A THESIS FOR THE DEGREE OF

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# Analysis of Antibiotic Susceptibility for *Lactobacillus* and *Bifidobacterium* and Their Antibiotic Resistant Genes

## *Lactobacillus*와 *Bifidobacterium*의 항생제 감수성 및 항생제 저항성 유전자 분석

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# Abstract

## Analysis of Antibiotic Susceptibility for *Lactobacillus* and *Bifidobacterium* and Their Antibiotic Resistant Genes

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*Lactobacillus* and *Bifidobacterium* are commonly used as probiotics. For their safe use on human consumption under antibiotic treatment, their resistance to various antibiotics and gene transferability to the other enteric bacteria need to be assessed. In this study, the susceptibility and the minimum inhibitory concentration (MIC) for 11 strains of *Lactobacillus* and six strains of *Bifidobacterium* were assessed against 17 antibiotics — penicillin G, carbenicillin disodium salt, methicillin, ampicillin sodium salt, dicloxacillin sodium salt sulfate, gentamicin, streptomycin sulfate salt,

kanamycin, cephalothin sodium salt, tetracycline, polymyxin B sulfate salt, bacitracin, erythromycin, metronidazole, chloramphenicol, clindamycin hydrochloride, and phosphomycin disodium salt. International Organization for Standardization (ISO) standard broth microdilution method was used in liquid medium. Additionally, the Etest method and the disc diffusion method were applied on agar medium. For the bacteria whose whole genome sequencing were already performed, the annotated antibiotic resistant genes were co-related with actual susceptibility of the corresponding strain. According to the susceptibility tests, β-lactam group antibiotics showed the higher MIC values in lactic acid bacteria susceptibility test medium (LSM) than de Man, Rogosa and Sharpe (MRS) medium. Most of the experimental *Lactobacillus* and *Bifidobacterium* showed low MIC values for β-lactam group, even though several strains possessed *penP* genes coding for penicillin resistance. For aminoglycoside group, MIC measured in MRS medium was higher than the MIC measured in LSM medium, particularly for *Bifidobacterium*. Most of the *Lactobacillus* strains were resistant against kanamycin. For all antibiotic susceptibility method, all *Lactobacillus* and *Bifidobacterium* were shown to have resistance against polymyxin B. Also, for tetracycline and chloramphenicol, several species were found to possess corresponding resistance gene and have high MIC values for those antibiotics, compared to the other species

not possessing the resistance genes. The results of this study are expected to give an insight into a safe and intelligent commercial application of experimental lactic acid bacteria.

**Keywords : minimum inhibitory concentration, MIC, lactic acid bacteria, antibiotics**

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# 1. Introduction

Since penicillin was discovered by Alexander Fleming in 1928, various antibiotic substances were found and being widely used as chemotherapeutic agents [1]. The word "antibiotic" is defined as "inhibiting the growth or the metabolic activities of bacteria and other micro-organisms by a chemical substance of microbial origin" [2], and antibiotic resistance means the ability to resist against the antibiotic.

Many lactic acid bacteria species including *Bifidobacterium,* which is regarded to be generally recognized as safe (GRAS) and colonizes the human large intestine and contribute to human gut health [3], exert antibiotic resistance. Analysis of antibiotic susceptibility is useful for the verification of the safety of probiotics and antibiotic resistance.

As a criterion for susceptibility to certain antibiotics, MIC value of the target bacteria is informative. MIC is defined as the lowest concentration of antibiotics that can inhibit the growth of bacteria [4]. MIC for lactic acid bacteria was also scrutinized in various studies but MIC values can widely fluctuate depending on the cultivation environment, for example, because of incubation time, the amount of inoculum or for some components in media antagonistic to certain antibiotics. Thus, some standardization for a measuring method is required and the ISO established a method to

determine MIC for lactic acid bacteria. Accordingly, this study adopted the method described in the ISO standard and determined MIC values with the standard method [5]. The MIC value for 11 strains of *Lactobacillus* and six strains of *Bifidobacterium* were determined in broth medium and agar medium.

Also, the Etest method was used to determine MIC values in agar medium. Etest is a novel susceptibility testing method, which involves the placement of a plastic strip containing a defined continuous gradient of an antimicrobial drug on the surface of inoculated agar [6].

For the antibiotics that cannot be purchased as Etest strip, for a legal issue, a disc diffusion test was performed to determine MIC value in agar medium. The disc diffusion test is the most frequently used procedure for determining the susceptibility of clinical strains to antimicrobial agents [7]. With diameters of clear zone measured by this method, the MIC value can be determined by linear regression [8].

To investigate antibiotics susceptibility for lactic acid bacteria, in this study, the widely used 17 antibiotics were chosen. Depending on their mechanism of action, their groups can be classified as in Table 1.

**Table 1. The groups of antibiotics used in this study and the genes related to the antibiotic resistance**

| Groups | Antibiotics | Genes for resistance |
|---|---|---|
| β-lactams | Penicillin G | *penI, penP, blaI* [9] |
| | Carbenicillin disodium salt | |
| | Methicillin | *murE* [10], *mecA, blaI* [11] |
| | Ampicillin sodium salt | *amp* [12] |
| | Dicloxacillin sodium salt | |
| | sulfate | |
| Aminoglycosides | Gentamicin sulfate | *aac*, *ant*, *aph* [13] |
| | Streptomycin sulfate salt | *aadA*, *aadE* [14] |
| | Kanamycin sulfate | *aph* [15] |
| Cephems | Cephalothin sodium salt | |
| Tetracyclines | Tetracycline | *tetA, tetW, tetM, tetX* [16] |
| Peptides | Polymyxin B sulfate salt | *pmrA, pmrB, phoP, phoQ* [17] |
| | Bacitracin | *bacA* [18] |
| Macrolides | Erythromycin | *ermE, ermF, ermG* [19] |
| Synthetic antimicrobial group | Metronidazole | |
| The other group | Chloramphenicol | *catA* [20] |
| | Clindamycin hydrochloride | |
| | Phosphomycin disodium salt | |

For β-lactam group antibiotics, β-lactamase is known to provide bacteria with resistance to β-lactam antibiotics. Several genes such as *penP* and *blaI* are encoding β-lactamase. β-lactam antibiotics interacts with penicillin binding protein (PBP), which is a protein that mediates bacterial peptidoglycan synthesis by forming cross-linking between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). Thus, PBP is essential for bacterial growth. However, every β-lactam group antibiotic including penicillin has high affinity with PBP. Their β-lactam ring opens and reacts covalently with a certain serine in an active site of PBP and irreversibly, then, inactivates PBP [21]. Methicillin is also a β-lactam group antibiotic, which was developed to inhibit penicillin resistant bacteria. The *mecA* gene encoding PBPa2, which has low affinity to all β-lactams, gives methicillin resistance to bacteria [11, 22].

*aph* gene is associated with aminoglycoside resistance and encodes aminoglycoside *O*-phosphotransferase (APH), which catalyze the ATP-dependent phosphorylation of specific aminoglycoside hydroxyl groups. There are several classes of these enzymes which have been classified primarily on the basis of substrate specificity. The largest family of APHs are those that catalyze the modification of kanamycin at the 3′-hydroxyl group [23].

Tetracycline represses protein synthesis by binding to ribosome in

bacteria. Many bacteria have resistance to tetracycline by expressing one of these proteins: membrane protein (*tetA* to *E*, *K*, *L*) that pumps tetracycline out of bacteria; 72 kDa protein (*tetM*, *tetW*) that blocks tetracycline from binding to 30S ribosome; and enzyme (*tetX*) that inactivates tetracycline directly [16].

Bacitracin resistance comes from *bacA* gene. Bacitracin interacts tightly with undecaprenyl pyrophosphate in the bacteria and then inhibits formation of undecaprenyl phosphate. However, bacA protein gives resistance to bacteria by catalyzing dephosphorylation of undecaprenyl pyrophosphate then converting it into undecaprenyl phosphate [24]. Polymyxin B, another peptides group antibiotic, is a bactericidal for gram-negative, but known to have little or no effect on gram-positive, because of their thick cell wall [17].

Chloramphenicol inhibits peptidyl transferase activity by hampering the binding of transfer RNA to the A site. And *catA* gene is known to repress the activity of chloramphenicol [25].

On the other hand, as increasing misuse and overuse of antibiotics is becoming global concern, World Health Organization (WHO) emphasizes its importance through many reports every year. These spread of antimicrobial resistance can occur through not only misuse and overuse of the antibiotics but also antibiotic resistant bacteria by horizontal gene

transfer.

One of the greatest safety concerns for commercially-produced lactic acid bacteria is that some of the microorganisms supplied in the form of diets may act as the donor of antibiotic-resistant plasmids to intestinal pathogens [26] [27] [28]. Several reports have found that in the presence of antibiotic treatment, some strains survive in the human gastrointestinal tract due to the transferred resistance of plasmids [28] [29] [30] [31]. A variety of microbial genes can be transferred to enteric bacteria in the intestine via plasmids, resulting in the spread of antibiotic resistance [28, 32]. Therefore, ensuring the safety of a probiotic strain is necessary prior to the mass production of lactic acid bacteria for commercial purposes [28].

Thus, in this study, for the newly isolated lactic acid bacteria, genomic annotation to the known antibiotic resistance gene was performed by using CLgenomics and RAST service, and co-related to the actual susceptibility of the corresponding strain. The results are expected to give us an insight into a safe and intelligent commercial application of experimental lactic acid bacteria.

# 2. Materials and Methods

## 2.1. Materials

### 2.1.1. The bacterial strains and culture condition

The bacterial strains used in the study are listed below (Table 2). The bacteria were stored at -70°C with MRS broth (BD, New Jersey, USA) supplemented with 50% glycerol. Before the bacteria were used for the study, they were subcultured for 18 h for the activity.

### 2.1.2. Antibiotic reagents

The antibiotics used in the study are listed below (Table 3). Ampicillin sodium salt was purchased from USP (MD, USA) and the other antibiotics were all purchased from Sigma (MO, USA). The tetracycline and chloramphenicol were dissolved in 100% ethanol and the others were dissolved in distilled water. All antibiotics used in this study were filtered and sterilized using 0.2 μm membrane filter (Pall Corporation, Michigan, USA).

**Table 2. 17 bacterial strains used in this study**

| Bacterial strains |
|---|
| *Lactobacillus plantarum* PH3A |
| *Lactobacillus fermentum* PH3B |
| *Lactobacillus acidophilus* KCTC 3168 |
| *Lactobacillus plantarum* KFRI 708 |
| *Lactobacillus fermentum* EPS22 |
| *Lactobacillus paracasei* CH88 |
| *Lactobacillus fermentum* G7 |
| *Lactobacillus sakei* KOK |
| *Lactobacillus brevis* GABA100 |
| *Lactobacillus rhamnosus* GG |
| *Lactobacillus casei* IBS041 |
| *Bifidobacterium longum* KCCM 91563 |
| *Bifidobacterium pseudocatenulatum* SS29 |
| *Bifidobacterium longum* RD47 |
| *Bifidobacterium lactis* AD011 |
| *Bifidobacterium pseudocatenulatum* INT57 |
| *Bifidobacterium bifidum* ATT |

**Table 3. Antibiotic reagents used in this study**

| Antibiotics used for broth microdilution test |
|---|
| Penicillin G (Sigma, Lot#111H0079) |
| Carbenicillin disodium salt (Sigma, Lot#126M4775V) |
| Methicillin (Sigma Lot#BCBR6817V) |
| Ampicillin sodium salt (USP, Lot#1105SHZL0512B0211Z) |
| Dicloxacillin sodium salt sulfate (Sigma, Lot#SZBD263XV) |
| Gentamicin sulfate (Sigma, Lot#SLBP2417V) |
| Streptomycin sulfate salt (Sigma, Lot#8944V) |
| Kanamycin sulfate (Sigma, Lot#066M4019V) |
| Cephalothin sodium salt (Sigma, Lot#056M4884V) |
| Tetracycline (Sigma, Lot#046M4809V) |
| Polymyxin B sulfate salt (Sigma, Lot#126M4071V) |
| Bacitracin (Sigma, Lot#017M4007V) |
| Erythromycin (Sigma, Lot#24H0050) |
| Metronidazole (Sigma, Lot#MKBZ3056V) |
| Chloramphenicol (Sigma, Lot#SLBN6556V) |
| Clindamycin hydrochloride (Sigma, Lot#021M1533V) |
| Phosphomycin disodium salt (Sigma, Lot#096M4031V) |
| **Antibiotics used for Etest** |
| Ampicillin (Thermo Fisher Scientific, Lot#2154184) |
| Ciprofloxacin (Thermo Fisher Scientific, Lot#2158308) |
| Clindamycin (Thermo Fisher Scientific, Lot#2168848) |
| Erythromycin (Thermo Fisher Scientific, Lot#2132361) |
| Gentamicin (Thermo Fisher Scientific, Lot#2168852) |
| Linezolid (Thermo Fisher Scientific, Lot#2158302) |
| Tetracycline (Thermo Fisher Scientific, Lot#2119199) |

## 2.2. MIC determination using ISO standard

The procedure for measuring MIC was performed based on broth microdilution method in ISO standard [5]. LSM broth medium was made of 90% of Iso-Sensitest (IST) broth (Mbcell, Seoul, Korea) and 10% of MRS broth (BD, New Jersey, USA). Especially for medium for the growth of *Bifidobacterium*, additional 0.03% (w/v) L-cysteine HCl (Sigma) was added. All media was sterilized at 121°C and gauge pressure 0.1 MPa for 15 min. Then they were cooled and stored at -4°C before using. All antibiotics were also prepared by following ISO standard instruction.

For the serial dilution procedure, 96 well plates (Corning, New York, USA) were used. In 96 well plates, the first column of plates was used for positive control; only bacteria was inoculated in the media without antibiotics. And the last column of plates was used for negative control, filled with only 50 μL of medium; neither bacteria nor antibiotics was added. This negative control was used to check contamination of medium. Then, 50 μL of antibiotics solution was injected to 96 well plates with various concentrations as shown in the Table 4.

Absorbance ($OD_{600}$) for bacteria was equalized to 0.2 to make initial concentration of each bacteria strains amounting to $3 \times 10^8$ CFU/mL. Absorbance was measured by using spectrophotometer (Spectramax 190,

Molecular Devices Corporation, Sunnyvale, CA, USA). Then, the prepared bacteria were diluted 500 times with LSM broth medium. Finally, 50 μL of inoculum was injected to each well. All bacteria were incubated at 37°C for 48 h. Six strains of *Bifidobacterium* were cultured anaerobically with 90% of $N_2$, 5% of $CO_2$, 5% of $H_2$ gas composition by using Whitley jar gassing system (Don Whitley Scientific, Shipley, UK).

After culture, all negative control samples were checked for contamination. When any contamination was detected, the whole plate was discarded. Finally, comparing with negative control, MIC was determined by selecting the minimum concentration for well which showed no visible growth.

For several bacterial strains, replication tests were conducted to show the reproducibility of the test. Their MIC values were compared to the MIC values of the previous study. The results are shown on the appendices.

Also, the result of MIC values are compared with the cut-off values on European Food Safety Authority (EFSA) guidance [33]. Regarding the resistance or sensitivity, for the bacteria whose MIC value is higher than the cut-off value, the corresponding bacterial strain is considered resistant. For the bacteria whose MIC value is equal or lower than the cut-off value, the corresponding bacterial strain is considered sensitive.

**Table 4. Layout of 96 well plates concentration (μg/mL)**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Penicillin G | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Carbenicillin disodium salt | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Methicillin | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Ampicillin sodium salt | N | 0.032 | 0.063 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | P |
| Dicloxacillin sodium salt sulfate | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Gentamicin sulfate | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Streptomycin sulfate salt | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Kanamycin sulfate | N | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | P |
| Cephalothin sodium salt | N | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | P |
| Tetracycline | N | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | P |
| Polymyxin B sulfate salt | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Bacitracin | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Erythromycin | N | 0.016 | 0.032 | 0.063 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | P |
| Metronidazole | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |
| Chloramphenicol | N | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | P |
| Clindamycin hydrochloride | N | 0.032 | 0.063 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | P |
| Phosphomycin disodium salt | N | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | P |

## 2.3. MIC determination using ISO standard with MRS

The whole procedure is identical with ISO standard except using MRS broth medium instead of using LSM broth medium. Especially for medium used for the growth of *Bifidobacterium*, additional 0.03% (w/v) L-cysteine HCl (Sigma) was added. All media was sterilized in 121°C and gauge pressure 0.1 MPa for 15 min and cooled and stored at -4°C before using. Six strains of *Bifidobacterium* were cultured anaerobically with 90% of $N_2$, 5% of $CO_2$, 5% of $H_2$ gas composition by using Whitley jar gassing system (Don Whitley Scientific, Shipley, UK).

## 2.4. MIC determination using Etest

MRS broth supplemented with 1.5% agar (BD, New Jersey, USA) was solidified in the Petri dishes. Especially for medium for *Bifidobacterium*, additional 0.03% (w/v) L-cysteine HCl (Sigma) was added. 150 μL of grown bacteria with concentration of $3 \times 10^9$ CFU/mL in MRS medium was added to agar plates and spread. Then 8 kinds of M.I.C. Evaluator strips (Thermo Fisher Scientific, Basingstoke, United Kingdom) were placed on the agar plate. All bacteria were incubated at 37°C for 48 h. The six strains of *Bifidobacterium* were cultured anaerobically with 90% $N_2$, 5% $CO_2$, 5% $H_2$

gas composition by using Whitley jar gassing system (Don Whitley Scientific, Shipley, UK). After 48 h, the scale on the strip was read as a MIC value, which intersects the border line between the area on which bacteria have grown and the area on which bacteria have not been grown.

## 2.5. MIC determination using disc diffusion test

MRS broth supplemented with 1.5% agar was solidified on the Petri dish. For medium used for *Bifidobacterium*, additional 0.03% (w/v) L-cysteine HCl (Sigma) was added. 150 μL of grown bacteria with concentration of $3 \times 10^9$ CFU/mL in MRS broth was added to agar plates and spread. Then six discs (BBL, Blank Paper Disc) were placed on the agar plate. Subsequently, 10 μL of antibiotic solution (Table 5) was added right on to each disc with micro pipette. The bacteria were incubated at 37°C for 48 h. Six strains of *Bifidobacterium* were cultured anaerobically with 90% $N_2$, 5% $CO_2$, 5% $H_2$ gas composition by using Whitley jar gassing system. After 48 h, diameter of clear zone, the area on which bacterial growth was inhibited, was measured in millimeter unit. The natural logarithm values of measured diameter were used with concentration of antibiotics to get linear regression models. From this model, the value of MIC is determined as the zero intercept of a linear regression of the squared size of these inhibition zones, plotted against the natural logarithm of the antibiotic concentration [8].

**Table 5. Concentration of antibiotic solution used in the disc diffusion test**

| Antibiotics | μg/mL |
|---|---|
| Penicillin G | 5,120 |
| Carbenicillin disodium salt | 5,120 |
| Methicillin | 5,120 |
| Ampicillin sodium salt | 320 |
| Dicloxacillin sodium salt sulfate | 5,120 |
| Gentamicin sulfate | 5,120 |
| Streptomycin sulfate salt | 5,120 |
| Kanamycin sulfate | 20,480 |
| Cephalothin sodium salt | 1,280 |
| Tetracycline | 1,280 |
| Polymyxin B sulfate salt | 5,120 |
| Bacitracin | 5,120 |
| Erythromycin | 1,600 |
| Metronidazole | 5,120 |
| Chloramphenicol | 1,280 |
| Clindamycin hydrochloride | 320 |
| Phosphomycin disodium salt | 5,120 |

## 2.6. Analysis of the antibiotic resistance gene location

The genomic DNA of the pure culture bacteria was extracted using MG™ Cell Genomic DNA Extraction SV miniprep (MGmed, Seoul, Korea). Whole genome sequencing and analysis were completed by using an Illumina MiSeq sequencer and a Nextera XT library preparation kit (Illumina, CA, USA). Nextera XT sequencing indices were used for multiplexing, and the participants were free to choose any sample index combination.

The bioinformatics analysis was completed by using CLgenomics in ChunLab Co., Ltd. (Seoul, Korea) and RAST (http://rast.nmpdr.org) service. With Miseq FASTQ formatted raw data, genome annotation was performed by RAST tool kit (Release version 1.3.0) [34-36]. And the NCBI protein BLAST(version BLAST+ 2.8.1) analysis was also performed to compare the results. Then, the location of the gene was checked, if the bacteria have genes that related to a certain antibiotic resistance.

# 3. Results

## 3.1. MIC values determined by using ISO standard

MIC values determined by using broth microdilution method in ISO standard with LSM broth are below (Tables 6, 7). Table 6 represents the result for *Lactobacillus* and Table 7 represents the result of *Bifidobacterium*. The superscript "R" and "S" in the tables denote resistant and sensitive, individually, based on the guideline of EFSA [33].

**Table 6. MIC values (μg/mL) of *Lactobacillus* spp. measured in LSM broth**

| Antibiotics | *L. plantarum* PH3A | *L. fermentum* PH3B | *L. acidophilus* KCTC 3168 | *L. plantarum* KFRI708 | *L. fermentum* EPS22 | *L. paracasei* CH88 | *L. fermentum* G7 | *L. sakei* KOK | *L. brevis* GABA100 | *L. rhamnosus* GG | *L. casei* IBS041 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Penicillin G | 4 | 4 | 2 | 4 | 1 | 1 | <0.5 | 2 | 8 | 1 | 1 |
| Carbenicillin disodium salt | 32 | 16 | 32 | 32 | 4 | 8 | 4 | 8 | 128 | 16 | 8 |
| Methicillin | 32 | 32 | 256 | 64 | 16 | 8 | 16 | 32 | >256 | 8 | 8 |
| Ampicillin sodium salt | 1 S | 1 S | 2 R | 4 R | 0.5 S | 4 S | 1 S | 4 | 16 | 4 S | 64 R |
| Dicloxacillin sodium salt hydrate | 4 S | 4 S | 32 S | 16 | 2 S | 1 S | 1 S | 8 | 64 | 2 | 2 |
| Gentamicin sulfate | 8 S | 8 S | 8 S | 16 S | 4 S | 16 S | 8 S | 8 | 8 | 16 S | 32 S |
| Streptomycin sulfate salt | 32 S | 16 S | 32 R | 16 | 32 S | 32 S | 32 S | 64 | 64 | 16 S | 32 S |
| Kanamycin sulfate | 128 R | 128 R | 128 R | 128 R | 128 R | 256 R | 256 R | 32 | 256 | 256 R | 256 R |
| Cephalothin sodium salt | 64 | 32 | 8 | 16 | 16 | 32 | 8 | 32 | 64 | 64 | 64 |
| Tetracycline | 16 S | 16 R | 8 R | 8 S | 4 S | 1 S | 8 S | 4 | 16 | 0.5 S | 1 S |
| Polymyxin B sulfate salt | 256 | 256 | 16 | >256 | 32 | >256 | 64 | 256 | >256 | >256 | >256 |
| Bacitracin | 128 | 256 | 4 | 64 | 4 | 128 | 2 | 128 | 32 | 64 | 16 |
| Erythromycin | 1 S | 1 S | <0.125 S | 0.25 S | 0.5 S | 1 S | 0.5 S | 1 | 0.25 | 0.25 S | <0.125 S |
| Metronidazole | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |

**Table 6. (continued)**

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Chloramphenicol | 8 S | 8 R | 4 S | 4 S | 4 S | 8 R | 4 S | 4 | 4 | 8 R | 4 S |
| Clindamycin hydrochloride | <0.03 S | <0.03 S | 0.0625 S | 0.0625 S | <0.03 S | 0.0625 S | <0.03 S | 0.125 | 0.0625 | 0.25 S | <0.03 S |
| Phosphomycin disodium salt | 256 | >256 | >256 | >256 | 256 | >256 | 256 | >256 | >256 | >256 | >256 |

**R = Resistant, S = Sensitive; Determined by EFSA guidance [33]; No superscript was added if the guidance has no reference values for the corresponding strain.**

**Table 7. MIC values (μg/mL) of *Bifidobacterium* spp. measured in LSM broth**

| Antibiotics | *B. longum* KCCM 91563 | *B. pseudocatenulatum* SS29 | *B. longum* RD47 | *B. lactis* AD011 | *B. adolescentis* INT57 | *B. bifidum* ATT |
|---|---|---|---|---|---|---|
| Penicillin G | <0.5 | <0.5 | 1 | <0.5 | 1 | 1 |
| Carbenicillin disodium salt | 2 | <0.5 | 8 | 8 | 16 | 1 |
| Methicillin | 8 | <0.5 | 128 | 32 | 128 | 1 |
| Ampicillin sodium salt | 0.5 S | 0.25 S | 8 R | 1 S | 1 S | 0.5 S |
| Dicloxacillin sodium salt hydrate | 4 | <0.5 | 8 | 16 | 64 | <0.5 |
| Gentamicin sulfate | 32 S | 8 S | 8 S | 32 S | 64 S | 64 S |
| Streptomycin sulfate salt | 16 S | 8 S | 64 S | 64 S | 128 S | 8 S |
| Kanamycin sulfate | 128 | 128 | 128 | 256 | 1024 | 128 |
| Cephalothin sodium salt | 16 | 1 | 8 | 16 | 32 | 2 |
| Tetracycline | 0.5 S | 0.25 S | 16 R | 4 S | 0.25 S | <0.125 S |
| Polymyxin B sulfate salt | 64 | 16 | >256 | 128 | >256 | 256 |
| Bacitracin | 2 | 1 | 16 | 1 | 256 | 32 |
| Erythromycin | <0.125 S | <0.125 S | 0.5 S | 0.5 S | 8 R | 64 R |
| Metronidazole | 256 | 4 | >256 | 256 | >256 | 128 |

**Table 7. (continued)**

| | | | | | | |
|---|---|---|---|---|---|---|
| Chloramphenicol | 1 S | 1 S | 4 S | 4 S | 4 S | 4 S |
| Clindamycin hydrochloride | <0.03 S | <0.03 S | <0.03 S | <0.03 S | 2 R | <0.03 S |
| Phosphomycin disodium salt | 256 | 128 | >256 | >256 | >256 | 64 |

**R = Resistant, S = Sensitive; Determined by EFSA guidance [33] ; No superscript was added if the guidance has no reference values for the corresponding strain.**

## 3.2. MIC values determined by using ISO standard with MRS broth

MIC values determined by using broth microdilution method in ISO standard with MRS broth are below (Tables 8, 9). Table 8 represents the result for *Lactobacillus* and Table 9 represents the result of *Bifidobacterium*. For β-lactam group antibiotics, the MIC values measured on MRS broth were lower than the MIC values on LSM broth. However, the MIC values measured on MRS broth tended to be higher than the MIC values on LSM broth for aminoglycoside group antibiotics.

**Table 8. MIC values (μg/mL) of *Lactobacillus* spp. measured in MRS broth**

| Antibiotics | *L. plantarum* PH3A | *L. fermentum* PH3B | *L. acidophilus* KCTC 3168 | *L. plantarum* KFRI 708 | *L. fermentum* EPS22 | *L. paracasei* CH88 | *L. fermentum* G7 | *L. sakei* KOK | *L. brevis* GABA100 | *L. rhamnosus* GG | *L. casei* IBS041 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Penicillin G | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| Carbenicillin disodium salt | 4 | 4 | 4 | 8 | 2 | 2 | 2 | 8 | 16 | 2 | 2 |
| Methicillin | 8 | 4 | 256 | 16 | 32 | 4 | 8 | 16 | 256 | 2 | 2 |
| Ampicillin sodium salt | 0.125 | 0.125 | 1 | 0.125 | 0.25 | 1 | 0.25 | 1 | 2 | 1 | 0.5 |
| Dicloxacillin sodium salt hydrate | 2 | 2 | 16 | 2 | 8 | 1 | 1 | 4 | 16 | 1 | <0.5 |
| Gentamicin sulfate | 128 | 256 | >256 | 128 | >256 | 256 | 256 | 256 | >256 | 256 | 256 |
| Streptomycin sulfate salt | >256 | >256 | >256 | 256 | >256 | 256 | >256 | >256 | >256 | 128 | 256 |
| Kanamycin sulfate | >1024 | >1024 | 1024 | >1024 | >1024 | >1024 | 1024 | 256 | >1024 | 1024 | 1024 |
| Cephalothin sodium salt | 2 | 2 | 1 | 4 | 2 | 8 | 1 | 8 | 4 | 4 | 4 |
| Tetracycline | 8 | 8 | 16 | 8 | 4 | 2 | 8 | 4 | 32 | 1 | 1 |
| Polymyxin B sulfate salt | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Bacitracin | 256 | 256 | 128 | 256 | 8 | 128 | 16 | 128 | 128 | 256 | 32 |
| Erythromycin | 2 | 2 | 2 | 2 | 1 | 0.5 | 2 | 2 | 4 | 0.5 | 0.5 |
| Metronidazole | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
| Chloramphenicol | 2 | 2 | 4 | 2 | 4 | 4 | 2 | 1 | 2 | 4 | 2 |
| Clindamycin hydrochloride | <0.03 | <0.03 | 0.125 | 0.125 | <0.03 | 0.25 | <0.03 | 0.125 | 0.125 | 0.5 | 0.0625 |
| Phosphomycin disodium salt | 256 | 256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |

**Table 9. MIC values (μg/mL) of *Bifidobacterium* spp. measured in MRS broth**

| Antibiotics | *B. longum* KCCM 91563 | *B. pseudocatenulatum* SS29 | *B. longum* RD47 | *B. lactis* AD011 | *B. adolescentis* INT57 | *B. bifidum* ATT |
|---|---|---|---|---|---|---|
| Penicillin G | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| Carbenicillin disodium salt | 2 | 1 | 4 | 2 | 4 | <0.5 |
| Methicillin | 16 | 4 | 128 | 64 | 64 | 1 |
| Ampicillin sodium salt | 0.25 | 0.25 | 2 | 0.25 | 0.5 | <0.03 |
| Dicloxacillin sodium salt hydrate | 4 | <0.5 | 4 | 8 | 32 | <0.5 |
| Gentamicin sulfate | >256 | 2 | >256 | 256 | 128 | >256 |
| Streptomycin sulfate salt | 256 | >256 | >256 | >256 | 256 | 256 |
| Kanamycin sulfate | >1024 | >1024 | >1024 | >1024 | >1024 | >1024 |
| Cephalothin sodium salt | 16 | 4 | 8 | 4 | 8 | 1 |
| Tetracycline | 1 | 1 | 64 | 8 | 32 | 1 |
| Polymyxin B sulfate salt | >256 | 64 | >256 | >256 | >256 | 256 |
| Bacitracin | 32 | 16 | 128 | 16 | >256 | 128 |
| Erythromycin | <0.125 | 0.25 | 4 | 2 | 16 | 0.25 |
| Metronidazole | >256 | 4 | >256 | >256 | >256 | 4 |
| Chloramphenicol | 0.5 | 1 | 4 | 4 | 4 | 1 |
| Clindamycin hydrochloride | <0.03 | 0.0625 | 0.0625 | 0.0625 | >16 | <0.03 |
| Phosphomycin disodium salt | 256 | 256 | >256 | >256 | >256 | 128 |

## 3.3. MIC values determined by using Etest

MIC values determined by using Etest method are below (Tables 10, 11). Table 10 represents the result for *Lactobacillus* and Table 11 represents the result of *Bifidobacterium*. On the agar medium, most *Lactobacillus* and *Bifidobacterium* showed lower MIC values on gentamicin, compared to the MIC values measured on the broth medium.

**Table 10. MIC values (μg/mL) of *Lactobacillus* spp. measured by using Etest**

| Antibiotics | *L. plantarum* PH3A | *L. fermentum* PH3B | *L. acidophilus* KCTC 3168 | *L. plantarum* KFRI 708 | *L. fermentum* EPS22 | *L. paracasei* CH88 | *L. fermentum* G7 | *L. sakei* KOK | *L. brevis* GABA100 | *L. rhamnosus* GG |
|---|---|---|---|---|---|---|---|---|---|---|
| Ampicillin | 0.5 | 0.25 | 0.25 | 0.12 | 0.12 | 2 | 0.5 | 8 | 1 | 32 |
| Ciprofloxacin | >256 | 8 | 8 | >256 | 16 | 4 | 8 | 4 | >256 | 16 |
| Clindamycin | 0.015 | 0.015 | 0.03 | 0.06 | 0.03 | 0.25 | 1 | 0.06 | 1 | 0.015 |
| Erythromycin | 1 | 0.12 | 0.5 | 1 | 0.12 | 0.06 | 1 | 0.12 | 2 | 0.12 |
| Gentamicin | 64 | 32 | 64 | 32 | 16 | 128 | 16 | 64 | 16 | 64 |
| Linezolid | 1 | 0.5 | 0.5 | 1 | 0.5 | 0.5 | 4 | 0.5 | 1 | 2 |
| Tetracycline | 16 | 2 | 2 | 8 | 4 | 1 | 0.5 | 1 | 8 | 1 |

**Table 11. MIC values (μg/mL) of *Bifidobacterium* spp. measured by using Etest**

| Antibiotics | *B. longum* KCCM 91563 | *B. pseudocatenulatum* SS29 | *B. longum* RD47 | *B. adolescentis* INT57 |
|---|---|---|---|---|
| Ampicillin | 0.25 | 0.12 | 0.06 | 1 |
| Ciprofloxacin | >256 | >256 | >256 | 4 |
| Clindamycin | 0.015 | 0.03 | <0.015 | 0.5 |
| Erythromycin | 1 | 1 | 1 | 0.12 |
| Gentamicin | 64 | 32 | 64 | 256 |
| Linezolid | 2 | 2 | 2 | 2 |
| Tetracycline | 4 | 4 | 8 | 1 |

## 3.4. MIC values determined by using disc diffusion test

MIC values determined by using Etest method are below (Tables 12, 13). Table 12 represents the result for *Lactobacillus* and Table 13 represents the result of *Bifidobacterium*. Diameters of six discs were measured and the MIC values were obtained by using the diameter through linear regression. The values that exceptionally high or low were denoted as N/D. Phosphomycin has shown no clear zone for every discs and their data could not be used to calculate the MIC values.

**Table 12. MIC values (μg/mL) of *Lactobacillus* spp. measured by using disc diffusion test**

| Antibiotics | L. plantarum PH3A | *L. plantarum* PH3B | *L. acidophilus* KCTC 3168 | *L. plantarum* KFRI 708 | *L. fermentum* EPS22 | *L. paracasei* CH88 | *L. fermentum* G7 | *L. sakei* KOK | *L. brevis* GABA100 | *L. rhamnosus* GG | *L. casei* IBS041 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ampicillin sodium salt | 0.42 | 0.07 | 0.01 | N/D* | 0.14 | 1.86 | 0.01 | 8.24 | N/D* | N/D** | 5.67 |
| Dicloxacillin sodium salt hydrate | 6.64 | 33.77 | 0.54 | N/D* | 5.94 | 0.26 | 0.06 | 5.78 | 169.70 | 0.01 | 1.13 |
| Tetracycline | 21.60 | 18.43 | 2.92 | 29.63 | 4.71 | 9.75 | 1.07 | 6.13 | 0.82 | 0.85 | 2.95 |
| Erythromycin | 4.33 | 3.13 | 0.17 | N/D* | 0.12 | 2.00 | 0.30 | 5.84 | 0.46 | 37.50 | 0.59 |
| Chloramphenicol | 34.35 | 36.81 | 10.97 | 16.34 | 5.29 | 14.86 | 4.30 | 19.26 | 8.60 | 8.91 | 34.53 |
| Clindamycin hydrochloride | N/D* | 0.02 | N/D* | 0.03 | N/D* | 0.39 | N/D* | N/D* | 0.01 | 0.13 | 0.65 |
| Phosphomycin disodium salt | N/D** | N/D** | N/D** | N/D** | N/D** | N/D** | N/D** | N/D** | N/D** | N/D** | N/D** |

**N/D* = Too low to detect, N/D** = Too high to detect**

**Table 13. MIC values (μg/mL) of *Bifidobacterium* spp. measured by using disc diffusion test**

| Antibiotics | *B. longum* KCCM 91563 | *B. pseudocatenulatum* SS29 | *B. longum* RD47 | *B. lactis* AD011 | *B. adolescentis* INT57 | *B. bifidum* ATT |
|---|---|---|---|---|---|---|
| Ampicillin sodium salt | 6.48 | 0.99 | 5.61 | 2.10 | 0.86 | 0.91 |
| Dicloxacillin sodium salt hydrate | 21.88 | 24.00 | 13.65 | 13.48 | N/D* | 0.33 |
| Tetracycline | 1.84 | 1.43 | 11.67 | 1.13 | 10.59 | 0.05 |
| Erythromycin | 0.03 | 0.02 | 0.67 | 0.07 | 8.65 | N/D** |
| Chloramphenicol | 7.19 | 19.45 | 21.22 | 10.21 | 130.71 | 3.79 |
| Clindamycin hydrochloride | 0.01 | N/D* | N/D* | 0.02 | 1.45 | 0.06 |
| Phosphomycin disodium salt | N/D** | N/D** | N/D** | N/D** | N/D** | N/D** |

**N/D* = Too low to detect, N/D** = Too high to detect**

## 3.5. Antibiotic resistance genes and their locations

Table 14 shows the antibiotic resistance genes located in the listed bacterial strains. The results were obtained using CLgenomics in ChunLab Co., Ltd. (Seoul, Korea) and RAST (http://rast.nmpdr.org) service. Also, the results from NCBI protein BLAST are shown on the third column to show the similarity for the antibiotic resistance gene.

**Table 14. The identified antibiotic resistance genes for nine lactic acid bacteria**

| Bacterial strains | Identified antibiotic resistant genes from CLgenomics | BLAST result and homology of the corresponding gene | | | The size of contig (bp) |
|---|---|---|---|---|---|
| | | Protein description | Query coverage | Identity | |
| *L. plantarum* PH3A | *penP* | Beta-lactamase | 93% | 99% | 132,943 |
| | *aadA* | Adenylyl transferase | 99% | 99% | 364,802 |
| | *bacA* | Undecaprenyl-diphosphate phosphatase | 100% | 99% | 112,349 |
| | *catA* | Chloramphenicol acetyltransferase | 100% | 100% | 472,456 |
| *L. fermentum* PH3B | *penP* | Beta-lactamase class A | 99% | 94% | 56,871 |
| | *aadA* | Adenylyl transferase | 99% | 99% | 50,347 |
| | *bacA* | Undecaprenyl-diphosphate phosphatase | 100% | 99% | 63,513 |
| | *catA* | Chloramphenicol acetyltransferase | 100% | 100% | 9,194 |
| *L. fermentum* EPS22 | *bacA* | Undecaprenyl-diphosphate phosphatase | 100% | 100% | 2,120,282 |
| *L. paracasei* CH88 | *penP* | Beta-lactamase class A | 100% | 100% | 3,086,873 |
| | *bacA* | Undecaprenyl-diphosphate phosphatase | 100% | 99% | 3,086,873 |

**Table 14. (continued)**

| | | | | | |
|---|---|---|---|---|---|
| *L. fermentum* G7 | *penP* | Zinc ribbon domain-containing protein | 100% | 99% | 3,507 |
| | *bacA* | Undecaprenyl-diphosphate phosphatase | 100% | 100% | 42,540 |
| *L. brevis* GABA100 | *bla* | Beta-lactamase class A | 99% | 65% | 32,514 |
| | *aph* | Aminoglycoside phosphotransferase | 100% | 99% | 32,819 |
| *B. pseudocatenulatum* SS29 | *murE* | murE1 protein | 99% | 69% | 2,189,313 |
| | *bacA* | Undecaprenyl-diphosphatase UppP | 100% | 100% | 2,189,313 |
| *B. longum* RD47 | *tetM* | MFS transporter | 100% | 99% | 117,675 |
| *B. bifidum* ATT | *penP* * | Beta-lactamase class A | 100% | 100% | 124,811 |
| | *penP* * | Beta-lactamase | 100% | 100% | 186,504 |
| | *aphA* | Aminoglycoside 3'-phosphotransferase | 100% | 100% | 1,648 |
| | *bacA* | Undecaprenyl-diphosphatase UppP | 100% | 100% | 41,965 |

**The data was obtained by using whole genome sequencing and analysis were completed by using an Illumina MiSeq sequencer. The size of contig represents the size of contig on which corresponding antibiotic resistance gene exists.**

*** two genes in two contigs separately**

# 4. Discussion

## 4.1. Comparison of MIC values on different culturing condition

### 4.1.1. Comparison of LSM medium and MRS medium

Depending on the antibiotics group, the MIC measured on MRS broth (Tables 8, 9) showed a little different patterns from the MIC measured on LSM broth (Tables 6, 7). For the β-lactam group, most MIC measured on MRS broth was lower than MIC measured on LSM broth. This trend appears on ampicillin and penicillin G apparently. Also, some of the *Lactobacillus* species had slightly high MIC for penicillin G, compared with the result (<0.032 – 2 μg/mL) of a previous study [37].

For the aminoglycoside group, MIC measured on MRS medium was apparently higher than MIC measured on LSM medium, especially for kanamycin. This trend is clear for *Bifidobacterium*, which is more sensitive to aminoglycoside than *Lactobacillus*. It can be attributed to the fact that some ingredients in MRS medium may inhibit the activity of antibiotic reagents. Unlike LSM medium which is made from a mixture of 90% IST medium containing a mixture of various known chemical compounds and 10% MRS medium, MRS medium is an undefined medium and contains

beef extract and yeast extract about which trace components is not known clearly. On the other hand, in the study reported by Klare at el., which showed the similar result with the present study, a possibility was proposed that low pH of MRS medium could decrease the activity of aminoglycoside group antibiotics [38].

For metronidazole, all 11 strains of *Lactobacillus* were resistant to metronidazole at the range of >256 μg/mL on both MRS broth and LSM broth. Also, for kanamycin, most *Lactobacillus* showed resistance to kanamycin and have high MIC than the cut-off value. However, according to Table 14, none of the *Lactobacillus* with high MIC for kanamycin were revealed to have neither the metronidazole nor the kanamycin resistant gene. Likewise, all 17 lactic acid bacteria used in this study showed very high MIC for polymyxin B at both LSM broth and MRS broth.

### 4.1.2. Comparison of broth medium and agar medium

The results of liquid medium and agar medium were also compared with respect to the MIC values. Overall, the result of the Etest (Tables 10, 11) tended to have higher MIC for ampicillin than the result of liquid medium tests. Also, in general, broth microdilution method showed much higher MIC for erythromycin and gentamicin than the MIC measured on

the Etest and disc diffusion test. These trends for ampicillin, erythromycin and gentamicin are similar to the results of the previous study which used Mueller-Hinton broth [39]. And as all 17 lactic acid bacteria showed very high MIC for polymyxin B at the broth microdilution method, they also showed high level of MIC agar medium test. However, this result does not mean that they expressed the polymyxin B resistance gene at high level, because polymyxin B is known to be hard to move across thick cell wall of gram-positive bacteria [17].

For the aminoglycoside group, a disc diffusion test was also performed. However, all diameters for the six discs cannot be measured. Their diameter (data not shown) sharply decreased as the concentration of antibiotics decreases. Thus, the diameter data measured could not be used for the regression.

## 4.2. Relationship between antibiotic susceptibility and antibiotic resistant gene on individual strains

According to the Table 14, it was revealed that many of the *Lactobacillus* and *Bifidobacterium* have the *penP* gene which is the β-lactamase gene giving penicillin resistance to bacteria. However, little

difference was found in MIC values between the strains that have *penP* gene and the strains that do not have *penP* gene.

The reason why those bacteria show the low MIC despite of existence of the corresponding antibiotic resistant gene might be attributed to either low expression of the resistance gene or the antagonistic interaction between the antibiotic agent and a composition of the culture medium. For β-lactam group, the MIC values for most of the lactic acid bacteria with *penP* gene used in this study were shown to be low both on LSM medium and MRS medium. It is likely that the *penP* genes were not efficiently expressed rather than a certain component in the medium interrupted the antibiotic activity.

On the other hand, according to the previous study, *L. reuteri* was the most penicillin G resistant species among 8 *Lactobacillus* species and the 66% of *L. plantarum* strains have shown resistance against penicillin G [40]. However, none of the *L. plantarum* strains used in the present study showed high MIC for penicillin G.

*L. brevis* GABA100 showed high MIC for carbenicillin, dicloxacillin and methicillin on broth microdilution tests and also showed high MIC for penicillin G and ampicillin on ISO standard method. Considering that all these antibiotics belong to β-lactam group antibiotics and *L. brevis* GABA100 was turned out to have β-lactamase gene (Table 14), *L. brevis*

GABA100 is thought to express β-lactamase gene at high level.

*B. longum* RD47 showed high MIC for tetracycline among the six strains of *Bifidobacterium,* which is 16 μg/mL. From the gene location result (Table 14), *tetM* gene was found from *B. longum* RD47 on its chromosomes. However, *B. longum* KCCM 91563, which belongs to the same species with *B. longum* RD47, showed relatively low MIC value for tetracycline. Thus, considering that antibiotic treatment can result in the change of human gut microbiota and decrease the number of *Bifidobacterium* species [41], *B. longum* RD47 might have a higher chance to survive in the gut of a patient being treated with some tetracycline, which may give a better probiotic effect assuming that the tetracycline sensitive harmful bacteria diminish.

Two strains of *Lactobacillus* (*L. plantarum* PH3A and *L. fermentum* PH3B) contained the *catA* gene which is related to chloramphenicol resistance and showed relatively high MIC than the other lactic acid bacteria. However, the MIC value of *L. plantarum* PH3A was not greater than the EFSA cut-off value (8 μg/mL) [33]. Actually many of *L. plantarum* species are known to have high MIC for chloramphenicol [42]. On the other hand, *L. fermentum* species have low EFSA cut-off value (4 μg/mL). Therefore, *L. fermentum* PH3B showed somewhat high resistance to chloramphenicol compared to the ordinary *L. fermentum* species.

For metronidazole, all *Lactobacillus* species and four *Bifidobacterium* species showed especially high MIC values regardless of the kind of broth medium. Bolton *et al*. measured the faecal concentration of metronidazole from the patients with *Clostridium difficile* colitis. Metronidazole was detectable in all nine watery samples (mean 9.3 ± 7.5 μg/g wet weight; range 0.8 – 24.2 μg/g), in all seven semiformed samples (mean 3.3 ± 3.5 μg/g wet weight; range 0.5 – 10.4 μg/g), and six of 13 formed faecal samples (mean 1.23 ± 2.8 μg/g; range 0 – 10.2 μg/g) [43]. Likewise, Johnson *et al*. reported that metronidazole was detected at a level of 1.5 μg/g wet weight from the stool of one of their patients [44].

The range of faecal metronidazole concentration on their results is less than the present study's MIC values of metronidazole for all *Lactobacillus* species and four of six *Bifidobacterium* species on both LSM medium and MRS medium. Thus, for those who are being treated with metronidazole, viability of *Lactobacillus* and *Bifidobacterium* species on their gut still might be able to be maintained.

The other study showed that the proportion of intestinal *Bifidobacterium* species can be easily affected and decrease by the metronidazole treatment [43]. However, no research was done on *Lactobacillus* species. Considering the result of the present study implying that *Bifidobacterium* species are relatively more vulnerable to

metronidazole than *Lactobacillus* species on both LSM medium and MRS medium, a further research on the survival of *Lactobacillus* species on the metronidazole treated patients might be worth to be performed.

## 4.3. Identification of location of antibiotic resistance gene and their transferability

According to the previous study, it is known that most horizontal gene transfer can occur via plasmid [45]. Thus, to examine the safety evaluation of the experimental *Lactobacillus* and *Bifidobacterium* used in the present study, the analysis of the whole genome sequences was performed for the experimental bacteria and their contigs was analyzed to check whether they have antibiotic resistance gene or not.

From the Table 14, several antibiotic resistance genes were identified to exist. Most of the genes were revealed to be located in large contigs (more than 30,000 bp), which is thought to be a large piece of the entire chromosome of corresponding bacteria. However, several genes were located in a very small contig — a *aphA* gene of *B. bifidum* ATT was found in the 1,648 bp contig, a *penP* gene of *L. fermentum* G7 was found in 3,507 bp contig and *catA* gene of *L. fermentum* PH3B was found in 9,192

bp contig. After analyzing the three contigs with NCBI BLAST service, it was found that none of those contigs contain any replication origin. Thus, those contigs apparently did not belong to plasmids. Therefore, their transferability to the other bacteria would be low. Still, for the safety concerns, the absence of the antibiotic resistance gene carrying plasmid needs to be verified for *B. bifidum* ATT, *L. fermentum* G7 and *L. fermentum*.

On the other hand, for those bacteria which have the antibiotic resistant gene in its large contigs (more than 30,000 bp), their resistance genes are expected to be hardly transferred to the other bacteria. Thus, those antibiotics, if applied for consumption, are thought to prevent pathogenic bacteria from growing and increase the possibility of growth of the corresponding experimented bacteria on the intestinal tracts of the hosts.

Several strains were found to have two copies of genes (Table 14). However, those strains that have two copies of same antibiotic resistance gene did not show high MIC values for the antibiotic compared with the strains that have a single copy of the corresponding antibiotic resistance gene.

# 5. Conclusion

The MIC of the 11 strains of *Lactobacillus* and the six strains of *Bifidobacterium* was determined for 17 antibiotic reagents. To determine the MIC, two types of medium (broth medium and agar medium) were used to see the difference between those two different environments. For susceptibility and resistance, some tendencies for each bacterial strains were revealed regardless of what types of medium were used. For example, high MIC for phosphomycin, polymyxin B and kanamycin was shown and especially for metronidazole, *Lactobacillus* showed more resistance than *Bifidobacterium*. The results on the present study are thought be helpful to compare or evaluate MIC values newly measured on LSM broth medium or MRS broth medium with those from the other studies.

For 11 strains of the newly isolated bacteria, the possibility was examined that antibiotic resistance gene is located in plasmid or chromosome, using whole genome sequencing data. Except for the genes found in three small contigs, most of the antibiotic resistance genes are thought to exist in their chromosomes. Thus, for those bacteria known to have no plasmid containing any gene related to antibiotic resistance in this study, it might be hard for their resistance to be transferred to the other enterobacteria.

The conclusion of this study can be summarized as below:

1) *Bifidobacterium* showed lower MIC for β-lactam group antibiotics than *Lactobacillus*. Most *Lactobacillus* strains were resistant to kanamycin and most lactic acid bacteria showed high MIC against polymyxin B and phosphomycin. MIC measured on MRS medium was higher than MIC measured on LSM medium, for aminoglycoside group antibiotics. The susceptibility trends for lactic acid bacteria can be useful for the development of the probiotic strains and the salient use of antibiotics for the patients.
2) Comparing the MIC values measured in the present study with the results of the previous studies which determined the faecal concentration of antibiotics, many species of lactic acid bacteria of the present study are thought to be able to survive in the intestine of subjects being treated with metronidazole.
3) By analyzing the contigs and the existence of replication origin for several lactic acid bacteria use in this study, it was found that their resistance genes are expected to be hardly transferred, which may be regarded as a desirable probiotic property.

# Appendices

**Appendix 1 Repetitively measured MIC values (μg/mL) for *B. longum* BB536 and the MIC values from the other study**

| **Antibiotics** | Repetition 1 | Repetition 2 | Ref [28] |
|---|---|---|---|
| penicillin G | <0.5 | <0.5 | 0.125 |
| carbenicillin disodium salt | 1 | <0.5 | 2 |
| methicillin | <0.5 | <0.5 | 4 |
| ampicillin sodium salt | 0.25 | <0.03 | 0.25 |
| dicloxacillin sodium salt hydrate | 2 | 1 | 4 |
| gentamicin sulfate | 4 | 8 | 32 |
| streptomycin sulfate salt | 8 | 16 | 32 |
| tetracycline | 0.25 | 0.25 | 1 |
| bacitracin | <0.5 | <0.5 | N.D. |
| erythromycin | <0.125 | <0.125 | 0.125 |
| metronidazole | 1 | 1 | 8 |
| chloramphenicol | 4 | 1 | 2 |
| clindamycin hydrochloride | <0.03 | <0.03 | <0.032 |
| phosphomycin disodium salt | 128 | 128 | 128 |

**Appendix 2 Repetitively measured MIC values (μg/mL) for *B. bifidum* ATT and the MIC values from the other study**

| **Antibiotics** | Repetition 1 | Repetition 2 | Ref [28] |
|---|---|---|---|
| penicillin G | 1 | 1 | 0.063 |
| carbenicillin disodium salt | 1 | 2 | 0.5 |
| methicillin | 1 | 1 | 0.5 |
| ampicillin sodium salt | 0.5 | 0.25 | 0.063 |
| dicloxacillin sodium salt hydrate | <0.5 | <0.5 | 1 |
| gentamicin sulfate | 64 | 64 | 256 |
| streptomycin sulfate salt | 8 | 16 | 32 |
| cephalothin sodium salt | 2 | 1 | 2 |
| tetracycline | <0.125 | 0.25 | 1 |
| polymyxin B sulfate salt | 256 | 256 | 512 |
| bacitracin | 32 | 16 | N.D. |
| metronidazole | 128 | 256 | 64 |
| chloramphenicol | 4 | 4 | 2 |
| clindamycin hydrochloride | <0.03 | <0.03 | >16 |
| phosphomycin disodium salt | 64 | 64 | 32 |

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# 국문초록

*Lactobacillus* 와 *Bifidobacterium* 은 사람의 장 건강에 기여하는 프로바이오틱스로서 주목을 받고 있다. 따라서 이들의 안전성 및 항생제 저항성, 유해균으로의 수평적 유전자 전이에 대한 평가는 산업적으로 유용하게 이용될 수 있다. 본 연구에서는 다양한 생태학적 조건에서 새롭게 분리 및 동정된 8 종의 *Lactobacillus* 와 5 종의 *Bifidobacterium* 균주, 그리고 표준 균주 6 종에 대한 항생제 감수성과 MIC 에 대해 시험하였다. 항생제는 총 17 종을 사용하였다. 그리고 총 17 종의 균주들의 항생제 감수성을 알아보기 위해 ISO 에서 제시한 표준 방법을 이용하여 액체 배지에서의 감수성을 확인하였고, 고체 배지 상태에서의 감수성을 확인하기 위해 Etest strip 방법과 디스크 확산 방법을 사용하였다. 17 종의 균주 중 유전체 분석이 완료된 균주 10 종에 대하여 각각의 항생제에 저항성 또는 감수성을 나타내는 유전자의 존재 여부를 확인하였다. 항생제 감수성 실험 결과, β-lactam 그룹에서는 LSM 배지에서 측정한 MIC 결과가 MRS 배지에서 측정한 MIC 결과보다 값이 높았다. Aminoglycoside 그룹에서는 MRS 배지에서 측정한 MIC 가 LSM 배지에서 측정한 결과보다 높았으며, 이러한 경향은 *Bifidobacterium* 에서 더 크게 나타났다. 또한, 대부분의 *Lactobacillus* 가 kanamycin 에 저항성이 있는 것으로 나타났다. 모든 실험 조건에서 모든 *Lactobacillus* 와 *Bifidobacterium* 은 polymyxin B 에 대한 저항성을 가지는 것으로 나타났다. 한편, tetracycline 과 chloramphenicol 저항성 유전자를 가지고 있는 균주는

해당 저항성 유전자를 가지고 있지 않은 균주에 비해 대부분 MIC 가 높게 측정되었다. 그러나 *penP* 유전자를 가지고 있지만, β-lactam 그룹에 대해 낮은 MIC 를 보인 균주도 있었다. 한편, 이 균주들이 가지는 대부분의 항생제 저항성 유전자는 염색체에 위치할 가능성이 높은 것으로 드러났으며, 이들은 산업적 프로바이오틱스로서의 활용이 용이할 것으로 기대된다.

**주요어 : 최소저해농도, 유산균, 항생제**

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