A Dissertation for the Degree of Master

Molecular characterization of *Bifidobacterium* spp. from Korean infant feces using culturomic approach

컬쳐로믹스 분석기술을 이용한 한국 유아 분변 유래 *Bifidobacterium* spp.의 분자적 특성 연구

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Graduate School of Department of Agricultural Biotechnology Seoul National University
Animal Science major

Hayoung Kim

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Advisor: Prof. Younghoon Kim

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Graduate School of Department of Agricultural Biotechnology Seoul National University
Animal Science major

Hayoung Kim

Confirming the master's thesis written by
Hayoung Kim
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Chair _____(Seal)

Vice Chair _____(Seal)

Examiner _____(Seal)

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지도교수 김영훈

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위 원 _____ (인)

Abstract

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Hayoung Kim

Program in Animal Science and Biotechnology

Department of Agricultural Biotechnology

Graduate School of Seoul National University

Through a variety of mechanisms, the human gut microbiome plays a crucial role in controlling human health and disease. *Bifidobacterium* is a probiotic famous for its prevalence in the gastrointestinal tract of infants as well as its beneficial properties. Using the fructose-6-phosphoketolase assay, we were able to isolate 11 *Bifidobacterium* candidates from fecal samples. Then we used the Matrix-assisted

laser desorption ionization time-of-flight mass spectrometry analysis and 16S rRNA sequencing to confirm them. Finally, *Bifidobacterium* was identified in seven isolates including *B. animalis* B7, *B. animalis* C1, *B. animalis* D2, *B. pseudocatenulatum* E6, *B. longum* E8, *B. pseudocatenulatum* E9, and *B. longum* F5. After that, we looked into their ability to work as a probiotic. The ability of *Bifidobacterium* strains from infant feces was confirmed through acidic and bile tolerance, antibiotic sensitivity, antibacterial activity, adhesion ability, and *C. elegans* experiments.

In the acid tolerance assay, *B. animalis* C1 and *B. animalis* D2 had a about 80% survival rate, while *B. longum* E8, *B. pseudocatenulatum* E9, and *B. longum* F5 had a survival rate of more than 80% in the bile tolerance assay. All *Bifidobacterium* strains were susceptible to ampicillin, chloramphenicol, and penicillin in the antibiotic sensitivity assay, and all isolates were also susceptible to tetracycline except *B. animals* B7 and *B. animals* D2. Additionally, adhesion ability assay revealed that *B. animalis* C1, *B. animalis* D2, and *B. pseudocatenulatum* E6 had similar adhesion ability in *C. elegans* model, and all strains had the potential to inhibit the pathogenic bacteria including *S. typhimurium* 5L1344 and *E. coli* ATCC 35153. Finally, it was demonstrated that all Bifidobacterium strains extended the lifespan of *C. elegans*, moreover, they had a significant impact on the host's immune defense system against pathogenic bacteria such as s. aureus Newman.

Therefore, we used the Fermentation of the intestinal microbiota model assay and *in vivo* experiment to see if *Bifidobacterium* strains had an effect on gut microbiota. The fermentation of the intestinal microbiota model assay demonstrated that *B. animalis* D2, *B. pseudocatenualtum* E6, and *B*. *longum* F5 influenced the synthesis of metabolites like lactic acid and acetic acid. In the mouse model, *B. animalis* D2, *B. pseudocatenualtum* E6, and *B*. *longum* F5 extended intestinal length and significantly decreased levels of inflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6 by alleviating antibiotic-induced gut microbiota dysbiosis. Finally, we demonstrated that *B. animalis* D2, *B. pseudocatenualtum* E6, and *B*. *longum* F5 were influenced fecal microbiota, microbial metabolites, and immune regulation.

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List of abbreviations

ALT: Alanine amino transferase

AST: Aspartate amino transferase

B7*: Bifidobacterium animalis* B7

BB12: *Bifidobacterium animalis subsp. lactic* BB-12

BHI: Brain heart infusion

BS: *Bifidobacterium* selective medium

C1: *Bifidobacterium animalis* C1

C. elegans: *Caenorhabditis elegans*

CFU: Colony-forming unit

D2: *Bifidobacterium animalis* D2

DNA: Deoxyribonucleic acid

E6: *Bifidobacterium pseudocatenulatum* E6

E8: *Bifidobacaterium longum* E8

E9: *Bifidobacterium pseudocatenulatum* E9

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

F5: *Bifidobacterium longum* F5

F6PPK: Fructose-6-phosphate phosphoketolase

FBS: Fetal bovine serum

FIMM: Fermentation of the intestinal microbiota model

HDLC: High-density lipoprotein cholesterol

IL-1β: Interleukin 1β

IL-6: Interleukin 6

IBD: Inflammatory bowel disease

LB: Luria-Bertani broth

LGG: *Lacticaseibacillus rhamnosus* GG

MALID-TOF/MS: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

MRS: De Man, Rogasa and Sharpe

NGM: Nematode growth medium

PB: Phosphate buffer

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

rRNA: Ribosomal ribonucleic acid

SCFA: Short-chain fatty acid

SEM: Scanning electron micrograph

TCHO: Total cholesterol

TG: Thyroglobulin

TNF-α: Tumor necrosis factor α

Units and marks

°C: Degree Celsius

%: Percent

g: Gram

mg: Milligram

ml: Milliliter

h: Hour

min: Minute

µm: micrometer

Chapter I.

Literature review

1.1. Gut microbial community

1.1.1. Factors that influence the gut microbiome

Human gut microbiota is composed of a lot of commensal microorganisms, for example, bacteria, viruses, fungi, and protozoa. The gut microbiome is influenced by many factors such as diet, age, ethnicity, geography, and disease. Especially, bacteria are the most well-known among them because many studies have been conducted associated with human health (Mann et al., 2020; Singh et al., 2017). For instance, diet is the easiest factor to regulate the gut microbiome, although the gut microbiome has resilience. In a previous study, a low-energy diet such as fat or carbohydrate-restricted diet had an impact on the ratio of Bacteroidetes: Firmicutes. Moreover, this effect occurred by losing weight dramatically (Xu & Knight, 2015). Furthermore, an animal-base diet altered the proportion of microorganisms such as Bacteroides, and Bilophila which have tolerance in bile, as well as Firmicutes that

digest the plant polysaccharides (David et al., 2014). On the other hand, the gut microbiome has been shown to alter with age. Huang, S., et al. proved that gut, oral, and skin microbiome indicated distinct differences according to age. Especially, *Bifidobacterium* and *Blautia* genus or *Lachnospiraceae* and *Ruminococcaceae* families correlate with age when they are researched through amplicon sequence variants (Huang et al., 2020). Thus, the gut microbiome was changed through many factors which are relevant to everyday life.

1.1.2. Effect of the gut microbiome on human health

The gut microbiome helps the host metabolic requirements by supporting nutrient digestion and homeostasis (Mann et al., 2020). First, saccharolytic bacteria which are present in the gut microbiome break carbohydrates down into beneficial metabolites such as monosaccharides and SCFAs. For example, butyrate which is produced by carbohydrate-degrading bacteria is abundant in healthy individuals (Venema, 2010). Furthermore, butyrate produced by the gut microbiome has a beneficial effect on obesity and insulin resistance in an animal model of metabolic syndrome (Bridgeman et al., 2020). Second, the gut microbiome has a significant proteolytic ability, digesting consumed food into peptides, amino acids, and branched-chain fatty acids (BCFAs). Here, the amino acid is important compounds

because of their functional aspect including the regulation of immune response, muscle growth, and aging (Grohmann & Bronte, 2010; Timmerman & Volpi, 2008; Wu, 2009, 2013). For instance, the gut microbiome that decreased the abundance of adverse bacteria inhibits hyperlipidemia through regulation of amino acid metabolism which has been linked to dyslipidemia (Yan et al., 2022). Last, the gut microbiome synthesizes some vitamins such as vitamin K, and vitamin B. Especially, *Bifidobacterium* has biosynthetic properties of folate, and riboflavin in the vitamin B group besides niacin and pyridoxine (LeBlanc et al., 2013). Here, the vitamin B group has a lot of useful functions including cognitive function and kidney disease (Mydlík & Derzsiová, 1997; Ontario, 2013). In conclusion, the gut microbiome is quite important because it affects nearly any part of the body.

1.1.3. Beneficial effect of probiotic

Probiotics are defined as “live microorganisms that provide a health benefit to the host when consumed in proper doses” (Shanahan, 2010). Probiotics influenced human health and disease. For instance, probiotics prevented the atopic disease as a primary treatment through their ability to correct the increased intestinal permeability (Kalliomäki et al., 2001). Moreover, probiotics mitigate inflammatory bowel disease by competing with pathogens, modulating the immune response,

enhancing the intestinal barrier activity, and inducing the T cell apoptosis (Rioux & Fedorak, 2006). In addition, probiotics engineered for human diseases including metabolic disorders, infectious diseases, and killing tumors are being studied (Chua et al., 2017). Probiotics have a beneficial effect on livestock including broilers and piglets besides humans. For example, probiotics are used as growth promoters instead of antibiotics. In a previous study, probiotic treatment to broiler chickens induced increasing the phagocytosis of enterocytes, moreover, it raised the serum IgE and IgM levels (Higgins et al., 2007). Furthermore, probiotics are applied in the swine industry to improve feed consumption, digestibility, growth, and meat quality (Barba-Vidal et al., 2019).

1.2. Approach to identification of gut microbiome

1.2.1. Metagenomics

New approaches to exploring the bacteria that live in the human gut have recently opened due to advances in sequencing technology and the development of metagenomics and bioinformatics techniques. Researchers had only investigated culturable bacteria before the development of the metagenomics technique, however, following the development of metagenomics, they were able to conduct in-depth research on the gut microbiome using the metagenomics (Lepage et al.,

2013). Especially, the next-generation sequence (NGS) technologies allow the evaluation of lots of microorganisms in various environments without the use of bacterial culture. A recent metagenomic analysis of oral microbiome in periodontitis patients and healthy people indicated that there were differences in the oral microbiome in the two groups, as well as that periodontitis patients' oral microbiome shared similar bacterial species. The major difference between healthy persons and periodontitis patients was that periodontitis patients had a lot of gram-negative bacteria, whereas healthy people had a lot of gram-positive bacteria (Xu & Gunsolley, 2014). Thus, metagenomics is being researched as a diagnostic tool because it indicates differences depending on diseases and it doesn't have responsibility for the difficulty of practicing medicine and diagnostic mistakes (Greninger, 2018).

1.2.2. Metabolomics

Numerous genes in the gut microbiome are involved in protein synthesis, signaling molecule formation, and antimicrobial chemical production. For example, they convert complex indigestible compounds like dietary fibers into short-chain fatty acids that are essential to the host's health (Daliri et al., 2017). In this respect, the huge number of varied metabolites produced by the gut microbiome interacts with

the host in a variety of ways (Misheva et al., 2021). For instance, Fromentin et al.(2022) demonstrated ischemic heart disease patients showed different metabolites including dysmetabolism-associated metabolites such as 4-butyrobetaine, linoleylcarnitine, and trimethylamine compared with healthy groups. Similarly, *Burkolderia pseudomallei* which might be associated with the cause of the endocarditis were enriched in ischemic heart disease patients (Fromentin et al., 2022). Moreover, the gut microbiome regulates metabolic pathways such as taurine, sphingolipid, and ceramide metabolism, furthermore, these changes influenced atherosclerosis. In addition, metabolomic differences are distinguished according to the nature of the disease (Liu et al., 2019). In summary, the metabolites of the gut microbiome were associated with necessary compounds for humans, in addition to the occurrence of disease.

1.2.3. Culturomics

Culturomics is a culturing technique that identifies bacterial species by using different culture conditions, MALDI-TOF mass spectrometry, and 16s rRNA sequencing. The culturomics approach is developed to identify fastidious microorganisms by using MALDI-TOF mass spectrometry which is used for high-throughput screening. Especially, anaerobic bacteria which have difficulty in

culturing *in vitro* conditions can verify through the anaerobic system including an anaerobic gas pack, anaerobic laboratory bench as well as specialized reagents (Lagier et al., 2018). A recent study found 136 bacteria species from different stool, small intestine, and colonic samples through the culturomics approach, furthermore 86 species of them have never been found in the human intestine (Lagier et al., 2016). Moreover, there are differences between healthy stool samples and anorexia nervosa stool samples in the culturomics approach. Eleven bacteria species that were never isolated from the gut microbiome were detected in anorexia nervosa stool samples. These findings extend the scope of the gut microbiome associated with anorexia nervosa, then it can help further research (Pfleiderer et al., 2013).

1.3. *Bifidobacterium*

1.3.1. Characteristic of *Bifidobacterium*

Bifidobacterium genus was the earliest microorganisms to colonize the human intestinal tract, and they are considered to provide health benefits to the host. Since the first bifidobacterial genome was revealed in 2002, the quantity of publicly obtainable bifidobacterial genome sequences has continuously increased (O'Callaghan & Van Sinderen, 2016). The genus *Bifidobacterium* is a member of the family *Bifidobacteriaceae* and the phylum *Actinobacteria*. They are most typically found in the gastrointestinal tracts of mammals, Moreover, they decrease

during adulthood and continue to decline in the old. Their increased abundance during childhood is related to their potential involvement as a microbial regulator of the immune system as well as the host's gut physiology (Duranti et al., 2019). The general characteristic of the *Bifidobacterium* genus includes gram-positive, non-spore, non-motile, Y- or V-shaped, moreover, they grow in anaerobic conditions that produce lactic acid and acetic acid without releasing CO_2. Their optimal growth temperature is around 36°C to 38°C and their optimal pH values are between 6.5 to 7. Furthermore, *Bifidobacterium* can produce riboflavin, amino acids, and thiamin (Bahmani et al., 2019).

1.3.2. Beneficial effects of *Bifidobacterium*

Bifidobacterium has been used as an ingredient in many functional products owing to its health-promoting capabilities. *Bifidobacterium* influences several diseases which are related to the gastrointestinal tract including colorectal cancer, diarrhea, inflammatory bowel disease, and necrotizing enterocolitis. In a previous study, *Bifidobacterium breve* A1 has an effect on Alzheimer's disease except for colonic diseases through behavior tests including the Y maze test and passive avoidance test, moreover, bifidobacterial acetate mitigated the cognitive impairment in Alzheimer's disease mice (Kobayashi et al., 2017). Moreover, *Bifidobacterium* improves kidney function and postpones chronic kidney disease by producing short

chain fatty acids and changing the gut microbiome (Iwashita et al., 2018). Furthermore, the treatment of *Bifidobacterium animalis* subsp. *lactis* V9 significantly reduced the alanine transaminase (ALT) and aspartate aminotransferase (AST) levels. These results lead to the improvement of hepatic steatosis (Yan et al., 2020). It is clear that the gut microbiome modulates host immune development and activity, especially, *Bifidobacterium* regulates the host immunomodulatory processes through direct contact with the host and metabolic activity *in vivo* (Konieczna et al., 2012).

1.4. Simulator of the human intestinal microbial ecosystem

1.4.1. *In vitro* human digestion system

Digestion is a complicated process that provides nutrients while also releasing chemicals in the intestinal system that can be useful or harmful to human health. As a result, learning about the fate the destiny of food in the digestive tract can help us learn more about how food affects our health. Food digestion studies involving *in vivo* models are complex, expensive, and often unethical. As a response, a number of *in vitro* models have been constructed. Specifically, Dynamic digestion systems can be monocompartmental or multicompartmental that imitate physiological reality and replicate what occurs in the gastrointestinal tract of

humans or other animals. In the mono-compartmental system, there are three models including the dynamic gastric model (DGM), human gastric simulator (HGS), and the artificial colon (ARCOL). First, DGM was created to solve the requirement for a system that could accurately represent both the biochemical and mechanical processes that occur throughout human gastric digestion. Second, HGS was created to allow for the monitoring of gastric food digestion in a system with physiologically similar physical and chemical conditions. This system focuses on gastric digestion. Lastly, ARCOL is a single-stage fermentation model that mimics the human and animal colonic environment. ARCOL is one of the few wireless systems among available colonic *in vitro* models that provides for the maintenance of anaerobic conditions through unique work.

Next, there are four multicompartmental systems including DIDGI, the gastric and small-intestinal model (TIM), simulator of the human intestinal microbial ecosystem (SHIME), and simulator of the gastro-intestinal tract (SIMGI). The DIDGI system was developed at INRA to track the disintegration and kinetics of food hydrolysis during a mimicked digestive process. It concentrates on the stomach and small intestine, which are the upper sections of the digestive tract. Second, TIM began developing *in vitro* gastrointestinal models in 1992, moreover, it is still ongoing, with simulations of newborn gastrointestinal problems and the construction of the elderly gastric model ‘TIMagc’. Third, SHIME represents the

adult human gastrointestinal tract which is made up of a series of five reactors that simulate the various regions. Lastly, SIMGI is a computer-controlled *in vitro* gastrointestinal model intended to imitate the physiological processes that occur during digestion in the stomach and small intestine, in addition to replicate the intestinal microbiota that is essential for metabolic bioconversions in the large intestine (Dupont et al., 2019).

1.4.2. Simulator of the human intestinal microbial ecosystem

SHIME is an acronym for Simulator of the Human Intestinal Microbial Ecosystem, and the name has been licensed jointly by ProDigest and Ghent University since 2010. The realization that fecal bacteria differ significantly from the *in vivo* colon microbiota in terms of community composition and metabolic activity led to the development of multi-compartment simulators of the human gut (Van de Wiele et al., 2015). The first two reactors use the fill-and-draw approach to imitate various processes in food absorption and digestion, with peristaltic pumps supplying a specified amount of SHIME nutritional medium (3x/day) and pepsin to the stomach and pancreatic enzymes with bile juice to the small intestine. Moreover, a particular software makes the simulation of the three small intestine sections such as duodenum, jejunum, and ileum. Lastly, three reactors mimic the ascending,

transverse, and descending colon after being inoculated with fecal bacteria (Dupont et al., 2019). The SHIME system applies to identify many experiments, for example, gut health, transferability of antibiotic resistance gene, and effect of nutrients (Lambrecht et al., 2019; Rovalino-Córdova et al., 2020; Sivieri et al., 2013).

Chapter II.

Molecular characterization of *Bifidobacterium* spp. from Korean infant feces using culturomic approach

The results of this chapter are in preparation for publication in the journal of agricultural and food chemistry.

2.1. Introduction

The human gut microbiome plays an important role in regulating human health and disease through a variety of mechanisms (Heintz-Buschart & Wilmes, 2018). The gut microbiome, for example, encodes a number of genes that conduct a variety of metabolic functions. A host's immune system is also developed by the gut microbiome, which produces a protective response to commensal bacteria and an inflammatory reaction to pathogenic bacteria. Furthermore, the gut microbiota can alter neurological, hormonal, and immunological signals and provides a potential route to the brain (Bull & Plummer, 2014). On the other hand, dysbiosis of the gut

microbiome is linked to disorders such as inflammatory bowel disease (IBD), allergies, metabolic syndrome, and obesity (Carding et al., 2015). Changes in the composition of the gut microbiome, in particular, can be used to detect the presence of illnesses. As a result, the composition ratio of helpful bacteria in the gut and the balance of intestinal bacteria are crucial to human health.

Bifidobacterium is a kind of probiotic that is known for its presence in the gastrointestinal tract of newborns and for its positive effects (Di Gioia et al., 2014). *Bifidobacterium*, for instance, inhibits colon cancer by secreting compounds that have anticancer properties (Bahmani et al., 2019). In healthy adults, *Bifidobacterium* reduces IL-6, which has beneficial effects on inflammatory and oxidative biomarkers (Bernini et al., 2018). *Bifidobacterium* also affects the host's energy metabolism by increasing the levels of short-chain fatty acids (Horiuchi et al., 2020). Until now, more research is being conducted to determine the functional effect of *Bifidobacterium* and isolate novel *Bifidobacterium* strains. as part of these experiments, an approach to isolating anaerobic bacteria is developed.

As previous stated, the human gastrointestinal tract contained a large number of bacterial cells, however the majority of them cannot be identified or cultured (Turroni et al., 2008). Because establishing a state like the human digestive system is difficult. As a result, several efforts such as culturomics, metagenomics, and metabolomics are being made to better understand the gut microbiome. Nowadays,

culturomics and metagenomics are being used to better understand the human microbiome and its properties (Mun et al., 2021).

The gut microbiome composition of infant feces was identified using culturomic and metagenomics studies, and seven *Bifidobacterium* strains were isolated. As a result, we evaluated their acid and bile tolerance, antibiotic susceptibility, antibacterial activity, and adhesion ability to see if they might be used as probiotics. The *in vivo* investigations were then carried out utilizing *C. elegans* and animal models to verify functional capabilities.

2.2. Materials and methods

2.2.1. Isolation and identification of *Bifidobacterium* from infant feces.

2.2.1.1. Isolation of *Bifidobacterium*

Bifidobacterium strains including *B. animalis*, *B. pseudocatenulatum*, and *B. longum* were isolated from Korean infant feces. The infant feces sample under the age of 3 were collected using a sterile tube. Infant feces were pooled regardless of age, sex, and delivery mode. However, it is divided based on the 12 months breast feeding period. Homogenized feces samples were serially diluted. The solution was plated on *Bifidobacterium* selective (Tejero-Sariñena et al.) agar (MB cell, Seoul, Korea) with 5% Horse Blood Defibrinated (MB cell, Seoul, Korea) and incubated at 37℃ for 3 days under anaerobic conditions. Anaerobic conditions were achieved using an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI, U.S.A) and a Whitley jar gassing system (Don Whitley Scientific Ltd., Shipley, United Kingdom).

2.2.1.2. Fructose-6-phosphate phosphoketolase (F6PPK) assay

The Fructose-6-phosphate phosphorketolase (F6PPK) assay was used to classify the selected isolates as *Bifidobacterium* at the genus level (Vlkova et al., 2002). Colonies grown on BS medium are inoculated to MRS medium with 0.05% L-

cysteine HCl and incubated for 3 days at 37℃. The supernatant is removed by centrifuging at 12,000×g for 5min and washing with 1ml of 0.05M phosphate buffer (pH 6.5) containing cysteine 3 times. The cell concentration was sonicated in ice for 20min with 1ml PB buffer, and the 60µl sonicate was added to 10µl fructose-6-phosphate (80mg/ml). 20µl of a mixture containing 6mg/ml NaF and 10mg/ml iodoacetic acid was added and incubated at 37℃ for 30min. after incubation, 13.9% (wt/vol) hydroxylamine hydrochloride was added and incubated for 10min at room temperature. Then 40µl of 15% (wt/vol) trichloroacetic acid and 40µl of 4M HCl were added. Finally, 40µl of 50mg/ml FeCl3 in 0.1M HCl was added to the solution (De Vries & Stouthamer, 1967). The F6PPK assay showed a pink, violet, or yellow color. A negative sample remained yellow, whereas the presence of the F6PPK enzyme in the sample was indicated by the pink and violet color (Vlková et al., 2005).

2.2.1.3. Identification of *Bifidobacterium* isolates by Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis and 16s rRNA sequencing

To determine the species level, isolates that showed a positive reaction in the F6PPK assay were prepared for MALDI-TOF/MS analysis (Oh et al., 2018). MALDI-TOF/MS analysis was performed using a Bruker Autoflex (Bruker

Datonics, Bremen, Germany). Each colony was applied directly to the stainless-steel target and allowed to dry at ambient temperature. The bacteria were then treated with 1µl of 70% formic acid and dried at room temperature. 1µl matrix HCCA (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) was added to the spot after the formic acid had dried completely (Veloo et al., 2014). The reliability of the MALDI-TOF/MS results is classified by the score (Table 2).

16S rRNA sequencing was used to identify *Bifidobacterium* strains that had been confirmed by MALDI-TOF/MS analysis. 16S rRNA sequencing was performed on the Illumina MiSeq platform at Macrogen (Seoul, Korea). The 27F/1492R primers (forward, 5'-AGA GTT TGA TCM TGG CTC AG-3'; revers, 5'-TAC GGY TAC CTT GTT ACG ACT T-3') and 784F/907R primers (forward, 5'-GGA TTA GAT ACC CTG GTA-3'; revers, 5'-CCG TCA ATT CMT TTR AGT TT-3') were used to amplify the V4 region of 16S rRNA gene.

Table 1. Score standard for identification of *Bifidobacterium* by MALDI-TOF/MS.

Range	Description	Symbols	Color
2.3~3.0	Highly probable species identification	+++	Green
2.0~2.29	Secure genus identification, probable species identification	++	Green
1.7~1.99	Probable genus identification	+	Yellow
0~1.69	Not reliable identification	-	Red

2.2.1.4. Scanning Electron Microscope (SEM) analysis

The structural shape of *Bifidobacterium* was determined using scanning electron microscopy (SEM). NICEM at Seoul National University provided electron micrographs using a scanning electron micrographs using a scanning electron microscopy JEOL-5410 LV (Tokyo, Japan). To prevent electron charging, all *Bifidobacterium* were coated with gold (purity, 99.99%) (Kang et al., 2019; Kim et al., 2005).

2.2.2. Functional experiment for isolated *Bifidobacterium* strains

2.2.2.1. Acid and bile tolerance

The acidic and bile tolerance experiments were carried out using the previously modified methods (Liu et al., 2020). Seven *Bifidobacterium* strains were isolated from infant feces and cultured anaerobically at 37°C for three days on MRS broth medium with 0.05 % L-cysteine HCl to examine acidic and bile tolerance. MRS broth was adjusted to pH 2.5 with 6N HCl to make an acidic medium. Pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, MO, USA) was added at a concentration of 1,000 units/mL after autoclaving. Using 0.45-m pore size syringe filters, the pepsin solution was filter-sterilized. 100μl of bacteria culture solution were mixed with 10ml of acidic medium, which was then anaerobically incubated

at 37°C for 3 h.

MRS broth with oxgall powder (Acumedia, Lansing, MI, USA) was prepared to make a bile medium. All *Bifidobacterium* were inoculated into a 10ml bile medium that had been adjusted to a 0.5% (w/v) concentration and anaerobically incubated for 24 h at 37°C. To calculate the cell density of the 0h control, some of the acidic and bile medium containing bacteria were diluted immediately. After 3 hours and 24 hours, the rest of the acidic and bile medium was diluted. All diluted solutions were anaerobically plated on MRS agar with 0.05 % L-cysteine HCl for 3 days at 37°C. Finally, by comparing the viable cell population of 0h and 3h, the survival rate (%) was estimated. Positive controls included *Lacticaseibacillus rhamnosus* GG (LGG) and *Bifidobacterium lactis* BB-12 (BB12).

Survival rate (%) = ($CFU_{\text{after 3h(acid) and 24(Tejero-Sariñena et al.) incubation}}$/$CFU_{\text{0h incubation}}$)

2.2.2.2. Antibiotic sensitivity

To evaluate antibiotic sensitivity, MRS agar plates were inoculated with 100µl of each *Bifidobacterium* strain cultured for 3 days in MRS broth. The antibiotic discs, which included ampicillin (10 g), chloramphenicol (30 g), kanamycin (30 g),

penicillin (10 g), tetracycline (30 g), and vancomycin (30 g), were then placed on the surface of the MRS agar plates. Under anaerobic conditions, plates were incubated for 3 days at 37°C. Antibiotic resistance was then assessed using inhibitory zone diameters, which were divided into three categories: resistant (R), intermediate resistant (IR), and susceptible (S). The degree of inhibition was divided into 1) Zone diameter ≤14mm (R), 2) Zone diameter 15-19mm (IR), and 3) Zone diameter >20mm (S) (Barry et al., 1979; Mayrhofer et al., 2008).

2.2.2.3. Antibacterial activity

With a slight modification of Sandra et al., 2012, the *Bifidobacterium* isolates were examined for the production of antibacterial substances such as bacteriocins and organic acids using four pathogenic bacterial strains: *Listeria monocytogenes* EGD-e, *Staphylococcus aureus* Newman, *Salmonella Typhimurium* SL1344, and *Escherichia coli* ATCC 35150. The pathogenic bacteria strains were incubated for 24 hours at 37°C in LB medium, while *L. monocytogenes* EGD-e was incubated for 48 hours at 30°C. *Bifidobacterium* strains were inoculated as a 5µl spot on LB agar with pathogenic bacteria on top. The plates were then incubated anaerobically for 3 days at 37°C. The antibacterial activity was divided into four categories. The inhibitory zone of four categories was include non (-), 5-10mm, weak (+), 11-

17mm, moderate (++), and >17mm, strong (+++) (Lin et al., 2020; Tejero-Sariñena et al., 2012).

2.2.2.4. Adhesion ability using HT29 cell line and *C. elegans* model

We utilized the *C. elegans fer-15; fem-1* model for the adhesion ability experiment because it cannot produce progeny at 25°C without phenotypic changes. For worm maintenance, nematode growth medium (NGM) agar was used, and *E. coli* strain OP50 was treated for feed. Using a sodium hypochlorite-sodium hydroxide solution, eggs were collected, and synchronized L1 worms were grown at room temperature. We measure live bacterial cells in worm intestines to confirm bacterial colonization in the *C. elegans* intestinal tract. Individual bacterial strains were exposed to *C. elegans* for 24 hours on NGM, after which 10 worms were selected at random. They were placed on gentamycin (25g/ml) brain heart infusion agar (BHI) (Sigma-Aldrich) plates for 5 minutes. Worms were mechanically disrupted using a pestle (Kontes Glass, Vineland, NF, USA) after being transferred to an Eppendorf tube containing M9 buffer with Triton X-100. The diluted worms were plated on MRS agar with 0.05 % L-cysteine HCl and incubated for three days at 37°C. As positive and negative controls, LGG and *E. coli* OP50 were used (H. Kim et al., 2021).

The ability of the strain to adhere to the HT-29, human intestine cell, was evaluated with some modifications to the previously described method (Mandal, H., et al. 2016). In briefly, HT-29 cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1X antibiotic/antimycotic (Gibco) at 37℃ in an atmosphere of 5% CO2. For the adhesion assay, monolayers of HT-29 cells were washed twice with DPBS (Gibco), and 109 CFU/ml of *Bifidobacterium* strains mixed in RPMI without antibiotic/antimycotic was added. Then, bacteria treated cells were incubated at 37℃ in an atmosphere of 5% CO2 for 2 hours. After 2 hours, cells were washed 5 times with DPBS to remove non-adherent bacteria. To dissociation cell, we used 0.25% Trypsin-EDTA (Gibco) and serial dilutions of the dissociated cells were plated on MRS containing L-cysteine HCl.

2.2.2.5. *C. elegans* lifespan and killing assay

L4 stage *C. elegans fer-15; fem-1* worms were transferred onto the NGM plate with a platinum wire after 100µl of concentrated *Bifidobacterium* was plated on 35mm NGM agar plates. In total, 90 worms were used for each bacterial species in three separate plates, which were all incubated at 25°C. Daily, live worms were counted and relocated once a day. As a negative control, *E. coli* OP50 was used.

L4 stage worms were placed on conditioning with *Bifidobacterium* strains and *E. coli* OP50 for 24 hours to conduct a *C. elegans* killing assay. On NGM agar, pathogenic bacteria such as S. aureus Neman and *E. coli* O157; H7 EDL933 were inoculated. L4 stage worms were placed onto plates with pathogenic bacteria and incubated at 25°C after being exposed to *Bifidobacterium* strains. During the assay periods, live worms were transferred to new pathogenic bacterial plates every day.

2.2.3. FIMM assay

2.2.3.1. Media composition

For each isolated *Bifidobacterium*, the FIMM model, which was modified from the Simulator of the human intestinal microbial exosystem (SHIME) model, was used as an *in vitro* digesting system (Siciliano et al., 2010). We used three fermentation phases that are analogous to the stomach, small intestine, and colon in humans. FIMM stomach medium was adjusted to pH 2.5 with 15.7% NaCl (175.3g/L), 3.0% NaH2PO4 (88.8g/L), 9.2 KCl (89.6g/L), 18 % CaCl2H20 (22.2g/L), 10% NH4Cl (30.6g/L), 6.5% HCl (37g/L), 2.5% pepsin, and 3% mucin. The small intestinal medium was composed of a 2:1 mixture of stomach medium and pancreatin solution, which contained 12.5g of NaHCO3, 6% bile, and 0.9% pancreatin. Finally, 0.1% arabinogalactan, 0.2% pectin, 0.1% xylan, 0.4% starch,

0.04% glucose, 0.3% yeast extract, 0.3% proteose peptone, 0.1% mucin, and 0.05% L-cysteine HCl are included in the colon stage medium. Furthermore, the colon stage media contains 10% infant fecal slurry to simulate a human colon with commensal microorganisms.

2.2.3.2. FIMM assay

The overnight *Bifidobacterium* strains were inoculated into 10ml of stomach medium and incubated at 37°C for 1 hour with shaking. The stomach medium was centrifuged for 5 minutes at 13,000 rpm after 1 hour. The bacterium cell was washed in small intestine stage media before being inoculated into a 100ml small intestine medium. After then, the small intestine medium was centrifuged and washed using colon medium as mentioned previously. Then, the washed bacteria cell was inoculated to colon medium.

The colon medium was adjusted to pH 7.0 and incubated for 24h at 37°C with shaking. Finally, the colon medium was divided into supernatant and bacterial cell for metabolites and metagenome analysis. The FIMM assay progressed in an anaerobic condition by using the anaerobic chamber and whitley jar gassing system, and N_2 purge system. Furthermore, the pH alteration of the FIMM medium was calculated for 24h every 6h by using an orion star A211 pH meter

(Thermoscientific, USA).

2.2.4. *In vivo* study

2.2.4.1. Experimental animal model

Male 5 weeks C57BL/6 mice (n= 30) that were specific pathogen free were purchased from the Animal Center for Pharmaceutical Research, Seoul National University (Seoul, Republic of Korea). Animal procedures were performed in accordance with the procedures of the Seoul National University Laboratory Animal Maintenance Manual. The mice were bred and housed with controlled temperature and humidity. All of the mice were randomized and grouped for the experiment. Therefore, total 6 groups were used for examination. The groups were divided based on the treatment 1)*Bifidobacterium animalis* D2, 2)*Bifidobacterium longum* F5, 3)*Bifidobacterium pseudocatenulatum* E6, 4)LGG, 5) Normal, and 6) Antibiotic control. All groups were treated antibiotic cocktail every other day during 10 days before bacteria treatment. *Bifidobacterium* and LGG treatment groups were administrated 1×10^8 of bacterial cells every other day during 10 days after antibiotic cocktail treatment. On the other day, the antibiotic control group was treated with antibiotic cocktails every other day during experiment period continuously.

2.2.4.2. Reagents

Based on a previous study, broad-spectrum antibiotics affected the gut microbiome. The antibiotic cocktail containing Ampicillin (100mg/kg), vancomycin (50mg/kg), metronidazole (100mg/kg), neomycin (100mg/kg), and amphotericin B (1mg/kg) were provided into sterile PBS to induce dysbiosis. These antibiotics had broad spectrum capacity and were well-documented impacts on gut microbiota. After the treatment for 10 days, the animals were anesthetized with 1.25% v/v Avertin (2,2,2-tribromomethyl alcohol) with tertiary amyl alcohol (2-methyl-2-butanol).

2.2.4.3. Colon length measurement

After sacrifice, the colonic tissues were collected from the mice. From the back of the cecum to the rectum, colon samples were collected, washed in PBS. For the measurement of colon length, the colon length was measured using Image J software1.8.0 (ImageJ bundled with 64-bit Java).

2.2.4.4. Serum biochemical analysis

Blood samples were collected from the postcaval veins and centrifuged at 600×g

for 20min, The serum was collected and stored at –70℃. The metabolic biomarkers which could identify from serum such as alanine amino- transferase (ALT), aspartate aminotransferase (AST), high-density lipoprotein cholesterol (HDLC), thyroglobulin (TG), and total cholesterol (TCHO) were analyzed using a Dri-chem Analyzer (Fujifilm, Tokyo, Japan). Serum IL-1β (ab197742, Abcam, China), IL-6 (Ab222503, Abcam, China), and TNF-α (ab208348, Abcam, China) levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit.

2.2.5. Metagenome analysis

Metagenome analysis from infant fecal samples were conducted by the described methods previously(H. Kim et al., 2021). The extraction of DNA performed by using DNeasy PowerSoil Pro Kit(QIAGEN, Germany) as described the manufacturer's instructions. Polymerase chain reaction(PCR) generated DNAs according to 16S metagenome sequencing library protocols (Illumina, San Diego, CA, USA). The V4 region of the 16S rRNA genes (primer set: forward, 5′-CCT ACG GGN GGC WGC AG-3′; reverse, 5′-GAC TAC HVG GGT ATC TAA TCC-3′) was analyzed using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). After concentrations of the index PCRs were measured by Qubit (Invitrogen, Carlsbad, CA, USA), equimolar PCR amplicons were pooled and sequenced using

the MiSeq system platform (Macrogen, Seoul, South Korea) based on the standard Illumina sequencing protocols. Then, the FASTQ files obtained from MiSeq data were analyzed through Mothur(v. 1.14). In Mothur analysis, reads integrated by using the make contig command, and reads were quality-filtered by the screen.seqs command(refer). Taxonomic classification was analyzed using the Greengenes-formatted database released in 2013 to eliminate sequences that were not categorized as Archaea or Mitochondria and operational taxonomic units were classified using 97% sequence similarity. Moreover, the Mothur SOP manual were used for processing of sequence data through the Mothur pipeline(https://nothur.org/wiki/miseq_sop/).

2.2.6. Metabolites analysis

Fecal samples were dissolved in 0.85% sodium chloride and filtered through a 0.2 μm PVDF membrane. The solution was then vortexed for 1 minute with ice-cold methanol. The supernatant was collected after centrifugation at 10,000rpm for 10 minutes at 4°C. At 30 °C for 90 minutes, the extract was derivatized with 60μL of a 20 mg/mL methoxyamine hydrochloride in pyridine (Sigma, St. Louis, MO, USA), followed by 100μL of N,O-Bis (trimethylsilyl)trifluoroacetamide (BSTFA; Sigma) at 60 °C for 30 minutes. Internal standards were added to the extract in the

form of a combination of fatty acid methyl esters and fluoranthene. A Thermo Trace 1310 GC (Waltham, MA, USA) was used in conjunction with a Thermo ISQ LT single quadrupole mass spectrometer for the GC–MS analysis (Waltham, MA, USA).

2.3. Results

2.3.1. Isolation and identification of *Bifidobacterium* from infant feces

2.3.1.1. Isolation of *Bifidobacterium*

To isolate the *Bifidobacterium* strains, we verified the microbial composition of infant feces which is known for having various *Bifidobacterium* taxon (Sakanaka et al., 2019). When we analyzed infant feces having a breast feeding period more than 12 months, the *Bifidobacterium* genus had the highest abundance, followed by *Akkermansia* (Figure 1A). On the other hand, Infant feces having a breast feeding period less than 12 months had diverse genus composition compared to other infant feces samples. In order taxa, *Clostridiales* were abundant instead of *Bifidobacteriales* (Figure 1B). These results probably depend on the breast feeding period of infants. When we cultured fecal samples to BS medium, we could isolate 11 *Bifidobacterium* candidates through F6PPK assay.

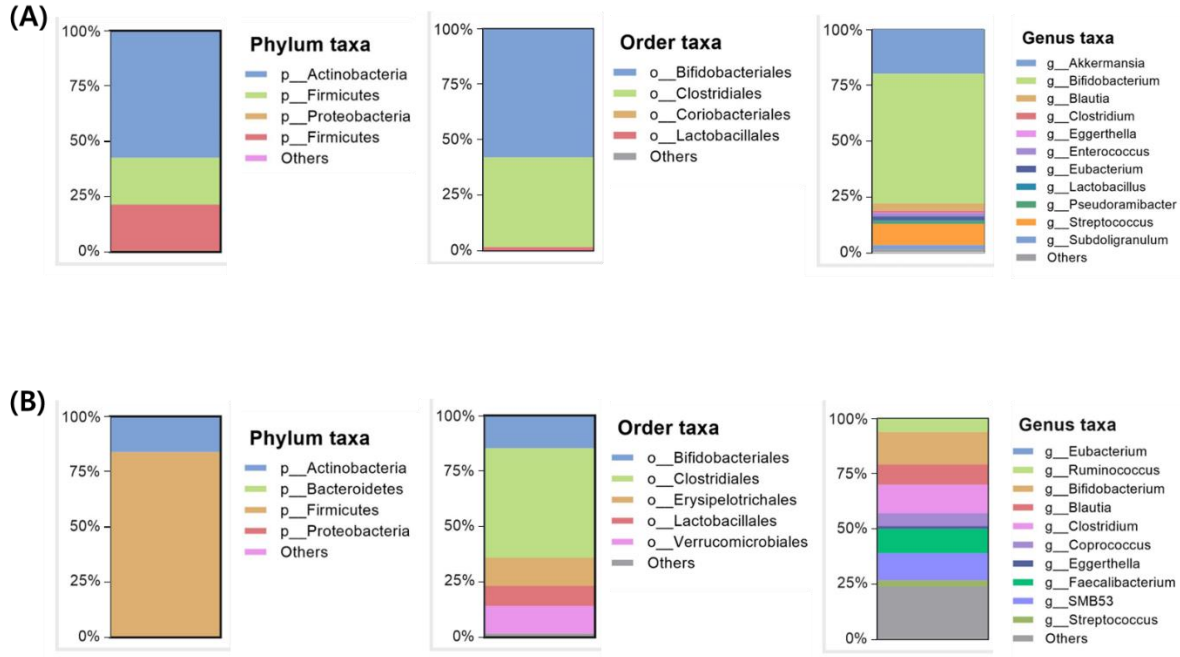

Figure 1. Next-generation sequencing of the 16S rRNA gene library demonstrates the relative abundance composition of the infant's fecal sample in a phylum, order, and genus taxa.

Distribution of microbial composition detected in infant fecal samples. Bar graph displaying the proportion of phyla, taxa, genera composition of samples. Fecal microbial composition of (A)infant feces having a breast feeding period more than 12 months and (B)infant feces having a breast feeding period less than 12 months were presented individually. The relative bacterial abundance was represented by percentage (%).

2.3.1.2. Identification of *Bifidobacterium*

We confirmed them using MALDI-TOF analysis which can be used for the early identification of bacteria from protein mass patterns (Lay Jr, 2001). MALDI-TOF analysis presented that 9 strains have protein mass patterns similar to *Bifidobacterium*. All 9 strains had a score over 1.7 (Table 2). 16S rRNA sequencing was performed to identify whether the candidates were *Bifidobacterium* through genome sequencing after MALDI-TOF analysis. Conclusionally, 7 isolates were revealed as a *Bifidobacterium* (Table 2). Identified *Bifidobacterium* isolates include *B. animalis* B7, *B. animalis* C1, *B. breve* D2, *B. pseudocatenulatum* E6, *B. longum* E8, *B. pseudocatenulatum* E9, and *B. longum* F5.

In addition to 16S rRNA sequencing for identification, the phylogenetic tree was consistent with the 16s rRNA sequencing results. When we analyzed the *Bifidobacterium* strains, we could confirm that they were clustered in each same species in the phylogenetic tree by using 16S rRNA sequence (Figure 2).

Table 2. Identification of *Bifidobacterium* using MALDI-TOF and 16S rRNA sequencing

Strains	MALDI-TOF			16S rRNA sequencing	
	Identification	Score	Symbols	Identification	Per. Ident(%)
B7	*Bifidobacterium animalis*	2.307	+++	*Bifidobacterium animalis*	99.56
C1	*No peacks found*	-	-	*Bifidobacteriu animalis*	99.42
D2	*Bifidobacterium animalis*	1.831	+	*Bifidobacterium animalis*	99.28
E2	*Bifidobacterium breve*	1.662	-	*Staphylococcus aureus*	99.42
E3	*Bifidobacterium catenulatum*	1.801	+	*Enterococcus faecalis*	99.57
E6	*Bifidobacterium catenulatum*	2.022	++	*Bifidobacterium pseudocatenulatum*	99.71
E8	*Bifidobacterium longum*	1.591	-	*Bifidobacterium longum*	99.71
E9	*Bifidobacterium catenulatum*	1.635	-	*Bifidobacterium pseudocatenulatum*	99.53
F5	*Bifidobacterium longum*	1.672	-	*Bifidobacterium longum*	99.28

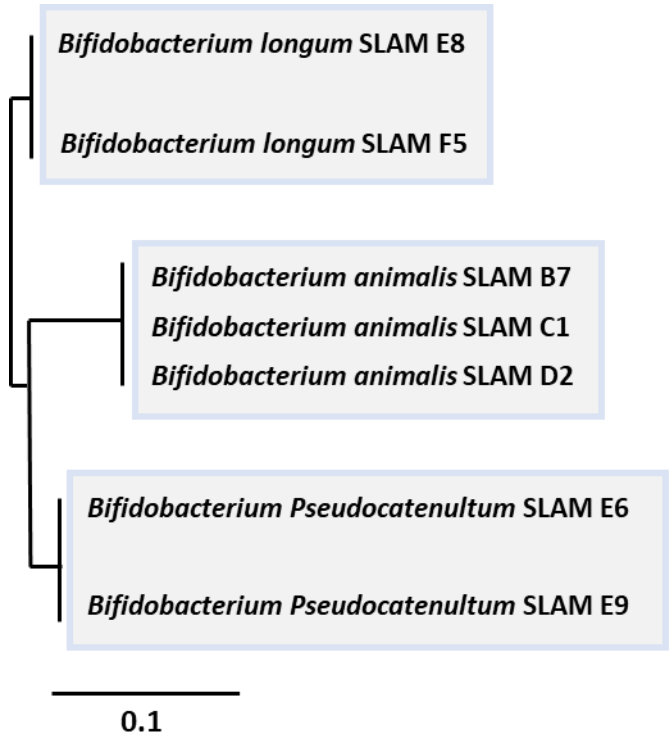

Figure 2. Neighbor-joining phylogenetic tree of 7 strains of *Bifidobacterium* isolated from infant's fecal sample.

Phylogenetic tree of the genus *Bifidobacterium* isolated form infant fecals showing *Bifidobacterium* strains clustered with the same species including *B. longum*, *B. animalis*, *B. pseudocatenulatum* by the neighbour-joining method based on 16S rRNA gene sequences. Bar, 0.1 sequence divergence.

2.3.1.3. Morphological identification of *Bifidobacterium*

SEM examinations were conducted to confirm their structure and morphology. 7 *Bifidobacteirum* strains show regularly and irregularly rods of about 1µm long, with some shorter forms. Moreover, they had a morphology like clubbed and we could also observe the sites where rupture of the outer cell wall layer occurred (Figure 3). Most bacterial cell show a stress-like phenotype because it was harvested during the stationary phase (Kang et al., 2019; Takade et al., 1983).

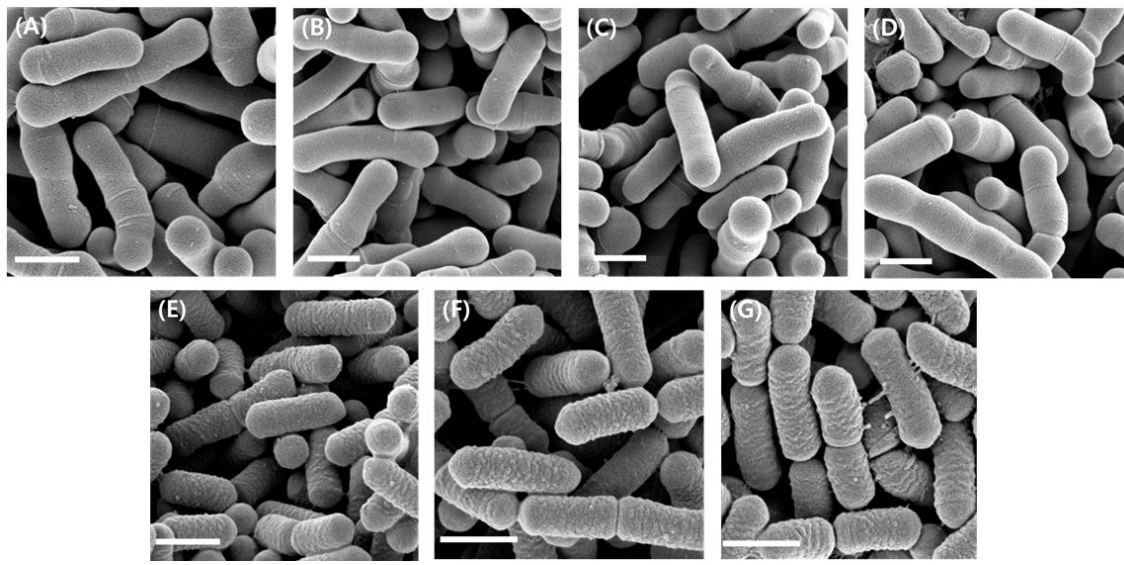

Figure 3. Scanning Electron Microscope image of isolated 7 *Bifidobacterium* strains.

The *Bifidobacterium* strains incubated in MRS containing L-cystein HCl medium at 37℃ for 4 days. Bar, 0.1㎛. (A)*B. animalis* B7, (B)*B. animalis* C1, (C)*B. breve* D2, (D)*B. pseudocatenulatum* E6, (E)*B. longum* E8, (F)*B. pseudocatenulatum* E9, and (G)*B. longum* F5.

2.3.2. Functional experiment for isolated *Bifidobacterium* strains

2.3.2.1. Acid and Bile tolerance

Probiotics should survive in acidic and bile conditions to adhere to the human mucus layer (Tuo et al., 2018). We verified the abilities of *Bifidobacterium* to survive gastrointestinal conditions in a timely manner. Isolated *Bifidobacterium* strains survived after 3h of exposure to acid MRS reflecting the stomach condition (Figure4). Especially, *B. animalis* C1 and *B. breve* D2 showed outstanding survival rates (about 76%). Moreover, *B. animalis* B7, *B. pseudocatenulatum* E9, and *B. longum* F5 have a notable abilities like LGG and BB12. Although *B. pseudocatenulatum* E6 and *B. longum* E8 fall short of positive controls including LGG and BB12, they had survival rates higher than 69%, suggesting that they have a tolerance to an acid condition.

When we confirmed the bile tolerance, the abilities of *Bifidobacterium* strains have a different tendency compared with the acid tolerance activity. All *Bifidobacterium* strains had lower ability than LGG, whereas they have comparable to BB12. These results were due to the different mechanisms used by *Lacticaseibacillus* and *Bifidobacterium* to endure the effect of bile (Tejero-Sariñena et al., 2012). Some *Bifidobacterium* strains including *B. animalis* C1, *B. longum* E8, *B. pseudocatenulatum* E9, and *B. longum* F5 had bile tolerance activity similar to

BB12. The others including *B. animalis* B7, *B. breve* D2, and *B. pseudocatenulatum* E6 showed 81%-82% of survival rates which were lower than BB12. In conclusion, isolated *Bifidobacterium* strains from infant fecal presented outstanding survival capacity in acidic and bile tolerance experiments.

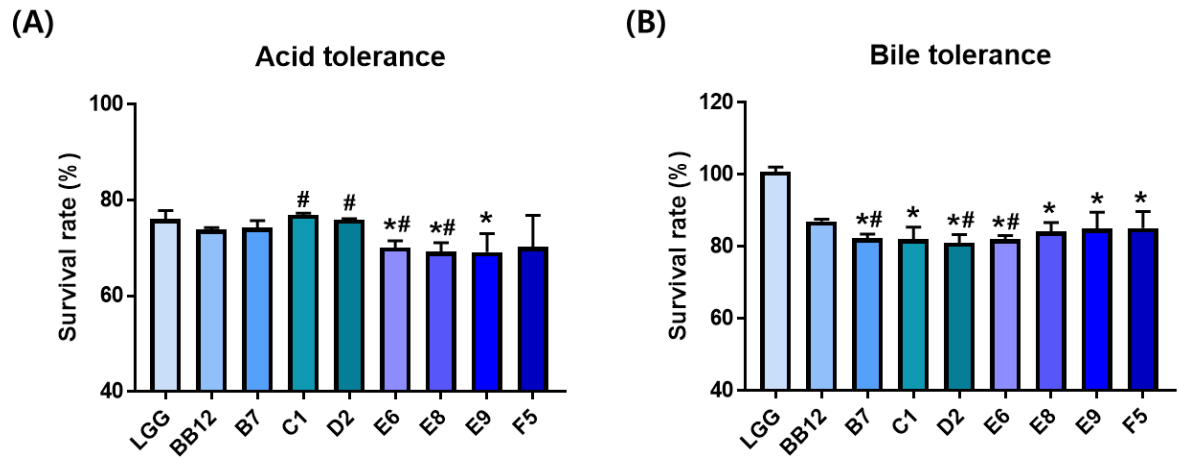

Figure 4. The survival cells of *Bifidobacterium* strains after (A)acid stress in pH 2.5 and (B)bile stress in 0.5% bile (w/v) concentration.

L. rhamnosus GG was used as a positive control strain in these experiments. B7, *B. animalis*; C1, *B. animalis*; D2, *B. breve*; E6, *B. pseudocatenulatum*; E8, *B. longum*; E9 *B. pseudocatenulatum*; F5, *B. longum*. Statistical analysis was performed using ANOVA and differences were considered significant when p value was below 0.05 (* or #). P value in * is compared with LGG, and the p value in # is compared with BB12.

2.3.2.2. Antibiotic sensitivity

Antibiotic sensitivity was confirmed by susceptibility of bacteria to antibiotic through disc diffusion assay. The antibiotics including ampicillin, chloramphenicol, kanamycin, penicillin, and tetracycline and vancomycin were used for this study. All *Bifdiobacterium* strains had different patterns except kanamycin. Especially, *B. longum* E8, *B. longum* F5, and LGG had significant results because it was susceptible to most of the antibiotics except kanamycin and vancomycin. Moreover, *B. animalis* B7 and *B. breve* D2 showed an identical pattern in antibiotic sensitivity. These strains were susceptible to antibiotics including ampicillin, chloramphenicol, and penicillin, then they resistant to kanamycin and tetracycline. Furthermore, *B. animalis* C1 was not susceptible to any antibiotics. Although the *B. pseudocatenulatum* strains including E6 and E9 were the same species, they showed different tendencies in antibiotic sensitivity.

Table 3. Antibiotic susceptibility pattern of *Bifidobacterium* strains and *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactic* BB-12 by using antibiotic discs including ampicillin (10 g), chloramphenicol (30 g), kanamycin (30 g), penicillin (10 g), tetracycline (30 g), and vancomycin (30 g).

Variable	Ampicillin	Chloramphenicol	Kanamycin	Penicillin	Tetracycline	Vancomycin
B. animalis B7	S	S	R	S	R	I
B. animalis C1	I	I	R	I	I	R
B. breve D2	S	S	R	S	R	I
B. pseudocatenulatum E6	I	I	R	S	S	R
B. longum E8	S	S	R	S	S	R
B. pseudocatenulatum E9	S	I	R	S	S	R
B. longum F5	S	S	R	S	S	R
B. animlais subsp. *Lactis BB-12*	S	S	R	S	S	S
L. rhamnosus GG	S	S	R	S	S	R

Antibiotic discs included ampicillin (10 µg), chloramphenicol (30 µg), kanamycin (30 µg), penicillin (10 µg), tetracycline (30 µg), and vancomycin (30 µg).

Antiabiotic resistance was evaluated by disc diffusion (Inhibition zone diameter);

S: >20 (mm), I: 15-19 (mm), R: ≤ 14 (mm).

S, suceptible; I, intermediate; R, resistant

2.3.2.3. Antibacterial activity

Antibacterial activity is necessary to eliminate or inhibit pathogenic bacteria by competing for attachment (Vine et al., 2004). Here, we investigated antibacterial activity using 4 pathogenic bacteria including *L. monocytogenes* EGD-E, *S. aureus* Newman, *S. typhimurium* SL1344, and *E. coli* ATCC35150. As a result, *Bifidobacterium* strains showed remarkable antibacterial activity against *E. coli* ATCC35150 and *S. typhimurium* SL1344. Moreover, *B. animalis* C1 and *B. animalis* D2 had antibacterial activity against *S. aureus* Newman. Lastly, only *B. longum* E8 has antibacterial activity against *L. monocytogenes* EGD-e except for *L. rhamnosus* GG. The antibacterial activity of *Bifidobacterium* is different depending on the strains, but usually isolated *Bifidobacterium* strains from infant fecal had antibacterial activity against 2 or 3 pathogenic bacteria.

Table 4. Inhibitory effect of *Bifidobacterium* strains, LGG, BB12 against pathogenic bacteria including *Listeria monocytogenes* EGD-e, *Staphylococcus aureus* Newman, *Salmonella Typhimurium* SL1344, and *Escherichia coli* ATCC 35150.

Variable	*L. monocytogenes* EGD-e	*S. aureus* Newman	*S. typhimurium* SL1344	*E. coli* ATCC 35150
B. animalis B7	-	-	++	+
B. animalis C1	-	+	+	++
B. breve D2	-	+	+	++
B. pseudocatenulatum E6	-	-	+	++
B. longum E8	+	-	+	++
B. pseudocatenulatum E9	-	-	+	+
B. longum F5	-	-	+	++
B. animlais subsp. *Lactis BB-12*	-	-	+	++
L. rhamnosus GG	-	-	+	+++

Pathogenic bacteria included *Listeria monocytogenes* EGD-e, *Staphylococcus aureus* Newman, *Salmonella Typhimurium* SL1344, and *Escherichia coli* ATCC 35150.

Antibacterial activity was evaluated by spot assay (Inhibition zone diameter); None (-): <5 (mm), Weak (+): 5-10 (mm), Middle (++); 11-17 (mm 0, Strong (+++); >17mm.

2.3.2.4. Adhesion ability using HT-29 cell line and *C. elegans* model

Adhesion ability is necessary for the survival and proliferation of probiotics. In this study, we identified the adhesion ability to HT29 cell and *C. elegans* model. When we used *C. elegans* model, we compared with LGG which is used as a commensal probiotic strain and *E. coli* OP50 as a negative control. In the *C. elegans* adhesion assay, *B. animalis* D2, *B. pseudocatenulatum* E6 had significant ability to adhere to *C. elegans* mucus layer. Moreover, *B. animalis* C1 had remarkable ability similar to LGG, while *B. longum* E8 had a lower ability compared to *E. coli* OP50. Lastly, *B. animalis* B7, *B. pseudocatenulatum* E9, and *B. longum* F5 had adhesion ability similar to *E. coli* OP50.

Next, we attempted to investigate the adhesion ability of *Bifidobacterium* strains by using HT29 cell. Unlike the *C. elegans* adhesion assay, *Bifidobacterium* strains had outstanding adhesion ability similar to LGG in the HT-29 adhesion. Especially, *B. animalis* B7 had higher ability compared with LGG. In conclusion, *Bifidobacterium* strains isolated from infant fecal had a notable ability to adhere to *C. elegans* and HT29 cell.

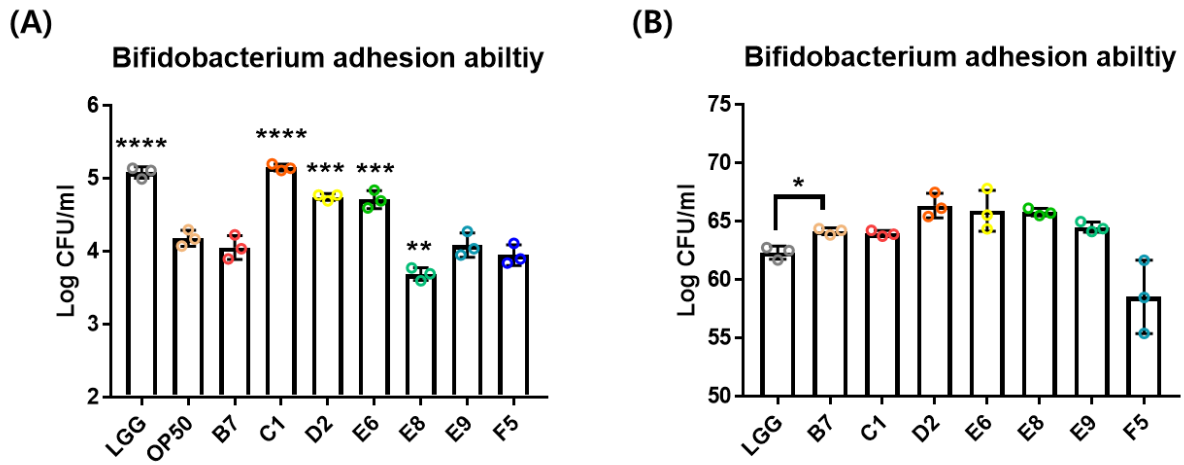

Figure 5. Adhesion ability of *Bifidobacterium* strains and *Lactobacillus rhamnosus* GG in the mucus layer on (A)HT29 cell line and (B)*Caenorhabditis elegans* model. B7, *B. animalis*; C1, *B. animalis*; D2, *B. animalis*; E6, *B. pseudocatenulatum*; E8, *B. longum*; E9 *B. pseudocatenulatum*; F5, *B. longum*. Statical analysis was performed using ANOVA, and differences were considered significant when p was below 0.0001 (****), 0.001 (***), 0.01 (**), and 0.05 (*). Data are expressed as the mean±SD of three independent experiments.

2.3.2.5. *C. elegans* lifespan

In previous experiments, we investigated that *Bifidobacterium* strains had a remarkable ability as a probiotic. Then we evaluated whether the *Bifidobacteirum* strains had beneficial effects on the *C. elegans* model (Figure 6). In the lifespan assay, *Bifidobacterium* strains including two strains of *B. animalis*, two strains of *B. pseudocatenulatum*, two strains of *B. longum*, and one strain of *B. animalis* were examined. In this study, we identified that all *Bifidobacterium* strains increased the lifespan similar to LGG which was used in a positive control LGG. Moreover, their effects were distinguished from *E. coli* OP50 excepting *B. animalis* C1.

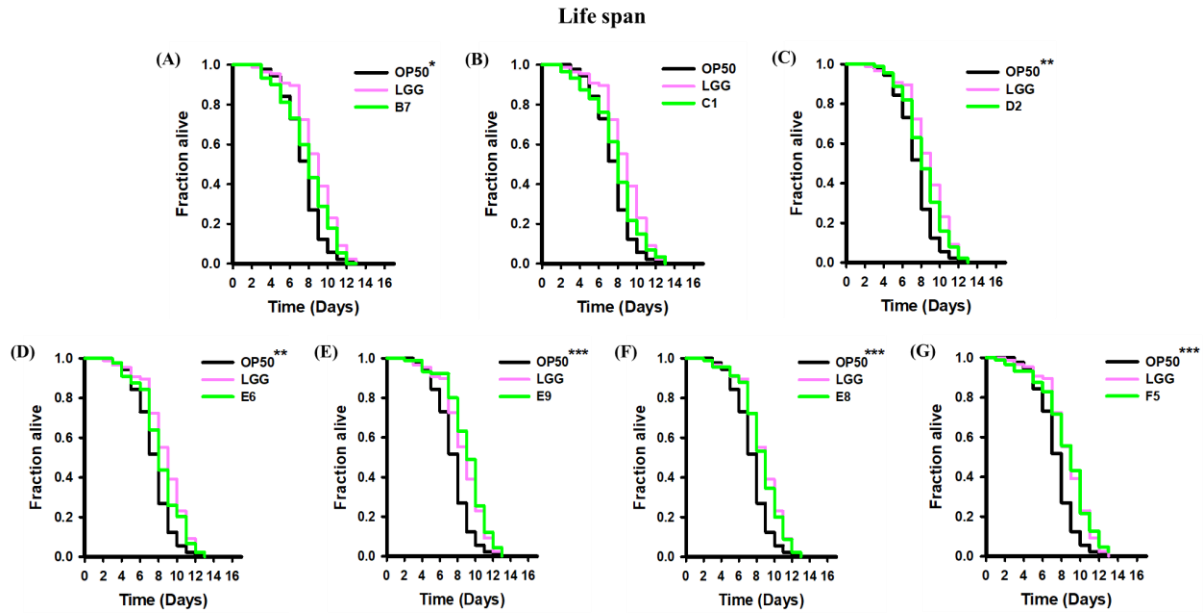

Figure 6. Enhanced lifespan of *C. elegans fer-15;fem-1* via conditioning with *Bifidobacterium* strains.

Life span assays (n=30 per plate) of *C. elegans* strains fer-15; fem-1 exposed to *Bifidobacterium* strains. Statistical analysis was performed using Kaplan-Meier method, and differences were considered significant when p was below 0.05. The p-value in black is compared with *E. coli* OP50, and the p-value in pink is compared with LGG. (A) B7, *B. animalis*, (B) C1, *B. animalis*, (C) D2, *B. animalis* (D) E6, *B. pseudocatenulatum*, (E) E8, *B. longum*, (F) E9 *B. pseudocatenulatum*, (G) F5, *B. longum*.

2.3.2.6. *C. elegans* killing assay

Bifidobacterium strains were examined killing assays to investigate whether the isolates affected host resistance against pathogenic bacteria in *C. elegans* model after lifespan analysis. The *C. elegans* was transferred to *S. aureus* Newman or *E. coli* O157:H7 EDL933 after exposure to *Bifidobacterium* isolates for 1 day. The pathogenic bacteria kill the nematode through an infection process in the intestine (Irazoqui et al., 2010). The *Bifidobacterium* strains could not extend the lifespan when the *C. elegans* was exposed to *E. coli* O157:H7 EDL933 compared with *E. coli* OP50. However, the *Bifidobacterium* strains including *B. animalis* D2, *B. pseudocatenulatum* E6 were similar to LGG control. Moreover, *Bifidobacterium* strains extended the lifespan when the *C. elegans* was exposed to *S. aureus* Newman. The *E. coli* OP50 treatment which indicates negative control showed 9 days lifespan extension after transfer to the *S. aureus* Newman. However, the *Bifidobacterium* strains treatment showed 11~14 days lifespan extension. The *Bifidobacterium* strains were significantly distinguished from *E. coli* OP50 treatment generally.

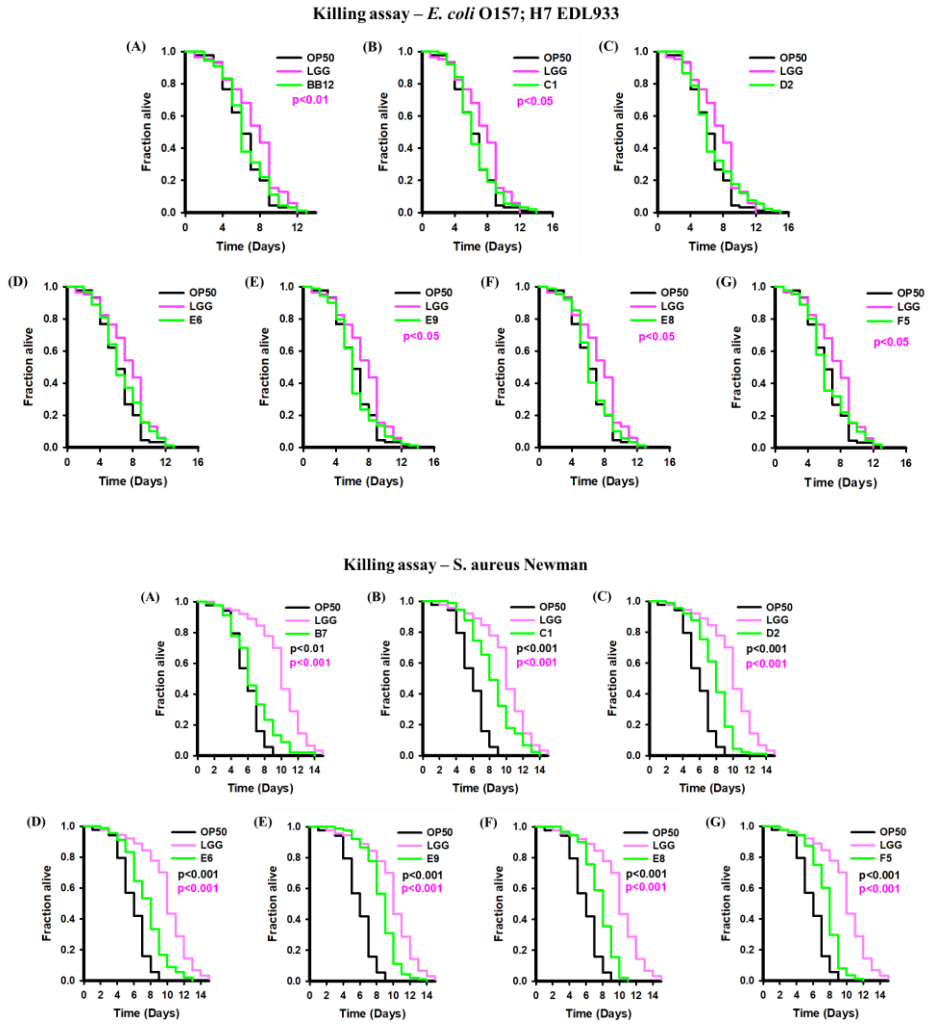

Figure 7. Immune stimulation of *C. elegans* via pre-conditioning with *Bifidobacterium* strains for 24 h prolonged the lifespan of *C. elegans fer-15;fem-1* infected with *Escherichia coli* O157:H7 and *Staphylococcus aureus* Newman.

Preconditioning with isolates from infant feces prolonged the life span of *C. elegans* infected with *E. coli* O157:H7 and *S. aureus*. Statistical analysis was

performed using Kaplan-Meier method, and differences were considered significant when p was below 0.05. The p-value in black is compared with *E. coli* OP50, and the p-value in pink is compared with LGG. (A) B7, *B. animalis*, (B) C1, *B. animalis*, (C) D2, *B. animalis* (D) E6, *B. pseudocatenulatum*, (E) E8, *B. longum*, (F) E9 *B. pseudocatenulatum*, (G) F5, *B. longum*.

2.3.3. FIMM assay

2.3.3.1. Short-chain fatty acid analysis

In comparison to normal control, *Bifidobacterium* strains have no significant differences in SCFAs except acetic and lactic acid. *B. animalis* D2 and *B. longum* F5 were similar to normal in acetic acid, whereas *B. pseudocatenulatum* E6 was lower (Figure 8A). *B. animalis* D2 and *B. longum* F5, on the other hand, significantly increased the level of lactic acid, but *B. pseudocatenulatum* E6 had no effect when compared to normal. It is assumed that there was no significant difference in the *B. pseudocatenulatum* E6 due to the large variance across samples (Figure 8B). There were no significant changes between treatments when we identified the colon stage media for pH alteration (Zhao et al., 2018). Because pH changes reflect SCFA production levels, these findings are in accordance with the findings that there are no differences in most SCFAs except acetic and lactic acid.

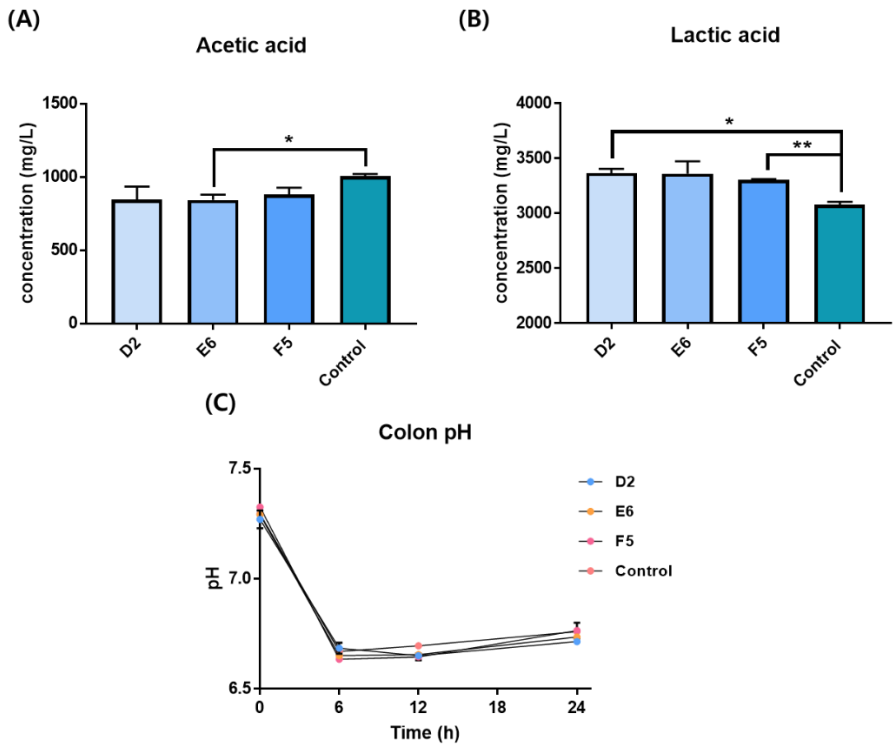

Figure 8. Short chain fatty acids concentrations including (A)acetic acid and (B)lactic acid and (C)pH changes of FIMM assay colon medium of each *Bifidobacterium* strains.

SCFAs alteration after treatment of *Bifidobacterium* strains including *B. animalis* D2, *B. pseudocatenulatum* E6, and *B. longum* F5 in FIMM assay. (A) Acetic acid and (B)lactic acid alteration were showed and (C) pH alteration was indicated. Statical analysis was performed using ANOVA, and differences were considered significant when p was below 0.01 (**), and 0.05 (*). Data are expressed as the mean±SD of three independent experiments.

2.3.3.2. Metagenome analysis

At the phylum level, Firmicutes were most abundant in every FIMM assay sample. However, *B. animalis* D2 treatment showed a reduction in Firmicutes in phylum level, despite other *Bifidobacterium* strains did not show noticeable changes (Figure 9A). At the Family level, *B. pseudocatenulatum* E6 and *B. longum* F5 have a microbial composition similar to control, however, *B. animalis* D2 was slightly different in *Lactobacillaceae* and *Bifidobacteriaveae* (Figure 9B). Moreover, *B. pseudocatenulatum* E6 and *B. longum* F5 showed similar microbial composition to the control, despite *B. animalis* D2 having a different composition. However, *B. pseudocatenulatum* E6 and *B. longum* F5 showed different patterns in microbial composition except for *Lactiplantibacillus* and *Bifidobacterium*. There are no remarkable changes in microbial composition because FIMM system did not reflect the human digestion system sufficiently. Because the change was observed in SCFAs that indicates metabolic changes, animal experiments were used to confirm the findings.

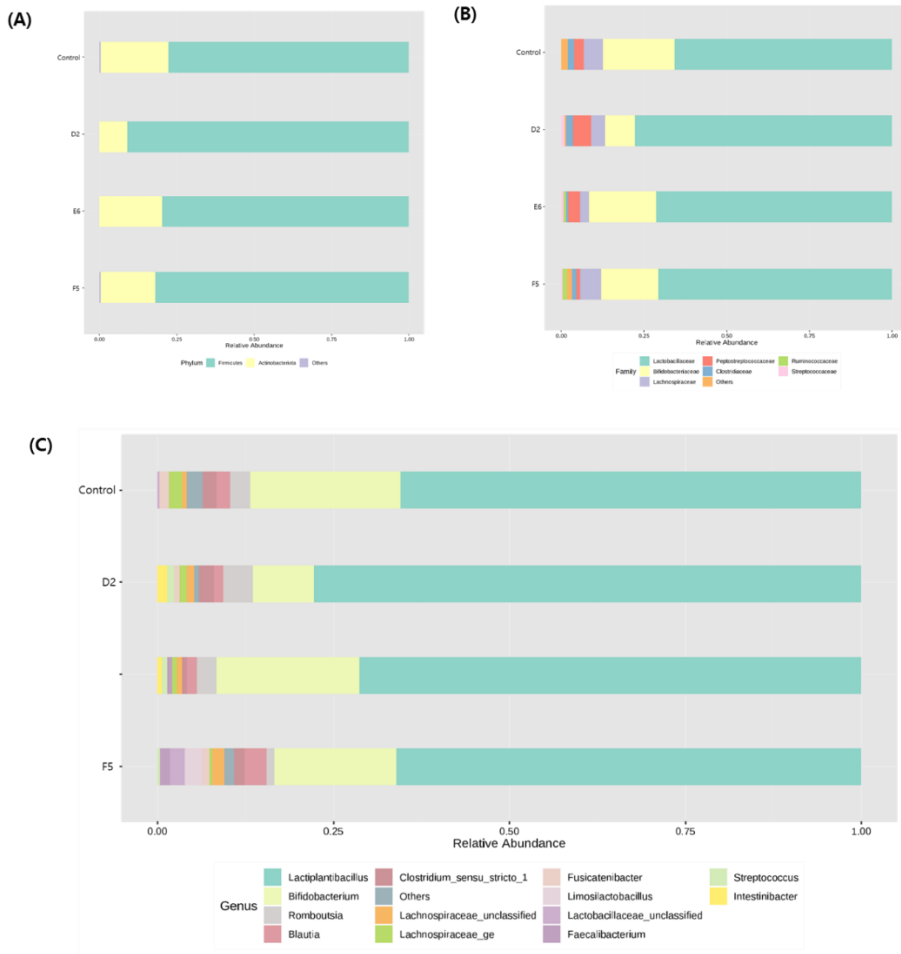

Figure 9. Next-generation sequencing of the 16S rRNA gene library demonstrates the relative abundance composition of FIMM fecal slurry sample in a phylum, order, and genus taxa.

Distribution of microbial composition detected in FIMM assay samples. Bar graph displaying the proportion of (A) phyla, (B) taxa, (C) genera composition of samples. D2, *B. animalis*; E6, *B. pseudocatenulatum*; F5, *B. longum*.

2.3.4. *In vivo* study

2.3.4.1. Phenotypic analysis

To determine the potential effect of *Bifidobacterium* on mice, we characterized the *Bifidobacterium* strains treatment groups compared to control group. In body weight gain results, all groups including the control, antibiotic, and *Bifidobacterium* treatment group were not different significantly. It is probably due to large variance of normal group. However, colon length associated with the gut microbiome showed changes compared in comparison with antibiotic treatment groups. Especially, *B. pseudocatenulatum* E6 and *B. lonugm* F5 treatment extended the colon length more than normal group. In addition, *B. pseudocatenulatum* E6 has an ability similar to LGG.

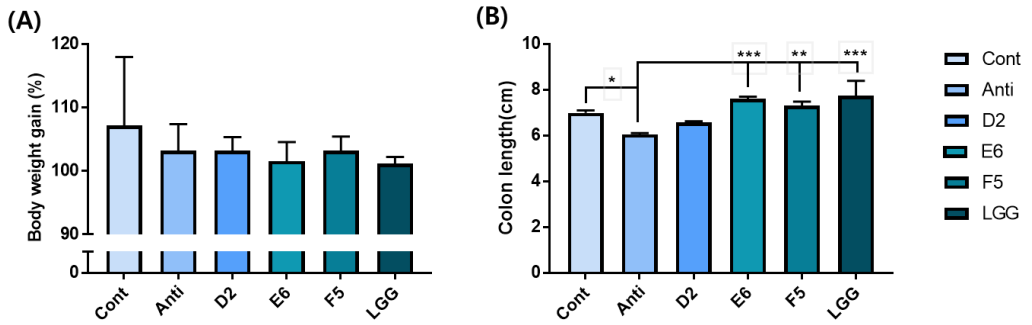

Figure 10. Phenotypic characteristics including body weight, and colon length in mice after administration of *B. animalis* D2, *B. pseudocatenulatum* E6, *B. longum* F5, *Lactobacillus rhamnosus* GG.

Phenotypic characteristic of (A) body weight gain and (B) Colon length D2, B. animalis; E6, *B. pseudocatenulatum*; F5, *B. longum*. Statical analysis was performed using ANOVA, and differences were considered significant when p was below 0.001 (***), 0.01 (**), and 0.05 (*). Data are expressed as the mean±SD of three independent experiments. Cont, normal control; Anti, antibiotic control; D2, B. animalis; E6, *B. pseudocatenulatum*; F5, *B. longum* LGG, *Lactobacillus rhamnosus* GG.

2.3.4.2. Serum biochemical analysis

The serum biochemicals including AST and ALT were investigated through Dri-chem Analyzer. Antibiotic treatment showed a tendency to increase, however, they did not present a significant difference. By the way, *Bifidobacterium* strains had an effect on reducing the ischemic injury score remarkably. Specifically, *B. animalis* D2 and *B. longum* F5 had outstanding results in both AST and ALT scores (Figure 11A).

Next, we identified serum lipid concentration by assessing the TG/TCHO and HDLC/TCHO ratio. Antibiotic treatment presented a lower amount of serum lipid and glucose. In specific, all *Bifidobacteirum* treatment groups indicated a higher TG/TCHO ratio than antibiotic treatment as well as LGG treatment group. Furthermore, *B. anim*alis D2 and *B. longum* F5 also influenced to HDLC/TCHO ratio (Figure 11B, C).

In the ELISA assay, we figured out the concentration of inflammatory cytokines including TNF-α, IL-1ß, and IL-6. Overall, *Bifidobacterium* strains showed a tendency to reduce the examined cytokines. While *B. longum* F5 presented a reduced tendency, it was not different significantly compared with antibiotic treatment. However, *B. animalis* D2 and *B. pseudocatenulatum* E6 had a distinct difference in IL-1ß and IL-6.

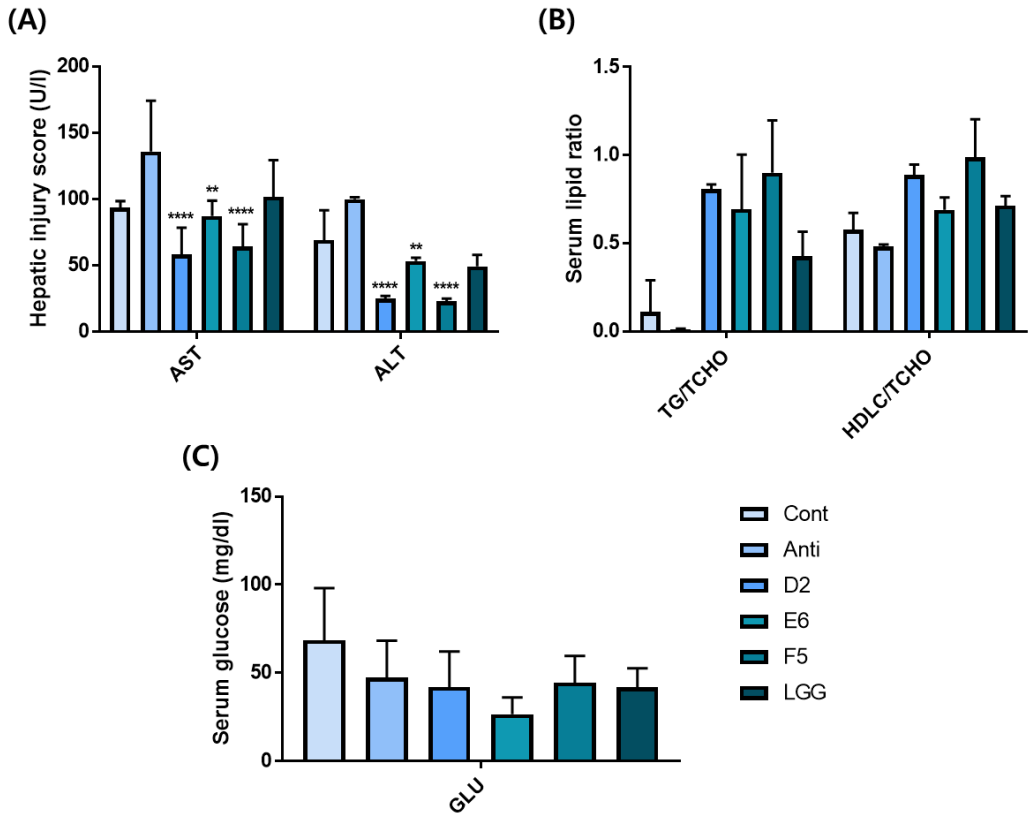

Figure 11. Serum metabolic biomarkers including ALT, AST, HDLC, TG, TCHO, GLU by using Dri-chem Analyzer after administration of *B. animalis* D2, *B. pseudocatenulatum* E6, *B. longum* F5, *Lactobacillus rhamnosus* GG.

Serum metabolic biomarkers (A) AST and ALT, (B) TG/TCHO and HDLC/TCHO, (C)GLU. Statical analysis was performed using ANOVA, and differences were considered significant when p was below 0.0001 (****), 0.01 (**). Data are

expressed as the mean±SD of three independent experiments. Cont, normal control; Anti, antibiotic control; D2, *B. animalis*; E6, *B. pseudocatenulatum*; F5, *B. longum* LGG, *Lactobacillus rhamnosus* GG.

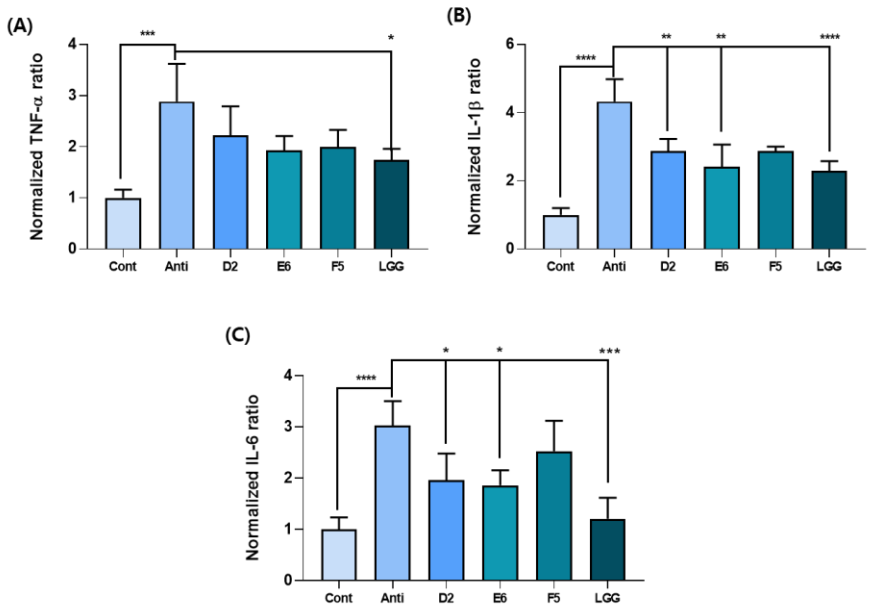

Figure 12. Serum Metabolic biomarkers including IL-1β, IL-6, and TNF-αBy using Elisa after administration of *B. animalis* D2, *B. pseudocatenulatum* E6, *B. longum* F5, *Lactobacillus rhamnosus* GG.

Serum Metabolic biomarkers (A) TNF-α, (B) IL-1ß and (C) IL-6. Statical analysis was performed using ANOVA, and differences were considered significant when p was below 0.0001 (****), 0.001 (***), 0.01 (**), and 0.05 (*). Data are expressed as the mean±SD of three independent experiments. Cont, normal control; Anti, antibiotic control; D2, *B. animalis*; E6, *B. pseudocatenulatum*; F5, *B. longum* LGG, *Lactobacillus rhamnosus* GG.

2.3.4.3. Metabolites analysis

Fecal samples of *in vivo* experiments were tested for metabolites analysis. *Bifidobacterium* treatment groups were separated with each strain, moreover, they separated with LGG and antibiotic treatment group. It means *Bifidobacterium* treatment influenced to metabolites associated with intestine.

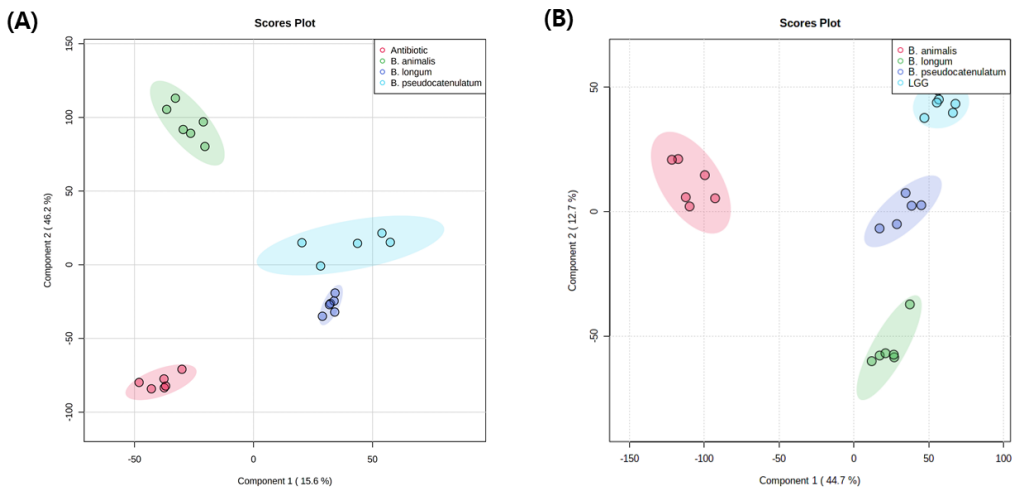

Figure 13. PCA score plot depicts the clustering patterns from the metabolite concentration data of *Bifidobacterium* strains and *Lactobacillus rhamnosus* GG.

PCA score plot revealed a separated cluster of each 5 groups. The PLS-DA score plot suggests that it is possible to discriminate between *Bifidobacterium* groups. Anti, antibiotic control; D2, *B. animalis*; E6, *B. pseudocatenulatum*; F5, *B. longum* LGG, *Lactobacillus rhamnosus* GG.

2.3.4.4. Metagenome analysis

When we investigated the fecal microbiota composition, proteobacteria increased in antibiotic treatment group contrary to bacteroidota in the phylum level. On the contrary, *Bifidobacterium* treatment groups maintained composition like normal group. Specifically, bacteroidota and firmicutes were abundant compositions, moreover, proteobacteria existed in small proportion. Furthermore, Muribaculaceae were prevalent in *Bifidobacteirum* treatment groups like normal at the family level. On the other hand, Enterobacteriaceae increased in antibiotic treatment group. Lastly, Muribaculaceae were observed in *Bifidobacterium* treatment groups matched with the family level result. Moreover, Enterobacteriaceae was enriched in antibiotic treatment group correlated with family level (Figure 14).

The diversity of microbiota represented intestinal homeostasis. In this respect, antibiotic treatment group had lower diversity than normal. However, *B. pseudocatenulatum* E6 and *B. longum* F5 recovered the diversity of fecal microbiota in the Chao1 and Shannon index. Moreover, all groups showed separated clusters individually (Figure15).

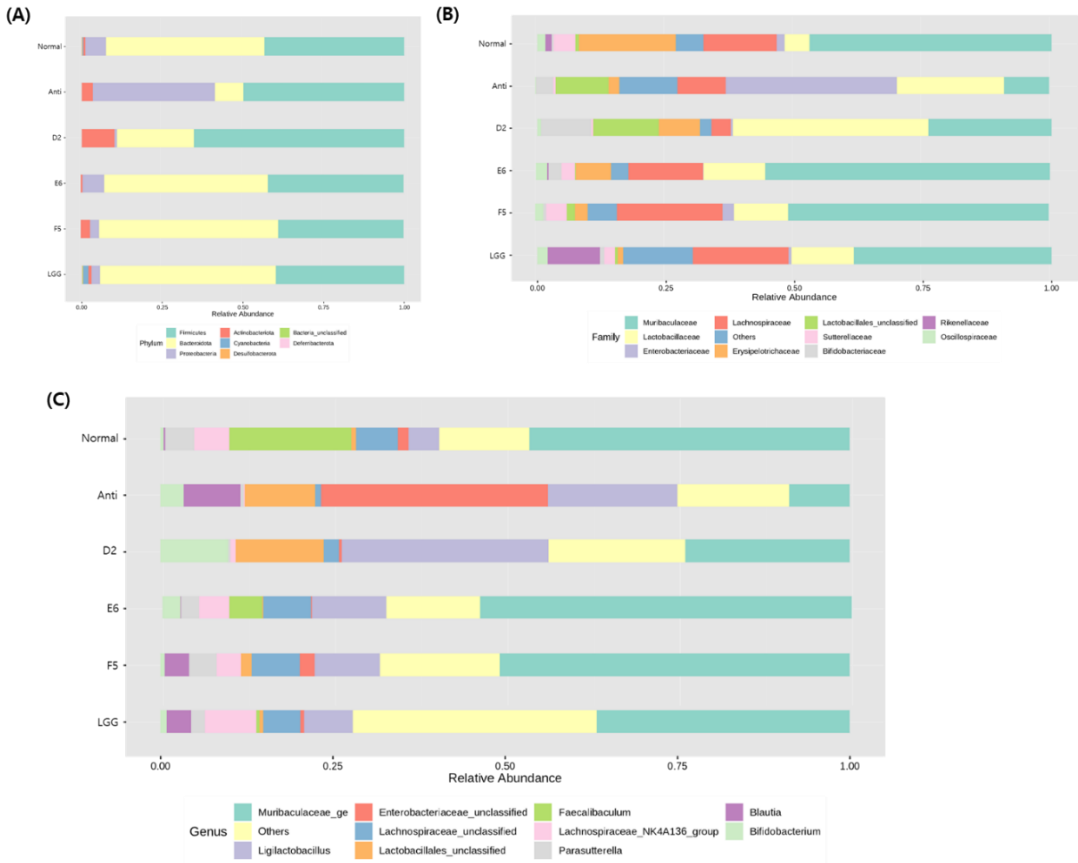

Figure 14. Next-generation sequencing of the 16S rRNA gene library demonstrates the relative abundance composition of the mice fecal samples in a phylum, order, and genus taxa.

Distribution of microbial composition detected in *in vivo* samples. Bar graph displaying the proportion of (A) phyla, (B) taxa, (C) genera composition of samples. Cont, normal control; Anti, antibiotic control; D2, *B. animalis*; E6, *B. pseudocatenulatum*; F5, *B. longum* LGG, *Lactobacillus rhamnosus* GG.

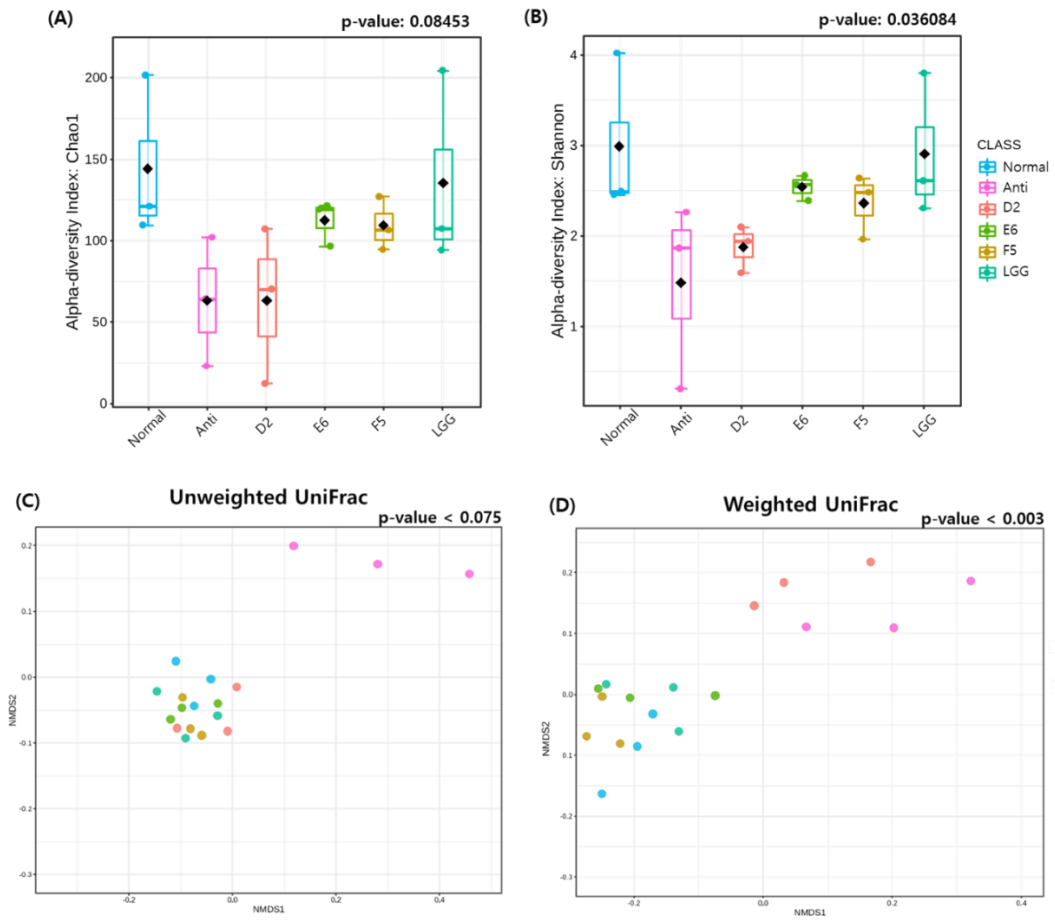

Figure 15. Analysis of different microbial α-diversity including Chao 1 and Shannon indices and β-diversity indices in the six experimental groups.

The Chao1 (A) index was used as richness estimators. The Shannon (B) index was used as diversity estimators. Principal Coordinates Analysis (PCoA) was

based on the Unweighted UniFrac (C) and weighted UniFrac (D) distance matrix generated from all the samples in each group. Cont, normal control; Anti, antibiotic control; D2, *B. animalis*; E6, *B. pseudocatenulatum*; F5, *B. longum* LGG, *Lactobacillus rhamnosus* GG.

2.4. Discussion

Human gut microbiota plays a crucial role in the human health, however, a lot of their functional characteristics did not defined specifically (Bäckhed et al., 2012). Therefore, many researches in various field are being progressed until now. For example, type 2 diabetes and inflammatory bowel disease could detected by alteration of gut microbiome composition to the metabolic syndrome associated to metabolic syndrome (Qin et al., 2014). In this sense, gut microbiome may be a tool of the diagnosis of disease. Moreover, gut microbiome have an impact on the brain as a way of induction of inflammation in central nervous and production of neurotoxic metabolites regards the gut-brain axis (Galland, 2014). Furthermore, gut microbiome functional modules including amino acid transporters and vitamic metabolism were change in cardiovascular diseases (Yoshida et al., 2018). Thus, gut microbiome is still being studied in various areas.

In this respect *Bifidobacterium* is one of the important components in human gut microbiome that has beneficial functions in human health. *Bifidobacterium* has feature that is reduction of composition as people get older. Especially, *Bifidobacterium* has reduction point in accordance with ageing with prevalence (Kato et al., 2017). Meanwhile, *Bifidobacterium* is abundant in infant gut microbiome to have an effect on immune response in infant having an immature

immune system (Huda et al., 2019; Mullié et al., 2004). Moreover, *Bifidobacterium* has a number of beneficial effect on human such as metabolic syndrome, antiobesity, immunoregulatory effect, and cognitive decline (An et al., 2011; Bernini et al., 2016; Groeger et al., 2013; Kobayashi et al., 2019). Threrfore, we isolated *Bifidobacterium* which has a beneficial effect on humans and identified its characteristics in this study.

We investigated microbial composition of infant fecals before we isolate the *Bifidobacterium* to identify the existence of them. When we confirmed the composition, two infants had different microbial composition. Infant G has a lot of *Bifidobacterium* genus, whereas infant N has various genus. It is due to their difference in breast feeding period, not by the age, sex, delivery mode. Because *Bifidobacterium* is dominant in breast fed infant fecal microbiota, while formula-fed infant has fecal microbiota more complex, with *Bifidobacterium* spp., Streptococcus spp., and Enterobacteria (Di Gioia et al., 2014). After we confirmed the composition of infant fecal, we isolated the *Bifidobacterium* from the fecal sample. When we isolated the *Bifidobacterium*, we can isolate 11 candidates through F6PPK assay. F6PPK assay detect the F6PPK enzyme that is involved in the *Bifidobacterium* to identify the *Bifidobacterium* (Vlková et al., 2005). Next, we conducted MALDI-TOF/MS analysis and 16S rRNA sequencing to identify the candidates specifically. When we analyze them, total 7 *Bifidobacterium* strains

were confirmed including three strains of *Bifidobacteriu*m *animalis*, two strains of *Bifidobacterium pseudocatenulatum*, *Bifidobacterium longum*. Moreover, the *Bifidobacterium* strains were clustered in each same species in phylogenetic tree.

To verify the ability as a probiotic, we conducted functional experiments including tolerance of digestive condition, antibiotic sensitivity, antibacterial activity, adhesion ability and *C. elegans* experiments. In acid tolerace test, *B. animalis* strains including C1 and D2 has significant ability compared with LGG. Acidic condition is always provided to probiotics through stomach and probiotic fermentative processes. In previous study, only *B. animalis* spp. showed high ability to endure under the acid condition, whereas the acidic condition limit the growth of *Bifidobacterium* species (Sanz, 2007). Similar to the previous study, *B. animalis* strains showed stronger acid tolerance than other *Bifidobacterium* strains. Furthermore*, B. pseudocatenulatum* E9 and *B. longum* F5 had a similar ability to BB12. BB12 is the most commonly used as a probiotic among the Bifidobacterium and it has magnificent acid and bile tolerance (Jungersen et al., 2014). Because, *B. pseudocatenulatum* E9 and *B. longum* F5 showed bile tolerance similar to BB12, they has a capability as a probiotics.

Antibiotic usage is essential for many disease, however, antibiotic resistance in pathogenic bacteria is observed increasingly. Therefore, probiotics should not have

an antibiotic resistance and transfer the antibiotic resistance (Ouwehand et al., 2016). In this sense, we examined antibiotic resistance of isolated *Bifidobacteirum* strains. All *Bifidobacterium* strain didn't have an antibiotic resistance except kanamycin and vancomycin. In general, *Bifidobacterium* has antibiotic resistance in kanamycin, therefore, kanamycin used for selection of *Bifidobacterium* (Mayrhofer et al., 2011). Some *Bifidobacterium* strains showed resistance to vancomycin, in the same way that the number of *Bifidobacterium* derived from Korean has resistant to vancomycin recently (Moon et al., 2006).

After the probiotic passed through acid and bile condition in human digestive system, they must be attached to mucosal layer (Saito, 2004). To confirm the adhesion ability, we conducted experiments using *C. elegans* model and HT29 cell line. As a results, *B. animalis* C1, *B. animalis* D2, and *B. pseudocatenulatum* E6 showed significant adhesion ability compared with *E. coli* OP50 in *C. elegans* model. Moreover, *B. animalis* B7 has remarkable adhesion ability in HT29 cell line. In general, most of *Bifidobacterium* strains had a higher tendency compared with LGG in HT-29 and some *Bifidobacterium* has similar ability with LGG in *C. elegans* model.

Because the *C. elegans* model has a lot of biological processes similar to human, *C. elegans* model were used as a drug-target interation and a number of human

diseases (O'Rourke et al., 2009). *C. elegans* model is often used in laboratory, because of its convenient characteristic such as short life span, self-fertilize, and high reproductive rate (Ruszkiewicz et al., 2018). In *C. elegans* lifespan, all *Bifidobacterium* strains extended *C. elegans* life span compared with *E. coli* OP50. In the killing assay, *Bifidobacterium* strains showed different results in each pathogenic bacteria. In this study, *Bifidobacterium* strains had the ability to reduce the S. aureus Neman infection, whereas they had weak ability to attenuate the *E. coli* O157:H7. In conclusion, *C. elegans* was influenced by *Bifidobacterium* strains in lifespan extension and S. aureus Newman infection.

There is little question that the gut microbiota plays a significant role in host health. Microbiota, which include bacteria, viruses, and fungi, are found throughout the gastrointestinal system and have a two-sided influence on human metabolism depending on their composition. Gut dysbiosis is a disruption or imbalance of changes in the kinds of bacteria that may contribute to the development of illnesses such as systemic inflammation, which is one of the most detrimental representative conditions that gut microbiota effects the host (Marchesi et al., 2016; Tremaroli & Bäckhed, 2012). Gut dysbiosis can be caused by a variety of factors, including dietary changes, heredity, stress, medicine, illness, and so on. As a result, a large number of studies have been conducted to prevent or cure microbial imbalances, with probiotics playing a prominent role, probiotics are living microorganisms that,

when administered, provide health advantages to the host (Marchesi et al., 2016; Myers & Hawrelak, 2004). In this sense, we administrated three strains of probiotic *Bifidobacterium* and *Lacticaseibacillus rhamnosus* GG to antibiotic-cocktail induced gut dysbiosis mice model in this study.

Antibiotics are detrimental to pathogenic bacteria but when they are treated to normal condition, the commensal bacteria are damaged and even the short-term administration of antibiotics may shift the microbial composition and disrupt the host metabolism, inducing systemic inflammation (Sun et al., 2019). To begin with, we examined the phenotypic changes in gut dysbiosis mice model induced by antibiotics. Antibiotics treatment reduced the body weight gain and shortened the colon length compared to normal, which is one of the representative biological markers in the assessment of colonic inflammation (Lee et al., 2021; Miao et al., 2020; Sun et al., 2019). The short term treatment of probiotic *Bifidobacterium* and LGG could not make changes to the body weight gain, however, it was enough to recover the shortened colon length. All *Bifidobacterium* were able to lengthen the colon length, especially the *B. pseudocatenulatum* E6 and *B. lonugm* F5 were significant, suggesting the similar colonic inflammation recovery potential to the control probiotics, LGG.

Gut microbiota dysbiosis is frequently associated with liver failure via the gut-liver axis. Gut dysbiosis and inflammation increase intestinal permeability, allowing inflammatory bacterial metabolites and bacteria to enter the systemic circulation and cause liver injury. Since the liver is essential in lipid and metabolism, when it is injured, the lipid circulation mediated by lipoprotein production is disrupted, resulting in aberrant lipid homeostasis (Gong et al., 2021; S.-E. Kim et al., 2021; Lange et al., 2016; Poteres et al., 2020). As a result, the antibiotics cocktail treated group demonstrated the tendency to increase in hepatic injury and the serum TG/TCHO and HDLC/TCHO ratios were the lowest among all groups, indicating that liver function failure occurred with lowered storage and transport of fatty acid within cells. On the other hand, *Bifidobacterium* and LGG treatment alleviated the hepatic injury and restored the serum lipid ratio. Though the TG/TCHO ratio was significantly increased, so did HDLC/TCHO ratio, which could be interpreted as restoration of lipoprotein synthesis mechanism.

However, the glucose level of all treatment groups were decreased compared to normal, and probiotic treatments could not rescue the glucose level significantly. Still, the conditions of diets low in fat distribution and the previous researches demonstrated the high utilization of glucose effect of *Bifidobacterium* and LGG under hyper-lipid should be considered (Aoki et al., 2017; Briczinski et al., 2008; Kim et al., 2013).

Then, using serum inflammatory cytokines, we assessed the systemic inflammation caused by antibiotic gut dysbiosis and the relieving impact of probiotics. Antibiotics substantially raised systemic inflammation, whereas *Bifidobacterium* and LGG treatment reduced the amount of pro-inflammatory cytokines. *B. animalis* D2 and *B. pseudocatenulatum* E6 were particularly effective in decreasing IL-1ß and IL-6 levels, indicating their significance in defending against inflammatory stimuli and regulating immunological response.

Along with the physiological changes induced by antibiotic gut dysbiosis, probiotics treatment made meaningful changes in fecal microbial compositions. Gut microbiota is known to utilize sophisticated intercommunications to sustain their niches. Therefore, the microbial network composed of diverse species with intrinsic function is considered to be an essential indicator of the homeostasis (Li & Tian, 2016; Ohland & Jobin, 2015). In metabolites analysis, *Bifidobacterium* treatment group exhibited distinctive cluster of fecal metabolites compared with antibiotics treatment and LGG. This finding suggests that both *Bifidobacterium* and LGG were effectively colonized and benefits the host but they worked in a distinguished way from each other, which makes *Bifidobacterium* a distinct probiotic strain.

In metagenome analysis, all probiotic groups fecal microbiota were clustered apart from the antibiotic groups and antibiotic treatment reduced in-group microbiota diversity while *B. pseudocatenulatum* E6 and *B. longum* F5 recovered the diversity of fecal microbiota comparable to the normal group. In details, antibiotics group fecal microbiota was abundant in *Enterobacteriaceae*, which was studied to be abnormally increased in both human and mouse with inflammatory bowel disease (Altomare et al., 2019; Li & Tian, 2016; Munyaka et al., 2016). On the other hand, *Bifidobacterium* treatment reduced *Enterobacteriaceae*, while increased the compositon of *Ligilactobacillus* and *Muribaculaceae*, which is also known as s24-7, which both demonstrate functional probiotic characteristics such as enhancing longevity, alleviating immune response, anti-cancer and diversification of commensal bacteria (Chung et al., 2021; Fijan, 2014; Gupta et al., 2021; Jin et al., 2021; Mu et al., 2021; Smith et al., 2019).

Chapter III.

Conclusion and further stud

In conclusion, the present results suggest that infant feces-derived *Bifidobacterium* strains can survive in digestive conditions and adhere to the mucus layer. Moreover, *Bifidobacterium* influenced *C. elegans* lifespan extension and protection from bacterial pathogenicity. Moreover, FIMM assay showed metabolic changes such as lactic acid whereas it presents no significant changes in metagenome analysis. Lastly, *in vivo* experiments indicated that the composition and metabolites of gut microbiota were changed, moreover, *Bifidobacterium* regulates the immunological reactions. These results proved that *Bifidobacterium* strains isolated from infant feces are safe for human consumption. This study confirmed that *Bifidobacterium* strains had a beneficial effect on immune-related factors, but it did not confirm how they performed that. Therefore, more investigation is necessary to show how changes in the composition of the gut microbiome and metabolites are related to immune response. Since Bifidobacterium can alleviate a variety of diseases by regulating immune response changes, it is important to investigate if the immunomodulatory response identified in this study can actually ameliorate a

variety of ailments. Furthermore, the functional effect of *Bifidobacterium* not only effects of immune response but also other beneficial effects must be studied.

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Summary in Korean

장내 미생물 균총은 다양한 메커니즘을 통해 인간의 건강과 질병을 조절하는 중요한 역할을 한다. 특히, *Bifidobacterium*은 혐기성 균주로 다른 프로바이오틱스 균주와 비교하였을 때 다양한 종이 밝혀져 있지는 않지만 유아의 장내에 주로 존재하는 것으로 알려져 있어 다양한 기능의 프로바이오틱스 균주로 많이 사용되고 있다. 프로바이오틱스로서 *Bifidobacterium* 균주를 개발하기 위해서는 다양한 *Bifidobacterium* 균주를 분리하고, 프로바이오틱스로서 기능성과 장내 미생물에 미치는 영향을 평가하는 연구가 진행되어야 한다. 따라서 본 연구는 유아 분변에서 다양한 *Bifidobacterium* 균주를 분리하고 프로바이오틱스로서 균주가 갖는 기능성을 평가하며, 다양한 모델을 활용하여 장내 미생물 균총에 미치는 영향을 확인하였다.

*Bifidobacterium*을 분리하기 위해 모유 수유 기간이 상이한 유아의 분변에서 장내미생물 균총의 구성을 확인하였고, *Bifidobacterium*의 composition이 상대적으로 우수하였던 12개월 이상 모유 수유한 유아의 분변에서 분리를 진행하였다. 분리된 9개의 후보 균주를 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry(MALDI-TOF/MS) 분석과 16S ribosomal RNA sequencing을 통해 확인한 결과, *Bifidobacterium animalis* 3종, *Bifidobacterium lognum* 2종, 그리고 *Bifidobacterium*

pseudocatenulatum 2종을 분리하였다. 분리된 *Bifidobacterium* 균주는 계통도 분석과 Scanning electron microscope(SEM)를 통해 계통학적, 형태학적 특성을 확인한 결과 동종 간에는 계통학적 유사성을 가지고 있었으며, *Bifidobacterium*에 해당하는 rod-type의 형태학적 특성을 보였다. 분리된 모든 *Bifidobacterium* 균주는 기능성 평가를 진행하여 프로바이오틱스로서 기용성을 확인하였다. 먼저 내산성, 내담즙성을 확인한 결과, 각각 비처리군 대비 약 70%, 80%의 생존율을 보였으며, 특히 내산성에서는 *Bifidobacterium animalis* B7, 내담즙성에서는 *Bifidobacterium longum* F5가 가장 높은 생존율을 보였다. 항생제 내성을 확인해보았을 때, kanamycin과 vancomycin, tetracycline을 제외한 모든 항생제에서 *Bifidobacterium* 균주가 저항성을 보이지 않았으며, tetracycline에서는 *Bifidobacterium animalis* B7과 *Bifidobacterium animalis* D2만 저항성을 나타냈다. 항균활성 실험을 진행한 결과, 모든 균주가 병원성 균인 *Salmonella typimurium* SL1344와 *Escherichia coli* ATCC 35150를 저해하는 것을 확인하였고, 장부착능을 확인한 결과, *Caenorhabditis elegans*(*C. elegans*) 모델에서는 *Bifidobacterium animalis* C1, D2, 그리고 *Bifidobacterium pseudocatenulatum* E6이 가장 높은 부착능력을 가지고, HT-29 세포주에서는 *Bifidobacterium animalis* B7이 대조 균주인 *Lactobacillus rhamnosus* GG 대비 유의적으로 부착능을 증가시켰다. 마지막으로 *C. elegans* 모델을 활용하여 *Bifidobacterium* 균주가 숙주에 긍정적인 효과를 가져오는지 확인해보았을 때, *Bifidobacterium animalis* C1을 제외한 모든 균주가 *Escherichia coli* OP50 균주 대비 *C. elegans*의 수

명을 연장시켰고, 병원성 균인 *Staphylococcus aureus* Newman에 노출된 *C. elegans*의 수명도 모든 *Bifidobacterium* 균주가 연장시키는 것을 확인하였다.

기능성 실험을 통해 총 3종의 *Bifidobacterium* 균주가 프로바이오틱스로서 가능성을 가지고 있음을 확인한 후, 인간의 소화기관을 모사한 시스템인 Fermentation of the intestinal microbiota model(FIMM)을 통해 *Bifidobacterium* 균주가 장내 미생물 균총에 미치는 영향을 확인하였다. 결과적으로 장내미생물 균총의 유의적인 변화는 나타나지 않았지만 *Bifidobacterium animalis* D2와 *Bifidobacterium longum* F5가 lactic acid를 약 300~400mg/L 유의적으로 증가시키는 것을 확인하여 *Bifidobacterium*의 처리가 대사체의 변화를 가져온다는 것을 확인하였다.

위의 결과를 토대로 유기체 내에서의 변화를 자세히 확인하기 위해 마우스 모델을 활용하여 *Bifidobacterium* 투여를 통한 효과를 확인하였다. *Bifidobacterium* 균주를 투여한 결과 *Bifidobacterium pseudocatenulatum* E6과 *Bifidobacterium longum* F5 투여군에서 장의 길이가 유의적으로 증가함을 확인하였다. 또한 혈액분석기와 Enzyme-linked immunosorbent assay(ELISA)를 통해 간 손상, 지방 대사, 염증성 사이토카인을 확인해본 결과, 지방 대사와 관련된 인자들에서는 유의적 차이가 나타나지 않았지만 간 손상을 나타내는 alanine amino transferase(ALT)와 aspartate amino transferase(AST)가 항생제를 투여한 그룹에서 증가한 반면

*Bifidobacterium*을 처리한 모든 그룹에서 유의적으로 감소하는 것을 확인할 수 있었다. 그리고 염증성 사이토카인인 Interleukin 1β(IL-1β)와 Interleukin 6(IL-6)의 수준 또한 *Bifidobacterium animalis* D2와 *Bifidobacterium pseudocatenulatum* E6이 항생제 투여 그룹과 비교하여 유의적으로 감소시켰다. 마지막으로 그룹별 대사체 분석과 유전체 분석을 진행하였을 때, 그룹별로 각각 다른 대사 산물을 만들어내는 것을 확인할 수 있었고, 유전체 분석을 통해 항생제를 처리한 그룹에서는 장내 미생물 불균형이 유발되었지만, *Bifidobacterium*을 처리한 그룹에서는 장내 미생물 군총의 균형이 회복되는 것을 확인할 수 있었다.

따라서 유아 분변에서 분리한 *Bifidobacterium* 균주는 프로바이오틱스로서 높은 생존율과 부착능을 보이며 *C. elegans* 숙주의 수명을 연장시킬 뿐 아니라 마우스 모델에서 장 길이를 증가시키고, 염증성 사이토카인을 감소시키는 등 항생제로 유발된 장내 불균형을 완화시키는 것이 확인되어 프로바이오틱스로서 사용되는데 효과적일 것으로 사료된다.