacterium* and *Ruminococcus* are usually found. Inflammatory bowel disease or the use of antibiotics can affect the defense mechanisms provided by the intestine’s bacterial community. For this reason many foods are scientifically intended to help strengthen the gut’s defense system. These ‘functional foods’ work by adding probiotic organisms and claim to have a health benefit above basic nutritional value. Functional Foods were first developed in 1980s in Japan (5).

Aging

The composition of the gastrointestinal (GI) microflora changes with the increased age of the host (6). This is mainly due to alterations in dietary habits and in GI physiology. Aging, for instance, is associated with decreased consumption of fiber, since fiber-rich foods tend to require more mastication than other foods (7, 8).

In addition, diet composition could be altered due to the decline in olfactory and gustatory sensitivity (9), and the decrease in cognition (10) which is sometimes brought on by aging. Moreover, the aging GI tract commonly undergoes pathophysiological processes leading to conditions such as gastric hypochlorhydria, intestinal dysmotility, and decreased colonic transit time (11). These conditions may promote the preferential growth of specific bacterial colonies, thereby altering the composition of the GI microbiota, which in turn affects intestinal homeostasis and function (12). Additionally, the pathophysiological changes seem to effect the regulation of essential groups of the gastrointestinal bacterial flora (13). An animal feeding study showed that the population of guts bacteria depends largely on age of animal, even with adding synbiotics into the diet (13). One important consequence of the changes associated with aging is the decrease in the number and diversity of beneficial microbiota in elderly humans (14) and in older animals (15).

Beneficial-Probiotic

According to the FAO/WHO, probiotics are live microorganisms that are similar to beneficial microorganisms found in the human gut, when given in adequate amounts grant a health benefit on the host. This word came from *pro* ("for") in Latin and *βιωτικός* (biotic) in Greek, the latter deriving from the noun *βίος* (bios, "life") (16). Probiotics are found in fermented dairy products such as yogurt and kefir. It also is in granola bars, soy products and dietary supplements. Lactic acid bacteria (LAB) and *Bifidobacterium* are the most common types of microbes used as probiotics.

Probiotics aid in preventing and treating a wide variety of diseases, from acute gastroenteritis to intestinal neoplasia (17). According to the American

Gastroenterological Association, research shows that probiotics help boost immune system, prevent infection, strengthen the barrier of the intestine and inhibit or destroy toxins. The studies also show that probiotics also relieve the symptoms of irritable bowel syndrome symptoms like constipation, diarrhea, abdominal cramps and other disorders.

To grant a significant health benefit, the ideal probiotic should be resistant to gastric acid digestion, to bile salts, and remain viable in the intestine in order to have greater immunologic effects and should be able to adhere to the intestinal epithelium wall (18, 19).

Some microbiotas are derived from the human intestine while others are nonhuman strains used in the fermentation of dairy products. Most probiotics are strains of *Bifidobacterium* or the *Lactobacillus* species. Some other bacteria like *Streptococcus, Bacillus*, and *Enterococcus* were also used but there are less prefer since the might contain some pathogen like *Enterococcus* (20). Yeasts such as *Saccharomyces* genus are also known for probiotics properties.

Research shows that probiotics have also managed the symptoms of diarrheal diseases. Statistically, the prevention of antibiotic-associated diarrhea could be done by supply the mixture of yeast *Saccharomyces boulardii* and the bacterium *Lactobacillus acidophilus* combined with *L. bulgaricus, L. rhamnosus* strain GG [American Type Culture Collection (ATCC) 53103; LGG], and *Enterococcus faecium* strain SF68. These help to reduce the length of diarrhea by >30 h (21).

Another research study has shown the effect of a probiotic mix (containing 3×10^{11} CFU *L. bulgaricus, L. casei, L. plantarum, L. acidophilus, Bifidobacterium longum, B. breve, B. infantis*, and *S. thermophilus*) preventing flares of chronic

pouchitis in patients with inflammatory bowel disease (22) and that by using of a different probiotic mix [*B. lactis* Bb12 and *Lactobacillus reuteri* (ATCC 55730) at 1 × 10^7 CFU/g in a cow milk formula] could prohibit diarrheal in infants attending childcare (23).

Even though probiotic mostly have the major clinical effects on gastrointestinal disorder, but some were shown to effect non-gastrointestinal diseases such as the treatment and prevention of atopic eczema (24).

Lactobacilli and *Bifidobacterium* represent two important groups of probiotic bacteria in intestinal micro flora. They have unique properties which provide the benefits to the host body in several ways.

Bifidobacterium

Bifidobacterium is gram-positive, anaerobic, nonmotile, nonsporeforming rods of variable appearance somewhat irregular or branched rod shaped bacteria. *Bifidobacterium* has a hexose metabolism through a phosphoketolase pathway or bifid shunt, and uses the key enzyme frutose-6-phosphate phosphoketolase (F6PPK). It is generally used as a diagnostic test for this *Bifidobacterium* as it is not found in other gram-positive intestinal bacteria. *Bifidobacterium* is to “commensal relationship” in human–microbe interactions. In newborn infants, colonization inside gastrointestinal tract begins the moment after birth (25). Mode of delivery, initial diet, geographical location and type of delivery dictate the colonization pattern (26). Generally, *Bifidobacterium infantis, B. brevi,* and *B. longum* are the largest group of bacteria in the intestine of infants. The number of *Bifidobacterium* remains relatively stable representing 3–6% of the fecal flora and started to decline in advanced age (27). *Bifidobacterium* occupy a large percentage compared to other microflora in the

gastrointestinal region, said to be the 3rd or 4th largest group in adults because they can use many source of molecule for energy (28). For example, *B. longum* genome codes for variety of enzymes that use for catabolism of oligosaccharides, nondigestible fiber, host-derived glycoproteins and glycoconjugates (29).

The different species of *Bifidobacterium* have seen a great increase in commercial and consequent scientific interest in lately, due to the ability to relive many types of disorders.

Antibiotics have an effect on the intestinal microflora community by decreasing the ability to colonize and promoting the growth of putrefactive microbes like *Clostridium* and *Klebsiella* species. *Bifidobacterium*, such as *B. longum*, has shown to reduce the incidence and duration of antibiotic-associated diarrhea (30, 31).

Research shows that the traveler's diarrhea incident can be reduce by using the combination of *Bifidobacterium* and other probiotic strains from 71 to 43% in tourists travelling to Egypt by given capsules of *S. thermophilus*, *Lactobacillus bulgarius*, *Lactobacillus acidophilus* and *B. bifidum* (32).

Research also shows that *Bifidobacterium* has anti-inflammatory capacity *in vitro* by inhibiting LPS-induced NF-κB activation (33). Further research also shows that *Bifidobacterium* has therapeutic effects in allergy and inflammatory disorders by activating MAPK, GSK3 and PI3K in order to modulate DC biological functions (34).

For individual who suffer lactose intolerance, *Bifidobacterium longum* was shown to have a potential probiotic treatment effect on the relieving of the symptoms (35).

The population of *Bifidobacterium* is high in infants and starts decline with age. Hence, infants intestinal microflora have the antagonistic activities and ability to

resist certain enteric infections and work more effectively compared to adults with a lower count of indigenous *Bifidobacterium* using the mechanism such as the production of various acids, hydrogen peroxide or bacteriocins, the competition for nutrients or adhesion receptors, anti-toxin action and stimulation of the immune system (36). With age, *Bifidobacterium* population is inversely proportional to the number of *Clostridium perfringens* detected in the elderly (37).

After lung cancer in men and breast cancer in women, colorectal cancer is the second most common cancer in Europe via epidemiological study data. Indirect result of some studies show that probiotic microflora help prevent, or delay the onset of certain cancers. The reason is the increasing levels of putrefactive microbes and a decrease in the levels of *Bifidobacterium* that cause by a diet high in meat and fat but low in fiber (38). The fecal enzymes such as beta-glucuronidase, azoreductase, urease and nitroreductase convert procarcinogens into carcinogens and may be a factor on an increased risk for colorectal cancer (39). *Bifidobacterium*‘s conjugated linoleic acid production is also believed to yield anticarcinogenic effects (40). *Bifidobacterium longum* and *B. breve* are believed to help prevent DNA damage by carcinogens (41). *Bifidobacterium longum* has also been recognized as aiding the reduction of aberrant crypt foci (ACF) occurrence in rats (42), which induce the anti-tumor activity.

A mixture of four *Lactobacillus* strains, three *Bifidobacterium* species (*B. breve*, *B. infantis*, *B. longum*) and a *Streptococcus thermophilus* strain have shown to have an effect on therapeutic and prophylaxis of inflammatory bowel syndrome (IBD) (43). IBD, a disruption in bowel habits and mucosal inflammation, is the overlapping phenotypes of Crohn’s disease and ulcerative colitis. The causes are still unclear but could be due to the genetic disposition and intestinal microflora.

There is evidence to suggest that the IBD symptom could be alleviated by the modification of the composition of microflora (44).The mechanism, still under investigation, could be the mucosa‘s cytokine transcription factors and regulation response to invasive microbes as well as interaction with mucosal regulatory T cells (45).

Another health benefit related to *Bifidobacterium* is the prevention and relief of constipation, especially in elderly. Probiotics have also been used to enhance the growth of *Bifidobacterium* in large intestine and have been found to have laxative effect (46). In another study*, B. bifidum* was shown to have the ability to relieve severe premenstrual syndrome (PMS) in relation to the gastrointestinal symptoms such as constipation, diarrhea and abdominal pain (47).

Since another duty of GI tract, beside absorption and digestion, are the defense barrier against antigens from microorganism and food, and then we can assume that probiotic is directly impacting the host’s immunity function. For example, *B. lactis* can induce the natural immune function by dietary consumption (48) and *B. bifidum* also shows the possibility to increase the immunomodulation effect in combination with other probiotic strains when consumed in cheese (49). *B. infantis* show an immunoregulatory role in the repression of Th2 cytokines during antigen sensitization (50).

Lastly, probiotic *Bifidobacterium* shows some evidence that it might assist on reducing the serum cholesterol that would lead to lower chances of cardiovascular disease (51). Probiotic intake increases the production of the enzyme bile salt hydrolase which shows decrease the serum cholesterol levels (52).

Lactobacillus

Lactobacillus is a gram-positive facultative anaerobic (microaerophilic), rod-shaped bacteria. They are the primary of the lactic acid bacteria group, which converts lactose and other sugars to lactic acid. In humans, they are present as a small group of gut flora and also found in vagina (53). *Lactobacillus* produces lactic acid, which also lowers the pH of the fermenting substance that is used in food productions such as yogurt, cheese, pickled, and starter culture for sourdough. In beer production, *L. casei* and *L. brevis* are commonly used as a beer spoilage organism.

According to the Beth Israel Deaconess Medical Center and UCLA in 2009, They believed that some strains of Lactobacillus spp. and other lactic acid bacteria can help prevent tumor and cancer especially a colonic tumors (54).

A mixture of *Lactobacillus* species, *Enterococcus* species, and *S*. *boulardii* were shown to improve the infective diarrhea in both adults and children (55).

Other benefits by oral administration are shown to decreased the chance of DNA adducts formation, ameliorated DNA damage and prevented putative preneoplastic lesions such as aberrant crypt foci in the gastrointestinal tract (56).

Lactobacilli is believed to help improve the gut micro flora and combat against unwanted bacteria with regular consumption, for example the production of natural agent “bulgarican” from *L. acidophilus* and *L. bulgaricus* .Bulgarican help fight against the spreading of other unwanted bacteria species in foods and in the human gut. Active *Lactobacilli* micro-flora has ability to alter the pH by producing lactic acid in large intestine, as a result, these created the uninhabitable environment for spoilage bacteria. It also helps destroyed other undesirable microbe like moulds, mould spores and yeast, especially Candida form. *Lactobacilli* can also be used to

restore physiological balance of the vaginal and helps protect the ecosystem from other bacterial infection (56).

Other benefits of *Lactobacillus* is the production of enzymes which when exposed to food, help break down the structure of that substance so the nutrients are easy to absorb by human digestive system and often increase the biological value of foods. The enzyme activity also benefits greatly in aged population where digestive efficiency tends to weaken when age progress (56).

Gram positive bacteria cell wall

The gram-positive cell wall consists of tough mesh, several rows and layer of peptidoglycan and teichoic acid, cross linked with lipoteichoic acid molecule and surface proteins. These structures provide strength and rigidity while maintaining elasticity and flexibility to offset intracellular turgor pressure associated with the maintaining of cell shape and preventing against osmotic lysis.

Peptidoglycan, made 60-90% of the cell wall structure and thicker in gram positive bacteria, is made of rigid glycan chains of N-acetylglucosamine (GlcNAc) and N-acetylemuramic acid (MurNAc), connected by b-1, 4 glycosidic bonds and cross linked by flexible peptide bridge making a glycan numerous interconnecting layer 20-80 nm thick (57).

Teichoic acid, which is a unique gram positive bacteria cell wall, is a polyribitol phosphate and glycerol phosphate, cross linked to peptide glycan making the outer layer. Teichoic acid attaches the cations, like magnesium and sodium, in order to maintain the stability and rigidity for the bacteria cell wall.

Lipoteichoic acid, cytoplasmic membrane lipids, works as a lipid link to the teichoic acid. These substances are combined to form the covalent multilayered structure.

Even though peptidoglycan presents in both gram positive and gram negative bacteria cell wall, gram positive cell wall contain more in quantity, chain length, the degree of cross link and the thickness (58). The lyses of a strong peptidoglycan barrier pose a challenge to overcome in order to disrupt the cell wall (59).

Other characteristics of the gram positive bacteria cell wall that interfere with the lysis process are; The petidoglycan layer are thick and abundant, and also contain many cross-linked 50 nm wind glycan strands that provided even more strength. Second, the covalent bond of the teichoic acid to the lipoteichoic acids causes interwoven in the cell wall. Third, each strain and species of the bacterium are different depending on the type of surface protein on the outer layer of peptidoglycan. Lastly, the viscous material that is presents in the periplasm region between the peptidoglycan layer and cytoplasmic membrane helps provide another barrier for the cell (60).

Microbiota development and characterization in the human host still rests largely on culture-dependent methods (11). These conventional methods have many drawbacks. First, their sensitivity is rather low. Second, they are quite time consuming and therefore not very cost-effective. Third, a cultural method requires the use of a fresh sample and to perform operations after sampling. Some might be limited to a difficult condition, like anaerobic culturing, while PCR is not. Next, PCR sample can be preserved in the freezer and transported in the distant future. Forth and most importantly, since only certain bacterial species and strains are amenable to culture, the results could be biased (certain bacterial populations might be

overestimated, while others might be missed). Thus, during the last decade, developments in molecular biology have led to the application of fast and reliable alternative culture-independent methods (61). For instance, in one study by De Vrese et al., quantitative real time PCR was utilized to estimate the levels of certain bacterial populations in the intestines of mice (62).

The aim of this study was to establish a method to detect the gastrointestinal tract microbiota, either by fecal or colonic tissue DNA extraction.

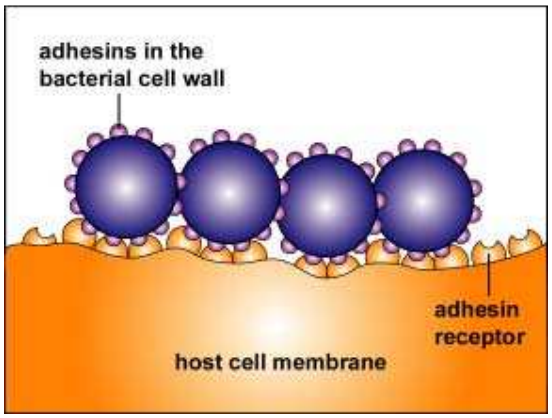

Figure1: Adhesins in the bacterial cell wall bind to receptor molecules on the surface of a susceptible host cell.

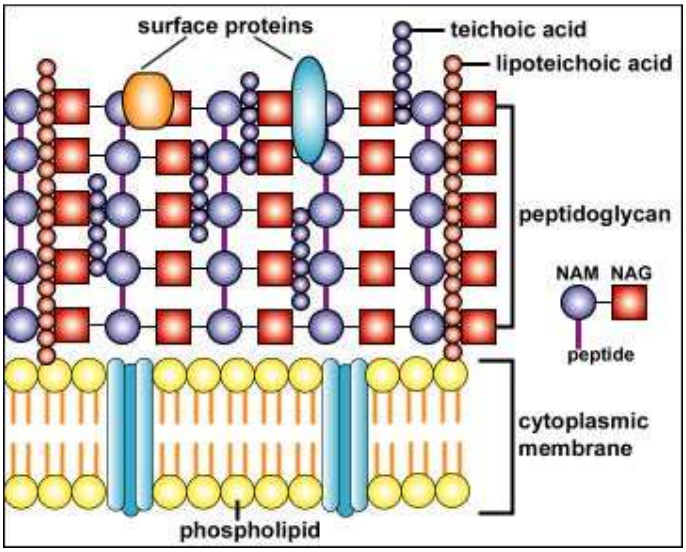

Figure2: The Gram-positive cell wall appears as dense layer typically composed of numerous rows of peptidoglycan, and molecules of lipoteichoic acid, wall teichoic acid and surface proteins.

MATERIALS AND METHODS

Mice

C57BL/6, specific pathogen-free male and female mice were used. The caring of the animals was in accordance with the NIH guidelines for the use and care of laboratory animals, and the animal protocol was approved by the Institutional Animal Care and Use Committee of the Wayne State University Animal Investigation Committee. Animals were housed, two mice per cage and kept in a 12-hour dark-light cycle, controlled environmental temperature of 22 $\pm$ 2^{o}C. Animals had free access to distilled water at all times. The amount of food provided was ad libitum. Food consumption and physical activity change were monitored daily.

Fecal sample collection

Fresh fecal samples were obtained from mice between the ages of 3 months to 1 year old. For collection, the mice were transferred to a cage that contains a one-centimeter-wired-mesh grating floor for one hour. To prevent the integration of feces and urine, the paper towel was used as a lining on the bottom of the cage for absorption. The mesh allows for collection of fresh feces to avoid mixing with older feces from cages and to avoid contamination from bedding material. Moreover, this can also prevent the autocoprophagy. The feces were then collected and stored at -20^{o}C until further analysis.

Colonic tissue sample collection

Mice were euthanized via cervical dislocation method. They were then aseptically dissected and colon tissue was removed. The feces inside the intestine were collected for DNA extraction. The colon was rinsed and washed from the inside

out of the intestine using forceps and syringe (20G) with PBS solution (Fisher Scientific Inc. Fairlawn, New Jersey).

The colon was collected using several different methods to assess the optimal and the best form of tissue collection for bacteria quantization. First, we transferred the colon tissue directly to a 15 ml tube containing 2 ml of DNAzol direct solution, manually homogenized then incubated at 95^{o}C for 15minute, homogenized, vertex and spun down.

Second, we rinsed the intestine with PBS buffer and the tissue was transferred onto a microscope slide on ice. The intestine was cut open by using two forceps holding one end of the intestine with angled serrated forceps and squeezing out the epithelial cells with the other forceps by running the smooth-edged forceps slowly to the other end of the tube. The epithelia cells were suspended in 1 ml of DNAzol direct solution. The rest of colon muscularis was also transferred into 2 ml of DNAzol direct solution. We then continued with the rest of the extraction protocol using the same process as the first condition.

Strain culture

In order to generate a positive control for *Bifidobacterium* experiments, we purchased DNA of *B. adolescentis* cat#15703D, *B. breve* cat#15700D-5 and *B. infantis* from ATCC (Manassas, VA). In addition, we grew a culture of *Bifidobacterium breve*; ATCC#15701- freeze-dried culture, which also was obtained from ATCC. Aseptically rehydration and inoculation of the culture was made using Difcoreinforced Clostridial medium (Becton, Dickinson and Company. Franklin Lakes, NJ). Inoculum broth was incubated at 37°C for 24 hours under anaerobic

conditions generated using the GasPak EZ anaerobe container system (Becton, Dickinson and Company. Franklin Lakes, NJ).Then the bacteria were cultured on agar plates using the same media and conditions. This provided a means for the evaluating PCR primer and DNA extraction method efficiency.

DNA extraction

DNA was extracted from feces for PCR detection. In addition DNA was extracted from entire colon and colonic scraping for PCR detection as well.

ZR Fecal DNA kit (ZYMO RESEARCH CORP. Orange, CA Catalog no.D6010)

The protocol was adapted from the manufacturer's instruction manual. The principle of this method was to lyses and centrifuged using ultra-high density beads that is fracture resistant and chemically inert.

We added up to 75 mg of fecal sample to a lysis tube, and then filled with 750 ul lysis buffer to the tube. Vertex for 10 minutes. Centrifuged the lysis tube in a micro centrifuge at ≥10,000 x *g* for 1 minute. Transferred up to 400 ul supernatant to a spin filter in a collection tube and centrifuged at 7,000 rpm (~7,000 x*g*) for 1 minute, added 1,200 ul of fecal DNA binding buffer to the filtrate. Then transferred 800 ul of the mixture to a column in a collection tube and centrifuged at 10,000 x*g* for 1 minute. Discarded the flow through from the collection tube and repeated the centrifuge for another 1 minute. Added 200 ul DNA pre-wash buffers to the column in a new collection tube and centrifuged at 10,000 x*g* for 1 minute. After that, added 500 ul fecal DNA wash buffer to the column and centrifuged at 10,000 x*g* for 1 minute. Transferred column to a clean 1.5 ml micro centrifuge tube and added 100 ul (25 ul minimum) DNA Elution Buffer directly to the column matrix, centrifuged at

10,000 x*g* for 30 seconds to elude the DNA. Transferred the eluted DNA to a spin filter in a clean 1.5 ml micro centrifuge tube and centrifuged at exactly 8,000 x*g* for 1 minute. DNA extracted was stored at -20°C until its use.

DNAzol Direct (Molecular Research Center, Inc. Cincinnati, OH)

In addition to ZR fecal kit, we also utilized DNAzol to determine which method gave the best quality DNA.

DNAzol direct is the reagent that was used for processing biological samples for the direct PCR, using alkaline solution containing polyethylene glycol and other additive to lyse the sample and releasing the DNA into the lysate. The combined effects of the alkaline pH and chaotropic properties induce the inactivation of PCR inhibitor such as protease and nucleic acid degradation enzyme.

To extract, we mixed up approximate 1-10 mg of fecal sample with 0.1 ml Nuclease-free water (Thermo Fisher Scientific Inc. Pittsburgh, PA). Hand-homogenized until mixed well then we added 0.1 ml of DNAzol Direct. To ensure the maximum yield, we incubated the mixture at 95 °C using heating block for 15 minutes. Vortexes the lysate for 30 seconds and transferred a 2 ul aliquot directly into the PCR mix, or froze at -20 °C until use.

For the bacteria colony, we picked one colony then mixed directly into 0.1 ml of DNAzol Directed and continued to incubation as explained above.

Quantitative reading of DNA/RNA

The quantitative of DNA/RNA were measured using The Thermo Scientific NanoDrop (series) spectrophotometer. The spectrophotometer measurement and the absorbance of the total molecule in the sample of interest, each wave length

represent different molecule; 230 indicates the presence of organic compound contaminants i.e. carbohydrates, phenol and EDTA. A260 shows the total of nucleotides, RNA, ssDNA, and dsDNA. While A280 exhibits the concentration of proteins content. The ratios of each value indicate the quality of sample:

260/280 ratio indicates the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA while a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is lower in either case, it may indicate the presence of contaminants that absorb, at or near, 280 nm i.e. protein, phenol or other.

260/230 ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values, commonly in the range of 2.0-2.2. If lower, then it may indicate the presence of contaminants, which absorb at 230 nm.

The procedure, according to the manufacturer’s manual, combines with the use of software program. Nuclease free water was used as standard blank sample, and for cleaning during each sample measurement. With the arm open, pipette 2 ul of sample directly onto pedestal. The pedestal automatically adjusted for an optimal path length (0.05 mm - 1 mm). When the measurement is complete, the surfaces are simply wiped with lint-free lab wipe and nuclease free water before measuring the next sample. Nucleic acid samples required purification prior to measurement.

RNase treatment

To determine zero contamination of RNA in the DNA extraction, we performed RNase treatment using RNase enzyme (QIAGEN Inc. Valencia, CA). The RNase was mixed into DNA extraction at the ratio of RNase: DNA=1:10. This was incubated on heating block for 30 minutes at 37°C then another 10 minutes at 70°C to denature

the enzyme and stop the treatment process. The product was being purified using QIAquick PCR Purification kit, the protocol as describe in the cloning and sequencing section, and was used to get rid of the excess enzyme. The product was kept on ice until further use.

Primers

After searching through multiple publications, PCR primers were chosen, as listed on Table 1. These primers were designed to target the 16s rRNA region of different members of *Bifidobaterium* species and *Lactobacillus* species. All of the oligonucleotides used were purchased from Invitrogen Corporation (Carlsbad, CA). All primers were tested for the primer concentration optimization. The higher primer concentration can increase the efficiency of PCR but can also lead to primer dimmer formation. The concentrations of each primer was adjusted by diluting with nuclease free water and mixed into 25 uL of total PCR reaction to reach the final concentration of 1.9, 1.0, 0.5, 0.1 and 0.05 uM. The 0.1uM final concentrations yielded the best results for amplifying of target DNA without primer dimmer formation in negative control or showed no evidence of secondary priming.

The specificity of primers were tested by amplifying DNA from the target organism purchased from ATCC which were diluted into 1:10 dilution by nuclease free water and used as positive control throughout the experiment.

Qualitative PCR

PCR

The experiment started off with normal PCR. The mixture of the PCR mastermix of 10.1 ul of nuclease free water, 2 ul 10x PCR buffer 10x, 0.8 ul of 2.5

mM dNTP mix, 0.4 ul each of 25 uM working concentration forward and reverse primer and 1.25 ul of $MgCl_2$ and 0.1 ul of Taq polymerase were mixed with 5 ul of DNA template then put in the thermocycler set the cycle parameter at (i) 2 minutes at 95°C 1 cycle then (ii) 30 cycles of 30 seconds at 95°C, 1 minute at 60±X°C (depend on primer-should be 2 degree lesser that the annealing temperature) and 1.50 minutes at 72°C. (iii) 1 cycle of extension step at 72°C for 5 minutes, the product was then put on hold at 4°C for 200 cycles or until use.

Touchdown PCR

Touchdown PCR was chosen since it was more suitable for varies annealing temperature of each primer. PCR master mix, Go Tag Green cat#M7122, was purchased from Promega Corporation (Madison, WI). Per reaction of PCR mixture contain 12.5 ul of master mix, 4.5 ul of nuclease free water, 2.5 ul of each forward and reverse primer at 1 uM working concentration and 2 ul of DNA template. 1 ul of DMSO (Thermo Fisher Scientific Inc. Pittsburgh, PA) was also added to improve the denaturation of CG-rich region in bacteria DNA double helix strands.

Amplification was performed using theMastercycler gradient (Eppendorf AG. Hamburg, Germany) with the following condition: (i) a hot start step of 2 minutes at 94°C and (ii) an initial step consisting of 1 minute at 94°C, 1 minute of 60-56°C with 2 cycles for each degree Celsius and 1 minute of 72°C.Then (iii) 30 cycles of 1 minute at 94°C, 1 minute of 55°C and 1 minute of 72°C.Final extension (iv) of 7 minutes at 72°C and put on hold at 4°C.

To ensure the integrity of the DNA, amplification products from PCR were subjected to gel electrophoresis using 2% (W/V) agarose gel (Promaga BioSciences.

San Luis Obispo, CA) and were observed by Ethidium bromide staining. UV imager for qualitative analysis was used to read the results.

Cloning and Sequencing

The PCR products were purified using QIAquick PCR Purification kit 250 (QIAGEN INC. Valencia, CA) following the manufacture protocol. Briefly, added 5 volumes of PBI buffer were added to 1 volume of PCR product and mixed. The mixture was transferred into the column that had been inserted into the 2 ml collection tube, then centrifuged for 60 seconds, discarded the flow-through. To wash, 0.75 ml PE buffer was added into the column then centrifuge for 60 seconds, discarded the flow-through. Centrifuged the column for an additional 1 minute and moved the column to the new 1.5 micro centrifuge tube. To eluted DNA, added 30 ul of nuclease free water to the center of the membrane, let the column stand for 1 minute and centrifuged for 1 minute. Increased DNA concentration by pipetted the flow-through and put back to the center of the membrane and centrifuged for 1 minute.

Purified DNA fragment was then inserted into plasmid and transform into 1-shot cells using TopoTA cloning kit (Invitrogen Corporation. Carlsbad, CA) according to the protocol. Briefly, the PCR mastermix of 37.5 ul of nuclease free water, 5 ul 10x easy A buffer, 4ul of 2.5 mM dNTP mix, 1ul each of 10 uM working concentration forward and reverse primer and 0.5 ul of Easy A enzyme were mixed with 1 ul of 100 ng of DNA template then put in the thermocycler set condition at (i) 4 minutes at 95°C then (ii) 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C. The last step of final extension of (iii) 7 minutes at 72°C, the product was then put on hold at 4°C until use. The 3’ overhangs were added to the PCR product

on ice using 1 unit of TAQ (0.5uL). Heated 8-10 minutes at 72°C and returned to ice. Added 1 ul of salt solution and 1 ul of vector to 4 ul of PCR product and incubated 5 minutes at room temperature then returned to ice. To transform the vector into 1-shot cells, 2 ul of cloning reaction were added and mixed gently, put on ice for 5 minutes, heat shocked in 42°C water bath and immediately put back to ice. The next step was to incubate the cells with 250 ul of SOC in 37°C shaking incubator for 1 hour then plated using x-gal as the indicator, let the cells grow for 24 hours at 37°C. Tested the fidelity of the cloning, the screening PCR were then performed. The mixtures of 15.1 ul of nuclease free water, 2.0 ul of 10x buffer, 1.2 ul of 25 mM working concentration of magnesium, 0.8 ul of 2.5 mM of dNTP mix, 0.4 ul each of screening forward and reverse primer and 0.1 L of Taq polymerase were mixed with the coloniesthat were picked by the pipette tip and process to the themocycler the same condition as above.

After the screening PCR was done, the plasmic cells of choice were going through the rapid isolation of plasmid DNA using Promega Wizard Plus system (Promega Corporation. Madison, WI). This system can be used to isolate plasmid from *E. coli* hosts, which we were using as one-shot cell suitable for the plasmid is less than 20,000 bp in size. The principle of this method is to lyse bacteria cell to separate out the plasmid using column and a series of wash solutions.

The plasmid was then sent to Applied Genomics Technology Center (Detroit, MI) for sequencing. The predicted products of gene from each bacterium were used to compare for matching genome using the BLAST algorithm (www.ncbi.nlm.nih.gov). After linearized the plasmid and serial dilutions were performed and keep in -20°C until needed.

RESULTS

All primers were sensitive and specific for the strain they were designed to target when amplified with the diluted ATCC DNA. The sequence of the clone products were compared (http://blast.ncbi.nlm.nih.gov) and showed to be 96-100% similarity to the 16s r RNA sequence of *Bifidobacterium spp.* and *Lactobacillus spp.* as expected.

Total bacteria in the test samples were qualified by Touchdown PCR. Gel electrophoresis of PCR product proved the presence of bacteria DNA in test samples. The concentrations and quality of the sample solutions were also analyzed by spectrophotometer.

ZR fecal kit

After collecting 0.15 g of fecal sample, extracted following the manufacturers procedure, the products were inspected using Nanodrop spectrophotometer (Thermo scientific Inc. Pittsburgh, PA). The result shows that DNA yield was not sufficient (lower than 250 ng/ul) and the 260/280 ratio were lower than 1.8. Then we repeated the experiment again using fresh fecal sample, which increased yield of DNA, storage sample in frozen form did not affect the quality of the sample. The experiment shows that 0.075 g was sufficient enough for DNA extraction. To improve texture and lysis ability, fecal material were soaked in nuclease free water for 10 minutes, then hand homogenized and vertex using bench top vertex at 8,000 xg for 5 minute. When finished the last step in protocol, transferred elute DNA to prepared IV-HRC spin filter one more time. The dilution of elution buffer has no effect to the extraction method.

During the quantization of extracted DNA by Nanodrop spectrophotometer, it was found that the 260/280 ratio failed to reach the acceptable range. Even though the reason might come from the preservatives that are used in the column and might not affect the usability of the product, but to ensure the quality, the columns were washed with 400 ul of nuclease free water to get rid of access reagent. The result showed that 260/280 ratio value had improved. (Figure 2)

Since some of the PCR gel results showed multiple bands, the possibility might be RNA contamination. The company claimed that this kit was designed for complete RNA hydrolysis. But to ensure the results, we extended time period when feces mixed with DNA binding buffer. Results still showed multiple bands and smear in PCR gel. So later on, RNase treatment was done on DNA extraction solution before running PCR with Beta-actin primer. The results showed that the additional band disappeared in some experiments, but some were still present.

The 20,25,30 and 35 cycles of PCR amplified with Beta-actin primer were performed in combination with the different dilution of extracted DNA and the results of PCR gel showed that there are linear relationship between the concentration of DNA presented in the samples and the intensity of the band when exposed to UV light.

Attempting to find the right annealing temperature, gradient PCR was performed on 55.1, 56.1, 56.8, 58.2, 58.9, and 60.0 °C using BiBre primer. The template was amplified twice. First, used ATCC DNA amplified with Bifid1 primer. Then inserted into plasmid, grew and amplified again using species specific primer. Results show that all of the annealing temperatures were suitable to amplify these bacteria DNA. (Figure.3)

Touchdown PCR was later used in the experiments but the DNA extraction using ZR fecal kit still failed to show the acceptable amplification signal.

DNAzol Direct

Since ZR Fecal kit failed to provide the DNA which can be amplified by PCR, DNAzol was chosen. First, the experiment was focused on the appropriate amount of feces used since too much sample can decrease the efficiency of the DNAzol solution. Experiments were attempted to find the appropriate among in range of 1-10 mg of fresh frozen mouse feces. The best yield came from using the pipette tip to touch the sample (same method when performing plasmid recombinant screening PCR), and mixed with 0.1 ml Nuclease-free water than added DNAzol direct solution. Next, we continue on the trial of various incubation temperatures, incubation time and the method of mixing (Table.3), results in the best condition which showed; for incubation temperature, between 85°C- 90°C and 95°C, 95°C yield the best results and 15 minutes of incubation time is better than 10 minutes. For mixing method, several techniques of hand homogenized and different length of vertex time were used. The results showed that the more the sample broke down, the better extraction of DNA. So we decided to use 30 seconds on bench top vertex. Centrifuge at 32000 rpm for 1 minute after the extraction process helped improve the quality also.

After performing touchdown PCR using the template DNA extracted via this method, amplified with both genus specific and species specific primers; results shown in Table 3. At first, the PCR gel results showed band in negative control which were thought of as contamination during PCR mixture preparation. But later on we

found out that the reason was from miscalculation of primer concentration resulted in primer dimerization.

To ensure that there is no PCR inhibitor present in the fecal material, experiment was formulated by mixing the fecal material with *Bifidobacterium breve* 15701from www.ATCC.org, which grew into live cell culture. PCR gel results show the DNA amplification of BiBre and Bifid1 primer on mixture of feces with live cell culture and dry cell, but failed to show signal on fresh feces, fresh frozen feces and feces that were collected directly from young mice colon incision. (Figure 4-a, b)

The experiment was repeated again using Lacto and Bifid2 primer. Results showed that, with touchdown PCR, both primers could detect the presence of *Lactobacillus spp*. and *Bifidobacterium spp*. in fecal material that was collected directly from mice colon incision. Bifid2 primer could also detected bacteria sample that was collected by scraping mucosa of mouse colon, but not present in Lacto primer PCR result. Both primers could not amplify DNA of the whole colon without incision. (Figure 5-a, b)

Touchdown PCR was performed at 45 cycles on serial dilution of *Bifidobacterium breve* colony to check the sensitivity of Bifid1 primer and BiBre primer. The results show that G.Bibif1 primer could detect the presence of *Bifidobacterium breve* as low as the dilution of 10^{-5} and for BiBre primer, it was the dilution of 10^{-6}. (Figure 6-a, b)

The sensitivity of Bifid2 primer was also checked by detecting the serial dilution of *Bifidobacterium breve* cell culture DNA, the result shows that it could detect the DNA presence as low as dilution of 10^{-8}. (Figure 6-c)

The experiment continues further to see the lowest concentration of diluted fecal DNA sample that Bifid1 and BiBre primer could detect. The gel electrophoresis

results show that there are multiple bands present in both fresh and fresh frozen fecal samples. In BiBre primer can detect as low as 1:100 times dilution in both fresh and fresh frozen fecal samples. This is the unknown DNA concentration since the sample started off with pipette tip touching method, and the extracted DNA in DNAzol solution could be used in PCR mixture without unnecessary PCR purification step. In case of G.Bibif1 primer, results show multiple bands and could detect as low as 1:50 times dilution in fresh fecal sample but detected none in fresh frozen fecal sample. (Figure 7-a, b, c)

DNA extraction from fecal matter was used to test Lacto primer sensitivity. The results show, at the primer concentration of 0.1 uM, the last concentration detection was at 394 ag. (Figure 8)

The serial dilution of DNA extracted from fresh frozen feces was amplified with Lacto primer. The results show that primer could detect as low as 1:100 times dilution (started off with pipette tip touching method as DNAzol Direct protocol). (Figure 9)

TA Sub-Cloning and Sequencing

The primer accuracy were checked by compared both genus specific (Bifid1 primer) and species specific primers (BIA, BiBre, BiLon primer). PCR amplified results to known bacteria species ordered from www.ATCC.org ; *Bifidobacterium adolescentis* catalog number 15703D, *Bifidobacterium breve* catalog number 15700D-5 and *Bifidobacterium infantis* catalog number 15697D.

In genus specific primer shows 99% match with *B. longum* sub species *infantis, B. breve* and *B. adolescentis*. In case of species specific primer, the results

show that its 96% similar to *B. longum* sub species *infantis* and 100% similar to *B. breve* and *B. adolescentis* as expected.

The first sample using fresh feces collected from young mice, extracted DNA using ZR Fecal kit. Then the DNA was amplified by touchdown PCR, using Bifid1 primer. The product was cloned and sent for sequencing. The resulting sequence was compared against 16S rRNA of each species, and the sample sequence showed to be 96% matched to *Bifidobacterium thermacidophilum* subsp. *Thermacidophilum.*

While developing the DNAzol direct extraction method, the PCR product collected from young mice feces amplified with Bifid1 were sent to sequencing but the results show no signal. The size of this DNA fragment was 250-550 base pair.

In case of DNAzol Direct DNA extracting method, fresh frozen fecal sample was amplified with Lacto primer. Sequencing results show 99% match with *Lactobacillus reuteri* 16S ribosomal RNA gene.

Primers test

Sample sequencing results compared to a query sequence from database of bacteria using BLAST program. (http://blast.ncbi.nlm.nih.gov)

Bifid1 genus specific primer amplified on ATCC *Bifidobacterium breve* catalog number 15700D-5, results in 99% match Bifidobacterium breve strain 885 16S ribosomal RNA gene.

```
Query  1    ATGGCGGGGTAACGGCCCACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41   ATGGCGGGGTAACGGCCCACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGC  100

Query  61   CACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  101  CACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA  160

Query  121  CAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161  CAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAA  220

Query  181  ACCTCTTTTGTTAGGGAGCAAGGCACTTTGTGTTGAGTGTACCTTTCGAATAAGCACCGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  221  ACCTCTTTTGTTAGGGAGCAAGGCACTTTGTGTTGAGTGTACCTTTCGAATAAGCACCGG  280

Query  241  CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTATTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  281  CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTATTG  340

Query  301  GGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  341  GGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGT  400

Query  361  GGATCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  401  GGATCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGT  460

Query  421  AACGGTGGAATGTGTAGATATCGGGAAGAACACCAAGGGCGAA  463
            |||||||||||||||||||||||||||||||||| ||||||
Sbjct  461  AACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAA  503
```

BiLon species specific primer amplified on ATCC *Bifidobacterium infantis* catalog number 15697D, results in 96% match with *Bifidobacterium longum* gene for 16S rRNA strain: JCM 7011.

```
Query  4    CGCTTGCTCCCCGATAAAAGAGGTTTACAACCCGAAGGCCTCCATCCCTCACGCGGCGTC  63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  CGCTTGCTCCCCGATAAAAGAGGTTTACAACCCGAAGGCCTCCATCCCTCACGCGGCGTC  326

Query  64   GCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCT  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  GCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCT  266

Query  124  GGGCCGTATCTCAGTCCCAATGTGGCCGGTCGCCCTCTCAGGCCGGCTACCCGTCGAAGC  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  GGGCCGTATCTCAGTCCCAATGTGGCCGGTCGCCCTCTCAGGCCGGCTACCCGTCGAAGC  206

Query  184  CACGGTGGGCCGTTACCCCGCCGTCAAGCTGATAGGACGCGACCCCATCCCATACCGCGA  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  CACGGTGGGCCGTTACCCCGCCGTCAAGCTGATAGGACGCGACCCCATCCCATACCGCGA  146

Query  244  AAGCTTTCCCAGAAGACCATGCGATCAACTGGAA  277
            ||||||||||||||||||||||||||||||||||
Sbjct  145  AAGCTTTCCCAGAAGACCATGCGATCAACTGGAA  112
```

BiBre species specific primer amplified on ATCC *Bifidobacterium breve* catalog number 15700D-5 results in 100% match with *Bifidobacterium breve* gene for 16S rRNA strain: JCM 1192.

```
Query  1    ACAAAGTGCCTTGCTCCCTAACAAAAGAGGTTTACAACCCGAAGGCCTCCATCCCTCACG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404  ACAAAGTGCCTTGCTCCCTAACAAAAGAGGTTTACAACCCGAAGGCCTCCATCCCTCACG  345

Query  61   CGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  344  CGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTA  285
```

```
Query  121  GGAGTCTGGGCCGTATCTCAGTCCCAATGTGGCCGGTCGCCCTCTCAGGCCGGCTACCCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  284  GGAGTCTGGGCCGTATCTCAGTCCCAATGTGGCCGGTCGCCCTCTCAGGCCGGCTACCCG  225

Query  181  TCGAAGCCATGGTGGGCCGTTACCCCGCCATCAAGCTGATAGGACGCGACCCCATCCCAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224  TCGAAGCCATGGTGGGCCGTTACCCCGCCATCAAGCTGATAGGACGCGACCCCATCCCAT  165

Query  241  GCCGCAAAGGCTTTCCCAACACACCATGCGGTGTGATGGAGCATCCGG  288
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164  GCCGCAAAGGCTTTCCCAACACACCATGCGGTGTGATGGAGCATCCGG  117
```

BIA species specific primer amplified on ATCC *Bifidobacterium adolescentis* catalog number 15703D results in 100% match with *Bifidobacterium adolescentis* strain ATCC 15703 16S ribosomal RNA gene.

```
Query  1    GGAAAGATTCTATCGGTATGGGATGGGGTCGCGTCCTATCAGCTTGATGGCGGGGTAACG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208  GGAAAGATTCTATCGGTATGGGATGGGGTCGCGTCCTATCAGCTTGATGGCGGGGTAACG  267

Query  61   GCCCACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  GCCCACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGA  327

Query  121  GATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328  GATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC  387

Query  181  CTGATGCAGCGACGCCGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTTGACTG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388  CTGATGCAGCGACGCCGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTTGACTG  447

Query  241  GGAG  244
            ||||
Sbjct  448  GGAG  451
```

Fecal samples test

Bifid1 genus specific primer amplified on fecal sample results in 96% match with *Bifidobacterium thermacidophilum* subsp. *thermacidophilum* gene for 16S rRNA, strain: NBRC 106100.

```
Query  1    CTCCTGGAAACGGGTGGTAATGCCGGATGTTCCCGCGCCCCGCATGGGGTGCGGGGAGAG  60
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  124  CTCCTGGAAACGGGTGGTAATGCCGGATGTTCCCGCGCCCCGCATGGGGTGCGGGGAAAG  183

Query  61   CTTTTGCGGCGTGGGATGGGGTCGCGTCCTATCAGCTTGTTGGCGGGGTGAGGGCCCACC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  CTTTTGCGGCGTGGGATGGGGTCGCGTCCTATCAGCTTGTTGGCGGGGTGAGGGCCCACC  243

Query  121  AAGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  AAGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGG  303

Query  181  CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC  363

Query  241  AGCGACGCCGCGTGCGGGATGGGGGCCTTCGGGTTGTAAACCGCTTTTGTTTGGGAGCAA  300
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  364  AGCGACGCCGCGTGCGGGATGGAGGCCTTCGGGTTGTAAACCGCTTTTGTTTGGGAGCAA  423

Query  301  GCCCTTCGGGGTGAGTGTACCTTTCGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  GCCCTTCGGGGTGAGTGTACCTTTCGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGC  483

Query  361  GGTAATACGTAGGGTGCGAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484  GGTAATACGTAGGGTGCGAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGG  543

Query  421  TTCGTCGCGTCCGGTGTGAAAGTCCATCGCCTCACGGTGGATCTGCGCCGGGTACGGGCG  480
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  544  TTCGTCGCGTCCGGTGTGAAAGTCCATCGCCTAACGGTGGATCTGCGCCGGGTACGGGCG  603

Query  481  GGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGTAACGGTGGAATGTGTAGATATC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  604  GGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGTAACGGTGGAATGTGTAGATATC  663
```

```
Query  541  GGGAAGAACACCAAGGGCGAA  561
            |||||||||||||| ||||||
Sbjct  664  GGGAAGAACACCAATGGCGAA  684
```

Lacto genus specific primer amplified on fecal sample results in 99% match with *Lactobacillus reuteri strain* MF2-2 16S ribosomal RNA gene.

```
Query  300  CACCGCTACACATGGAGTTCCACTCTCCTCTTCTGCACTCAAGTCGCCCGGTTTCCGATG  359
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  676  CACCGCTACACATGGAGTTCCACTGTCCTCTTCTGCACTCAAGTCGCCCGGTTTCCGATG  617

Query  360  CACTTCTTCGGTTAAGCCGAAGGCTTTCACATCAGACCTAAGCAACCGCCTGCGCTCGCT  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  616  CACTTCTTCGGTTAAGCCGAAGGCTTTCACATCAGACCTAAGCAACCGCCTGCGCTCGCT  557

Query  420  TTACGCCCAATAAATCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACG  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  556  TTACGCCCAATAAATCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACG  497

Query  480  TAGTTAGCCGTGACCTCCTGGTTGGATACCGTCACTGCGTGAACAGTTACTCTCACGCAC  539
            |||||||||||||| | ||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  TAGTTAGCCGTGACTTTCTGGTTGGATACCGTCACTGCGTGAACAGTTACTCTCACGCAC  437

Query  540  GTTCTTCTCCAACAACAGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGCGGTGTTGC  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  GTTCTTCTCCAACAACAGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGCGGTGTTGC  377

Query  600  TCCATCAGGCTTGCGCCCATTGTGGAAGATTCCCTACTGCT  640
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  376  TCCATCAGGCTTGCGCCCATTGTGGAAGATTCCCTACTGCT  336
```

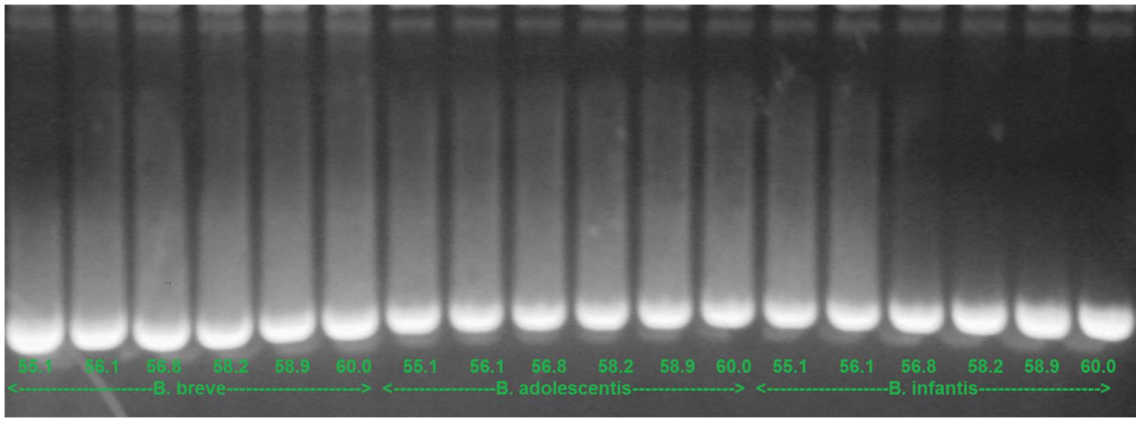

Figure 3 Electrophoresis gel of Gradient PCR in different annealing temperature, using BiBre primer amplified ATCC DNA of *B. breve, B.adolescentis* and *B. infantis* at 55.1, 56.1, 56.8, 58.2, 58.9 and 60.0 °C.

The figure shows that BiBre primer can be used at the annealing temperature ranging from 55.1-60.0°C to detect the presence of *B. breve, B.adolescentis* and *B. infantis.*

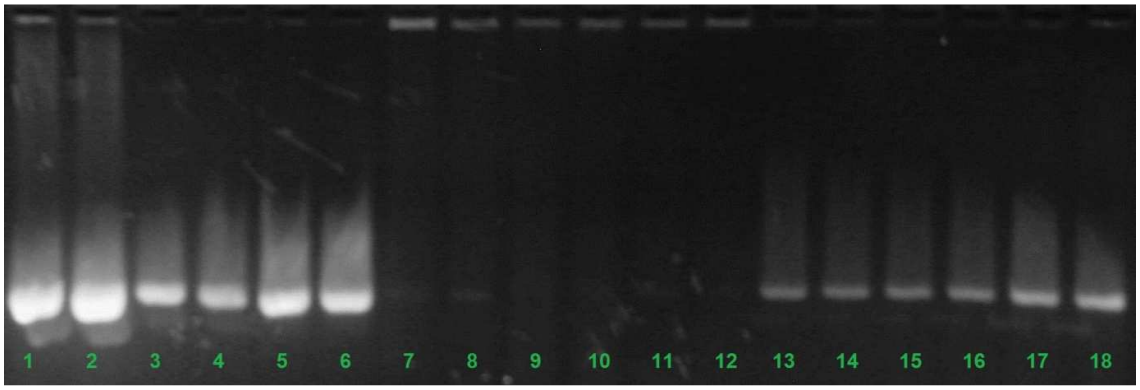

Figure 4-a Electrophoresis gel of Touch-down PCR amplification products, using BiBre primer, from live cell culture, dry cell culture and feces.

Samples used - Lane1,2) 1:2 dilution of ATCC *B. breve* DNA; Lane3,4)ATCC *B. breve* dry cell culture; Lane5,6) ATCC *B. breve* live cell culture; Lane7,8) fresh collected feces; Lane9,10) frozen fresh collected feces; Lane11,12) feces collected directly from colon incision; Lane13,14) fresh collected feces combined with dry cell culture; Lane15,16) frozen fresh collected feces combined with dry cell culture; Lane17) fresh collected feces combined with live cell culture; Lane18) frozen fresh collected feces combined with live cell culture.

From this figure, the results show that BiBre primer could detect the presence of B.breve in the form of DNA extraction, live and dry cell cultures with or without the presence of fecal material. This proves that there is no PCR inhibitor present in feces. This extracting method didn't yield the results that are usable for PCR when performed on fecal samples alone.

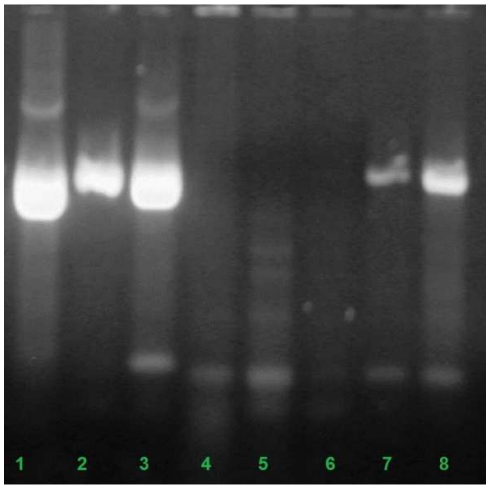

Figure 4-b Electrophoresis gel of Touch-down PCR amplification products, using Bifid1 primer, from live cell culture, dry cell culture and feces.

Samples used - Lane1) 1:2 dilution of ATCC *B. breve* DNA; Lane2) ATCC *B. breve* dry cell culture; Lane3) ATCC *B. breve* live cell culture; Lane4) fresh collected feces; Lane5) frozen fresh collected feces; Lane6) feces collected directly from colon incision; Lane7) fresh collected feces combined with dry cell culture; Lane8) fresh collected feces combined with live cell culture.

In the case of Bifid1 primer, the results also showed that it could be detected in the presence of B. breve in the form of DNA extraction, live and dry cell culture with or without the presence of fecal material. It didn't yield the results that are usable for PCR when performed on fecal samples alone.

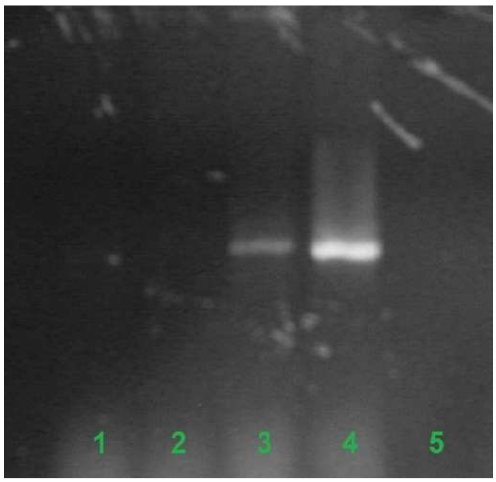

Figure 5-a Electrophoresis gel of Touch-down PCR amplification products, using Bifid2 primer, from mouse colon mucosa scraping, mouse whole colon and feces. Samples used – Lane1) fresh collected feces; Lane2) frozen fresh collected feces; Lane3) feces collected directly from colon incision; Lane4) mouse colon mucosa scraping; Lane5) mouse whole colon.

In the case of Bifid2 primer, the results showed that it could detect some Bifidobacterium in mouse colon feces and mouse colon mucosa but couldn't detect fecal material in both freshly collected and pre-frozen feces, the whole colon also did not yield the results.

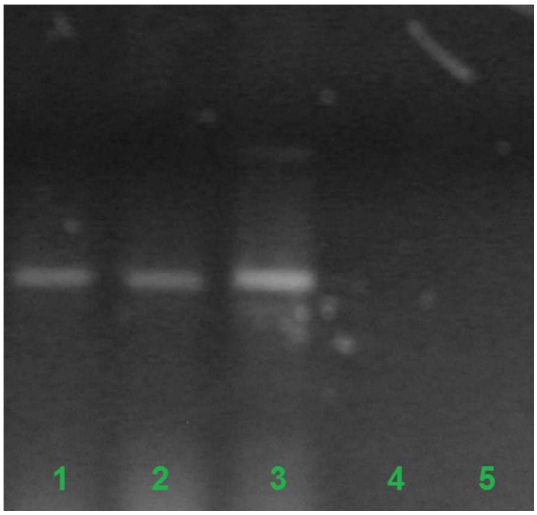

Figure 5-b Electrophoresis gel of Touch-down PCR amplification products, using Lacto primer, from mouse colon mucosa scraping, mouse whole colon and feces. Samples used – Lane1) fresh collected feces; Lane2) frozen fresh collected feces; Lane3) feces collected directly from colon incision; Lane4) mouse colon mucosa scraping; Lane5) mouse whole colon.

Lacto primer could detect the presence of Lactobacillus spp. DNA in the PCR product of freshly collected feces DNA, the pre-frozen feces and the feces that collected directlyfrom colon incision. The PCR results failed to show signal in the case of the sample of mouse mucosa and whole mouse colon.

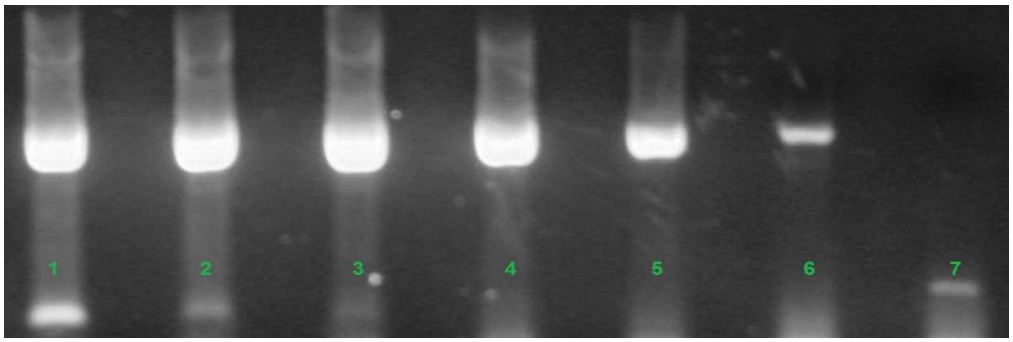

Figure 6-a Electrophoresis gel of Touch-down PCR amplification products, testing on Bifid1 primer sensitivity test, using *B. Breve* live colony as a template.

Samples used – Lane1) no dilution; Lane2) 10^{-1} dilution; Lane3) 10^{-2} dilution; Lane4) 10^{-3} dilution; Lane5) 10^{-4} dilution; Lane6) 10^{-5} dilution; Lane7) 10^{-6} dilution.

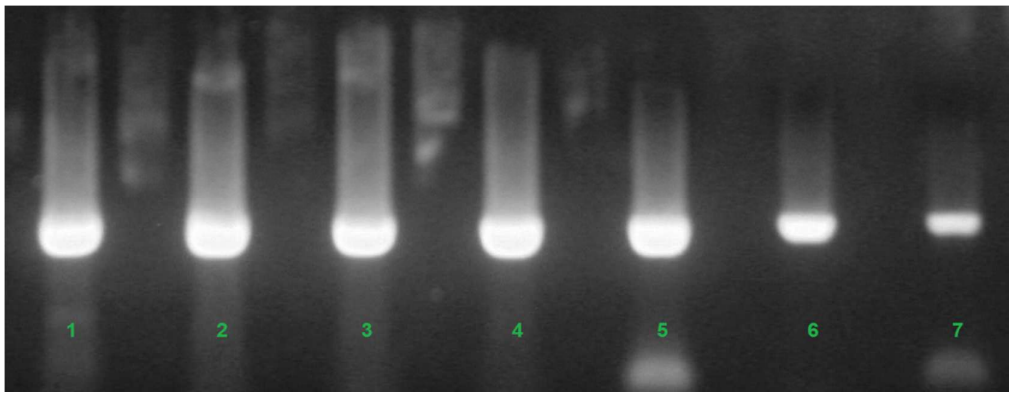

Figure 6-b Electrophoresis gel of Touch-down PCR amplification products, testing on BiBre primer sensitivity test, using *B. Breve* live colony as a template.

Samples used – Lane1) no dilution; Lane2) 10^{-1} dilution; Lane3) 10^{-2} dilution; Lane4) 10^{-3} dilution; Lane5) 10^{-4} dilution; Lane6) 10^{-5} dilution; Lane7) 10^{-6} dilution.

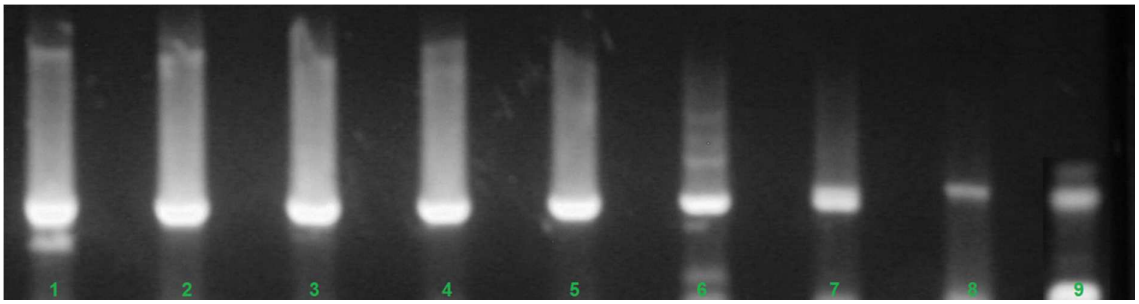

Figure 6-c Electrophoresis gel of Touch-down PCR amplification products, testing on Bifid2 primer sensitivity test, using *B. Breve* live cell colony as a template. Samples used – Lane1) no dilution; Lane2) 10^{-1} dilution; Lane3) 10^{-2} dilution; Lane4) 10^{-3} dilution; Lane5) 10^{-4} dilution; Lane6) 10^{-5} dilution; Lane7) 10^{-6} dilution; Lane8) 10^{-7} dilution; Lane9) 10^{-8} dilution.

Bifid1 primer and BiBre primer sensitivity test on B. Breve live cell colony showed that the primer could detect the presence of bacteria DNA as low as the dilution of 10^{-6}, while Bifid2 primer was at 10^{-8}. The concentration was unknown since the sample was collected by touching the pipette tip directly to the colony.

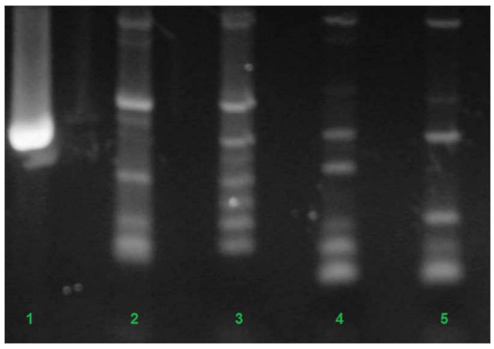

Figure 7-a Electrophoresis gel of Touch-down PCR amplification products, using BiBre primer, from fresh collected feces DNA extraction by DNAzol direct solution.

Samples used – Lane1) 1:10 dilution of ATCC B. Breve; Lane2) 1:10 dilution of DNA extraction; Lane3) 1:20 dilution of DNA extraction; Lane4) 1:50 dilution of DNA extraction; Lane5) 1:100 dilution of DNA extraction.

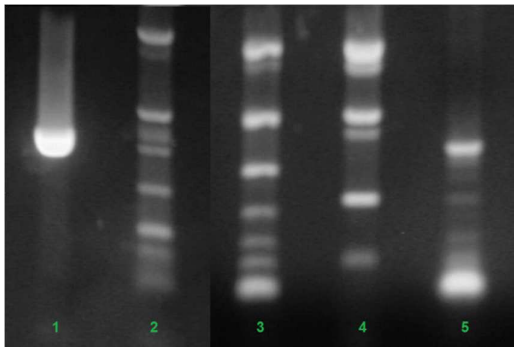

Figure 7-b Electrophoresis gel of Touch-down PCR amplification products, using BiBre primer, from frozen fresh collected feces DNA extraction by DNAzol direct solution.

Samples used – Lane1) 1:10 dilution of ATCC B. Breve; Lane2) 1:10 dilution of DNA extraction; Lane3) 1:20 dilution of DNA extraction; Lane4) 1:50 dilution of DNA extraction; Lane5) 1:100 dilution of DNA extraction.

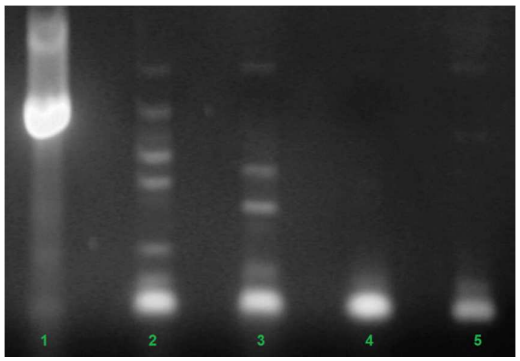

Figure 7-c Electrophoresis gel of Touch-down PCR amplification products, using Bifid1 primer, from fresh collected and frozen fresh collected feces DNA extraction by DNAzol direct solution.

Samples used – Lane1) 1:10 dilution of ATCC B. Breve; Lane2) 1:10 dilution of fresh collected feces DNA extraction; Lane3) 1:50 dilution of fresh collected feces DNA extraction; Lane4) 1:10 dilution of frozen feces DNA extraction; Lane5) 1:50 dilution of frozen feces DNA extraction.

When attempt to amplify the DNA extraction using Bifid1 primer and BiBre primer, the PCR results showed multiple bands and a decrease in the concentration. The template couldn't solve the problem.

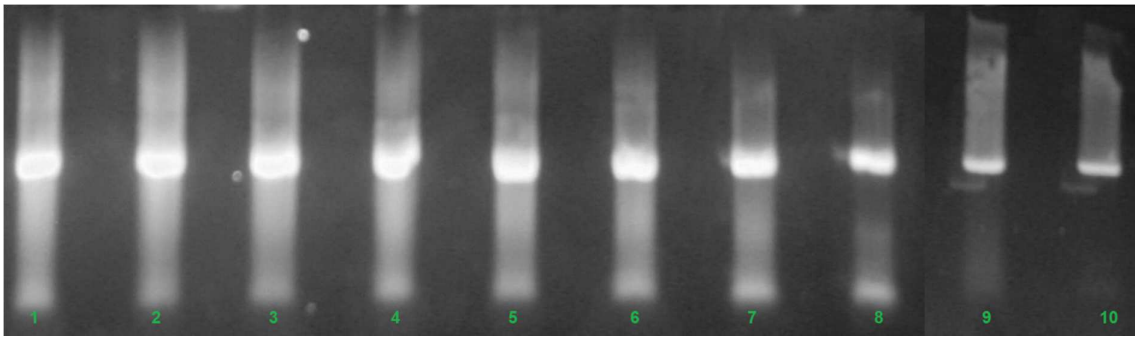

Figure 8 Electrophoresis gel of Touch-down PCR amplification products, testing on Lacto primer sensitivity test, using DNA extracted from feces in different concentration as a template.

Samples used – Lane1) 15.76 ng/ul; Lane2) 7.88 ng/ul; Lane3) 3.94 ng/ul.; Lane4) 3.94×10^{-1} ng/ul.; Lane5) 3.94×10^{-2} ng/ul.; Lane6) 3.94×10^{-3} ng/ul.; Lane7) 3.94×10^{-4} ng/ul.; Lane8) 3.94×10^{-5} ng/ul.; Lane9) 3.94×10^{-6} ng/ul.; Lane10) 3.94×10^{-7} ng/ul.

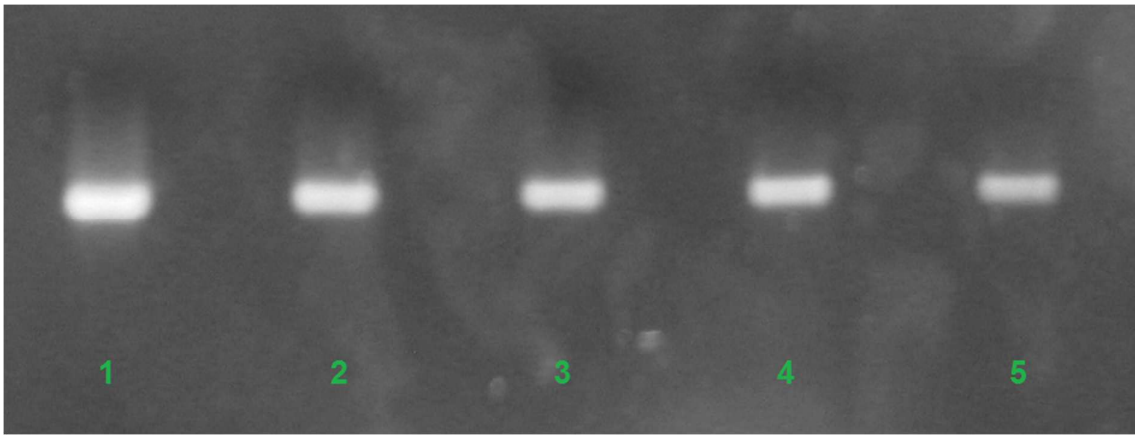

Figure 9 Electrophoresis gel of Touch-down PCR amplification products, using on Lacto primer ,on DNA extracted from frozen fresh collected feces using DNAzol direct solution.

Samples used – Lane1) 1:10 dilution; Lane2) 1:2 dilution; Lane3) 1:5 dilution; Lane4) 1:7 dilution; Lane5) 1:100 dilution of frozen feces DNA extraction.

Lacto primer could detect the presence of DNA extracted via this method even at the low concentration of DNA present. In primer sensitivity test (fig.8), the lowest concentration detected was at 3.94×10-7 ng/ul.

CONCLUSION

ZR Fecal kit

1. Fresh collected feces worked better than dry. Fecal sample could be frozen and stored at -20°C without decline in DNA quality.
2. To improve the bacteria-lysis ability, many texture improving techniques were used i.e.; hand homogenization, bench top vertex. The more the samples broke down, the better the results.
3. PCR method was started off with different annealing temperatures (gradient PCR) and number of PCR cycles. Later on, TDPCR was chosen in order to cover more temperature ranges and reduced the amplification of nonspecific sequences. For PCR reaction solution, Go Tag Green with DMSO gave the best amplifying results.
4. PCR results from Beta-actin primer and Bifid1 primer showed inconsistent results, either 2 bands or smear. The dilution of template and RNase treatment were tried but could not improve the outcome.
5. ZR Fecal Kit, after adjusting the protocol, could not extract bacteria DNA at acceptable spectrophotometer's 260/280 wave length ratio. The concentration of bacteria DNA was low, and could not show acceptable results on electrophoresis gel after PCR.
6. However, with the correct G.bidif1 primer concentration, mouse fecal DNA could be amplified by PCR. The sequence matched 96% to *Bifidobacterium themacidophilum.* For species specific primer, they fail to give acceptable PCR results.

DNAzol Direct

1. The most suitable among mouse fecal samples, either fresh or frozen, was obtained by touching the pipette tip to the sample (same method as screening PCR in plasmid cloning procedure). But this DNA extraction method needed to extract daily since the DNAzol Direct solution is not suitable to store DNA sample.
2. The most efficient incubation condition was 95°C for 15 minutes on heating block, then vertex for 30 seconds followed by centrifuge at 32000 rpm for 1 minute.
3. DNAzol Direct DNA extract method was preferred. Yield from template DNA could be amplified in PCR and showed results in electrophoresis gel and it required fewer steps in protocol.
4. There was no PCR inhibitor in feces. Experiments by mixing ATCC culture into fecal sample and results in amplification signal on PCR gel.
5. Fecal DNA extraction by this method, when amplified with Lacto genus specific primer, yielded 99% match with sequence of *Lactobacillus reuteri* in database.

The reaction sensitivity test

The sensitivity of reactions was evaluated by serial dilution of ATCC DNA: *B. adolescentis, B. breve*, and *B. infantis.*

1. Bifid1 genus specific primer, with template from all 3 species, could detect DNA up to a dilution of 10^{-10}, which contained approximately 10 ag of DNA.
2. For species specific primers,

   -BIA primer could detect DNA up to a dilution of 10^{-8}, or 1 fg of DNA.

   -Both BiLon and BiBre primer could detect DNA up to a dilution of 10^{-7}, or 10 fg of DNA.

The serial dilution of ATCC *Bifidobacterium breve* culture was also used as a template to evaluate the sensitivity of the reaction.

1. Bifid1 genus specific primers could detect DNA up to the dilution of 10^{-5} or approximate amount of 1 pg of DNA, while Bifid2 was 10^{-8} or approximate amount of 1 fg of DNA.
2. BiBre species specific primer could detect DNA up to the dilution of 10^{-6}, or approximate amount of 100 fg of DNA.

The PCR product of Bifid1 primer amplified against ATCC bacteria DNA:- resulted in 99% match with all 3 bacteria sequences (*B. breve*, *B. Adolescentis*, and *B. Longum*). When using BiBre and BIA primers amplified against B. breve and B. infantis ATCC DNA, the sequences were 100% similar to each species in bacteria database. For BiLon primer, the results yield 96% match.

DISCUSSION

During the sample collection, we used both young and old mice. The disparity in age of the mice might play an important role in the occurrence of bacteria detected in fecal samples, due to unequally late development of the young mice's intestinal ecosystems (67).

The presence of bacteria in the feces might not represent the type or quantity of bacteria that inhabitant the colon. From the experiments, some types can be detected more in colons but not in feces. The reason might be attributable to the surface protein called adhesion in the bacteria cell wall, which aids the binding of that bacterium to the receptor molecule on the surface of a susceptible host cell. This type of contact enables the bacteria to adhere, colonize, and resist physical removal like flushing.

For example, *Bifidobacterium bifidum* has been shown to have the highest adhesion to the intestinal epithelial cell (IEC), using the specific lipoprotein called Bifidobacterial outer protein A (bopA) that helps enhanced adhesion to IEC (68).

Factors that determine or help promote the colonization of each different bacterium include the mobility which helps increase the chance of connection to host and spreading of the colonies, in addition to how well the bacteria can adhere and resist physical removal. Some bacteria can invade the host cell and fight for nutrients with other species. If the bacteria can defence against the host's innate immune defuses system, such as phagocytosis, and adapt to the immune defences, then it can increase the chance to colonize.

Many factors could come into play in the regulation of the studied microflora, including local immune mechanism, the substrates supplied by the mucosa,

interactions between different microbial species, initial digestion transit time, pH, and the local supply of oxygen (58).

Primer design

16S ribosomal RNA is the species-specific signature sequences of prokaryotic ribosome used for identifying the bacteria. It consists of 30 small subunits, approximately 1.5 kb in length. This region acts as a framework and defines the placement of ribosomal protein. 3' end of 16S rRNA binds upstream to AUG start codon on mRNA initiate protein synthesis. It is also interact with 23S rRNA helping 2 ribosomal subunits to bind. This helps to stabilize the right codon-anticodon pairing in A site (ribosomal-decoding site). 16S rRNA is essential and present at least one copy in the genome (79).

The primer design targeted the 16S ribosomal RNA, because it is the conservative regions content, involves the bacteria species essential functions, is used for generating amplicons, and benefits greatly from the phylogenetic and taxonomic studies. In PCR technical, the V1-V9 regions of 16S rRNA genes are usually the target for primer design (67). The over-relaxed match of primer design can cause PCR failure.

ZR Fecal Kit

While performing the DNA extraction using the ZR Fecal kit, as show in the sample number 21,22,23 in table 2, the appearance of fecal lysed solution was different between each sample regardless of whether they were collected from the same subject or not. This could be due to the characteristic difference between each fecal sample i.e. fiber content, moisture content, food particle etc.

The sample quantitative DNA reading via Nano Drop spectrophotometer showed a value of 260/280 less than expected, possibly due to acidity in the solution. A simple change in the ionic strength would results in low 260/280 ratio (70). Another reason for this observation could be a slight shift in wavelength accuracy of the spectrophotometer, particularly the absorbance curve at 280 nm. For example, a 1 nm shift could affect as much as a 0.4 difference in the 260/280 ratio. (www.nanodrop.com). Lastly, the presence of nucleotides in the sample could affect the overall value of 260/280 ratio. When measuring each nucleotide individually, the values are as follows. Guanine equals 1.15, Adenine equals 4.50, Cytosine equals 1.51, Uracil equals 4.00, and Thymine equals 1.47 (71). The ratio would depend on the composition of the nucleic acid that contaminated the sample.

After extraction, samples were processed with the PCR. The results of electrophoresis gel, extracted DNA using ZR Fecal kit showed multiple bands that could be the contamination of RNA. The ZR Fecal kit claims that the reagent in the kit help gets rid of all the RNA. To ensure this, the samples were treated with RNase to prevent secondary priming. But that still did not solve the problem. The reasons might due to contamination in negative controls or the accumulation of primer. Some samples showed smear in the gel that could be non-specific binding of primer to template DNA.

Non-specific binding causes by “hair pins” or the DNA-DNA binding. The results of this secondary structure DNA folding and knotting can decrease the productivity yield. The trouble can be resolved by adding dimethyl sulfoxide (DMSO) or glycerol, which helps minimize the secondary structure DNA by preventing the DNA self-complementarity.

Other causes of non-specific binding include repeating of the DNA template, undesirable binding of primer and template, and incomplete primer binding which left 5’end unattached to the template. One way to solve these issues is to use touchdown PCR, which in this experiment did not always yield the best results. Another method that needs further experimentation includes using hot start polymerase enzyme. Nested PCR which could also helps lower the amplification on unexpected primer binding site. Moreover, non-specific binding of primers can be fixed by manipulation of annealing temperature and/or the magnesium ion concentration (72).

Another solution can be obtained from finding the right polymerase enzyme. For example, one can replace Taq polymerase enzyme with high-fidelity DNA polymerase. Normally, Taq polymerase has no 3’-5’ exonuclease activity which is prone to error amplification of DNA product, while these enzymes have engineered 3’-5’ exonuclease activity which provide error-proof reading during synthesis.

The concentrations of each reaction mixture are very important. Too much or too little of each part can cause PCR failure. From this experiment, primers concentration was 10 times too high, which results in primer dimerization. Primer dimerization causes the annealing of 3’ end from one primer to another, and then primer extends to complete the synthesis of the DNA strand. In this experiment, the solution was to find the optimum concentration of primer. Other conditions that

should be studied further include the increasing of $MgCl_2$ concentration, the increasing or decreasing of annealing temperature, and the use of different primers (the longer base pair trend to be more specific).

In this study, the primers that were used were based on previous studies, which had been designed and could be readily purchased. These primers could be improved to achieve a better result. For example, to decrease non-specific priming the primer sequence should have C-G content between 40 and 60%. The distribution of C-G should be divided equally throughout the primer strands, and C-G sequence should not position at 3' end of the primer.

There are other possibilities such as using too much deoxy nucleotides (dNTPs) which has the ability to bind to magnesium ion and can decrease the magnesium ion concentration in the solvent.

Magnesium ion should be added as the thermostable DNA polymerase co-factor. If the concentration is too low, this could impair the working ability of Taq polymerase. Using too much can cause double stranded DNA stabilization, preventing completion of the denaturing process of the DNA strands. In case of non-specific binding, high magnesium concentration aids the binding of incorrect template and primer.

DNAzol Direct

One challenge that needs to be overcome in recovery of DNA is finding the lysis method that could penetrate peptidoglycan, the heavily cross-linked structure in gram-positive bacteria cell wall. While conducting the experiment, many conditions and methods were used in order to cause interference of the cell wall. Some research found that glycoside hydrolyses enzymatic lysis could improve DNA yield.

For example, fecal material can be resuspended in bactozol enzyme solution or by using muramidases enzyme such as lysozyme and mutanolysin that hydrolyze the b-1, 4 glycoside linkages in GlcNAc and MurNAc of the glycan backbone (31). Another possibility for improving lysis DNA yield is to use Labiase from *Streptomyces filvissimus*, the enzyme that could lyse gram positive bacteria wall. This enzyme also contains lysozyme and β-N-acetyl-D-glucosaminidase (73).

Furthermore, since we directly used the mixture of fecal material with DNAzol direct in PCR mixture, it could be that the presences of multiple substances in fecal material inhibit the polymerase enzyme in the PCR analysis (74).The amount of fecal material that is suitable for both the extraction process and the PCR reaction is still in question.

DNAzol direct was chosen instead of ZR Fecal kit because of the ability to extract the usable DNA and to reduce the number of steps in the process. Qualification of *Bifidobacterium* was performed using touchdown PCR with specific primers. This method was used in this study to avoid the non-specific binding amplification by gradually declining the annealing temperature template and primer. The PCR gel results were not as expected, maybe due to the high concentration of primer that caused primer dimerization.

The present study has several strengths and weaknesses. Some strengths include: adequate sample size, controlled environment and dietary composition, adequate length of experimental period, and validation of results through use of two qualitative techniques (PCR and spectrophotometer) to analyze the same set of samples. One weakness is the lack of a method to verify the development of the pathophysiological changes that are claimed to be responsible for the variation in bacterial levels in the GI tracts of the mice. For example, it would have been helpful

to analyze the pH of the intestines of the studied mice, or to study the morphology of the intestinal wells. In addition, the selected measure of outcome (i.e. the *Bifidobacterium* DNA in the fecal material) might not truly represent all that occurs in the flora of the GI tract. The amount of DNA extraction from each fecal sample might solely indicate the amount of *Bifidobacterium* population in cecum of the animal rather than that of the entire GI tract. Finally, the environmental conditions in the distal bowel, such as depletion of preferred energy substrates, might not be suitable for the growth of microbiota. Therefore, the DNA results from collected fecal material might be affected by this limitation (75).

Aging might significantly influence gastrointestinal health, by inhibiting the growth of beneficial GI microflora. Therefore, further understanding of the regulation of gut microbiota will offer a basis for future studies to improve GI health in humans. *Bifidobacterium* plays an important role in the body, affecting the reactivity of the immune system and helps the other physiological functions (76). The lower amount or absence of *Bifidobacterium* may cause serious health consequences in the GI tract of the host. For example, colon cancer development can be promoted by the absence of protective *Bifidobacterium* strains. To this regard, dietary modifications such as supplementation of probiotics or prebiotics have been suggested to benefit in the prevention of diseases such as colon cancer. Indeed, probiotics would provide and immediately increase *Bifidobacterium* and prebiotics that would induce the growth and maintain the health of microbiota population (77). Further research in animals and humans, especially randomized controlled trials, is warranted.

Table 1. PCR primers used in this study

Primer (Bacteria species)	Oligonucleotide sequence (5'→3')	Amplicon size (bp)	Annealing temp (°C)
Lacto [63] Lactobacillus spp.	F:CACCGCTACACATGGAG R:AGCAGTAGGGAATCTTCCA	341	58
Bifid1 [64] Bifidobacterium spp.	F:CTCCTGGAAACGGGTGG R:GGTGTTCTTCCCGATATCTACA	549-563	58
Bifid 2 [65] Bifidobacterium spp.	F:TCGCGTC(C/T)GGTGTGAAAG R:CCACATCCAGC(A/G)TCCAC	243	58
BiBIF [64] B. bifidum	F:CCACATGATCGCATGTGATTG R:CCGAAGGCTTGCTCCCAAA	278	63
BiBRE [64] B. breve	F:CCGGATGCTCCATCACAC R:ACAAAGTGCCTTGCTCCCT	288	60
BiLON [64] B. longum	F:TTCCAGTGATCGCATGGTC R:TCSCGCTTGCTCCCCGAT	277	62
BIA [66] B. adolescentis	F:GGAAAGATTCTATCGGTATGG R:CTCCCAGTCAAAGCGGTT	244	55

Table 2. The ZR Fecal Kit Extraction Method

Texture improvement

sample	weight (mg)	condition	Hand homoginized	Bench Top Vertex
1	150	dry feces	no	no
2	150	dry feces	no,but soaked lysis buffer for 10 min	no
3	150	dry feces	no,but soaked lysis buffer for 30 min	no
4	75	dry feces	yes,and soaked in lysis buffer for 30 min	5 min
5	75	fresh feces	yes	5 min
6	150	fresh feces	yes	5 min
7	75	frozen fresh feces	yes	5 min
8	150	frozen fresh feces	yes	5 min
9	75	fresh feces	yes	5 min
10	75	fresh feces	yes	5 min
11	75	fresh feces	yes	5 min
12	75	fresh feces	yes	5 min
13	75	fresh feces	yes	5 min
14	75	fresh feces	yes	5 min
15	75	frozen fresh feces	yes	5 min
16	75	frozen fresh feces	yes	5 min
17	75	fresh feces	yes	5 min
18	75	fresh feces	yes	5 min
19	75	frozen fresh feces	yes	5 min
20	75	frozen fresh feces	yes	5 min
21	75	frozen fresh feces	yes	5 min
22	75	frozen fresh feces	yes	5 min
23	75	frozen fresh feces	yes	5 min

Procedure adjustment

Sample	Cell Lysis Improvement	DNA Binding Improvement
1	as protocal	1200 ul binding solution as protocal
2	as protocal	1200 ul binding solution as protocal
3	as protocal	1200 ul binding solution as protocal
4	as protocal	1200 ul binding solution as protocal
5	as protocal	1200 ul binding solution as protocal
6	as protocal	1200 ul binding solution as protocal
7	as protocal	1200 ul binding solution as protocal
8	as protocal	1200 ul binding solution as protocal
9	as protocal	1200 ul binding solution as protocal
10	as protocal	1200 ul binding solution as protocal
11	Vertex with lysis buffer 5 min	1200 ul binding solution as protocal
12	Vertex with lysis buffer 10 min	1200 ul binding solution as protocal
13	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	increased DNA binding solution to 1575 ul
14	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	increased DNA binding solution to 1800 ul
15	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	increased DNA binding solution to 1800 ul
16	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	increased DNA binding solution to 1800 ul
17	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	increased DNA binding solution to 1800 ul
18	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	increased DNA binding solution to 1800 ul
19	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	adding ,increased binding time to 5 min
20	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	adding ,increased binding time to 5 min
21	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	adding ,increased binding time to 5 min
22	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	adding ,increased binding time to 5 min
23	Vertex with lysis buffer 10 min,increase lysis buffer to 1000 ul	adding ,increased binding time to 5 min

Procedure adjustment (continue)

Sample	DNA Elution improvement
1	as protocol
2	as protocol
3	as protocol
4	repeated the last step by put the elute in the IV-HRC spin filter twice
5	repeated the last step by put the elute in the IV-HRC spin filter twice
6	repeated the last step by put the elute in the IV-HRC spin filter twice
7	repeated the last step by put the elute in the IV-HRC spin filter twice
8	repeated the last step by put the elute in the IV-HRC spin filter twice
9	adding,increased of 100 ul elution buffer to elude the DNA in final step
10	adding, increased of 50 ul elution buffer to elude the DNA in final step
11	adding, increased of 50 ul elution buffer to elude the DNA in final step
12	adding, increased of 50 ul elution buffer to elude the DNA in final step
13	adding, increased of 50 ul elution buffer to elude the DNA in final step
14	adding, increased of 50 ul elution buffer to elude the DNA in final step
15	adding,washed the 1x column with 400 ul with nuclease-free water
16	adding,washed the column with elute buffer twice
17	adding,washed the 1x column with 400 ul with nuclease-free water
18	adding,washed the 1x column with 400 ul with nuclease-free water twice
19	adding,diluted elute solution 1:10 with TE buffer
20	adding,diluted elute solution 1:10 with TE buffer
21	adding,diluted elute solution 1:10 with TE buffer
22	adding,diluted elute solution 1:10 with TE buffer
23	adding,diluted elute solution 1:10 with TE buffer

Results

Sample	Concentration (ng/ul)	260/280 Ratio
1	97.37	1.36
2	92.28	1.45
3	71.96	1.47
4	74.27	1.52
5	120.11	1.33
6	104.18	1.48
7	123.22	1.54
8	115.94	1.53
9	53.59	1.26
10	175.00	1.58
11	57.76	1.52
12	58.74	1.41
13	99.01	1.60
14	68.77	1.47
15	155.98	1.80*
16	101.46	0.93
17	44.74	1.58
18	141.42	1.82*
19	117.61	1.83*
20	170.87	1.85*
21	48.75	1.78
22	68.1	1.77
23	35.35	1.72

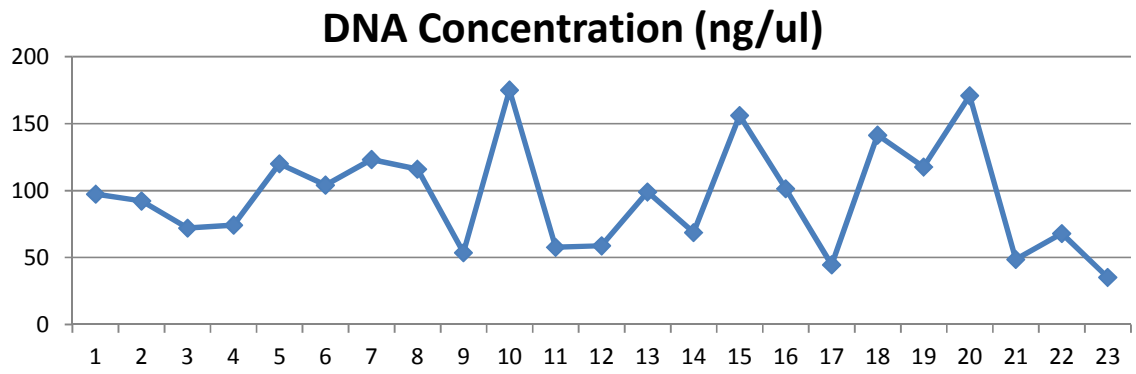

Figure 10.1 DNA Concentration detected by Nanodrop Spectrophotometer.

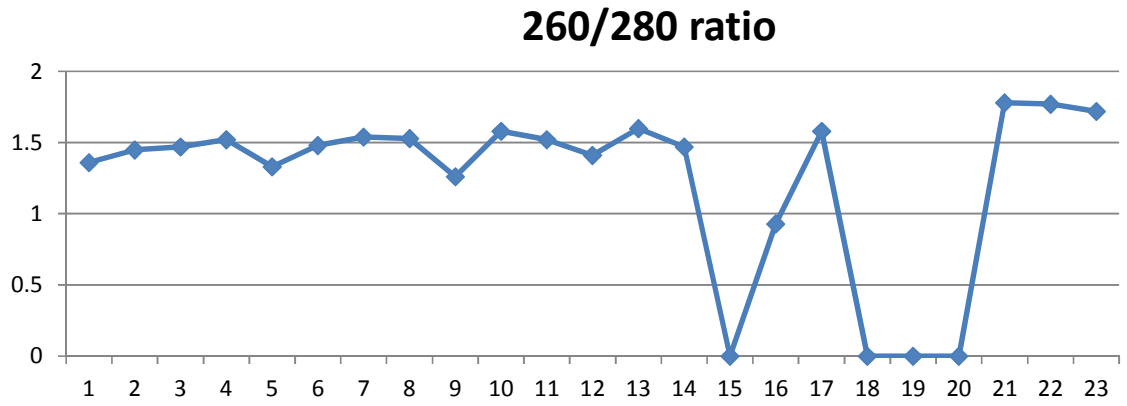

Figure 10.2 DNA Quality detected by Nanodrop Spectrophotometer

Table 3. The DNAzol Direct Extraction Method

Procedure adjustment

Sample	weight (mg)	incubation temperature (C °)	Vertex time (sec)
1	5.7	85	briefly
2	4.3	85	briefly
3	4.1	85	briefly
4	2.9	85	10
5	1.8	85	10
6	1.4	85	30
7	5.9	85	30
8	4.7	85	30
9	2.1	85	30
10	4.8	room temperature	30
11	4.4	room temperature	30
12	3.6	room temperature	30
13	5.0	90	10
14	5.4	90	30
15	4.9	90	10
16	5.0	90	30
17	4.8	95	30
18	2.6	95	30
19	4.8	95	30
20	4.6	95	30
21	2.9	95	30
22	3.1	95	30
23	4.8	95	briefly
24	10.0	95	briefly
25	12.6	95	briefly
26	touch with pipette tip	95	briefly
27	touch with pipette tip	95	briefly
29	touch with pipette tip	95	briefly

note: Sample 21-24 were centrifuged at 32000 rpm after vertex

PCR Results

Sample	amplified with g-bifid primer	amplified with BiBre primer
1	no	n/a
2	no	n/a
3	no	n/a
4	no	no
5	no	no
6	no	no
7	no	no
8	show but wrong size	no
9	show but wrong size	no
10	no	no
11	show but wrong size	no
12	no	no
13	show but wrong size	no
14	show but wrong size	no
15	show multiple bands	no
16	show multiple bands	no
17	show multiple bands	no
18	show multiple bands	smear
19	no	n/a
20	show	yes
21	show multiple bands	smear
22	show multiple bands	show multiple bands
23	no	no
24	show multiple bands	no
25	show multiple bands	n/a
26	show but wrong size	n/a
27	show but wrong size	n/a
29	show but wrong size	n/a

Table 4. Results of PCR for amplification of Lacto, Bifid1, Bifid2, and BiBre primer

Template DNA souce	Primer			
	Lacto	Bifid1	Bifid2	BiBre
Fresh collected feces	+	-	-	-
Fresh frozen feces	+	-	-	-
ATCC dry cell + Feces	N/A	+	+	+
ATCC live cell + Feces	N/A	+	+	+
Feces collected directly from colon incision	+	-	+	-
mouse colon mucosa	-	-	+	-
Whole colon	-	-	-	-

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ABSTRACT

DETECTION OF BENEFICIAL MICROBIOTA IN MOUSE COLON

by

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Lactic acid bacteria (LAB) and *Bifidobacterium* are the most common types of microbes used as probiotics. They are present in the human gastrointestinal tract and have a significant influence on our health and well-being. Microbiota plays an important role in host metabolism and provides a natural defense mechanism against invading pathogens. This experiment was focusing on establish a method to detect the gastrointestinal tract microbiota, either by fecal or colonic tissue DNA extraction.

The experiment comparing 2 types of DNA extraction; ZR Fecal kit and DNAzol direct. DNAzol direct was easy to use but was not suitable for long term DNA storage, hence the sample need to be extract fresh when needed. The extracted DNA, when amplified with Lacto genus specific primer show 99% match with sequence of *Lactobacillus reuteri* in database. For primer sensitivity test, Bifid1 genus specific primer could detect DNA up to a dilution of 10^{-10}, or approximately 10 ag of DNA. For species specific primers, BIA could detect DNA up to a dilution of 10^{-8}, or 1 fg of DNA. Both BiLon and BiBre primer could detect DNA up to a dilution of 10^{-7}, or 10 fg of DNA. The PCR product of Bifid1 primer amplified against ATCC

bacteria DNA:-resulted in 99% match with all 3 bacteria sequences (*B. breve*, *B. Adolescentis*, and *B. Longum*). When using BiBre and BIA primers amplified against B. breve and B. infantis ATCC DNA, the sequences were 100% similar to each species in bacteria database. For BiLon primer, the results yield 96% match.

The difference in age of the mice could play an important role in the presence of bacteria detected in fecal samples, due to unequally delayed development of the young mice's intestinal ecosystems. The presence of bacteria in feces might not represent the type or quantity of bacteria that inhabitant the colon. One challenge that needs to be overcome in recovery of DNA is finding the lyses method that could penetrate peptidoglycan, the heavily cross-linked structure in gram-positive bacteria cell wall. Also find the method to verify the development of the pathophysiological changes, that are claimed to be responsible for the variation in bacterial levels in the GI tracts of the mice, would make the results more accurate. Further large-scale research in animals and humans, especially randomized controlled trials, is warranted.

AUTOBIOGRAPHICAL STATEMENT

Aranya Linpisanl graduated from Chiang Mai University, Thailand, in 2003 with a Bachelor of Science Degree in Agro-industry, major in Packaging Technology. She currently completed her graduate requirements towards the accomplishment of a Master of Science degree in Nutrition and Food Science at Wayne State University (WSU).