理學碩士學位論文

# 대나무 종 *Sasa borealis* 잎의 항균효과와 파이로시퀀싱을 이용한 분변내 세균총의 변화 분석

**Utilizing extract obtained from leaves of *Sasa borealis* as anti-microbial agent and analysis of alteration in the fecal microbiome via pyrosequencing in ICR mice**

**2012年 8月**

서울大學校 大學院

生命科學部

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# Utilizing extract obtained from leaves of *Sasa borealis* as anti-microbial agent and analysis of alteration in the fecal microbiome via pyrosequencing in ICR mice

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*With Great Honor, I Dedicate This Thesis To My Family*

# ABSTRACT

## Utilizing extract obtained from leaves of *Sasa borealis* as antimicrobial agent and analysis of alteration in the fecal microbiome via pyrosequencing in ICR mice

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Bacteria are a large domain of prokaryotic microorganisms with a typical length in micrometers. Bacteria have a wide range of shapes, ranging from spheres to rods and spirals. There are in the region of ten times as many bacterial cells in the human flora as there are human cells in the body, with large numbers of bacteria on the skin and as gut flora (Sears CL, 2005). Most of the bacteria in human body are considered harmless by the protective effect of the immune system, and some even are thought to be beneficial. However, few species of bacteria display harmful effect to human being meaning it causing a disease. These are called pathogenic bacteria. Presence of pathogenic bacteria brings out the necessity of any substance that kills or slows down the growth of pathogenic bacteria; that is antibiotics.

Antibiotics display several ways in killing bacteria. At first, quinolone

antibiotics interfere with changes in DNA supercoiling by binding to topoisomerase II or topoisomerase IV. This leads to the formation of double-stranded DNA breaks and cell death in either a protein synthesis-dependent or protein synthesis-independent manner. β-lactams inhibit transpeptidation by binding to penicillin-binding proteins (PBPs) on maturing peptidoglycan strands. The decrease in peptidoglycan synthesis and increase in autolysin leads to lysis which kills the cell. Lastly, aminoglycosides bind to the small (30S) subunit of the ribosome and case misincorporation of amino acids into elongating peptides. Mistranslated proteins cause misfolding and it can lead to increased drug uptake (Kohanski *et al*., 2010).

However, number of pathogenic bacteria has evolved mechanisms to overcome these antibiotic-induced cell deaths. These mechanisms are able to either chemically modify the antibiotics or modify target sites so that it is not recognized by certain antibiotics. Due to these reasons, it is important to develop newly advanced antibiotics that can cause antibiotic-induced cell death.

In this study, anti-microbial activity of extract obtained from leaves of *Sasa borealis* was investigated. Bamboo (*Sasa borealis*) Leaf Extract (BLE) was tested out against numerous bacteria including Gram (+), Gram (-) and also Lactic Acid Bacteria (LAB). Without any concentration process, BLE showed strong inhibiting ability against both Gram (+) and Gram (-) bacteria. Lactic acid bacteria are considered a probiotic species and were not inhibited by BLE. With this data,

it was clear that extract obtained from leaves of *Sasa borealis* contains certain type of substance or molecule with strong antibiotic effect. For further research, ammonium sulfate precipitation was performed and using an acrylamide gel electrophoresis, substance with size of near 100kDa was discovered and thought to be bacteriocin secreted by *Sasa borealis*.

In a similar manner, bamboo leaf extract was tested to perceive if there are any cytotoxic effects against several cell lines. At first, BLE was tested against normal cells (murine splenocyte, RAW264.7, J774) and as a result, it did not show any cytotoxic effect. However, BLE showed solid cytotoxic effect against murine colon cancer cell line (CT-26), murine sarcoma cell line (S-180) and murine T-lymphoblast (EL4).

Bacterial 16S rRNA gene was analyzed via technique called pyrosequencing in order to explore intestinal microbial community in fecal sample of experimental mice. Most fecal bacteria belonged to two major bacterial phyla; that are Bacteroidetes and Firmicutes. Comparing HFD-treated group (Group 2) and BLE+LAB (*Lactobacillus casei* LS2, *Lactobacillus paraplantarum* GL and *Leuconostoc citreum* IH22) administered groups (Groups 3, 4, 5), there were quite significant difference in proportion of phyla Bacteroidetes and Firmicutes. Despite the fact that BLE+LAB administered groups had as low Bacteroidetes to Firmicutes ratios as STD-treated group, HFD treated group which became progressively obese had Firmicutes enriched microbiome. Accordingly, it

was clear that selectivity of BLE and LAB attributed to restoration of altered fecal microbiome.


- Keywords : Bamboo Leaf Extract, lactic acid bacteria, bacteriocin, antibiotics, anti-microbial effect, pyrosequencing, fecal microbiome
- Student ID number : 2009-22937

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# LIST OF APPREVIATION

| | |
|---|---|
| BLE | Bamboo Leaf Extract |
| CFU | Colony Forming Unit |
| ELISA | Enzyme Linked ImmunoSorbent Assay |
| EtBr | Ethidium Bromide |
| EtOH | Ethyl Alcohol |
| GL | *Lactobacillus paraplantarum* GL |
| HFD | High Fat Diet |
| IH22 | *Leuconostoc citreum* IH22 |
| LAB | Lactic Acid Bacteria |
| LPS | Lipopolysaccharide |
| LS2 | *Lactobacillus casei* LS2 |
| NO | Nitric Oxide |
| O.D. | Optical Density |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| STD | Standard Diet |

# CHAPTER I

# INTRODUCTION

# 1. Antibiotics

Many ancient cultures including Egyptians and even Greeks used selected mold or mold like substances and plant extracts to heal wounds and treat infections. The term antibiotic originates from Greek *anti* meaning 'against' and *bios* meaning 'life'. The term is often used synonymously with the term antibacterial; meaning that they are drugs or any substances or any compounds that kills or slows down the growth of bacteria. The term 'antibiotic' was coined by biochemist and microbiologist named Selman Abraham Waksman in 1942 to define any substances or compounds produced by microorganism that has a role of suppressing the growth of other microorganisms in high dilutions (SA Waksman, 1947).

Penicillin, one of the most popular antibiotics was discovered in 1929 and since then, other effective antimicrobials have been discovered and developed throughout years. These developments and efforts have greatly enhanced our clinical apparatus. Antibiotic-derived cell death is a complex process which begins with the interaction between a drug substance and its specific target sites in bacteria. It also involves alterations to the affected bacterium at their biochemical, molecular and structural levels (Kohanski *et al*., 2010).

Usual antibiotic induced cell death associates with formation of DNA breakage, arrest of RNA synthesis or with cell envelope damage which results in loss of structural integrity. There are numerous antibiotics in the filed of medicine and the three major types of drug are; Fluoroquinolones, β-lactams and Aminoglycosides.

The importance of antibiotic sometimes is forgotten. For the past few generations, antibiotics have been the cure to many bacterial-induced diseases including meningitis, endocarditis and so on. Likewise, antibiotics have played a major role in both pharmaceutical and livestock industries. The importance of

antibiotics is not only for curing or treating diseases in human but also for belongings near human disease-free.

### 1.1.Type of antibiotics

#### 1.1.1 Quinolone

Fluoroquinolone antibiotics work as DNA synthesis inhibitor and include nalidixic acid, ciprofloxacin, levofloxacin and gemifloxacin. The first generation of the quinolones begins with the introduction of nalidixic acid in 1962 which was used for treating urinary tract infections in human (Sanofi-Aventis U.S. LLC, 2008). Its' primary targets are topoisomerase II which is also known as DNA gyrase and topoisomerase IV. After binding to DNA gyrase or topoisomerase IV it forms double-stranded DNA breaks which leads to cell death. Numerous cellular pathways including DNA replication, SOS response, cell division, ATP generation, TCA cycle, Fe-S cluster synthesis are affected by such antibiotics. As result of Fenton reaction, hydroxyl radicals (OH) formation occurs and it damages DNA, lipids and proteins.

#### 1.1.2 βeta-lactam

Unlike quinolone antibiotic, β-lactam antibiotics induce cell death by inhibiting cell wall synthesis. Drugs included in β-lactam antibiotics are penicillins (ampicillin, oxacillin), cephalosporins (cefazolin, cefoxitin, ceftriaxone, cefepime) and carbapenems (imipenem). Penicillin-binding proteins (PBPs) are its primary target. β-lactams inhibit cell wall synthesis by hindering transpeptidation on maturing peptidoglycan strands (Kohanski *et al*., 2010). Irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis (Fisher J.F. *et al*., 2005).

Increase in autolysin and inhibition of peptidoglycan synthesis leads to lysis and cell death. Moreover, β-lactam antibiotics are the most frequently used antibiotic in the pharmaceutical field.

### 1.1.3 Aminoglycoside

Gentamicin, tobramycin, streptomycin and kanamycin are all frequently used antibiotics under category of aminoglycoside antibiotics. Aminoglycoside antibiotics work as protein synthesis inhibitor and targets small (30S) subunit of ribosome. It affects protein translation pathway which results in mistranslation and mismatching and also affects SOS response and TCA cycle. These antibiotics work against all pathogenic aerobic Gram (+), Gram (-) species and *M.tuberculosis*. Its cell-death pathway begins with binding to the 30S subunit of ribosome which causes misincorporation of amino acids into peptides. As a result, mistranslated proteins are formed and misfolded proteins incorporate into cell envelope. This results in increase drug uptake in cell envelope and cell dies. These antibiotics share similar pathway; through Electron Transport Chain (ETC), it stimulates NADH oxidation of TCA cycle. As a result superoxide formation is stimulated and this superoxide damages Fe-S clusters. Damaged Fe-S clusters results in forming ferrous irons through Fenton reaction. After all, DNA, lipid and protein are damaged by hydroxyl radical made out of Fenton reaction (Dwyer D. J. *et al*., 2010).

# 2. Bamboo

Bamboo is a group of evergreens in the grass family Poaceae, subfamily Bambusoideae, tribe Bambuseae. Bamboo species are one of the fastest growing plants in the world because of its unique rhizome-dependent system (Farrelly D., 1984). Bamboos share notable economic significance in South Asian, South East Asian countries for its use in many different categories. Structurally, internal region of the stem has hollow shape to it and cross section is scattered around the stem. There are more than 1400 species of bamboo divided under 70 genera worldwide. These species adapt well to diverse climate, covering cold regions to hot regions. Unlike other trees, bamboo stems arise from the ground and grow to their full height in just 3-4 months.

Flowering of bamboo occurs infrequently. Actually, bamboo species are known for their ‘mass flowering’ which means that they flower at intervals of 65 or over 100 years (Thomas R.S. *et al*., 1979). The reason behind this phenomenon is still mysterious, but lack of environmental impact is considered one reason behind it.

Bamboo in certain Asian country such as China plays an important role. The culture thinks bamboo as symbol of uprightness, integrity and even elegance. Additionally, in Japanese culture, bamboo is thought to be barrier against evil. That is the reason lots of Buddhist temples are surrounded by bamboo forest. Moreover, there are numerous myths and legends regarding bamboo around Asian cultures counting Andaman Islands, believing humanity arose from bamboo stem.

## 2.1. Uses

Different parts of bamboo are used in many different fields from its usability in culinary field to even medical field. In the culinary field, the shoots of bamboo are used in many different Asian dishes including broth. There is fresh

and canned version of bamboo shoots available in certain Asian cultures. Unlike the shoots of bamboo, leaves share important role in preserving nourishment. In the countries with hot climate, it is very important to preserve what they eat because it is very easy for their food to get rotten. Primary reason behind rotten food is microorganisms permeating the food material. Bamboo leaves are known to have antimicrobial effect and by surrounding the nourishment with the leaves of bamboo, it prevents most nourishment getting rotten and also keeps the moisture of the food (Singh V.K. *et al*., 2010). Bamboos also are used in construction field as an exterior holding material. It was well known for its use as scaffolding. In early Chinese years, bamboos were used to be materials for paper making.

### 2.1.1. Medical uses

Bamboo is currently used in Chinese culture as treatment for infections. From its antimicrobial effect, certain bamboo species is known to control electrolytes and body water balance (Ryou SH *et* al., 2012). Bamboo contains lots of amino acids, organic acids, cations and anions that are advantageous to human health. In addition, certain flavone C-glucosides in bamboo are known for its ability as antioxidant material (Zhang Y. *et al*., 2005). There are numerous studies and researches regarding its anticancer effect. Bamboo shares a unique characteristic of each part of it possessing different medical effects. First of all, leaves are most actively used in field of medicine. Recent study has quantified several flavonoids including orientin, isoorientin, vitexin, isovitexin using HPLC method (Wang J. *et al*., 2010). Flavonoid orientin ($C_{21}H_{20}O_{11}$) is known to have anti-apoptotic effect and isoorientin (or homoorientin) is known for its anti-oxidative effect. Moreover, vitexin ($C_{21}H_{20}O_{10}$) and isovitexin are known for reducing oxidative stress.

Bamboo shoot also is actively being researched over its role in immune system. One of the leading causes of death in West and many other developed countries are atherosclerosis and its related complications. Recent research have intensely studied hypolipidemic effect of bamboo shoot oil and found out that bamboo shoot oil significantly decreased total cholesterol, triacylglycerol, LDL-cholesterol, lipoprotein lipase and so on (Lu B. *et al*., 2010). Certain species of bamboo carries significant importance in field of medicine because of its capability in modulating immune system.

### 2.2. Bamboo species

Bamboo is a group of perennial grass in the grass family Poaceae. It is a large family with over 10,000 species. Under tribe *Bambuseae* (also known as Bamboo), there are over 1000 species. Clumpers and runners are two subgroups divided under species. Clumpers grow from the soil in a slowly expending tuft. Runners utilize underground rhizomes to produce shoots from parent plant.

#### 2.2.1. *Sasa borealis* (Hack.) Makino & Shibata

*Sasa borealis* is classified under family Poaceae, subfamily Bambusoideae, tribe *Bambuseae* and genus *Sasa*. It is one of the most abundant species found in several Asian countries including China and South Korea. They are under category of ornamental grasses and bamboo. Fully grown *Sasa borealis* has an approximate height range of 6-10ft (1.8-3m). Leaves of *Sasa borealis* are known to have beneficial effect to human immune system. In certain Asian cultures, dried leaves are used as tea and it is well known for its ability in modulating immune system.

### 2.3. Effectiveness of *Sasa borealis*

Various recent studies have identified countless beneficial effect of *Sasa borealis*. In the field of antibiotics, it is crucial to discover a new antibiotic other than what's out on the market. Number of pathogenic bacteria has evolved mechanisms to overcome antibiotic-induced cell deaths. These mechanisms are able to either chemically modify the antibiotics or modify target sites so that it is not recognized by certain antibiotics. In one recent study, two actinobacterial strains JR-43T and JR-4 inhabiting antibacterial activity against plant pathogenic bacteria were isolated from bamboo (*Sasa borealis*) rhizosphere soil (Lee HJ *et al*., 2012). Leaves of *Sasa borealis* also display improvement on insulin resistance by modulating inflammatory cytokine secretion in high-fat diet induced obese C57/BL6 mice. It modulates secretion of cytokine TNF-α, IL-6 and serum leptin (Yang JH *et al*., 2010). Additional study has reported that extract obtained from leaf of *Sasa borealis* showed protective effect AAPH-induced oxidative stress in LLC-PK1 cell (Hwang JY *et al*., 2011). Plant flavones occupying anti-oxidative effect are also included in *Sasa borealis*; those are C-glycoside derivatives, isoorientin and isoorientin 2"-O-alpha-L-rhamnoside along with tricin 7-O-beta-D-glucopyranoside and apigenin 6-C-beta-D-xylopyranosyl-8-C-beta-D-glucopyranoside. Bamboo species *Sasa borealis* clearly displays its ability in promoting immune system by modulating various pathways.

# 3. Bacteriocin

Bacteriocins are defined as toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s). They are typically considered to be narrow spectrum antibiotics, though this has been debated (Farkas-Himsley H, 1980). Bacteriocins were first discovered by microbial and viral genetics A.Gratia in year 1925. They are structurally, functionally and ecologically so diverse.

## 3.1. Classification of bacteriocin

Based on their producing strain, common resistance mechanisms, and mechanism of killing, bacteriocins are categorized in multiple ways. Large categories of bacteriocin are phenomenological related. Bacteriocins from Gram (+) bacteria, the colicins, the microcins, and the bacteriocins from Archaea are included in such categories. Alternative methods of classification include: method of killing (pore forming, dnase, nuclease, murein production inhibition, etc.), genetics (large plasmids, small plasmids, chromosomal), molecular weight and chemistry (large protein, polypeptide, with/without sugar moiety, containing atypical amino acids like lanthionine) and method of production (ribosomal, post ribosomal modifications, non-ribosomal) (Cotter PD *et al*., 2006).

### 3.1.1. Class I bacteriocin

Class I bacteriocins are simply the ones with small peptide inhibitors. Nisin and other lantibiotics fall under this class I bacteriocin. Nisin is an antibacterial peptide with 34 amino acid residues used as a food preservative. Lantibiotics are peptide antibiotics that contain the characteristic polycyclic thioether amino acids lanthionine or methyllanthionine. They are

produced by Gram (+) bacteria *Streptococcus* and *Streptomyces* and attack other Gram (+) bacteria.

### 3.1.2. Class II bacteriocin

Class II bacteriocins occupy size of about <10kDa and is a heat stable proteins. Class II bacteriocins are subdivided into 5 different classes. Class IIa bacteriocins also known as pediocin-like bacteriocins contains N-terminal consensus sequence. The C-terminal is responsible for species-specific activity, causing cell-leakage by permeabilizing the target cell wall. Class IIb bacteriocins require two different peptides like lactococcin G for its activities. In the case of class IIc bacteriocins, it involves cyclic peptides, which possesses the N-terminal and C-terminal regions covalently linked. At last, class IId bacteriocins cover all single peptide bacteriocins. Aureocin A53 is a major bacteriocin that falls under class IId bacteriocin. Aureocin A53 is a new tryptophan-rich anti-bacterial peptide that does not contain post-translationally modified amino acid residues.

### 3.1.3. Class III bacteriocin

Unlike class II bacteriocins, class III bacteriocins include proteins that are large (>10kDa) in size and heat-unstable. Subclass IIIa is also called a bacteriolysins kills target bacteria by cell-wall degradation which results in cell lysis. Most well known IIIa bacteriocin is lysostaphin with a size of 27kDa (Bastos M.C.F. *et al*., 2010). Subclass IIIb on the other hand kills the target cells by disrupting the membrane potential, which causes ATP efflux.

### 3.1.4. Class IV bacteriocins

Class IV bacteriocins include complex bacteriocins containing lipids carbohydrates. Sublanicin and Glycocin F (GccF) are the two independent groups that were established just recently (Oman T.J. *et al*., 2011).

### 3.2. Medical significance of bacteriocin

Bacteriocins play an important role in field of medicine and its related industries because they are made by non-pathogenic bacteria that normally reside in human body. Loss of these harmless bacteria may allow pathogenic bacteria to invade human body. Not only it has antibacterial effect, bacteriocins also are used as cancer treatment (Faskas-Himsley H. *et al*., 1985). Bacteriocins are also a diagnostic agent for some cancers (Cruz-Chamorro L. *et al*., 2006). Bacteriocins regardless of its characteristics aid human immune system by invading pathogenic bacteria.

# 4. Lactic Acid Bacteria (LAB)

Lactic acid bacteria are defined as Gram (+), catalase (-), acid-tolerant, generally non-sporulating, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. These bacteria are normally found in decomposing substance or lactic products, producing lactic acid as major metabolic product of fermentation (Schleifer & Ludwig, 1995). Lactic acid bacteria were first discovered in 1858 by microbiologist Louis Pasteur. There are 12 genus comprising the LAB; those are *Lactobacillus, Carnobacterium, Atopobium, Lactococcus, Pediococcus, Tetragenococcus, Leuconostoc, Weisella, Oenococcus, Enterococcus, Streptococcus* and *Vagococcus*.

## 4.1. Probiotics

Probiotics are defined as live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance (Fuller, 1989). It is also defined as viable microorganisms in sufficient numbers, which alter the microflora in a compartment of the host and that exhibit beneficial effects on the health of the host when they are ingested. Several studies discovered that orally delivered probiotics can transiently colonize the gastrointestinal tract and actively communicate with cells in immune system (Gill, 1998; Cross, 2002). LABs are also known to reduce serum cholesterol level (Liong *et al*., 2006).

Numerous studies have pointed out that the oral administration of certain lactic acid bacteria exerts the stimulation of the specific and non-specific immune response such as innate immunity (Salminen *et al*., 1998; Benyacoub *et al*., 2003). Probiotics are undeniably crucial in supporting a functional yet balanced immune systems and further employment of immune-modulatory bacteria in health care can be seen in combating microbial pathogens, including virus. It should be recognized that the probiotic strains possess the ability of maintaining

metabolically active state during gastrointestinal passage as the viability of probiotics is important for improvement of balanced intestinal bacterial flora. The intestinal bacterial flora also plays a key role in host defense to modulate both innate and adaptive immunity (Lambrecht & Hatcher, 1993; Nikoskelainen *et al*., 2003).

### 4.2. Effectiveness of Lactic acid bacteria

Strains of LAB are the most common microbes employed as probiotics. At first, LAB employs a role of regulating intestinal microbial balance. It prevents pathogenic bacteria attaching to an intestinal epithelial wall. Also, lactic acids, fatty acids and $H_2O_2$ which are some by-products resulted in LAB metabolism plays a role in inhibiting pathogenic bacteria. Besides, lactic acid and citric acid secreted from LAB are very effective against intestinal toxins in host.

LAB also stimulates immune response. LAB rapidly detects pathogens and viruses (Carmen *et al*., 2007; Silviya *et al*., 2007), promotes rapid differentiation of lymphocytes and induces β-defensins (Schlee *et al*., 2008). In addition, LAB improves NK cell response (Lisbeth et al., 2007).

Lastly, LAB improves nutritional values. The genus *Lactobacillus* secrets lactic acid while growing and proliferating and as a by-product it generates amylase, cellulose, lipase and protease which works as digestive enzyme. LAB also synthesizes vitamin B1, B2, B6, B12 and inhibits bacteria that produces enzyme which hinder vitamin B1 synthesis (Hatanaka et al., 1988). Nicotinic acid, inositol, vitamin K, E are also produced by lactic acid bacteria.

# 5. Sequencing

DNA sequencing includes several different methods and technologies used for determining the order of nucleotide bases (A, T, G and C) in a given DNA molecule. Knowledge of DNA sequence has become essential for biological science. In numerous fields including diagnostic, forensic science and biotechnology utilizes DNA sequencing technology very frequently. It is mostly used for applications in genome sequencing, metagenomics, epigenetics and discovery of non-coding RNAs and protein-binding sites. The first DNA sequences were obtained in early 1970s using laboratory method based on two-dimensional chromatography. After dye-based sequencing technologies and computerized analysis was introduced, DNA sequencing technologies has improved significantly over the years.

## 5.1. Sanger sequencing

Sanger sequencing, also known as dideoxy method or chain termination method was first developed by Fred Sanger in year 1975 in order to translate the human genome. First step of Sanger sequencing begins with continuous copying of template DNA so as to translate the order of Adenine (A), Thymine (T), Guanine (G) and Cytosine (C) in a DNA strand. This copying reaction is terminated by binding of modified nucleotide (di-deoxynucleotide (ddNTP) which has a fluorescent dye attached. Modified nucleotide ddNTP randomly binds to polymerizing DNA strand, meaning limited incorporation of the chain terminating nucleotide by the DNA polymerase results in a series of related DNA fragments that are terminated only at positions where that particular nucleotide is used. Then, the fragments are available for separation based on their read length using a polyacrylamide gel electrophoresis. Added ddNTP contains dye with various wavelengths matching to each base, so it is possible to distinguish the order of

DNA molecule by reading the order of each base A, T, G, and C after electrophoresis. One of the most substantial merits of using Sanger sequencing is that it is available to achieve the whole sequencing set (500~800bp) in a single reaction. New sequencing technologies with copious advantages (rate of data generation, data volume and etc.) over traditional Sanger sequencing have evolved. This will lead to reduced usage of old-fashioned Sanger sequencing which requires lengthier read time and physical labor, but still it definitely has its strength in read length. This is why Sanger sequencing is termed 'gold standard' of sequencing.

### 5.2. Pyrosequencing

New sequencing technologies have enabled the acquisition of gigabases of sequence information in just a few days. Pyrosequencing was developed by Pål Nyrén and Mostafa Ponaghi in 1996. Unlike the traditional Sanger sequencing that uses chain termination by dideoxynucleotide; this new technology recognizes pyrophosphate as a product to analyze the order of bases. Pyrosequencing was first commercialized in year 2005 by Biotage (for low-throughput sequencing) and Roche (for high-throughput sequencing). In this study, pyrosequencing platform by Roche 454 Life science was used. Traditional Sanger sequencing has limited read length in a single sequencing making it difficult to analyze in whole genome scale. However, pyrosequencing by Roche 454 Life science successfully analyzes 100,000,000 bases (100 megabases) in just about 7 hours.

This new Next Generation Sequencing (NGS) technology utilizes enzyme cascades of 4 enzymes; DNA polymerase, sulfurylase, luciferase and apyrase. Initially, double-stranded sample DNA is fragmented into 300-800bp of size by nitrogen gas. Addition of adaptor A and B which aids amplification and sequencing occurs at each end of fragmented double-stranded sample DNA. Mixing the DNA capture bead to DNA library is a next step to follow.

Complementary primer against partial nucleotide of adapter exists on DNA capture bead making possible for DNA fragment to bind to the bead. Afterward, amplification using emulsion PCR (emPCR) follows and each bead goes into each well with 4 enzymes.

Polymerase and primer binds to DNA fragment. When specific base is added to reaction solution, DNA polymerization take place and as a result, pyrophosphate (PPi) group falls off.

$$(DNA)_n + dNTP \xrightarrow{\text{Polymerase}} (DNA)_{n+1} + PPi$$

Right at this moment, reaction between pyrophosphate and APS (adenosine 5' phosphosulfate) occurs by sulfurylase resulting in generating ATP, and this ATP activates luciferase. Activated luciferase works as an oxidative substance that converts luciferin to oxyluciferin. Oxyluciferin emits light at such stage and the strength of the light emitting is proportional to the number of adding base. CCD camera detects the light emitting and using outputted peak on graph, it is possible to analyze the order of nucleotide bases.

$$APS + PPi \xrightarrow{\text{Sulfurylase}} ATP$$

$$\text{Luciferin} \xrightarrow{\text{Luciferase, ATP}} \text{Oxyluciferin} + \text{Light}$$

Apyrase on the other hand works as a degradation enzyme and degrades unincorporated dNTP and ATP. Single dNTP gets degraded and the next dNTP is added to reaction solution.

$$\text{dNTP} \xrightarrow{\text{Apyrase}} \text{dNDP} + \text{dNMP} + \text{phosphate}$$

$$\text{ATP} \xrightarrow{\text{Apyrase}} \text{ADP} + \text{AMP} + \text{phosphate}$$

### 5.2.1. Use of pyrosequencing

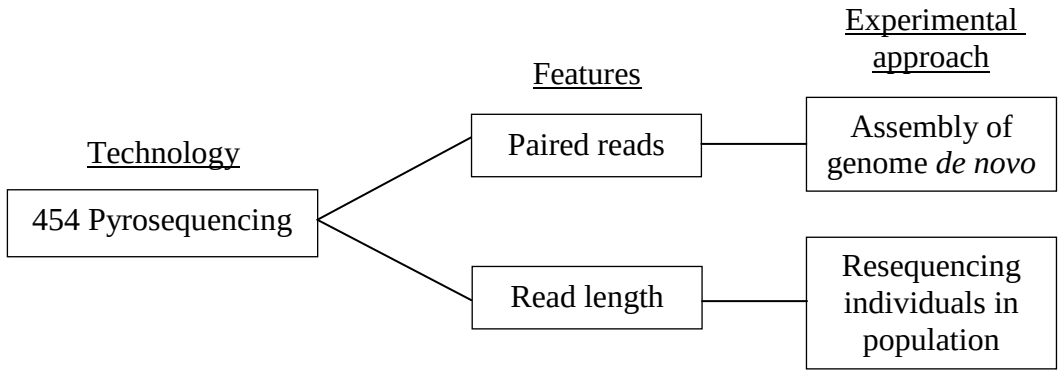
Just like other NGS technologies such as SOLiD, Solexa GA, Heliscope and Pacific biosciences, first wave of pyrosequencing aimed to resequence genomes in shorter time and at a lower cost than traditional Sanger sequencing.

Utilizing 454 pyrosequencing make it possible to sequence the genome of humans, animals and even virus easier and faster. In June of year 2006, Max Planck Institute has spent almost 2 years sequencing genome of Neanderthal species very related to Homo sapiens. Nonetheless, in year 2007, Baylor College of Medicine using 454 sequencing technology has sequenced complete human diploid genome of Dr. James Watson much faster and cost-effectively.

454 pyrosequencing distinct from traditional Sanger sequencing is able to detect even minute mutations. This makes it possible to detect specific phenomenon such as low frequency somatic mutation in cancer patient. 454 sequencing is also used in sequencing full-length mRNA transcripts and order of nucleotide bases of small RNA.

Metagenomics is the study of metagenomes, genomic substance recovered from environment. In other words, metagenomics is study of genomic contents in complex sample. Two main objectives of metagenomics are

identifying organisms in a given sample and discovering the role of those organisms in the environment. Samples can be collected from anywhere including microenvironment in human, soil and deep ocean. 454 pyrosequencing can identify the number or type of microorganisms in a sample. In this specific study, pyrosequencing was used as objective technology in researching numbers and types of microorganisms in fecal samples. It is possible to achieve reliable data with a simple sample preparation, analyzing software and pyrosequencing technology.




### 5.2.2. Disadvantage of using pyrosequencing

Disregarding numerous merits of pyrosequencing, there are some weaknesses to it. First of all, since such technology relies only on strength of emitting light, error could occur in situation when sequencing bases of homopolymers. This means when there are single nucleotide (A, T, G, or C) continuously present in DNA template, it show its limitation in accurately analyzing how many certain nucleotides are present. Analyzing cost and time required for analysis is friendly only when sequencing a single base; whole genome sequencing is extremely expensive making it not suitable for pedigree analysis of DNA sample.

# 6. Aims of this study

In present study, anti-microbial activity of extract obtained from leaves of *Sasa borealis* was researched. Over 20 bacteria including both Gram (+), Gram (-) and LAB were tested against BLE. Also, in order to observe the effect of BLE on immune cells, MTT assay was performed against murine splenocyte, macrophage, CT-26 cell line, S-180 cell line and EL-4 cells. In addition, bacterial 16S rRNA gene was analyzed via pyrosequencing in order to explore alteration in microbial community structures from the mouse fecal samples.

During the first trial, BLE significantly inhibited growth of majority of bacteria tested (>20 bacterial strains). Most significantly affected bacteria were *Rothia dentocariosa* G1201 and *Streptococcus mutans* CCARM 0079. On the other hand, BLE did not show any inhibiting effect against lactic acid bacteria. Through data collected from first trial, it was clear that BLE selectively hinder bacteria other than LAB. Most susceptible bacterial strains were selected for further research based on the data collected from first trial test.

On the second trial, both normal and heat killed (HK) *Lactobacillus paraplantarum* GL and *Lactobacillus casei* LS2 were prepared ($5 \times 10^7$ and $5 \times 10^{11}$). 200µl was orally injected to experimental mouse and as a result, heat killing the LAB had the same effect compare to normal LAB. Based on the data collected from second trial, it was decided that $10^{10}$ CFU/ml is appropriate LAB concentration for further research.

# CHAPTER II

# MATERIALS AND METHODS

# 1. *Sasa borealis* (Hack.) Makino & Shibata

## 1.1. *Sasa borealis* leaf extract

Hand picked bamboo leaves were purchased from Damyang, South Korea. It was appropriate for sample extraction since it was in moisture free condition. Bamboo leaves (500g) were first cut in pieces and immersed under 5% ethanol (EtOH) in room temperature (RT) for 72h for sample extraction. After collecting the supernatant, aqueous solution was centrifuged (6000rpm, 30min, 4℃) and filtered using 0.22μm microfilter (Whatman). In order to remove any remaining organic solvent, aspiration using rotary vapor system (Rotavapor, BUCHI #R-210) was followed. To test in various concentrations, extracted aqueous sample solution was diluted with 1X PBS. Various concentrations of diluted extract solution were kept frozen (-80℃) for further studies.

### 1.1.1. Unit decision

With the aim of collecting more reliable data, working unit of *Sasa borealis* leaf extract was decided. Based on the data from Minimum Inhibitory Concentration (MIC) assay against *Rothia dentocariosa* G1201, 1unit (concentration of 1/128 compared to the original solution) was set for further study.

## 1.2. Bradford assay

Quantification of proteins in extracted *Sasa borealis* leaf solution was done by method of Bradford. After placing 10μl of extract solution into 96 well plate, 200μl of Bradford dye solution (4X, 95% EtOH 100ml, phosphoric acid 200ml, Coomassie Blue R-250 350ml) that were diluted into 1X was added. After

5mins of incubation at room temperature, Optical Density (O.D.) was measured at 595nm using ELISA reader.

### 1.3. Anthrone test

Quantifying carbohydrates in an extract solution was performed by method of Spiro (1966). 1ml of anthrone reagent (anthrone 200mg in conc. $H_2SO_4$ 100ml) was supplemented into glucose solution (10-500µg/ml) and placed in ice water bath for 5mins. Ice-cold tubes were then reacted in 100℃ for 10mins before it was cooled again in ice bath. After the cooling process, optical density of the solution was measured at 620nm.

### 1.4. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was completed using NOVEX mine gel kit following the method of Laemmli (1970). Running gel and the stacking gel was made with the following ingredients:

| Running Gel (12%, 5ml) | | Staking Gel (3ml) | |
|---|---|---|---|
| $dH_2O$ | 1.7ml | $dH_2O$ | 2.1ml |
| 30% Acrylamide mix | 2.0ml | 30% Acrylamide mix | 0.5ml |
| 1.5M Tris (pH8.8) | 1.3ml | 1.0M Tris (pH6.8) | 0.38ml |
| 10% SDS | 0.05ml | 10% SDS | 0.03ml |
| 10% APS | 0.05ml | 10% APS | 0.03ml |
| TEMED | 0.002ml | TEMED | 0.003ml |

*Sasa borealis* extract solution and sample buffer (125mM Tris-Cl (pH6.8), 20% Glycerol, 2% SDS, 2% β-mercaptoethanol, 0.02% Bromophenol Blue) was mixed (1:1) together and the mixture was boiled for no longer than 10minutes before loading. Staking gel was operated with 80V and the running gel was operated with 110V. Mid-range pre-stained (blue) protein size marker (Elpisbio, S.Korea) was used in determining the size of proteins.

### 1.4.1. Silver Staining

Silver staining was performed under method of Oakley (1980). EzWay™ silver staining kit (Koma Biothech INC.) was utilized throughout the whole process. Gel was fixed with the fixing solution (EtOH 40ml, acetic acid 10ml, T.D.W. up to 100ml) for 15mins (2 times). After discarding the first fixing solution, gel was fixed with the second fixing solution (EtOH 50ml, T.D.W. up to 100ml) for 5mins (2 times). Second fixing solution was discarded and 25ml of sensitizing solution (0.02% Sodium thiosulfate) was added and the gel was incubated in the sensitizing solution for 2mins. Next step included discarding the sensitizing solution and washing the gel in 50ml of second fixing solution for 2mins. Then, gel was washed with 100ml T.D.W. for 1min (3 times) before it was stained in the staining solution (0.1% Silver nitrate) for 20mins. Using 100ml of T.D.W. for washing the gel (1min x 2 times), gel was incubated in 25ml of developing solution (0.04% Formaldehyde in 2% Sodium carbonate, 50% MeOH, 5% Formic acid) for no longer than 10mins. Once the appropriate staining intensity was achieved, 0.5ml of 100% acetic acid was added directly to the gel to stop developing.

### 1.4.2. Gel elution

Protein was eluted from the gel matrix by passive elution technique. First, excised gel pieces were placed in clean microcentrifuge tubes. Then, 1ml of

elution buffer (50mM Tris-HCl, 150mM NaCl, 0.1mM EDTA; pH7.5) was added so that the gel pieces are completely immersed. Gel was crushed using a clean pestle and incubated in a rotary shaker at 30℃ overnight. Final process comprised of centrifugation at 8,000 x g for 10mins and pipetting supernatant into a new microcentrifuge tube. An aliquot of the supernatant was tested for the presence of protein by subjecting it to SDS-PAGE.

### 1.5. Quantitative analysis of compounds of *Sasa borealis* by HPLC

The qualitative and quantitative analysis of sole compounds such as organic acids, amino acids, cations, anions and sugars present in *Sasa borealis* leaf extract were determined by High Performance Liquid Chromatography (HPLC). For organic acids and sugar analysis, Aminex 87H column and Ultimate 3000 (Dionex, USA) were utilized. 0.01N $H_2SO_4$ occupied as an eluent and oven temperature was set to 40℃. RI detector (Shodex RI-101, Japan) was used and runtime was set to 30mins. Standard sugar materials included glucose, arabinose, xylose, fructose, sucrose and citric, malic, succinic, ethanol, lactic, formic, acetic, butyric, oxalic, levulinic, 5-HMF, furfural, tartaric, pyruvate and propionic acids were standard materials for the organic acid analysis. For amino acid analysis, C18 column (4.6mm x 150mm, 5μm) and Agilent 1200LC was used. Mobile phase A included 20mM sodium phosphate monobasic (pH7.8) and mobile phase B included T.D.W., acetonitrile and methanol (10 : 45 : 45 (v/v)). Injection volume was set to 0.5μl and temperatures of column and sample were 40℃ and 20℃ each. Amino acid standard (Agilent 5061-3330) was used as standard material. In addition, cation analysis involved IonPac® CS12A as reference and Dionex ICS3000 (Dionex, USA). Column was Ionpac CS12A (4 x 250mm) and column oven temperature was at 30℃. Flow rate was set to 1ml/min and 20mM MSA (Methanesulfonic acid) worked as an eluent. 25μl was injected and

detection was done by CSRS URTRA (4mm) in recycle mode. In the case of anion analysis, every setting was identical except Ionpac AS20 anion-exchange column was used and 24mM KOH utilized as eluent. Also, ASRS URTRA II (4mm) in recycle mode was the detection setting for the anion analysis.

# 2. Cultivation of bacterial strains

## 2.1. Bacterial strains and media

Bacterial strains used in the study includes both Gram (+) bacteria (*Rothia dentocariosa* G1201, *Streptococcus mutans* CCARM 0079, *Bacillus subtilis*, Staphylococcus *saprophyticus* and *Staphylococcus epidermis*), Gram (-) bacteria (*Escherichia coli* JM109, *Serratia marcescens*, *Klebsiella pneumoniae* and *Salmonella typhimurium*) and LAB (*Lactobacillus paraplantarum* GL, *Lactobacillus casei* LS2 and *Leuconostoc citreum* IH22). All of bacteria other than *S.mutans* CCARM0079 and LAB were cultured in Trypticasein Soy medium (TSA, Conda, S.A) at 37℃ for 24hrs. *S.mutans* CCARM0079 was cultured in BHI (Difco, Detroit, MI) medium at 37℃ for 24hrs. Lactic acid bacteria were cultured in MRS medium at 30℃ for 48hrs. In the case of LAB, strains were first centrifuged (6000rpm, 15min, 4℃) at their stationary phase. Then, it was washed with phosphate buffered saline (1X PBS, pH7.2) three times before it was kept frozen (-80℃) with an appropriate concentration (CFU/ml).

### 2.1.1. PBS (Phosphate buffered saline)

Autoclaved (121℃, 1.2 ATM, 15min) PBS (Phosphate buffered saline) was prepared with following ingredients; it was used in dissolving sample precipitate and various washing situations.

| | |
|---|---|
| NaCl | 8g |
| KCl | 0.2g |
| $KH_2PO_4$ | 0.24g |
| $Na_2HPO_4 \cdot 12H_2O$ | 2.89g |
| T.D.W. | 1L |

### 2.1.2. Trypticasein soy agar (TSA)

Autoclaved (121℃, 1.2 ATM, 15min) TSA (Trypticasein soy agar) was prepared with following ingredients. It was used for cultivating most bacteria tested.

| | |
|---|---|
| Pancreatic Digest of Casein | 17g |
| Sodium chloride | 5g |
| Glucose monohydrate | 2.5g |
| Papaic Digest of Soy Bean | 3g |
| Dipotassium Phosphate | 2.5g |
| Agar | 15g |
| D.W. | 1L |

### 2.1.3. Brain heart infusion (BHI)

Brain heart infusion (BHI) medium was prepared in order to cultivate bacterial strain *Streptococcus mutans* CCARM0079. S.mutans CCARM0079 was

incubated at 37℃ for 24hrs. BHI medium was prepared with following ingredients.

| | |
|---|---|
| Calf Brains, Infusion from | 200g |
| Beef Hearts, Infusion from | 250g |
| Proteose Peptone | 10g |
| Dextrose | 2g |
| Sodium Chloride | 5g |
| Disodium Phosphate | 2.5g |
| Agar | 15g |
| D.W. | 1L |

### 2.1.4. MRS broth for LAB cultivation

In order to cultivate lactic acid bacteria for mouse oral injection, MRS medium was prepared with following ingredients and autoclaved (121℃, 1.2 ATM, 15min) before use.

| | |
|---|---|
| Bacterial Peptone | 10g |
| Beef Extract | 8g |
| Yeast Extract | 4g |
| Dextrose | 20g |
| Tween 80 | 1g |
| Ammonium Citrate | 2g |

| | |
|---|---|
| Sodium Acetate | 5g |
| Magnesium Sulfate | 0.2g |
| Manganese Sulfate | 0.05g |
| Dipotassium Phosphate | 2g |
| D.W. | 1L |

### 2.2. Counting Colony Forming Unit (CFU) of LAB

Lactic acid bacteria (CFU) were cultivated in MRS broth (Conda, S.A) at 30℃ for 48hrs. After incubation, centrifugation (6000rpm, 15min, 4℃) was performed. LAB were washed three times with PBS (1X) and serial diluted for CFU counting. Serial diluted LAB was inoculated on MRS agar plate and it was incubated at 30℃ for 48hrs. After incubation, colonies were counted and diluted to appropriate concentration ($5 \times 10^{10}$ CFU/ml). Diluted LAB were kept in sterilized 1.5ml tube at -80℃ for further use.

#### 2.2.1. MRS agar for LAB cultivation

To count the CFU of LAB, MRS agar plate was prepared. It shared identical ingredients as MRS broth but only 1.5% agar (Conda, S.A) was added. Media was also autoclaved (121℃, 1.2 ATM, 15min) before use.

| | |
|---|---|
| Bacterial Peptone | 10g |
| Beef Extract | 8g |
| Yeast Extract | 4g |
| Dextrose | 20g |
| Tween 80 | 1g |

| | |
|---|---|
| Ammonium Citrate | 2g |
| Sodium Acetate | 5g |
| Magnesium Sulfate | 0.2g |
| Manganese Sulfate | 0.05g |
| Dipotassium Phosphate | 2g |
| Agar | 15g |
| D.W. | 1L |

# 3. Antimicrobial activity of *Sasa borealis*

### 3.1. Agar well diffusion assay and paper disc diffusion assay

To test antimicrobial activity of extract obtained from leaves of *Sasa borealis*, agar well diffusion assay was performed. Each bacterial strain was inoculated using spreader on to appropriate agar plates and well was made with sterilized borer (9mm in diameter). The extract compound (150µl) was introduced into the well and plates were incubated at 30℃ for 48hrs. All samples were tested in triplicates. Microbial growth was determined by measuring the diameter of zone of inhibition.

Paper disc diffusion assay was also performed for determining the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to antimicrobial compound. Instead of making a well, 6mm filter paper disc was used. After placing the filter paper disc, discs were moistened with 150µl of BLE. Following instructions were identical as agar well diffusion assay.

### 3.2. Measuring Optical Density (O.D.) of bacterial strains

To determine the antimicrobial activity of extract obtained from leaves of *Sasa borealis*, Optical Density (O.D.) of culture supernatant was measured for each bacterial strain tested in the study. First, 200µl of appropriate culture media (TSB, BHI and MRS) was loaded into wells of 96 well plate. Then, extract solution (10% (v/v)) was added to each well before bacterial strains were inoculated. Plate was incubated overnight at 37℃ for O.D. measurement. Optical density measurement was done by ELISA reader (Molecular Devices, USA) with reading wavelength at 540nm.

### 3.3. Time-Kill assay

The time-kill assay was used for examining the rate at which concentrations of an extract solution kill a bacterial isolate. 0.1ml of inoculum containing 5 x $10^8$ CFU/ml was added to each tube containing 10ml of media (+ 10% *Sasa borealis* leaf extract (v/v)). Final organism concentration was approximately 5 x $10^6$ CFU/ml. Immediately after inoculation, 0.1ml was removed from each reaction tube to perform serial dilution bacterial plate counts for time zero. Then, tubes were incubated at 37℃. At the next sample time (4hrs), tube was removed from the incubator. Tubes were gently agitated and 0.1ml of aliquot was removed from each tube for bacterial plate counts. Steps were repeated for other necessary time points. Counting the colonies on each of the serial dilution was followed. Data was recorded for each bacterial strains and CFU/ml was converted into $\log_{10}$ data.

# 4. Cell culture and media

## 4.1. Cell lines

Murine splenocyte, murine RAW 264.7 macrophage cell line, J774 cell line, EL-4 cell line, Jurkat cell, CT-26 cell line and S-180 cell line were used in present study. Murine RAW 264.7 macrophage cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in either Dulbecco's Modified Eagle's Medium (DMEM, Gibco-Invitrogen, Grand Island, NY) or Roswell Park Memorial Institute medium (RPMI 1640, Gibco, NY). Medias were supplemented with 10% (v/v) heat-inactivated FBS (Hyclone, USA) and 20µg/ml of gentamycin (Sigma Chemical Co., USA) and incubated at 37℃ in humidified 5% $CO_2$ incubator.

### 4.1.1. HBSS (Hank's Balanced Salt Solution, pH7.2)

HBSS was used during subculture process as a rinsing solution. Autoclaved (121℃, 1.2 ATM, 15min) HBSS was made with following ingredients:

| | |
|---|---|
| $CaCl_2$ | 0.14g |
| KCL | 0.4g |
| $KH_2PO_4$ | 0.06g |
| $MgCl_2$ | 0.1g |
| $MgSO_4 \cdot 7H_2O$ | 0.1g |
| NaCl | 8g |
| $NaHCO_3$ | 0.35g |
| $Na_2PO_4$ | 0.09g |

| | |
|---|---|
| Dextrose | 1g |
| Phenol Red | 0.01g |
| T.D.W. | 1L |

# 5. Cell viability and measurement of anti-inflammatory effect

## 5.1. Cell cytotoxicity assay

The cytotoxicity of extract solution obtained from leaves of *Sasa borealis* on various cells was performed by MTT (Sigma, USA) colorimetric assay according to the method previously described (Mosmann, 1983). Cells with various densities were treated with indicated concentrations for either 24hrs or 48hrs based on the objective. Then, MTT (0.5μg/ml) was added (10% (v/v)) to the cell and continuously incubated for 4hrs. After 4hrs of incubation, 0.04N HCl / isopropanol solution was added to solubilize the formazan crystal. Optical density at 540nm was determined with microplate reader (Molecular Devices, USA). Cell viability was express as the optical density of formazan crystal formed in the cell. In order to calculate percentage value of cell proliferation, following equation was utilized: Cell proliferation (%) = (O.D. of treatment group / O.D. of control group) x 100.

## 5.2. Nitric Oxide (NO) assay

Murine macrophage cell line, RAW 264.7 cells were cultured in 96-well culture plates at density of ~1.0 x $10^6$ cells/well. DMEM (phenol red (-)) containing 10% FBS was used as culture media. Cells were incubated with

various concentrations of extract obtained from leaves of *Sasa borealis* and stimulated with 5μg/ml of LPS (*Escherichia coli* 011: B4, Sigma, USA) for 24hrs and 48hrs. After 48hrs of incubation, the culture supernatants were collected and analyzed for NO production via Griess reaction (Ding *et al*., 1988; Park *et al*., 1999; Kim *et al*., 2007). Nitrites are product from nitric oxide in the presence of $H_2O$ and $O_2$ and are accumulated in culture medium that reflects the amount of NO production. The concentration of nitrite was measured by mixing 100μl of culture supernatant with equal volume of Griess reagent (1% sulfanilamide in 50% $H_3PO_4$ (w/v), 0.1% N-1-napthylethylenediamine dihydrochloride (1:1)) in 96-well microtiter plates. The color development was measured at absorbance of 540nm. The amounts of nitrite presence were estimated according to the standard curve generated using a known concentration of sodium nitrite. All experiment was done in triplicate. LPS (5μg/ml, from *E.coli*, Sigma, USA) was used as positive control and the production of NO were measured utilizing microplate reader (Molecular Devices, USA) at 540nm.

# 6. Analysis of alteration in fecal microbiome community

## 6.1. Experimental animal

Experimental mouse was purchased from company OrientBio through institute of laboratory animal resources. 25 female ICR mouse was purchased and kept in plastic cage with unlimited access to forage and water. Temperature and humidity was controlled (22±2℃, 55±10%) thoroughly and lighting was provided every 12hrs. All of experimental mouse spent 7 days for adapting to environment before used.

### 6.1.1. *In vivo* experiment design

After 1week of stabilization, 5 mice were assigned as a group (5 groups total). Group 1 was provided with standard diet (Samtako, S.Korea) and 200µl of PBS was orally injected every other day. Group 2 was provided with High Fat Diet (HFD, STD with lard) and also 200µl of PBS was orally injected every other day. Groups 3, 4 and 5 were also provided with HFD, but also 100µl of *Lactobacillus casei* LS2, *Lactobacillus paraplantarum* GL and *Leuconostoc citreum* IH22 ($10^{10}$ CFU/mouse) and 100µl of *Sasa borealis* leaf extract (original = 128 units) were orally injected every other day. Every group was provided with unlimited access to forage and water. Groups 2, 3, 4 and 5 were provided with unlimited access to lard.

### 6.1.2. Composition of the standard diet

| | | |
|---|---|---|
| Main ingredients | Moisture | 11.10% |
| | Carbohydrate | 52.90% |
| | Protein | 18.00% |
| | Fiber | 6.00% |
| | Calcium | 0.70% |
| | Ash | 5.70% |
| | Fat | 5.60% |
| Energy | 4,310 cal/g | |

### 6.2. Genomic DNA extraction from mouse fecal sample

At day 56, fecal samples of each group were collected. Collected samples were kept frozen (-80℃) before usage. When using it, fecal samples were first untangled in 1X PBS (pH7.2). Samples were then dissolved with 1X PBS to obtain O.D. value between 0.8~1.0. DNA of fecal samples was extracted using G-spin™ genomic DNA extraction kit for bacteria (Intron, S.Korea).

### 6.3. Polymerase Chain Reaction (PCR)

DNA extracted from fecal samples collected from day 56 was amplified with test PCR process which makes barcode tagging PCR more efficiently. Efficient barcode tagging PCR is essential since is it is part of preparation step for pyrosequencing. To amplify 16S rRNA V3 region gene of countless microorganisms in fecal sample, 27f, 518r primer (Bioneer, Korea) was used.

| | |
|---|---|
| 27f | GAGTTTGATCMTGGCTCAG |
| 518r | WTTACCGCGGCTGCTGG |

PCR mixture includes: ① 0.25μl *i-Taq* polymerase 5U/μl (Intron, S.Korea),
② 5μl 10X PCR buffer with $MgCl_2$ (Intron, S.Korea),
③ 1μl 50 pM 27f primer,
④ 1μl 50 pM 518r primer,
⑤ 2μl template DNA,
⑥ T.D.W. ⟶ Final volume to 50μl.

PCR steps includes: ① Pre-denaturation at 94℃ for 5mins,
② Denaturation at 94℃ for 30secs,

③ Annealing at 55℃ for 45secs,

④ Extension at 72℃ for 90secs (repeat 20 times),

⑤ Final extension at 72℃ for 7mins.

Utilizing electrophoresis technique (100V, 30mins), PCR product was loaded on to a 1% agarose gel (Seakem GTG agarose, FMC bioproducts, USA) with TAE buffer (1X). Gel was stained with EtBr for 20mins and washed for 3mins before the bands were observed.

### 6.3.1. 10X TAE buffer

Autoclaved (121℃, 1.2 ATM, 15min) 10X TAE buffer was diluted every time it was used. It was made with the following ingredients:

| | |
|---|---|
| Tris base | 48.4g |
| Acetic acid glacial | 11.4g |
| 0.5M EDTA (pH8.0) | 20ml |
| T.D.W. | 1L |

### 6.3.2. TE buffer

Autoclaved (121℃, 1.2 ATM, 15min) TE buffer was used when diluting the primer. It was made with the following ingredients:

| | |
|---|---|
| 1M Tris-HCl (pH7.6) | 1ml |
| 0.5M EDTA | 20µl |
| D.W. | 100ml |

### 6.3.3. 5X Native sample loading buffer

10ml of 5X native sample loading buffer was made to use. 0.1g of Bromophenol blue was dissolved in 1ml of 1X PBS. Final concentration of the buffer contained 60mM Tris-HCl (pH6.8), 25% Glycerol, 2% SDS, 5% β-mercaptoethanol and 0.1% Bromophenol blue. The buffer was used in gel electrophoresis after PCR.

| | |
|---|---|
| 1M Tris-HCl (pH6.8) | 0.6ml |
| 50% Glycerol | 5ml |
| 10% SDS | 2ml |
| β-mercaptoethanol | 0.5ml |
| Bromophenol blue | 1ml |

## 6.4. Preparation of samples for sequencing

### 6.4.1. Barcode tagging PCR

Instead of using existing 27f and 518r primers, barcode tagged primer (barcode sequence and adapter link sequence added) was used in barcode tagging PCR (Table 1). Barcode tagging PCR is a preparation step for successful pyrosequencing of fecal samples collected. Such step makes it possible to organize data in groups so that it is much easier to analyze the sequencing data. Adapter link sequence which was added to primer during PCR must be designed with dissimilar nucleotide bases compare to bacterial 16S rRNA V3 region gene sequence. It catches distinction between barcode and target sequence. In the present study, nucleotide bases adenine (A) and cytosine (C) were used.

Barcode tagging PCR mixture was made of 0.25µl *i-Taq* polymerase 5U/µl (Intron, S.Korea), 5µl 10X PCR buffer wit $MgCl_2$ (Intron, S.Korea), 1µl 10mM dNTPs (2.5mM each, Intron, S.Korea), 1µl 50pM 27f barcode tagged primer, 1µl 50pM 518r barcode tagged primer, 2µl template DNA and was leveled to total volume of 50µl with T.D.W.

Barcode tagging PCR steps includes: ① Pre-denaturation at 94℃ for 5mins,
② Denaturation at 94℃ for 30secs,
③ Annealing at 55℃ for 45secs,
④ Extension at 72℃ for 90secs (20 times),
⑤ Final extension at 72℃ for 7mins.

Electrophoresis (100V, 30mins) using 1% agarose gel (Seakem GTG agarose, FMC biopriducts, USA) and 1X TAE buffer of PCR product was performed. EtBr was used for staining and 3mins of washing step followed before observing bands.

Table 1. List of barcode PCR primers for pyrosequencing

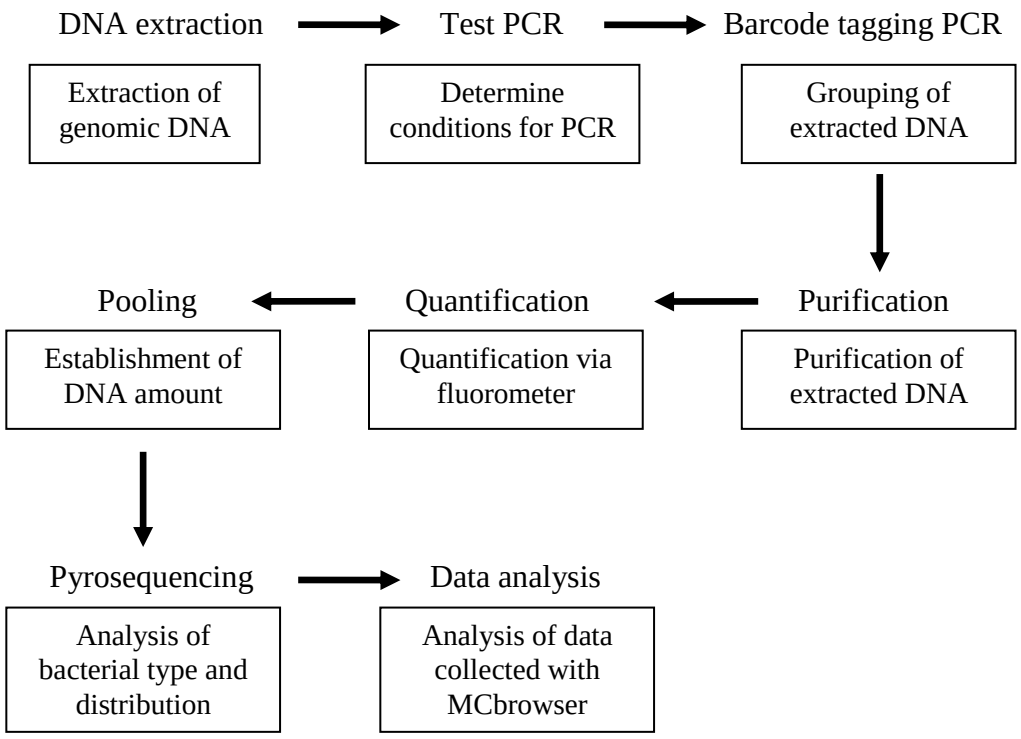
| Barcode | 27f, 518r |
|---|---|
| TCATCG | TCATCGACGAGTTTGATCMTGGCTCAG<br>TCATCGACWTTACCGCGGCTGCTGG |
| GAGTAC | GAGTACACGAGTTTGATCMTGGCTCAG<br>GAGTACACWTTACCGCGGCTGCTGG |
| TAGCAC | TAGCACACGAGTTTGATCMTGGCTCAG<br>TAGCACACWTTACCGCGGCTGCTGG |
| ACAGTG | ACAGTGACGAGTTTGATCMTGGCTCAG<br>ACAGTGACWTTACCGCGGCTGCTGG |
| CACATG | CACATGACGAGTTTGATCMTGGCTCAG<br>CACATGACWTTACCGCGGCTGCTGG |

### 6.4.2. Purification and quantification

Utilizing PCR quick-spin$^{TM}$ PCR product purification kit (Intron, S.Korea), PCR product was purified. Electrophoresis (100V, 30mins) using 1% agarose gel (Seakem GTG agarose, FMC biopriducts, USA) and 1X TAE buffer of PCR product was performed. EtBr was used for staining (20mins) and 3mins of washing step followed before observing bands.

Purified PCR product was quantified using fluorometer (TBS-380, Turner Biosystems, USA). This step occurred by measuring its optical density occupying picogreen (PicoGreen dsDNA Quantificatoin kit (MOP-P-11496)) as a dye. After quantification, DNA amount was set to 250ng for each sample before it was sequenced with Roche 454 GS FLX (Roche, Germany) platform. All of sequencing process was done through National Instrumentation Center for Environmental Management (NICEM) of Seoul National University.

### 6.4.3. Whole process of pyrosequencing



# CHAPTER III

# RESULTS

# 1. Preparation of *Sasa borealis* leaf extract

## 1.1. Partial purification of active compounds of *Sasa borealis*

Recent scientific studies on bamboo leaf tend to focus only on flavonoids or polyphenols. In present study, protein especially glycoprotein was the main material to be focused on. In order to achieve aqueous extract solution, dried leaves of *Sasa borealis* was completely immersed under 5% EtOH for 72hrs in room temperature. After collecting the supernatant, aqueous extract solution was centrifuged (6000rpm, 30min, 4℃) and filtered using 0.22μm microfilter (Whatman). To partially purify glycoprotein from an extract solution, saturated ammonium sulfate ($(NH_4)_2SO_4$) solution was slowly and deliberately added to the extract solution. When ammonium sulfate ($(NH_4)_2SO_4$) solution has reached 80% of total volume, mixture was kept in 4℃ for overnight. Mixture was then centrifuged (11000rpm, 20mins, 4℃) and the pellet of mixture were dissolved in 1X PBS. Collected pellets needed the process of removing salt via dialysis (Spectrum Laboratories, MWCO: 12-14000) for 48hrs at 4℃. Mixture of pellets were used as it is or concentrated with organic solvents. Both pellets and supernatant were tested for its antimicrobial activity (Figure 1).

## 1.2. Protein and carbohydrate contents of *Sasa borealis*

Amount of protein and carbohydrate contents in leaves of *Sasa borealis* were determined with Bradford assay and anthrone test (Table 2). The results indicate that there are copious amount of proteins and carbohydrates present in leaves of *Sasa borealis*. There were minor decrease in protein amount after ammonium sulfate ($(NH_4)_2SO_4$) precipitation and significant increase in carbohydrate contents. With the purpose of determining approximate size of protein, SDS-Poly Acrylamide Gel Electrophoresis was performed. Silver staining demonstrated a dark band near 100kDa (Figure 2).

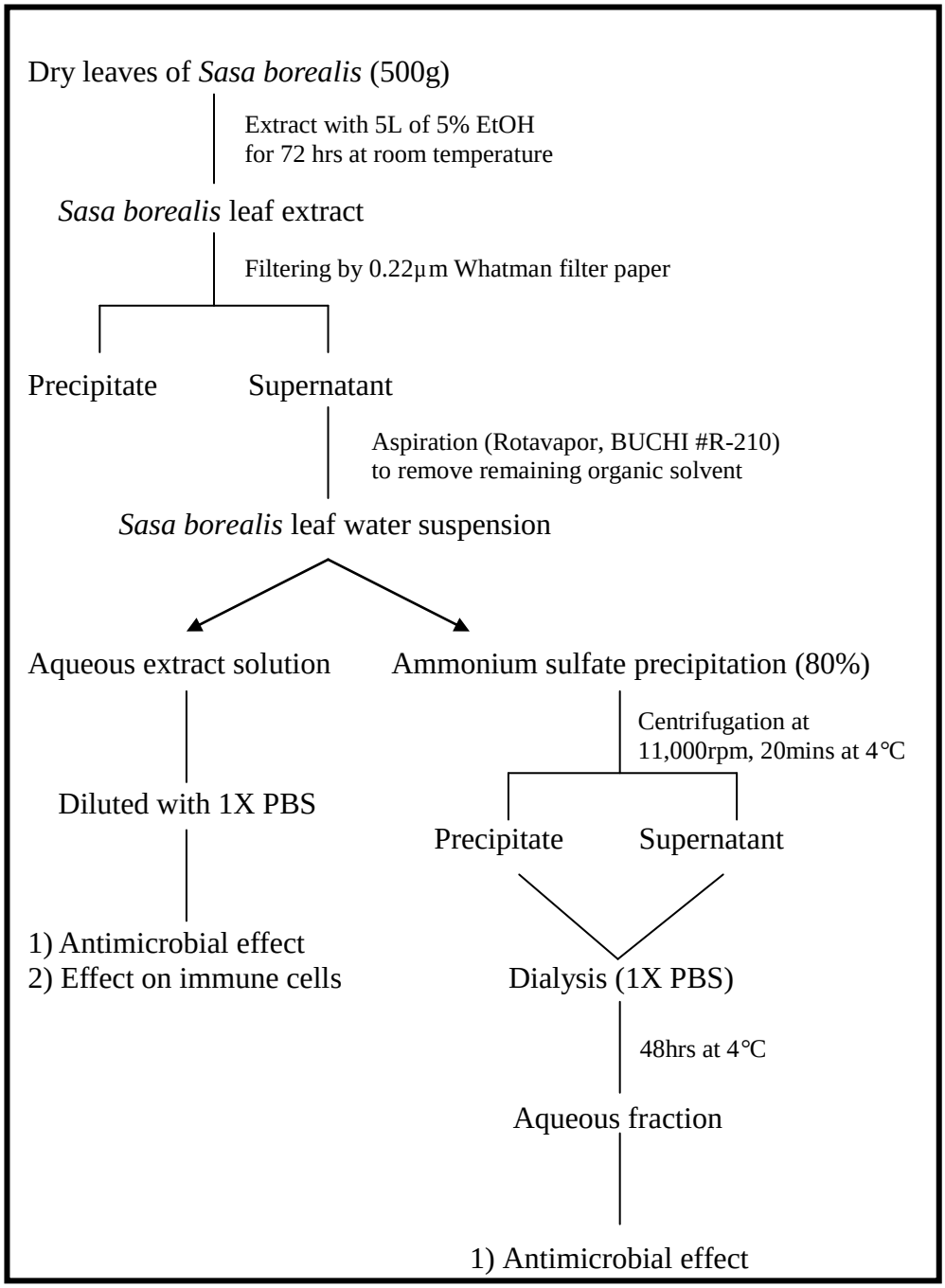



**Figure 1. Scheme of *Sasa borealis* leaf extract preparation.**

**Table 2. Protein and carbohydrate contents of *Sasa borealis*.**

| | Protein (μg/ml) | Carbohydrate (μg/ml) |
|---|---|---|
| 5% EtOH extract | 429.3 | 1938.2 |
| Ammonium sulfate precipitate | 378.7 | 4938.6 |

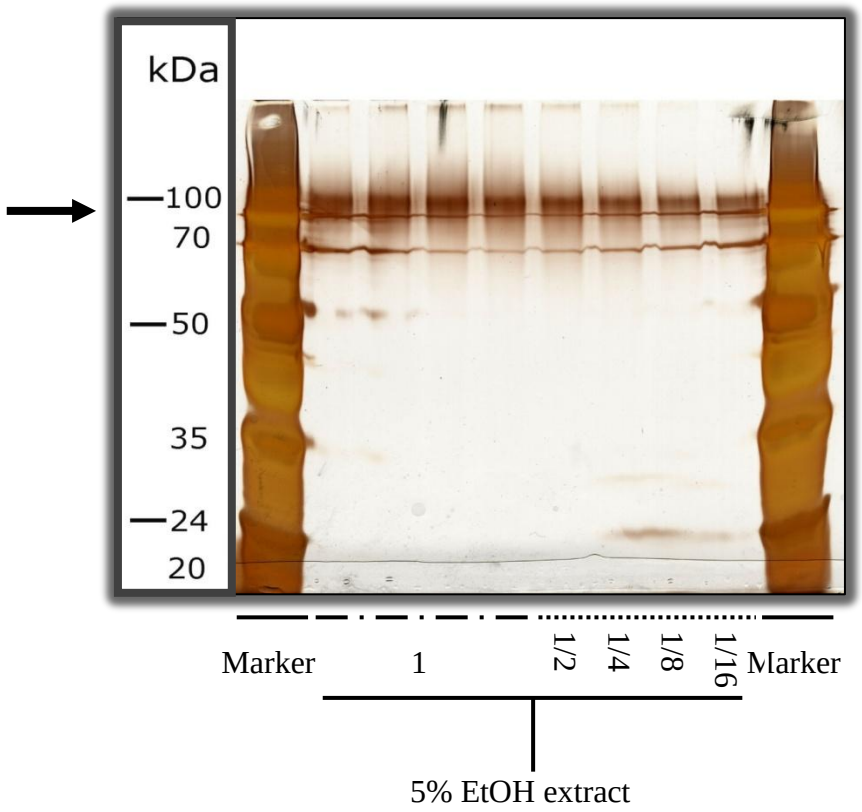



**Figure 2. SDS-PAGE of *Sasa borealis* leaf extract.**
Various concentrations of *Sasa borealis* leaf extract was loaded into wells 2 to 9 with equal loading volume of 60µl (20µl x 3) each. After running the gel, it was stained with Silver staining solution. Pre-stained protein size marker (ELPIS EBM-1031) was loaded into lanes 1 and 10 for a comparison.

# 2. Analysis of *Sasa borealis* (Hack.) Makino & Shibata

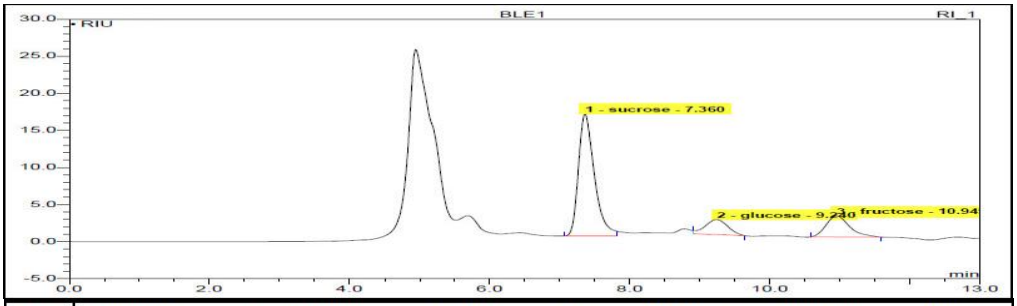
## 2.1. Analysis of compounds in *Sasa borealis*

Copious compounds in *Sasa borealis* were acquired via HPLC system. Each standard solution was dissolved into various concentrations (100 ~ 10000ppm) and utilized to determine which peak is which and what the relationship is between the peak area and the concentration of the standard. The peaks were identified by comparing the spectra from unknown peaks to the spectra from standards. Based on the HPLC analysis, aqueous extract obtained from leaves of *Sasa borealis* contained sucrose, glucose and fructose as its sugar contents (Figure 3A). Additionally, aqueous extract comprises of citric, malic, formic and acetic acid as its organic acid component (Figure 3B).

Amino acid component of *Sasa borealis* leaf extract was also analyzed via HPLC system. After 30mins of retention time, total of 20 amino acids were detected. Gamma-aminobutyric acid (GABA) was the highest amino acid content with 271.59 pmol/µl (Figure 4). Lastly, cation and anion contents of *Sasa borealis* leaf extract were examined. As a result, number of cations including sodium (Na), ammonium ($NH_4$), potassium (K) and magnesium (Mg) were detected. Alternatively, chloride (Cl), sulfate ($SO_4$) and phosphate ($PO_4$) were detected as anion contents of aqueous extract (Figure 5). Cations and anions were detected by Dionex ICS3000 (Dionex, USA) with the run time of 15 and 12mins each.

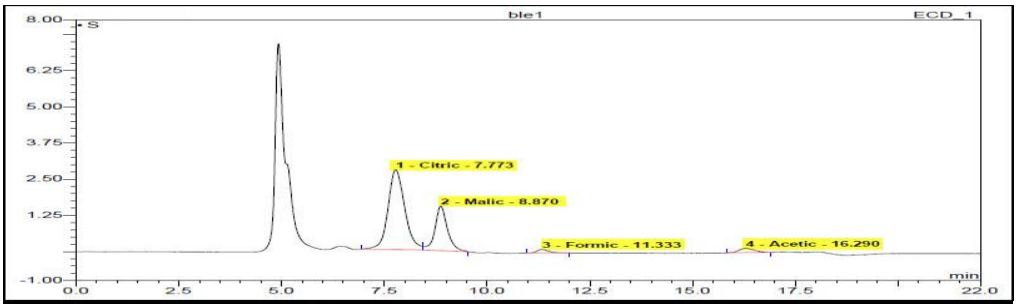
Several plant polyphenols are known to composite in bamboo leaves. Those polyphenols include orientin, homoorientin, vitexin, luteolin and so on. In this present study, plant polyphenol gallic acid was detected via HPLC (Figure 6). Gallic acid is a molecule with a molecular mass of 170.12g/mol and known to have antimicrobial against several bacterial strains including *Staphylococcus aureus* (Akiyama H. *et al*., 2001).

(A)



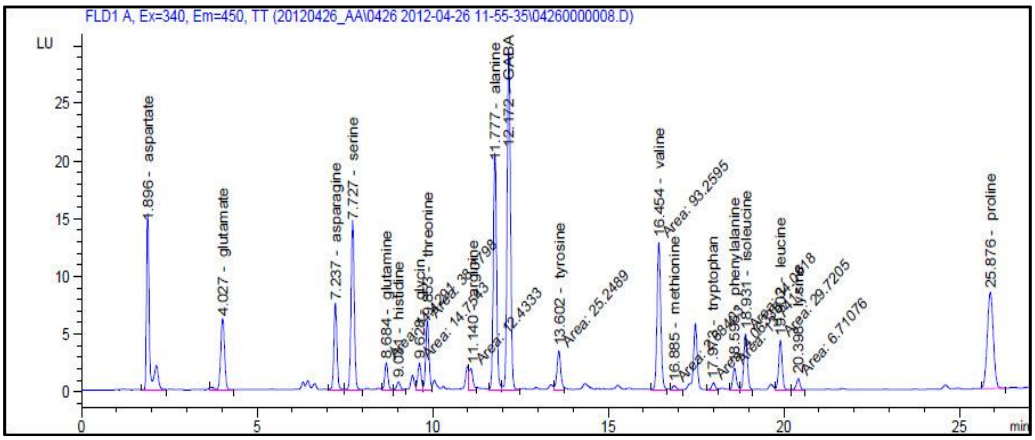

| No. | Ret.Time min | Peak Name | Height • RIU | Area • RIU*min | Rel.Area % | Amount mg/L | Type |
|---|---|---|---|---|---|---|---|
| 1 | 7.36 | sucrose | 16.420 | 4.327 | 70.04 | 1631.975 | BM * |
| 2 | 9.24 | glucose | 2.033 | 0.755 | 12.22 | 255.749 | MB* |
| 3 | 10.95 | fructose | 2.833 | 1.096 | 17.74 | 385.586 | BMB* |
| Total: | | | 21.287 | 6.178 | 100.00 | 2273.310 | |

(B)




| No. | Ret.Time min | Peak Name | Height • S | Area • S*min | Rel.Area % | Amount mg/L | Type |
|---|---|---|---|---|---|---|---|
| 1 | 7.77 | Citric | 2.754 | 1.294 | 66.78 | 416.726 | BM * |
| 2 | 8.87 | Malic | 1.538 | 0.543 | 27.99 | 220.230 | MB* |
| 3 | 11.33 | Formic | 0.118 | 0.037 | 1.93 | 10.716 | BMB* |
| 4 | 16.29 | Acetic | 0.147 | 0.064 | 3.29 | 27.079 | BMB* |
| Total: | | | 4.557 | 1.938 | 100.00 | 674.750 | |

**Figure 3. HPLC chromatogram of *Sasa borealis* leaf extract.**
(A) HPLC chromatogram of sugar contents of *Sasa borealis* leaf extract (Aminex 87P column, 300mm x 7.8mm / Bio-rad, USA). (B) Organic acid components of *Sasa borealis* leaf extract (Aminex 87H column).



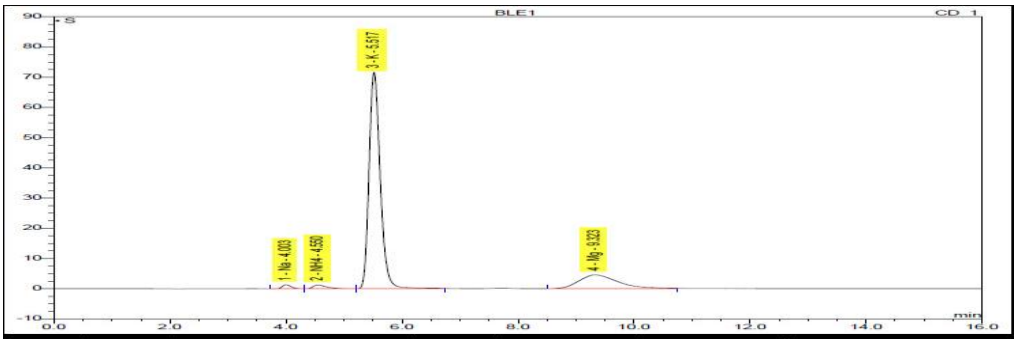

| RetTime [min] | Type | Area LU *s | Amt/Area | Amount [pmol/ul] | Grp | Name |
|---|---|---|---|---|---|---|
| 1.896 | BB | 94.37983 | 1.53559 | 144.92845 | | aspartate |
| 4.027 | BB | 47.30310 | 1.66001 | 78.52344 | | glutamate |
| 7.237 | BV | 46.14681 | 1.53730 | 70.94147 | | asparagine |
| 7.727 | VB | 93.54533 | 1.45809 | 136.39762 | | serine |
| 8.684 | FM | 14.42907 | 1.88510 | 27.20028 | | glutamine |
| 9.031 | VV | 4.65319 | 2.06280 | 9.59862 | | histidine |
| 9.628 | FM | 14.75435 | 1.39099 | 20.52320 | | glycin |

| RetTime [min] | Type | Area LU *s | Amt/Area | Amount [pmol/ul] | Grp | Name |
|---|---|---|---|---|---|---|
| 9.853 | FM | 38.37984 | 1.48335 | 56.93084 | | threonine |
| 11.140 | MF | 12.43330 | 1.28112 | 15.92851 | | arginine |
| 11.777 | BV | 135.65755 | 1.43460 | 194.61441 | | alanine |
| 12.172 | VB | 195.12627 | 1.39187 | 271.58955 | | GABA |
| 13.602 | MF | 25.24886 | 1.44438 | 36.46895 | | tyrosine |
| 16.454 | MF | 93.25953 | 1.06364 | 99.19487 | | valine |
| 16.885 | FM | 2.68403 | 1.19671 | 3.21201 | | methionine |
| 17.248 | | - | - | - | | norvaline |
| 17.973 | MF | 4.06335 | 1.53179 | 6.22421 | | tryptophan |
| 18.596 | FM | 13.74109 | 1.27816 | 17.56335 | | phenylalanine |
| 18.931 | MF | 34.06177 | 1.01277 | 34.49662 | | isoleucine |
| 19.902 | FM | 29.72050 | 1.23031 | 36.56536 | | leucine |
| 20.398 | MF | 6.71076 | 3.44584 | 23.12420 | | lysine |
| 21.723 | | - | - | - | | hydro proline |
| 24.985 | | - | - | - | | sarcosine |
| 25.876 | BB | 99.45349 | 1.05551 | 104.97377 | | proline |

| Totals : | 1388.99972 |
|---|---|

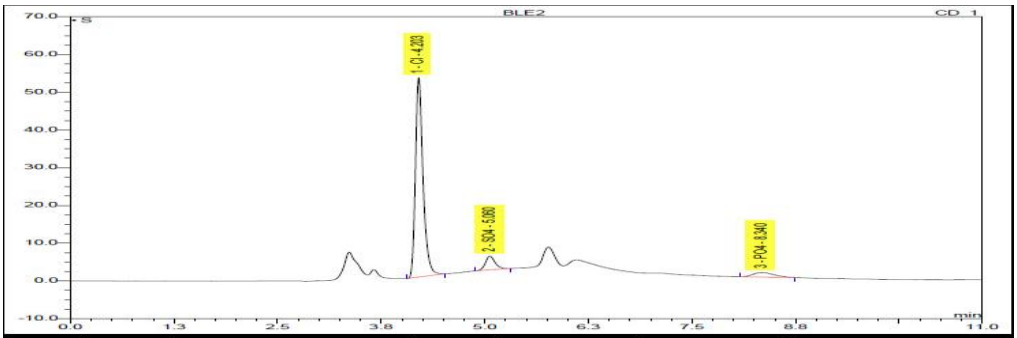
**Figure 4. HPLC chromatogram of *Sasa borealis* leaf extract.**
HPLC chromatogram of amino acid contents of *Sasa borealis* leaf extract (C18 column, 4.6mm x 150mm, 5µm). Amounts are expressed in pmol/µl.

(A)



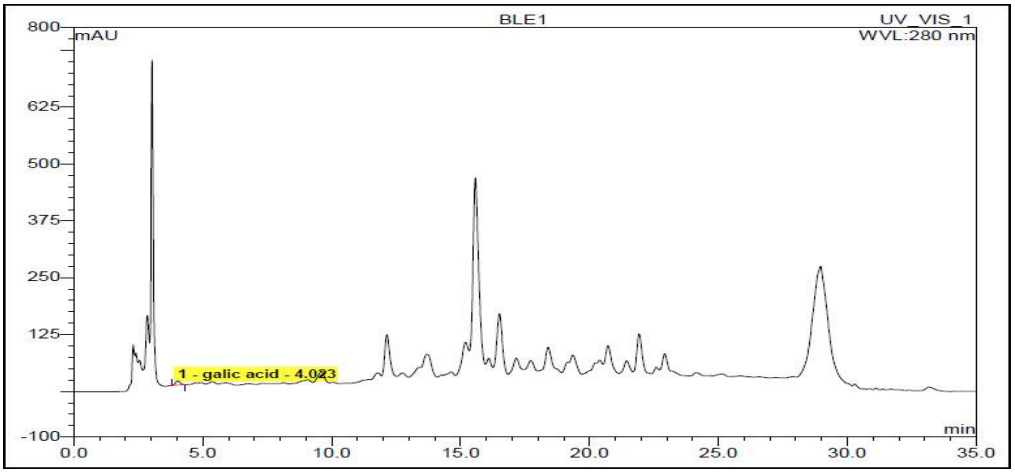

| No. | Time min | Peak Name | Type | Area • S*min | Height • S | Amount ppm |
|---|---|---|---|---|---|---|
| 1 | 4.00 | Na | BM * | 0.239 | 1.334 | 10.5673 |
| 2 | 4.55 | NH4 | M * | 0.364 | 1.278 | 8.5009 |
| 3 | 5.52 | K | M * | 16.051 | 71.626 | 845.8995 |
| 4 | 9.32 | Mg | BM * | 3.544 | 4.562 | 83.1986 |
| TOTAL: | | | | 20.20 | 78.80 | 948.17 |

(B)




| No. | Time min | Peak Name | Type | Area • S*min | Height • S | Amount mg/L |
|---|---|---|---|---|---|---|
| 1 | 4.20 | Cl | BMB* | 5.571 | 52.764 | 314.9334 |
| 2 | 5.06 | SO4 | BMB* | 0.497 | 3.643 | 38.4824 |
| 3 | 8.34 | PO4 | BMB* | 0.350 | 1.207 | 62.8901 |
| TOTAL: | | | | 6.42 | 57.61 | 416.31 |

**Figure 5. HPLC chromatogram of *Sasa borealis* leaf extract.**
(A) HPLC chromatogram of cation contents of *Sasa borealis* leaf extract (Ionpac CS12A column, 4 x 250mm / Dionex, USA). (B) Anion components of *Sasa borealis* leaf extract (Ionpac AS20 column, 4 x 250mm / Dionex, USA).




| No. | Ret.Time min | Peak Name | Height mAU | Area mAU*min | Rel.Area % | Amount mg/L | Type |
|---|---|---|---|---|---|---|---|
| 1 | 4.02 | galic acid | 9.399 | 1.976 | 100.00 | 1.697 | BMB* |
| Total: | | | 9.399 | 1.976 | 100.00 | 1.697 | |

**Figure 6. HPLC chromatogram of *Sasa borealis* leaf extract.**
HPLC chromatogram of plant polyphenol (gallic acid) content of *Sasa borealis* leaf extract (C18 column, 4.6mm x 150mm). Amounts are expressed in mg/L.

# 3. Antimicrobial effect of *Sasa borealis* leaf extract

## 3.1. Working unit determination

Antimicrobial ability of extract obtained from leaves of *Sasa borealis* was examined. Bamboo leaves are known for its antimicrobial activity against particular bacterial strains. First, it was necessary to determine working unit of aqueous extract from leaves of *Sasa borealis*. Such process was done by testing Minimum Inhibitory Concentration (MIC) of bacterial strain *Rothia dentocariosa* G1201. *Rothia dentocariosa* G1201 was selected since it was most susceptible bacterial strain against *Sasa borealis* leaf extract during pre-trial experiment. Such bacterial strain was inoculated into each well of 96-well culture plate with various concentrations of *Sasa borealis* leaf extract (serial diluted to 1/1024). Culture plate was then incubated at 30℃ for overnight long. After overnight incubation, Optical Density (O.D.) of culture supernatant was measured for unit determination. As of result, concentration of 1/128 was the lowest concentration of *Sasa borealis* leaf extract that inhibited the growth of *Rothia dentocariosa* G1201 (Figure 7). Thus, concentration of 1/128 was termed 1unit and the original solution was equivalent to 128 units.

## 3.2. Antimicrobial effect of *Sasa borealis* utilizing agar well diffusion method

Following the unit determination, 16 bacterial strains that were inhibited the most by *Sasa borealis* leaf extract during pre-trial experiments were selected. Bacterial strains included 5 Gram (+) bacteria, 4 Gram (-) bacteria and 6 lactic acid bacteria (Table 3). After the selection of bacterial strains, all of bacteria were spread on to an agar plate. Using a sterilized borer with appropriate pore size (9mm), wells were made for each agar plate. Then, 150µl of aqueous extract (10 units of antimicrobial extract) was inserted into each well for inhibition zone forming. Plates for each bacterial strain were incubated overnight (either 30℃ or

37℃) and diameter of inhibition zone was inspected the next day. Inhibition zone is known as area on an agar plate where growth of a control organism is prevented / inhibited by an antibiotic substance usually plated on the agar surface. As of result, bacterial strain *Rothia dentocariosa* G1201 exhibited largest inhibition zone with 35.5 ± 0.43mm in diameter. Both Gram (+) and Gram (-) were inhibited by aqueous extract from leaves of *Sasa borealis* (Figure 8, Table 4). Nonetheless, all of lactic acid bacteria examined did not show any suppression against bamboo leaf extract. Based on the data collected, it was clear that *Sasa borealis* leaf extract selectively inhibits the growth of bacteria excluding lactic acid bacteria.

### 3.3. Measurement of Optical Density (O.D.)

Alternative path of examining antimicrobial effect of *Sasa borealis* leaf extract was the measurement of O.D. of culture supernatant. The theory of optical density known to increase as bacterial strain grows was suitable for the purpose of such experiment. First of all, 200µl of culture media was inserted into wells of 96-well culture plate. Then, *Sasa borealis* leaf extract (1unit, 10% (v/v)) was introduced to culture media before each bacteria was inoculated. After inoculating each bacterial strain with the sterilized loop, culture plates were incubated at either 30℃ or 37℃ for overnight long. The result displayed significant inhibition of bacterial strains *Rothia dentocariosa* G1201, *Streptococcus mutans* CCARM0079 and *Escherichia coli* JM109. Lactic acid bacteria were not inhibited by extract tested (Table 4).

### 3.4. Antimicrobial activity of glycoprotein in *Sasa borealis* leaf extract

With the purpose of finding substance or molecule exhibiting antimicrobial effect, protein (glycoprotein) was partially purified from a *Sasa borealis* leaf extract with the method of ammonium sulfate precipitation. Both precipitate and supernatant was tested for its antimicrobial activity. Result

indicated that protein precipitate displayed suppressive activity against 8 out of 9 bacterial strains excluding lactic acid bacteria (Figure 9A). Bacterial strain *Serratia marcescens* only showed suppressive activity by supernatant (Figure 9B). By observing at the data collected, it was assure that *Sasa borealis* leaf extract contains at least 2 or more antimicrobial agents.

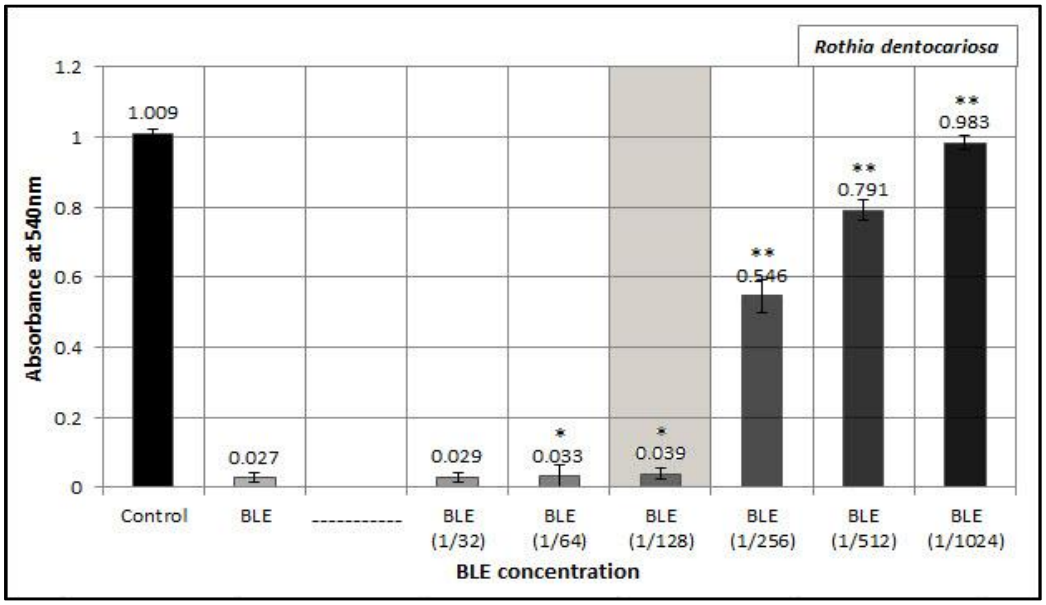



**Figure 7. Minimum inhibitory concentration (MIC) of *Sasa borealis* on *Rothia dentocariosa* G1201.**

Working unit of *Sasa borealis* leaf extract was determined. A bacterium was cultured with various concentrations of BLE (diluted in PBS) for 24h in 96-well culture plate. The optical density (O.D.) of culture supernatant was measured by ELISA reader at absorbance of 540nm. The result represents the average of three independent experiments ± SD. *$p < 0.05$, **$p < 0.01$ compared with the control.

**Table 3. List of bacterial strains used for antimicrobial activity test.**

| Strain | Cultivation condition |
|---|---|
| **Gram (+) bacteria** | |
| *Rothia dentocariosa* G1201 | Nutrient media (TSA), 30℃, Aerobic |
| *Streptococcus mutans* CCARM 0079 | Brain Heart Infusion (BHI), 37℃, Aerobic |
| *Bacillus subtilis* | Nutrient media (TSA), 37℃, Aerobic |
| *Staphylococcus saprophyticus* | Nutrient media (TSA), 37℃, Aerobic |
| *Staphylococcus epidermis* | Nutrient media (TSA), 37℃, Aerobic |
| **Gram (-) bacteria** | |
| *Escherichia coli* JM109 | Nutrient media (TSA), 37℃, Aerobic |
| *Serratia marcescens* | Nutrient media (TSA), 37℃, Aerobic |
| *Klebsiella pneumoniae* | Nutrient media (TSA), 37℃, Aerobic |
| *Salmonella typhimurium* | Nutrient media (TSA), 37℃, Aerobic |
| **Lactic Acid Bacteria (LAB)** | |
| *Lactobacillus paraplantarum* GL | MRS media, 30℃, Aerobic |
| *Lactobacillus casei* LS2 | MRS media, 30℃, Aerobic |
| *Lactobacillus plantarum* M1 | MRS media, 30℃, Aerobic |
| *Lactobacillus fermentum* JS1101 | MRS media, 30℃, Aerobic |
| *Leuconostoc gasicomitatum* MB | MRS media, 30℃, Aerobic |
| *Leuconostoc citreum* IH22 | MRS media, 30℃, Aerobic |
| *Leuconostoc kimchii* IH25 | MRS media, 30℃, Aerobic |

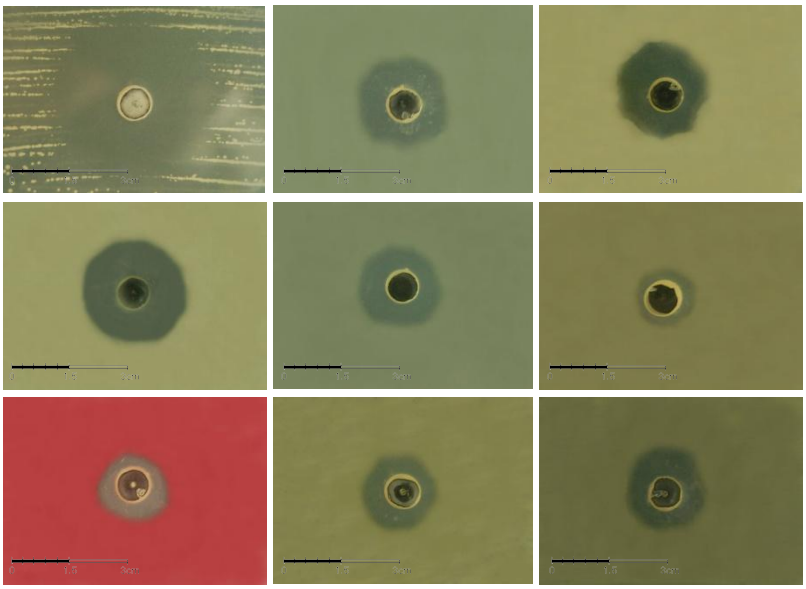


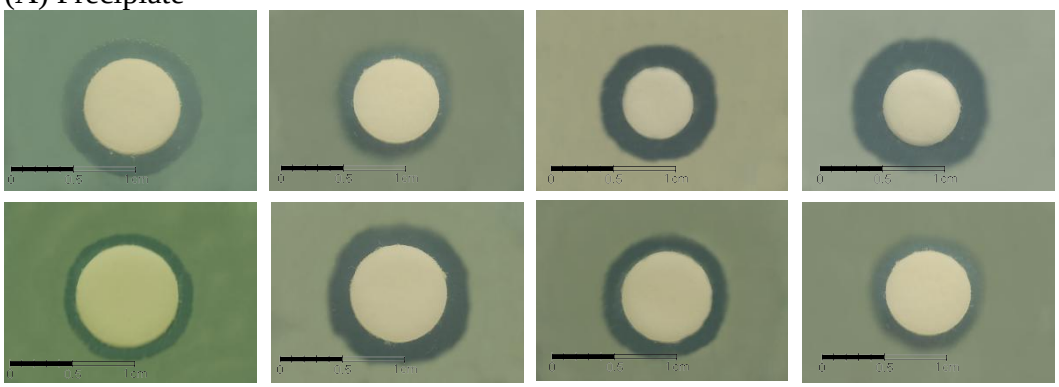
**Figure 8. Agar well diffusion assay of an extract (10 units) obtained from leaves of *Sasa borealis*.**
10 working unit of *Sasa borealis* leaf extract was inserted into wells made. Bacterial strains were co-cultured with *Sasa borealis* leaf extract for overnight long before diameter of inhibition zone was calculated. From left to right 1st row: *Rothia dentocariosa* G1201, *Streptococcus mutans* CCARM 0079, *Bacillus subtilis*; 2nd row: *Staphylococcus saprophyticus, Staphylococcus epidermis, Escherichia coli* JM109; 3rd row: *Serratia marcescens, Klebsiella pneumoniae, Salmonella typhimurium*.

**Table 4. Anti-microbial activity of extract obtained from leaves of *Sasa borealis*.**

| Strain | Optical density[1] (BLE 1 unit) | Inhibition zone (mm)[2]* (BLE 1 unit) | Inhibition zone (mm)[2]* (BLE 10 units) |
|---|---|---|---|
| Gram (+) bacteria | | | |
| *Rothia dentocariosa* G1201 | 0.039 | 31.5 ± 0.50 | 35.5 ± 0.50 |
| *Streptococcus mutans* CCARM 0079 | 0.249 | 14.5 ± 0.87 | 21.5 ± 0.50 |
| *Staphylococcus epidermis* | 0.618 | —[3] | 13 ± 0.25 |
| *Bacillus subtilis* | 0.672 | — | 13.5 ± 0.43 |
| *Staphylococcus saprophyticus* | 0.691 | — | 11 ± 0.87 |
| Gram (-) bacteria | | | |
| *Escherichia coli* JM109 | 0.321 | 13.5 ± 0.48 | 17 ± 0.83 |
| *Serratia marcescens* | 0.473 | 10 ± 0.25 | 14.5 ± 0.43 |
| *Salmonella typhimurium* | 0.692 | — | 10.5 ± 0.25 |
| *Klebsiella pneumoniae* | 0.770 | — | 10 ± 0.50 |
| Lactic Acid Bacteria (LAB) | | | |
| *Lactobacillus fermentum* JS1101 | 0.781 | — | — |
| *Leuconostoc citreum* IH22 | 0.984 | — | — |
| *Lactobacillus paraplantarum* GL | 0.991 | — | — |
| *Leuconostoc kimchii* IH25 | 0.999 | — | — |
| *Lactobacillus plantarum* M1 | 1.009 | — | — |
| *Lactobacillus casei* LS2 | 1.017 | — | — |
| *Leuconostoc gasicomitatum* MB | 1.025 | — | — |

[1] Optical density at 540nm.
[2] Diameter of inhibition/clear zone.
[3] Very weak or no inhibitory zone was formed.
* Values are means ± SD (mm) of three separate experiments

(A) Precipiate



(B) Supernatant

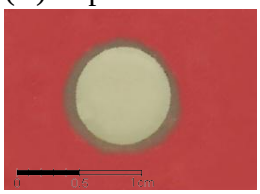


**Figure 9. Paper disc diffusion assay after ammonium sulfate precipitation.** (A) Antimicrobial activity of precipitate from ammonium sulfate ($(NH_4)_2SO_4$) precipitation was examined. From top left: *Rothia dentocariosa* G1201, *Streptococcus mutans* CCARM0079, *Staphylococcus saprophyticus*, *Staphylococcus epidermis*, *Escherichia coli* JM109, *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Bacillus subtilis*. (B) Bacterium *Serratia marcescens* was only inhibited by supernatant.

### 3.5. Protein purification

To determine whether protein in extract obtained from leaves of *Sasa borealis* has antimicrobial property, protein was eluted from the gel matrix after electrophoresis. Based on the silver staining data, dark band near 100kDa was observed. Excised gel was placed in microcentrifuge tubes. Then elution buffer (50mM Tris-HCl, 150mM NaCl, 0.1mM EDTA; pH7.5) was added to the gel. After crushing the gel, microcentrifuge tubes were incubated in a rotary shaker for overnight period. Supernatant was collected the following day and tested for its microbial activity. According to the data collected, purified protein significantly inhibited the growth of most bacterial strains tested. All except bacterial strain *Serratia marcescens* and LAB were resistant to purified protein (Table 5). Additionally single lane of gel piece was placed on to an agar plate after washing SDS (15min x 2 with TDW) in order to observe the diffusion of antimicrobial substance. As a result, bacterial strain (*Rothia dentocariosa* G1201) near 100kDa was strongly inhibited by the protein (Figure 10).

### 3.6. Antimicrobial activity of leaves and sprouts

As study progressed, it was unclear whether leaves are the only part that exhibits antimicrobial activity. To solve the uncertainty of it, both leaves and sprouts of *Sasa borealis* were tested to its ability of inhibiting bacterial growth (*Rothia dentocariosa* G1201). As a result, only leaves of *Sasa borealis* showed strong inhibition activity against bacterial strain examined. Sprouts did not show any antimicrobial effect when tested itself, but weak suppression of bacterial growth was observed when extracted together with the leaves (Figure 11).

**Table 5. Antimicrobial activity of purified protein (glycoprotein).**

| Strain | Optical density[(1)] | Inhibition zone (mm)*[(2)] |
|---|---|---|
| Gram (+) bacteria | | |
| *Rothia dentocariosa* G1201 | 0.035 | 25.5 ± 0.25 |
| *Streptococcus mutans* CCARM 0079 | 0.058 | 23 ± 0.73 |
| *Staphylococcus epidermis* | 0.142 | 12 ± 0.50 |
| *Staphylococcus saprophyticus* | 0.171 | 17.5 ± 0.87 |
| *Bacillus subtilis* | 0.218 | 13.5 ± 0.50 |
| Gram (-) bacteria | | |
| *Escherichia coli* JM109 | 0.071 | 13 ± 0.43 |
| *Salmonella typhimurium* | 0.141 | 16.5 ± 0.71 |
| *Klebsiella pneumoniae* | 0.186 | 11.5 ± 0.50 |
| *Serratia marcescens* | 0.925 | —[(3)] |
| Lactic Acid Bacteria (LAB) | | |
| *Lactobacillus fermentum* JS1101 | 0.837 | — |
| *Leuconostoc citreum* IH22 | 0.912 | — |
| *Lactobacillus paraplantarum* GL | 0.948 | — |
| *Leuconostoc kimchii* IH25 | 0.951 | — |
| *Lactobacillus plantarum* M1 | 0.984 | — |
| *Lactobacillus casei* LS2 | 1.010 | — |
| *Leuconostoc gasicomitatum* MB | 1.036 | — |

* Values are means ± SD (mm) of three separate experiments

(1) Optical density at 540nm.

(2) Diameter of inhibition/clear zone.

(3) Very weak or no inhibitory zone was formed.

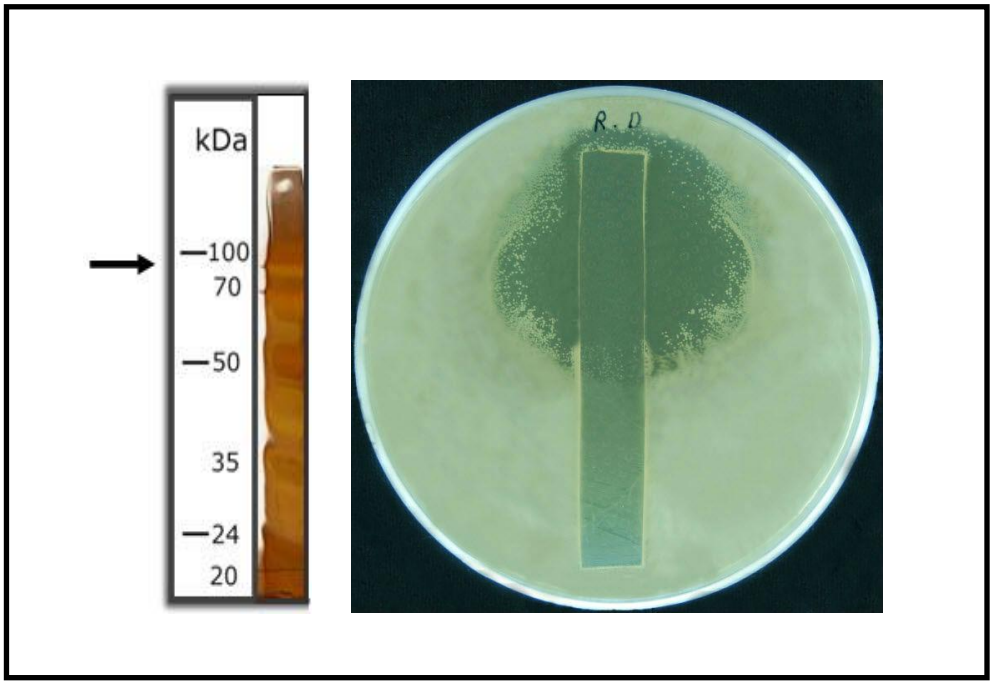



**Figure 10. Antimicrobial activity of purified protein (glycoprotein).**

After the gel electrophoresis, gel matrix was washed twice for 15mins with TDW in order to wash out the ionic detergent SDS. After washing, gel piece was plated on an agar plate that's already spread with a single bacterial strain (*Rothia dentocariosa* G1201). Following overnight incubation, zone of inhibition was observed.

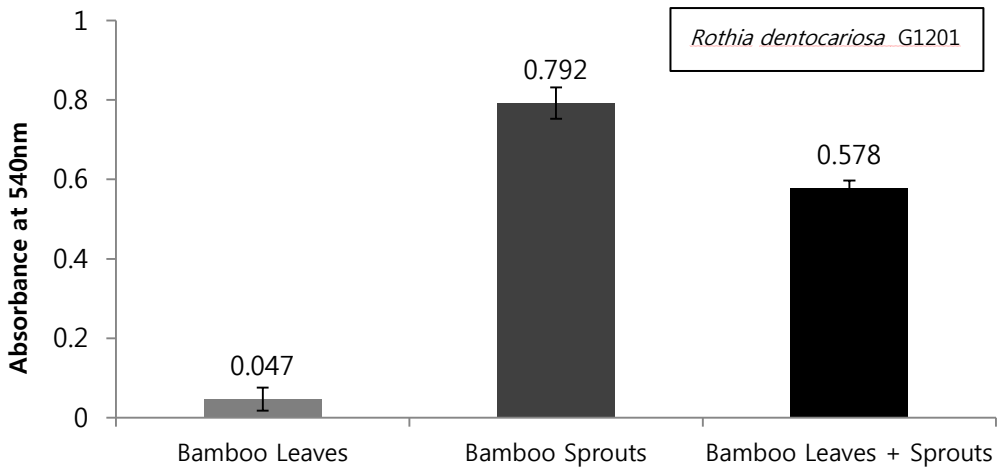



**Figure 11. Antimicrobial effect of *Sasa borealis* leaves, sprouts and mixture against *Rothia dentocariosa* G1201.**
A bacterium was cultured with aqueous solution extracted from *Sasa borealis* leaves, sprouts or mixture of both for 24h in 96-well culture plate. The optical density (O.D.) of culture supernatant was measured by ELISA reader at absorbance of 540nm. The result represents the average of three independent experiments ± SD.

### 3.7. Bactericidal effect of *Sasa borealis*

It is vital to distinguish the term bactericidal and bacteriostatic. The term bactericidal is used when an antimicrobial substance kills bacteria and, ideally, nothing else. Bacteriostatic antibiotics are the one which stops bacteria from reproducing, while not necessarily harming them otherwise. It was important to figure out whether extract obtained from leaves of *Sasa borealis* exhibits bactericidal or bacteriostatic characteristics. To do so, a bacterium *Rothia dentocariosa* G1201 and *Streptococcus mutans* CCARM0079 were co-cultured with or without *Sasa borealis* leaf extract (10 units, 10% (v/v)) in TSB medium at 30℃ for overnight long. Then, culture soup was centrifuged (6000rpm, 15mins) and washed twice with 1X PBS (pH7.2) before it was serial diluted for CFU count. Serial diluted bacteria was inoculated on fresh plate and overnight incubation followed. Result indicates that extract obtained from leaves of *Sasa borealis* kills both bacteria (w/ 96.91% and 96.76% effectiveness) meaning it exhibits bactericidal effect (Figure 12).

### 3.8. Time-kill assay

The time kill assay is used for examining the rate at which concentrations of an antimicrobial agent kill a bacterial isolate. Time-kill assays can be used to study both concentration-dependent and time-dependent bactericidal activities of antimicrobial agents. Prior to performing time-kill assay, there were several parameters that must be defined. First, MIC of the specific antimicrobial agent for the organism of interest needed to be determined. From the MIC, concentrations of antimicrobial agent to test were determined. In the present study, time-kill assay of various concentrations (1, 10, 128 units) of extract from leaves of *Sasa borealis* was examined. Target organism of interest was bacterium *Rothia dentocariosa* G1201. Sample time points were every 4hrs until 24hrs has reached. Results for the control and each agent concentration were plotted versus time from

drug exposure. Generally, a 3-$\log_{10}$ CFU/ml decreases in bacterial counts in antimicrobial solution compare with counts for the growth control. Comparing the data of three concentrations tested, 128 working unit (=original solution) of *Sasa borealis* leaf extract initiated to kill bacterial isolate the fastest. As time from drug exposure increased, all three concentrations leveled out in their ability of inhibiting bacterial isolate (Figure 13).

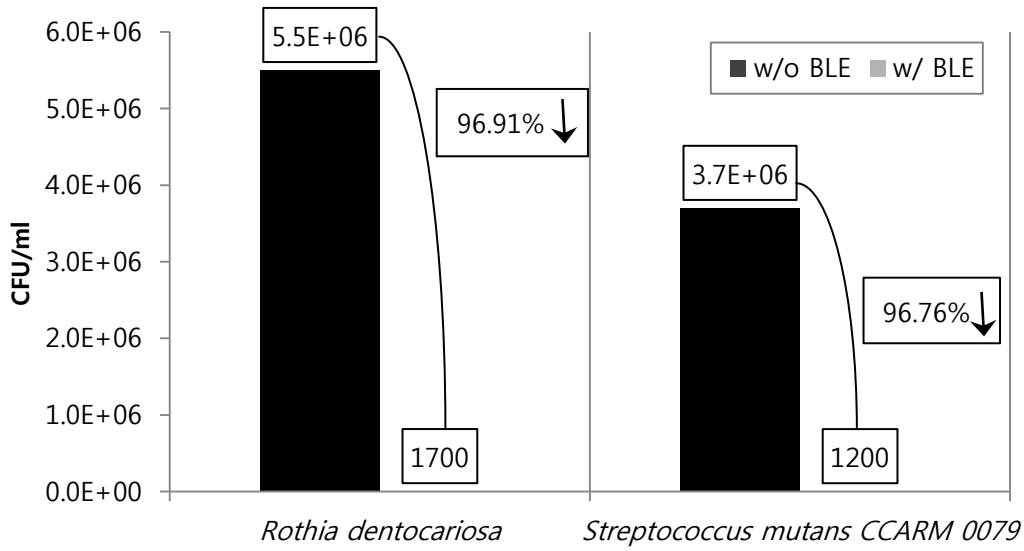



**Figure 12. Bactericidal activity of *Sasa borealis* against *Rothia dentocariosa* G1201 and *Streptococcus mutans* CCARM0079.**
Each bacterium was cultured with or without *Sasa borealis* leaf extract (10 units) for 24hrs in sterilized tube. After centrifugation, each bacterium was washed twice with 1X PBS (pH7.2) and the Colony Forming Unit (CFU) was counted on a fresh plate (TSA / BHI) after overnight incubation at either 30℃ or 37℃.

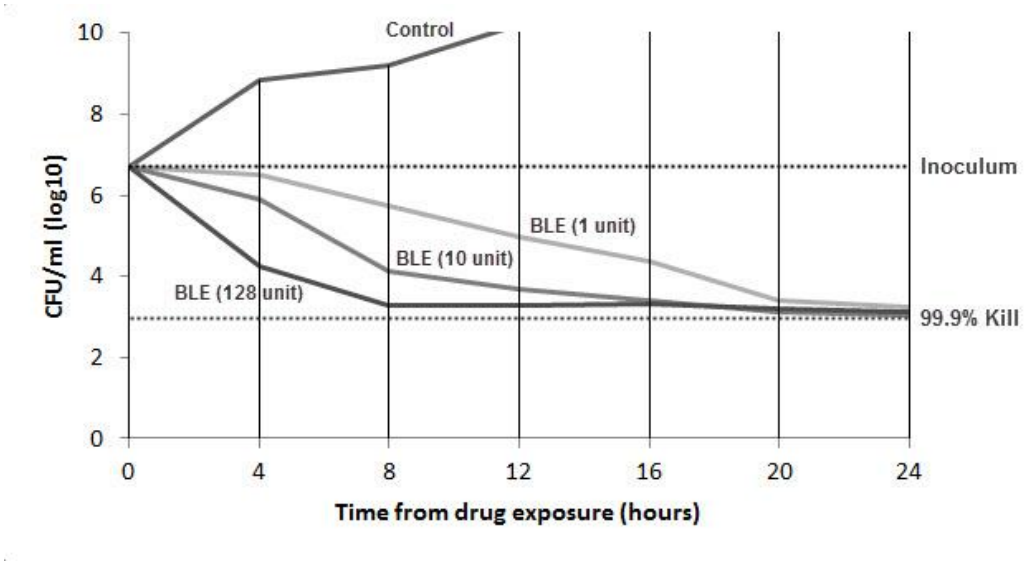



**Figure 13. Time-Kill curve of *Sasa borealis* against *Rothia dentocariosa* G1201.**

Time-kill curve. Broth culture inoculated with $5 \times 10^6$ CFU/mL was exposed to various concentrations of BLE (1, 10, 128 units). The graph above illustrates different time-kill curves for BLE 1, 10 and 128 units. The end point is the time to a bactericidal effect, which is considered a 99.9% kill.

# 4. Immuno-modulatory effects of *Sasa borealis*

## 4.1. Cell cytotoxicity

Cytotoxic effects of *Sasa borealis* was studied against numerous immune cells by MTT assay. First, J774 cells (murine macrophage cell line) and splenocytes isolated from mouse model were co-cultured with various concentrations of *Sasa borealis* leaf extract for 24hrs and 48hrs each and then analyzed by MTT assay respectively (Figure 14, 15). Proliferations of J774 cells and murine splenocytes were determined by Mosmman's method. It was evidently demonstrated that *Sasa borealis* leaf extract has no significant cytotoxic effect against J774 cells and murine splenocyte (Figure 14, 15).

Cytotoxic effect of *Sasa borealis* leaf extract was evaluated in RAW 264.7 cells also. Concentrations of 1, 5, 25, 125, 128 units of *Sasa borealis* leaf extract was tested against RAW 264.7 cells. Cell viability was expressed as the optical density of formazan formed in cells and calculated as follows: cell proliferation (%) = (O.D. of treatment group / O.D. of control group) x 100. It was clearly concluded that *Sasa borealis* does not exhibit cytotoxic effects in RAW 264.7 cells (Figure 16).

*Sasa borealis* leaf extract was examined for its cytotoxic effect against some disease causing cells such as CT-26 cell line (murine colon cancer cell line), EL-4 cell line (murine T-lymphoblast) and S-180 cell line (murine sarcoma cell line). Cell viability was measured by MTT assay. The results undoubtedly demonstrate significant cytotoxic effect against all of cells tested (Figure 17A, B and C). In order to achieve more profound data, trypan blue exclusion test was performed utilizing same cell lines. Such experiment was designed to observe effect of *Sasa borealis* leaf extract (10 units) on long-term cultivation of cells. Cells co-cultured with *Sasa borealis* leaf extract were considerably diminished quite quickly. Identical results were observed from all cell lines tested (Figure 18A, B and C).

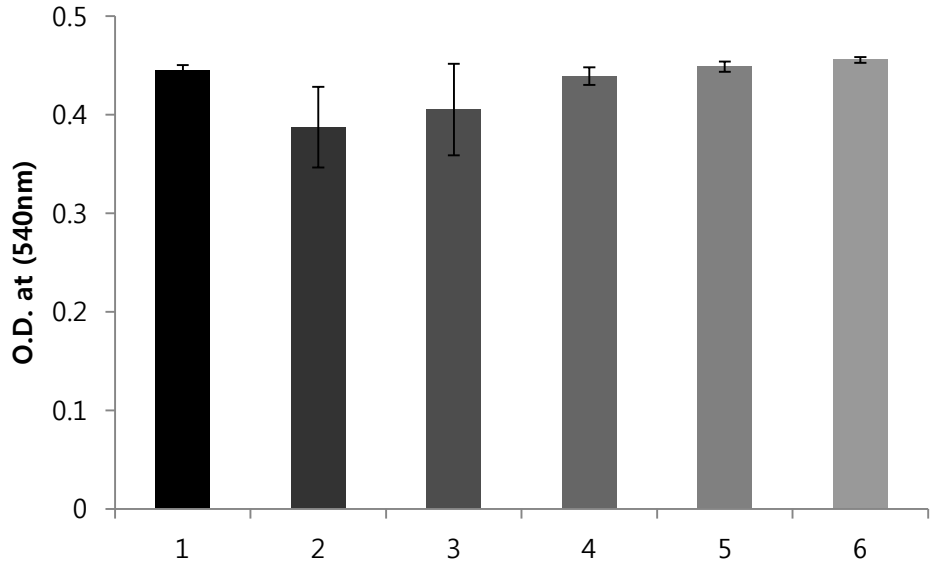



**Figure 14. The suppressive activity of *Sasa borealis* on J774.**
*Sasa borealis* leaf extract showed very weak suppressive activity on proliferation and viability of J774 (2.5 x $10^6$ cells/ml) cultured in RPMI 1640 medium for 48hrs. Viability of the cell was measured by MTT assay. The results were presented by mean SD.
(1: Control, 2: 128 units, 3: 125 units, 4: 25 units, 5: 5 units, 6: 1 unit)

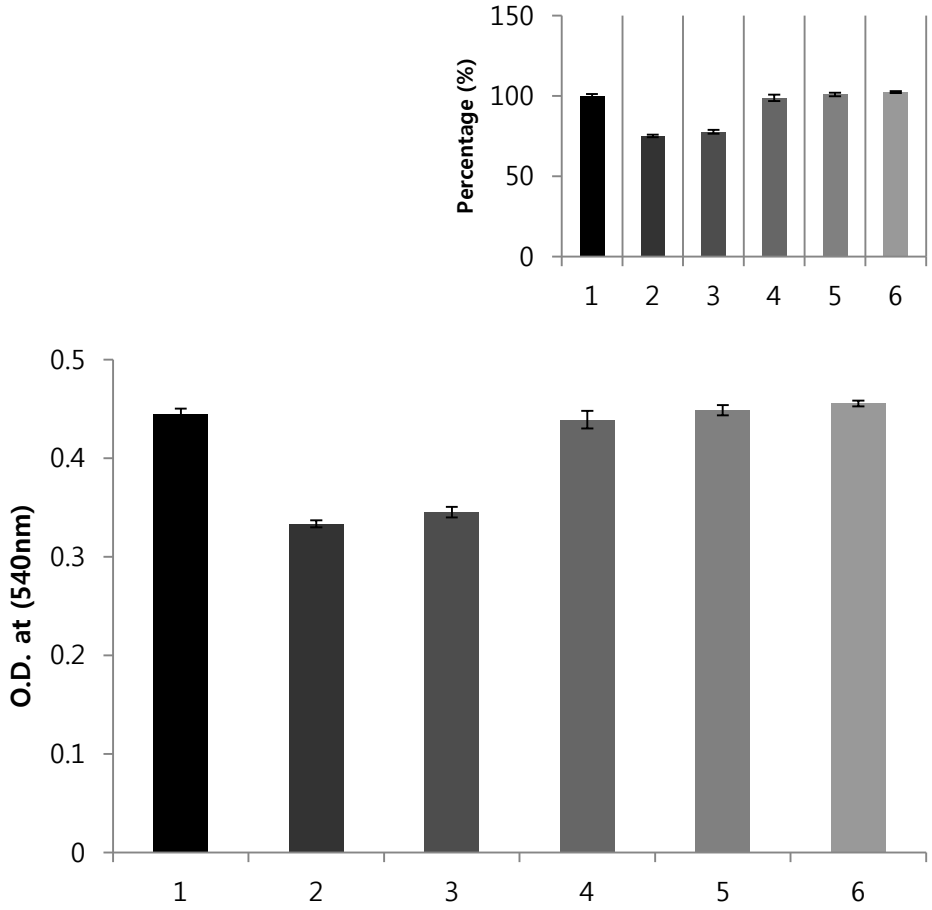



**Figure 15. The suppressive activity of *Sasa borealis* on murine splenocyte.**
*Sasa borealis* leaf extract showed weak suppressive activity on proliferation and viability of murine splenocyte ($2.5 \times 10^6$ cells/ml) cultured in RPMI 1640 medium for 24hrs. Viability of the cell was measured by MTT assay. The results were presented by mean SD.
(1: Control, 2: 128 units, 3: 125 units, 4: 25 units, 5: 5 units, 6: 1 unit)

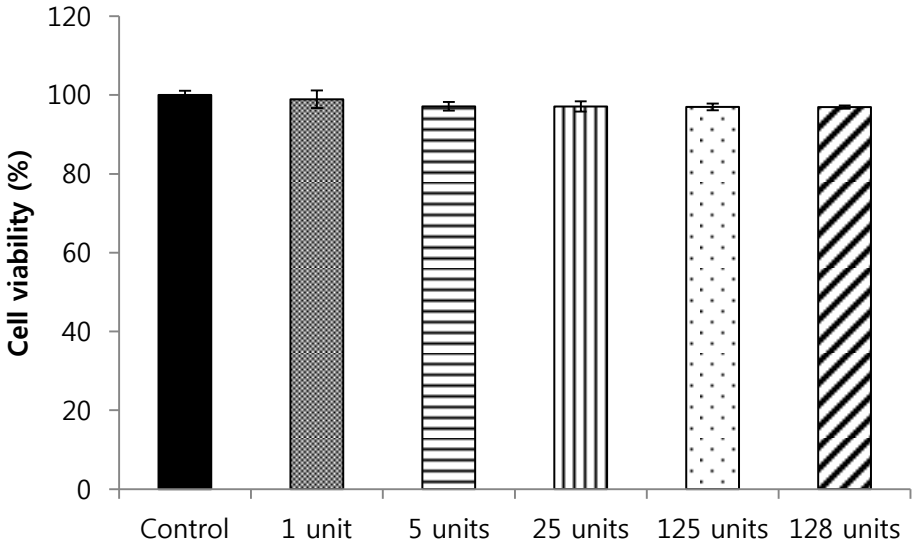
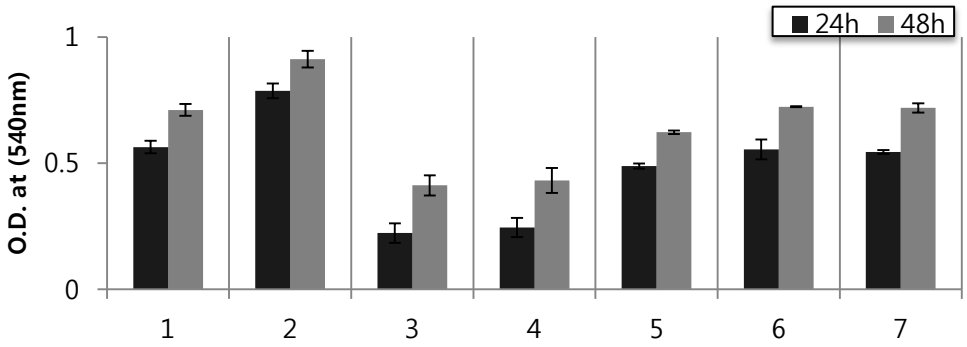
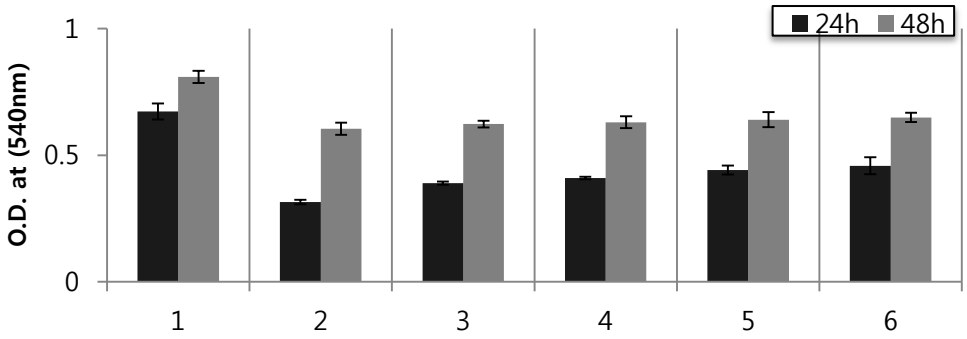



**Figure 16. Cytotoxic effect of *Sasa borealis* on RAW 264.7 cells.**
RAW 264.7 (2.5 x $10^6$ cells/ml) cells were treated with various concentrations of *Sasa borealis* leaf extract and cultured for 48hrs. Proliferation of RAW 264.7 cells were determined by Mosmman's method. Cell viability was expressed as the optical density of formazan formed in cells.
Cell proliferation (%) = (O.D. of treatment group / O.D. of control group) x 100.

(A)




(B)




(C)

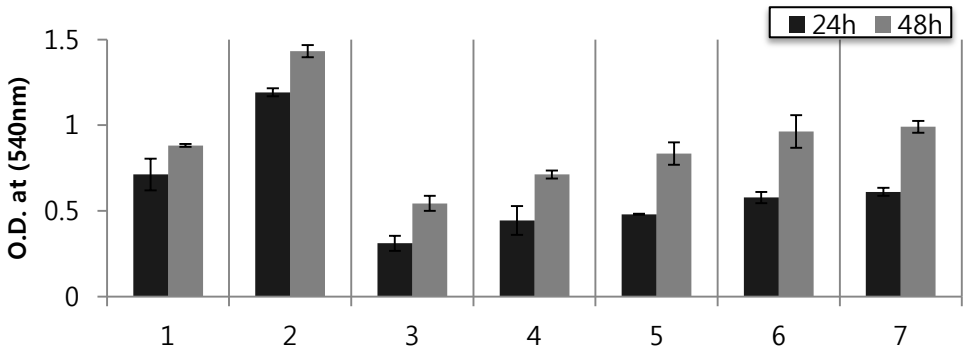



**Figure 17. Cytotoxic effect of *Sasa borealis*.**

Cells were treated with various concentrations of *Sasa borealis* leaf extract. The number of proliferating cells was indirectly measured by MTT assay. (A) CT-26 cells, murine colon cancer cell line (1 x $10^5$ cells/well). (B) EL-4 cells, murine T-lymphoblast (1 x $10^5$ cells/well). (C) S-180 cell, murine sarcoma cell line (1 x $10^5$ cells/well).

(A), (C): (1: Control, 2: LPS (5μg/ml), 3: 128 units, 4: 125 units, 5: 25 units, 6: 5 units, 7: 1 unit), (B): (1: Control, 2: 128 units, 3: 125 units, 4: 25 units, 5: 5 units, 6: 1 unit)

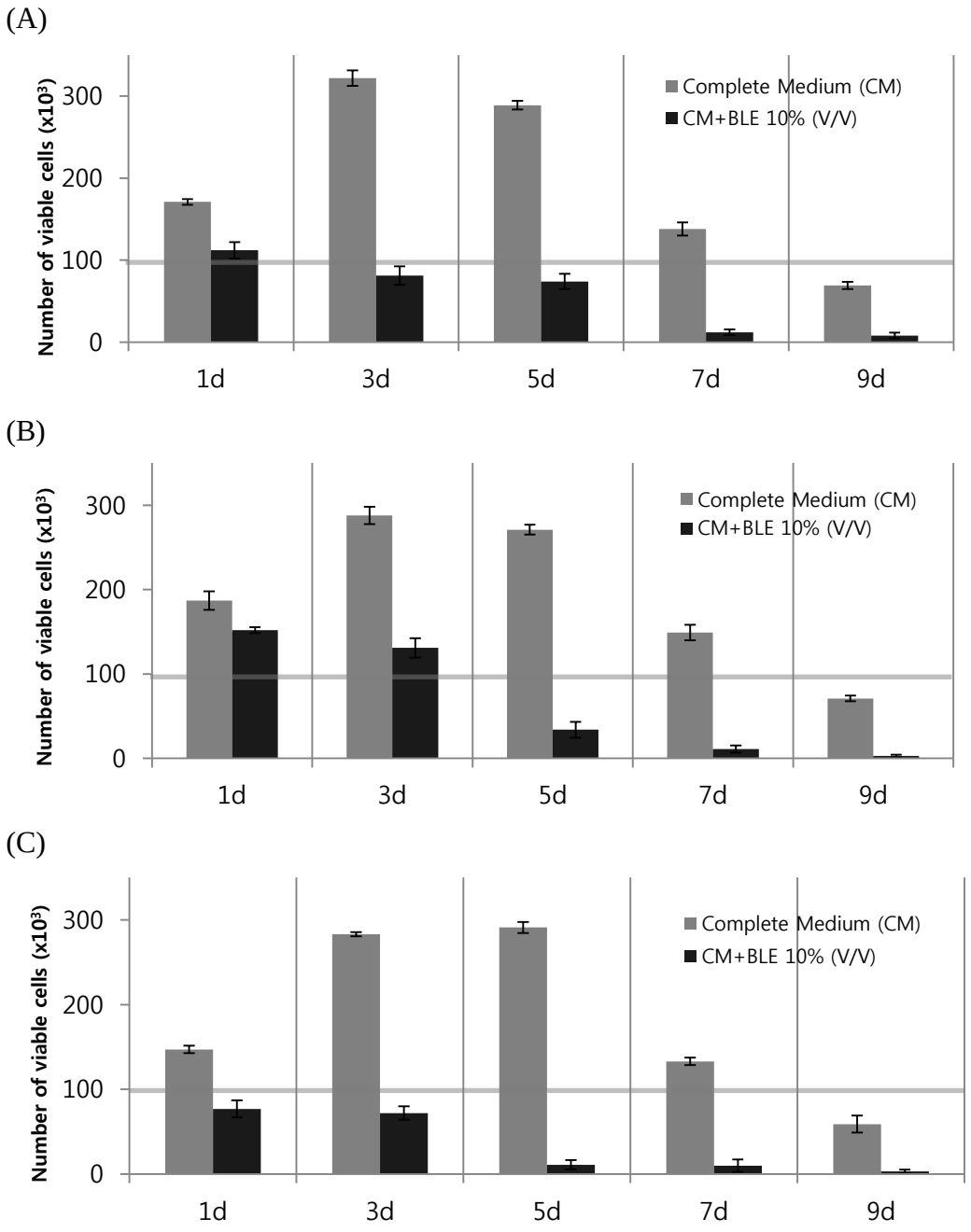


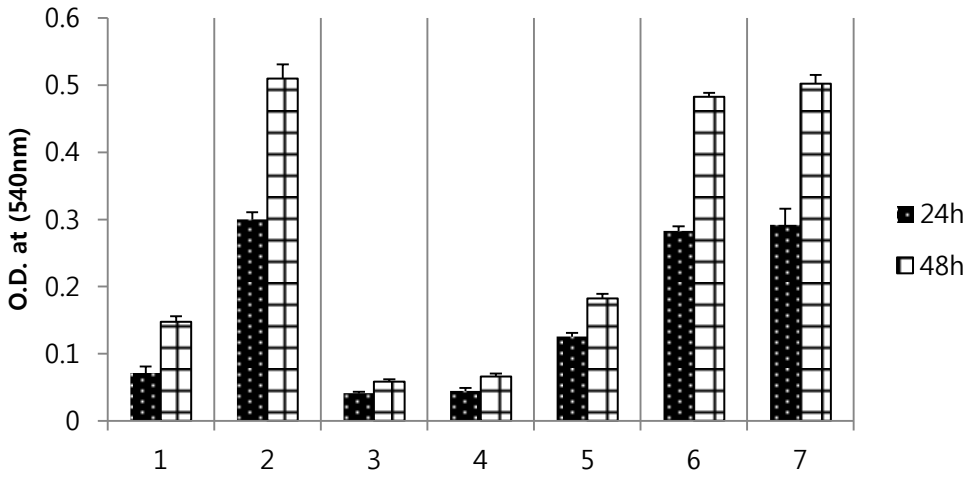

**Figure 18. Trypan blue exclusion test of cell viability.**
Each cell (1 x $10^5$ cells/well) was cultured in complete medium alone or with *Sasa borealis* leaf extract (10 units, 10% (V/V)). Viable and dead cell numbers on 1, 3, 5, 7 and 9days were determined by Trypan Blue Exclusion assay. The results were presented by mean SD.

### 4.2. Nitric oxide (NO) determination

Nitric Oxide (NO), produced endogenously from L-Arginine by nitric oxide synthetases, plays an important role in many physiological processes including vascular regulation, immune responses, and neural communication. NO is extremely unstable and undergoes repaid oxidative degradation to nitrite (NO2-) and nitrate (NO3-), which can be spectrophotometrically determined.

The level of NO in LPS-induced RAW 264.7 and J774 cells was measured as an index of an inflammatory response. Higher concentrations of *Sasa borealis* leaf extract were able to significantly inhibit LPS-induced nitric oxide (NO) production in both cells examined (Figure 19A, B). The results displayed in time and concentration-dependent manners.

(A)




(B)

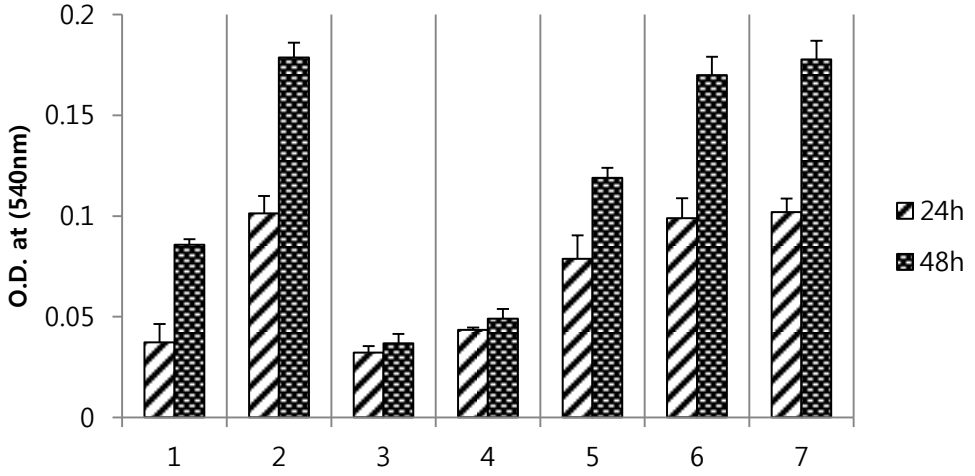



**Figure 19. The effect of *Sasa borealis* on nitric oxide (NO) production.**
Both cells were cultured with various concentrations of *Sasa borealis* extract or LPS or both for 24, 48h in RPMI1640. Culture supernatant was then incubated with equal volume of Griess reagent for 5min at 25˚C. The amount of NO in culture supernatant was measured by ELISA reader at absorbance of 540nm. The amount of NO released was quantified by comparison with sodium nitrite as standard. The results were presented by mean SD.
(A) RAW 264.7 cells, (B) J774 cells.
(1: Control, 2: LPS (5μg/ml), 3: LPS+128 units, 4: LPS+125 units, 5: LPS+25 units, 6: LPS+5 units, 7: LPS+1 unit)

## 5. Genomic DNA isolation from fecal samples

During the experiment, fecal samples were collected every other day until the end (day 56). Fecal samples were dissolved in and also diluted with 1X PBS (pH7.2) solution. Samples were diluted until it gave $O.D._{600}$ : 0.8~1.0 before genomic DNA was extracted utilizing G-spin$^{TM}$ genomic DNA extraction kit for bacteria (Intron, S.Korea). Out of all, using genomic DNA from fecal samples of day 56 as template DNA for PCR, dark band near 500bp was observed using 27f and 518r primer (Figure 20).

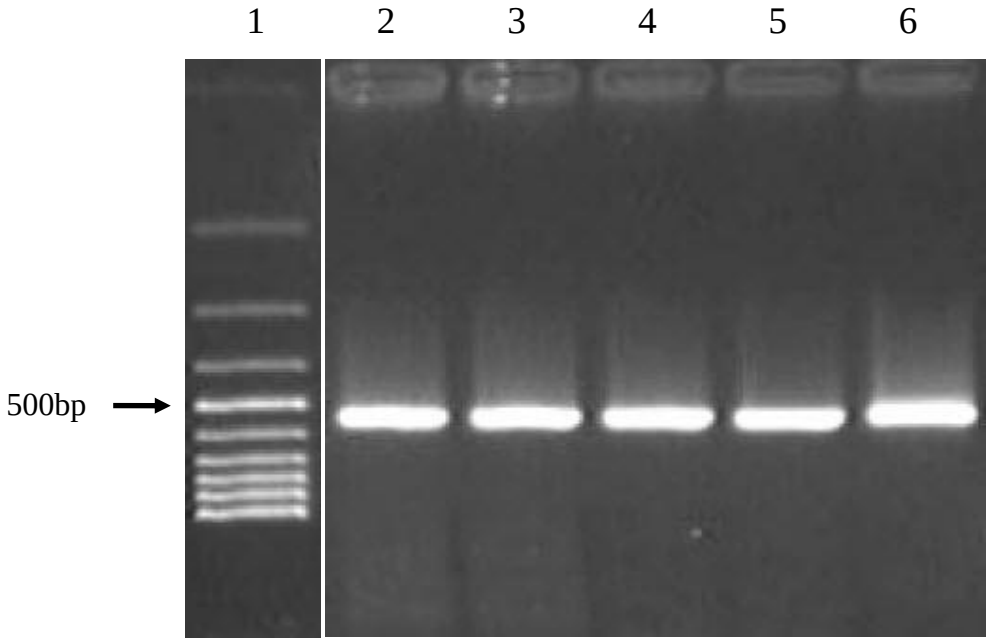



**Figure 20. Band pattern of genomic DNA after electrophoresis.**
Genomic DNA was extracted via G-spin$^{TM}$ genomic DNA extraction kit for bacteria (Intron, S.Korea). Lane 1 contained 100bp DNA ladder. Lanes 2, 3, 4, 5 and 6 were PCR amplification products from fecal samples of each group. (Lane 2: Group 1, Lane 3: Group 2, Lane 4: Group 3, Lane 5: Group 4, Lane 6: Group 5)

# 6. Analysis of fecal microbiome

After analyzing nucleotide bases of bacterial 16S rRNA gene extracted from fecal samples, groups provided with both *Sasa borealis* leaf extract and lactic acid bacteria exhibited larger number in sequence (Table 6). Sequence data were then analyzed into phylum level. As a result, group 2 provided with HFD showed increased number of percentage in Firmicutes and percentage of phyla Bacteroidetes has decreased compare to group 1 provided with STD (Table 7, 8). Several previous studies (Turnbaugh, 2006) have proven such fact as well. In the case of groups 3, 4 and 5, Bacteroidetes in microbiome have significantly increased and Fermicutes have decreased compare to group 2 (Figure 21).

Alteration in fecal microbiome can be induced by several external phenomenon such as obesity. Especially, microbial community in induced obesity model is known to express decrease in Bacteroidetes and increase in Fermicutes (Turnbaugh et al., 2006). It was important to recognize *Sasa borealis* leaf extract and lactic acid bacteria recover the negative effect from obesity.

**Table 6. Summary of reads from pyrosequencing.**
Microbial community structures in the mouse fecal samples after sequence trimming and assembly.

| | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| Number of sequences | 5765 | 5299 | 7428 | 7985 | 8112 |
| Number of contigs | 3878 | 3145 | 4021 | 5532 | 6732 |

**Table 7. The number of identified bacterial phyla from the fecal microbiome.**

| Name of phyla | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| Firmicutes | 3781 | 4023 | 4451 | 5106 | 5504 |
| Bacteroidetes | 1611 | 1029 | 2584 | 2232 | 2536 |
| Verrucomicrobia | 171 | 81 | 94 | 223 | 31 |
| Tenericutes | 59 | 36 | 101 | 178 | 7 |
| Proteobacteria | 84 | 20 | 24 | 42 | 9 |
| Unclassified | 35 | 59 | 95 | 111 | 17 |
| Actinobacteria | 22 | 37 | 37 | 47 | 4 |
| Acidobacteria | 1 | 11 | 9 | 2 | 0 |
| Cyanobacteria | 0 | 1 | 0 | 9 | 0 |
| Chlotoflexi | 0 | 2 | 1 | 0 | 0 |
| Nitrospirae | 0 | 0 | 7 | 0 | 0 |
| Thermotogae | 0 | 0 | 0 | 1 | 2 |
| TM7* | 1 | 0 | 21 | 34 | 2 |
| Lentisphaerae | 0 | 0 | 0 | 0 | 0 |
| TM6* | 0 | 0 | 0 | 0 | 0 |
| Planctomycetes | 0 | 0 | 4 | 0 | 0 |

*TM6 and TM7 are belonged to Candidatus_uncultured.

**Table 8. The percentage of identified bacterial phyla from the fecal microbiome.**

| Name of phyla | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| Firmicutes | 65.58 | 75.92 | 59.92 | 63.94 | 67.85 |
| Bacteroidetes | 27.95 | 19.42 | 34.79 | 27.95 | 31.26 |
| Verrucomicrobia | 2.97 | 1.53 | 1.27 | 2.79 | 0.39 |
| Tenericutes | 1.02 | 0.68 | 1.36 | 2.23 | 0.09 |
| Proteobacteria | 1.46 | 0.38 | 0.32 | 0.53 | 0.11 |
| Unclassified | 0.61 | 1.11 | 1.28 | 1.39 | 0.21 |
| Actinobacteria | 0.38 | 0.70 | 0.50 | 0.59 | 0.05 |
| Acidobacteria | 0.02 | 0.21 | 0.12 | 0.03 | 0.00 |
| Cyanobacteria | 0.00 | 0.02 | 0.00 | 0.11 | 0.00 |
| Chlotoflexi | 0.00 | 0.03 | 0.01 | 0.00 | 0.00 |
| Nitrospirae | 0.00 | 0.00 | 0.09 | 0.00 | 0.00 |
| Thermotogae | 0.00 | 0.00 | 0.00 | 0.01 | 0.02 |
| TM7* | 0.01 | 0.00 | 0.30 | 0.43 | 0.02 |
| Lentisphaerae | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| TM6* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Planctomycetes | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 |

*TM6 and TM7 are belonged to Candidatus_uncultured.

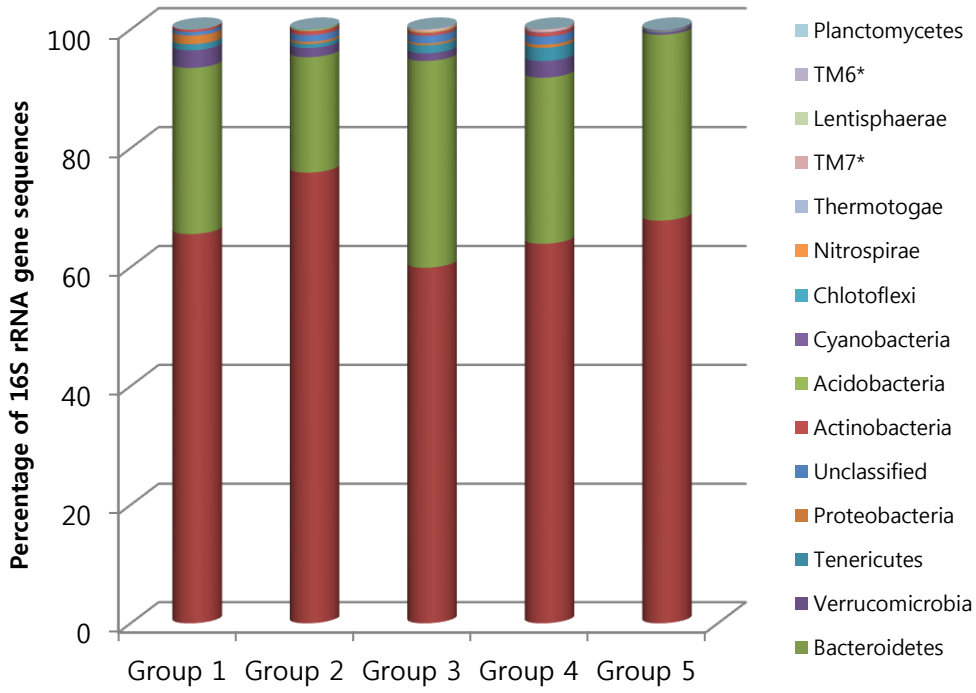



**Figure 21. A phylum level composition of the fecal microbiome of the mouse groups.**

Most fecal bacteria belonged to two main bacterial phyla which were Firmicutes and Bacteroidetes. The proportion of phyla Firmicutes and Bacteroidetes slightly differ between HFD provided group (group 2) and groups provided with *Sasa borealis* leaf extract and lactic acid bacteria.

*TM6 and TM7 are belonged to Candidatus_uncultured.

**Table 9. The percentage of most abundant bacterial phyla identified and its ratio.**

| | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| | STD | HFD | HFD with BLE + LS2 | HFD with BLE + GL | HFD with BLE + IH22 |
| Firmicutes (%) | 65.58 | 75.92 | 59.92 | 63.94 | 67.85 |
| Bacteroidetes (%) | 27.95 | 19.42 | 34.79 | 27.95 | 31.26 |
| Firmicutes / Bacteroidetes | 2.35 | 3.91 | 1.72 | 2.29 | 2.17 |

STD: Standard Diet, HFD: High Fat Diet, BLE: Bamboo Leaf Extract LS2: *Lactobacillus casei* LS2, GL: *Lactobacillus paraplantarum* GL, IH22: *Leuconostoc citreum* IH22

# CHAPTER IV

# DISCUSSION

Bamboos are some of the fastest growing plants in the world, due to a unique rhizome-dependent system. Bamboos are of notable economic and cultural significance in South Asia, South East Asia and East Asia, being used for building materials, as a food source, and as a versatile raw product. Different parts of bamboo are used in many different fields from its usability in culinary field to even medical field. Bamboo in many Asian countries means more that just evergreen plant. It is a symbol of uprightness, integrity and even elegance.

*Sasa borealis*, one of the most popular and abundant species of bamboo are known for its multiple beneficial effects. Recent study has proven its antimicrobial activity against plant pathogenic bacteria (Lee HJ *et al*., 2012). Leaves of *Sasa borealis* also display major improvement on insulin resistance by modulating inflammatory cytokine secretion (Yang JH *et al*., 2010).

Based on the data from pre-trial experiments, extract obtained from leaves of *Sasa borealis* exhibited great potential to be utilized as antimicrobial agent. When tested against over 20 bacterial strains ranging from Gram (+), Gram (-) and lactic acid bacteria, extract selectively inhibited the growth of Gram (+) and (-) bacteria. Further study was designed based on the working mechanism of extract selectively executing bacterial strains other than lactic acid bacteria which are known as probiotics.

In the present study, antimicrobial and immune-modulatory effects of extract obtained from leaves of *Sasa borealis* were examined. Additionally, alteration in microbial community in obesity-induced mouse was analyzed via pyrosequencing. Throughout the study, *Sasa borealis* leaf extract inhibited most of bacteria tested against. Most susceptible bacterium was *Rothia dentocariosa* G1201. Working unit of extract was distinguished based on the MIC data of *Rothia dentocariosa* G1201. To observe whether protein in a sample involves antimicrobial effect, ammonium sulfate ($(NH_4)_2SO_4$) precipitation was performed. As a result, precipitate (= pellet) inhibited 8 out of 9 bacterial strains tested.

Supernatant on the other hand inhibited one bacterium left telling that there are at least two antimicrobial substances in extract obtained from leaves of *Sasa borealis*. SDS-PAGE was performed to detect approximate size of protein that exhibits the antimicrobial activity. Based on the silver staining data, dark smeared band around 100kDa was observed. Protein was then partially purified using gel elution technique and the protein near 100kDa of size inhibited the growth of bacteria (*Rothia dentocariosa* G1201). It is uncertain why such molecule with large molecular weight exhibits in *Sasa borealis* but it could be thought as new bacteriocin found. Bacteriocins are defined as toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s). They are typically considered to be narrow spectrum antibiotics, though this has been debated (Farkas-Himsley H, 1980). Bacteriocins were first discovered by microbial and viral genetics A.Gratia in year 1925. They are structurally, functionally and ecologically so diverse.

Aqueous *Sasa borealis* leaf extract displayed various immune-modulating activities. It selectively killed disease causing cells (CT-26, EL-4, S-180) but did not show any significant cytotoxic effect against normal cells such as murine splenocyte and RAW 264.7 cells. Also extract solution significantly inhibited LPS-induced NO production. NO production was inhibited in a concentration-dependent manner.

Analysis of nucleotide bases of bacterial 16S rRNA from fecal samples of mouse was performed. After analyzing in phylum level, group 2 (HFD) showed decreased number in Bacteroidetes and increased number was observed for bacterial phylum Firmicutes. Ratio between Bacteroidetes and Firmicutes was highest in group 2 and for groups 3, 4 and 5 the ratio was similar to group 1. It is known that when obesity is induced, number of Bacteroidetes decreases and Firmicutes greatly increases. Based on the pyrosequencing data collected, *Sasa borealis* leaf extract solution and lactic acid bacteria have synergetic effect of

recovering alteration in microbial community close to a normal condition. Based on the data collected throughout the study, extract obtained from leaves of *Sasa borealis* definitely exhibits antimicrobial effect and also modulates immune system. If the mechanism of how *Sasa borealis* leaf extract works gets unveiled, it will be beneficial towards numerous fields in our lives.

# CHAPTER V

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# 국 문 초 록

## 대나무 종 *Sasa borealis* 잎의 항균효과와 파이로시퀀싱을 이용한 분변내 세균층의 변화 분석

사람들은 항상 세균에 노출되어 있다. 흔한 감기나 염증에서부터 목숨을 앗아 갈수 있는 질병까지, 세균이 인간에게 미치는 영향은 실로 엄청나다. 이에 사람들은 세균에 대항하는 물질, 즉 항생제를 끊임없이 연구하고 제조해 왔다. 하지만 사람들의 계속된 복용으로 인해 대다수의 항생제에 대한 내성이 점차 생겨나고 있다. 쉽게 말하자면, 세균들도 인간들의 공격에 대해 그 나름의 방식으로 대응, 변화한 것이다. 항생제는 주로 세균 세포의 세포벽 합성 혹은 단백질 합성을 억제하거나, 세포막의 투과성을 변동시키거나, 세균의 물질대사를 억제시키는 등의 기작으로 세균 세포를 사멸시킨다. 하지만 항생제의 오남용 혹은 지나친 복용으로 인해 내성이 생기는 경우가 있다. 내성균이 한번 생겨나면 그 내성균은 다른 균에도 내성을 전이시켜 내성균이 계속 늘어나게 하기 때문에 결국에는 항균 능력이 더 강한 항생제 혹은 다른 계열의 항생제로 바꾸어 사용해야 한다. 그렇기에 병원성 세균들이 내성을 가지지 않는 새로운 항생 물질을 발견하는 것은 매우 중요하다. 이에 본 연구에서는 대나무 *Sasa borealis* 종의 잎에서 항균 작용을 하는 물질을 발견하고, 대나무 잎의 항균 활성을 알아보았다. 또한 분변에서 추출한 bacterial 16S rRNA

유전자의 염기서열 분석을 통한 intestinal microbiome 분포의 변화를 분석하였다.

대나무는 전 세계적으로 92 개의 속과 1400 여 종이 존재한다. 우리나라에도 4 속 14 종의 대나무가 자라고 있다. 이 중 왕대, 오죽, 조릿대 (*Sasa borealis*) 등 몇 가지 종만이 사람들에게 알려져 있다. 가장 흔하게 볼 수 있는 대나무는 조릿대인데, 성인병에 효능이 있다고 알려져 있기는 하지만 그러한 사실에 대한 의학적 연구는 아직 진행이 미미한 상태이다. 한방에서도 대나무가 약으로 쓰여 온 경우는 있지만, 그 잎을 약재로 쓰지는 않았다. 본 실험에서는 조릿대 (*Sasa borealis*)를 재료로 사용하는데, 구매한 조릿대 잎은 잘 말려서 잘게 썰어 놓은 것으로 항균 물질을 추출하기에 적합하였다.

댓잎 추출물을 농축하지 않은 채로 여러 종류의 미생물 (20 가지 bacteria) 들에 처리하여 억제환 생성을 살펴보았더니, 그 중 대다수에 대하여 성장 억제 효과를 보였다. 반면, 전혀 영향을 받지 않는 몇몇 미생물들이 있다는 것을 볼 수 있었는데, 알고 보니 이들은 모두 유산균 (Lactic Acid Bacteria)에 해당하는 것이었다. 어떤 경우에라도 유산균들이 억제되지 아니하고 다른 불필요한 미생물들의 성장만 억제된다면, 이와 같은 물질을 유산균의 효능을 극대화 시키는 등의 용도에 활용할 수 있을 것이라 판단하였다. 유효성분만을 분리해 내기 위하여 앞에서 이야기한 것처럼 새로운 방법을 도입하여, 추출물에 황산암모니움 (Ammonium Sulfate)을 첨가하여 유효 성분을 침전시켰다. 상등액을 따로 분리해 놓고, 침전물은 황산암모니움 (Ammonium Sulfate)을 제거한 다음 다시 항균 효과를

점검하였다. 이때, 원액에 의하여 억제되던 미생물 가운데 몇 종류의 미생물들이 정제한 성분에 대하여 효과가 나타나지 아니하였다. 이것은 두 가지로 생각해 볼 수 있다. 우선은, 황산암모니움 (Ammonium Sulfate)으로 침전시키는 과정에서 약간의 분자 변형이 일어났는데, 이 변형으로 인하여 특정 미생물에 대해서는 항균효과를 보이지 않았을 수도 있다. 아니면, 이 미생물을 억제하기 위해서는 황산암모니움 (Ammonium Sulfate)으로 침전되지 않는 다른 성분이 필요할 수도 있을 것이다. 그래서 본 연구에서는 침전된 성분과 침전되지 않은 성분을 섞어 다시 항균 효과를 조사하였다. 이 과정에서 여전히 효과를 보이지 않은 미생물들도 존재하였으나, 두 가지 미생물들이 성장 억제되는 것을 확인할 수 있었다. 이들은 ***Bacillus subtilis*** 와 ***Serratia marcescens*** 였다. 이들 미생물에게는 두 가지 이상의 성분이 작용하여야만 성장이 억제될 수 있음을 의미하는 것이다. 그런데, 두 가지 성분을 섞어 주었음에도 불구하고 여전히 성장 억제 효과가 되살아나지 않은 미생물들은 결과적으로 또 다른 성분이 필요한 것이거나, 정제과정에서 항균물질이 어떤 이유로든 억제 효과를 잃어버리고 만 것이라고 밖에 생각할 수 없다. 앞선 결과를 통해, 부분적으로 정제한 댓잎의 항균 물질이 당을 포함하는 당단백질 (Glycoprotein)일 것이라는 것을 알게 되었고, Acrylamide gel 을 통한 분자량을 조사한 결과, 100kDa 정도 크기의 Bacteriocin 에 해당하는 물질을 찾았다고 잠정적으로 생각하게 되었다.

세균뿐만 아니라, 댓잎 추출물이 암세포나 육종 세포등 다양한 세포에는 어떤 영향을 끼치는지도 본 연구를 통해 알아보았다. 그 결과,

normal cell 에 해당하는 murine splenocyte 나 macrophage cell line 들에는 cytotoxicity 를 보이지 않았고 murine colon cancer cell line 인 CT-26 cell line 과 murine sarcoma cell line 인 S-180 cell line 그리고 murine T-lymphoblast cell line 인 EL-4 cell line 은 세포의 성장이 억제되었다.

분변에서 추출한 bacterial 16S rRNA 유전자의 염기서열 분석을 통한 intestinal microbiome 분포의 변화를 관찰, 분석한 결과, STD 나 HFD 만 제공한 그룹에 비해 댓잎 추출물과 유산균을 함께 경구 투여한 그룹에서 sequence 수가 더 많았다. HFD 만을 제공받은 그룹은 STD 를 제공받은 그룹에 비해 fecal microbiome 에서 Bacteroidetes 의 수는 감소하였고 Firmicutes 의 수는 증가하였다. 반면, 댓잎 추출물과 유산균을 경구 투여한 그룹은 HFD 만 제공받은 그룹에 비해 Bacteroidetes 는 크게 증가하였으며 Firmicutes 는 감소하였다. 따라서 HFD 만 제공받은 그룹은 fecal microbiome 에서 Bacteroidetes 에 대한 Firmicutes 의 비율이 가장 높았고, 댓잎 추출물과 유산균을 함께 투여한 세 그룹의 비율은 STD 만 먹인 그룹보다도 낮았으며, 댓잎 추출물과 *Lactobacillus paraplantarum* GL 을 경구 투여한 그룹에서 가장 두드러졌다. HFD 에 의해 유도된 비만의 결과, 장내 세균총 중 Bacteroidetes 는 감소하고 Fermicutes 는 증가한다고 밝혀져 있는데 (Ley RE *et al*, 2006), 불필요한 미생물을 사멸하고 유산균은 살리는 selectivity 를 가진 댓잎 추출물과 probiotic 로 알려진 유산균은 비만에 의한 fecal microbiome 분포의 변화를 정상상태로 회복시킨다는 사실을 확인하였다.

▫ 주요어 : 대나무 잎, 항균 효과, 박테리오신, 파이로시퀀싱, 분변내 세균총

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