Doctoral Dissertation

Elucidation on antibacterial activity of Bifidobacteria

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Elucidation on antibacterial activity of Bifidobacteria

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Doctor of Engineering

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CONTENTS

ABBREVIATIONS

µm	micrometer
AU	activity (Arbitrary Unit)
BBLA72	*Bifidobacterium bifidum* lamb 72
BLM	blood liver medium
BSA	Bovine Serum Albumin
CFS	Cell free supernatant
Da	Daltons
DNA	Deoxyribonucleic acid
EBFA	Enzymatic Bioanalysis / Food analysis
F6PPK	Fructose-6-P phosphoketolase
GAM	Gifu anaerobic medium
GIT	Gastro intestinal tract
g/L	Gram per liter
JCM	Japan collection of Microorganism
kDa	Kilodalton
LAB	Lactic Acid Bacteria
LB	Lysogeny broth
MMECA	Mongolian Medical Ethics Committee Approval
MRS	de Main Rogasa and Sharpe
mg	Milligram
min	Minute
ml	Milliliter
mmole/L	Millimole per liter
MW	Molecular weight
NCFS	Neutralized Cell Free Supernatant
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Negative decadal logarithm of the [H+] ion concentration
SDS	Sodium dodecyl sulfate

TSA	Trypticase soy agar
TSB	Trypticase soy broth
U	Unit
UV	Ultraviolet
UF	Ultrafiltration
w/v	Wet volume

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GENERAL ABSTRACT

Antibacterial activity of Bifidobacteria isolated from Mongolian infant feces was elucidated on pathogenic intestinal bacteria for the development of a new antibacterial Bifidobacteria by permission of the Mongolian Medical Ethics Committee Approval (MMECA). Forty-nine single colonies were totally obtained from 3 samples by using an enrichment BL agar medium. Among them, 29 isolates had Gram−positive, catalase−negative properties, and maul−like or Y−shaped morphology, and then, 20 *Bifidobacterium breve* and 9 *Bifidobacterium longum* strains were detected by the *B. breve* and *B. longum* specific primers. Organic acids produced by the isolated Bifidobacteria in their cell free supernatants were quantitatively analyzed by a spectrophotometric absorbance at 340 nm, suggesting that D−lactic, L−lactic, and acetic acids were produced, and the pH of the supernatants was at 3.86−4.55. The isolated Bifidobacteria showed antibacterial activity toward *Escherichia coli* and *Salmonella typhimurium* as high as that of standard Bifidobacteria, however, lower activity against *Staphylococcus aureus*. The antibacterial activity was probably due to the production of the organic acids.

We aimed high antibacterial potential probiotic found for the development of a new antibacterial peptide. Thirty-six single colonies were totally obtained 15 samples of the faeces and rumen cud collected from Mongolian young livestock animals of around the three provinces (Tuv, Khovd, Khuvsgul). The 5-type species

Bifidobacterium identified *B.bifidum B.catenulatum*, *B.ruminantum*, *B.longum*, *B.pseudocatenulatum* and 8 type species lactic acid bacteria *Enterococcus alcedinis*, *Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae*, *Enterococcus lactis*, *Enterococcus mundti*, *Lactobacillus reuteri*, and *Lactobacillus plantarum* by 16S rDNA sequencing analysis.

Isolated from the lamb rumen *B.bifidum* (BBLA72) was produced 29.6 mmol per litres acetic acid and 19.3 mmol per litres of lactic acid, thereby releasing pH from 7.0 to 4.57. However, organic acids were produced small in comparison with other strains, the antibacterial activity of the pH neutralized up to 7.0 cell-free supernatant was more significant than other strains.

BBLA 72 starts with early stationary phase and its antibacterial activity is the highest in 18 hours of bacterial culture. In the pH stabilization, bacterial resistance to 1-10 was active and 5, 6, and 7 did not significantly reduce the antibacterial activity.

The arbitrary unit was measured 25600 AU/ml against to *E.coli*. It is preliminary for this substance as a bacteriocin-like substance that weighs 2 kDa and 5 kDa, capable of withstanding a 100-degree heat to 30 minutes, proteinase K and trypsin enzyme.

Chapter I.

General Introduction

Bifidobacteria with Gram-positive and catalase−negative properties [1, 2] are known to have a potent antibacterial activity due to the production of organic acids. [3, 4] Bifidobacteria are found from the intestinal tract of mammalian [5, 6] and work as probiotics for promoting health. [7] Although the antibacterial activity of bifidobacteria is usually expressed by the produced organic acids [8-9] peptides called bacteriocin are also participated. [10-11]

Several papers appeared on the antibacterial activity of bifidobacteria by the production of organic acids. Makras et al, 2006 were found a strong antibacterial activity of bifidobacteria towards Gram-negative bacteria, *Salmonella* and *E. coli* strains, by the production of lactic and acetic acids and reported the inhibition of growth of Gram-positive bacteria by the production of a bacteriocin[8]. The agar spot test of bifidobacteria on several pathogenic bacteria showed the antibacterial activity and one of the main inhibitory reasons was reported by the production of organic acids [9] and. [1,2] Georgieva et al., 2015 described on a comparison of acidic and neutralized cell-free supernatants cultured by bifidobacteria, indicating that the acidic supernatant was active to several pathogenic bacteria and the neutralized supernatant also showed the antibacterial activity. [13] Biedrzycka et al., 2003

reported that acetate and lactate were the main products of Bifidobacteria fermentation with sugars. [14] The highest amount of lactate and acetate was obtained from the fermentation on lactose determined by a gas chromatography analysis.

Yildirim et al., 1999, reported the amino acid sequence of bifidocin B and characterization which is isolated from *B.bifidum* NCFSB 1454. Properties of Bifdocin B is studied which was sensitive for some enzymes but resistant to pH 2-10 and inhibited the growth of the food-borne, food-spoilage bacteria such as *Bacillus*, *Enterococcus*, *Lactobacillus*, *Listeria* and *Pediococcus.* [15]

Touré et al., 2003, obtained anti-listerial activity bifidobacteria isolated from infants faeces. The purified peptide properties were hydrophilic, the limit of the heat resistance was 100 °C for 5 min and sensitive to protease. [16]

Ueli von Ah., 2006, Isolated from baby faeces *Bifidobacterium thermophilum* RBL67 produced Thermophilicin B67. It's formed diameter of 13 mm, 11 mm, and 10 mm inhibited zone to Lactobacillus *acidophilus*, *listeria Ivanovic* and *Listeria innocua* respectively. [29]

Cheikhyoussef et al., 2010 partial sequenced of Bifidin I isolated from *Bifidobacterium infantis*. Antibacterial active was after growing 30 min about 99% inhibited of cell-free supernatant per ml of all indicator strains. Bifidin I was

purified by using a three-step purification procedure. Antibacterial activity of Bifidin I was after dialysis 52000 AU/ml. [17]

Cheikhyoussef et al., 2009b, another hand, Bifidin I also, has a potential antibacterial activity for Gram-positive bacteria such as *Staphylococcus*, *Streptococcus* and *Clostridium*, and Gram-negative bacteria such as *Salmonella*, *Shigella* and *E. coli*. As mentioned above, a strong antibacterial activity of bifidobacteria was mainly caused by the production of organic acids. Recently, we showed the antibacterial activity of lactic acid bacteria (LAB), *L. hilgardii* and *L. diolivorans*, which were isolated from a traditional fermented mare's milk, airag, in Mongolia and identified by the 16S rDNA analysis. Although the antibacterial activity of LAB was due to the production of organic acids, antibacterial peptides were also contained in the fermented supernatant.

In this work, we isolated and identified several bifidobacteria from Mongolian livestock's young animals by the universal primers for *B.catenulatum*, *B.longum*, *B.pseudocatenulatum*, *B.ruminantum* to select potent antibacterial strains. The antibacterial activity was evaluated by the ability of lactic and acetic acid productions and pH by the inhibition of multiplication on pathogenic strains, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*. Also, we found antibacterial peptide producing form of *Bifidobacterium pseudocatenulatum,* antibacterial activity AU/ml determined in enzyme treatment, heat treatment, pH 1-

10 resistance potential by AU/ml respectively Molecule mass of antibacterial peptide was determined by SDS-page result.

1.1. Genus of the Bifidobacterium

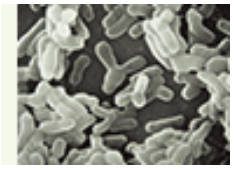
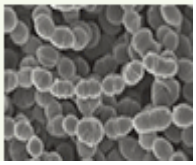
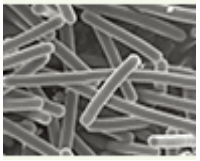
The discovery of the bifidobacteria was isolated from infants faeces by Henry Tissier in 1900, and that bacteria identified have cell special morphology shown Y-shaped characteristic. This bacterium was Gram-positive, catalase-negative and named it Bacillus bifidus. Bacterial taxonomic of the bacteria be included in the genus Bifidobacterium which is generally accepted but somewhat like Lactobacillus by Orla-Jensen (1924). Therefore, the 7th edition of Bergey's bacterial identification (Breed et al., 1957).

In the 8th edition of Bergey's Manual of Determinative Bacteriology (Rogosa, 1974) bifidobacteria were classified in the genus Bifidobacterium using the same name initially adopted by Orla-Jensen. The genus comprised eight species; it was included in the family of Actinomycetaceae of the order Actinomycetales.

1.2. Ecology

Bifidobacterium species are detected commonly on human and animals' intestinal tract and swage [1], [18], human vaginal tract, dental caries and oral cavity [2, 19].

Table 1. Difference between Bifidobacteria and lactobacilli

	Bifidobacteria	**Lactic acid bacteria**
Cell morphology	Rods and clubs or branched rods  Bifidobacterium	Cocci and rods  Lactococcus  Lactobacillus
Habitat	Intestines of human and animals	In nature, milk and dairy products, human and animals' intestines, fermented foods such as vegetables
Oxygen sensitive	Unable to live under oxygen (strict anaerobic)	Unable to live under oxygen (facultative anaerobic)
Main metabolites	Lactic acid and **acetic acid**	Lactic acid

1.3. Physiological properties of Bifidobacteria.

Bifidobacterium are anaerobic, Gram-positive irregular rods. The genus of the Bifidobacteria non-motile, non-spore forming and catalase negative.

Growth conditions of optimal temperature range between 37-41°C [5]. Optimum pH for growth for Bifidobacteria is ranges from 6,5-7,0. Generally observed at pH values under 4,0 and over 8,5 no growth. *Bifidobacterium* spp key enzyme is fructose-6-phosphate phosphohexoses. This enzyme split the hexose phosphate to

erythrose-4-phosphate and acetyl phosphate [2]. Lactic acid and acetic acid in a theoretical final ratio of 1.0:1.5 [5].

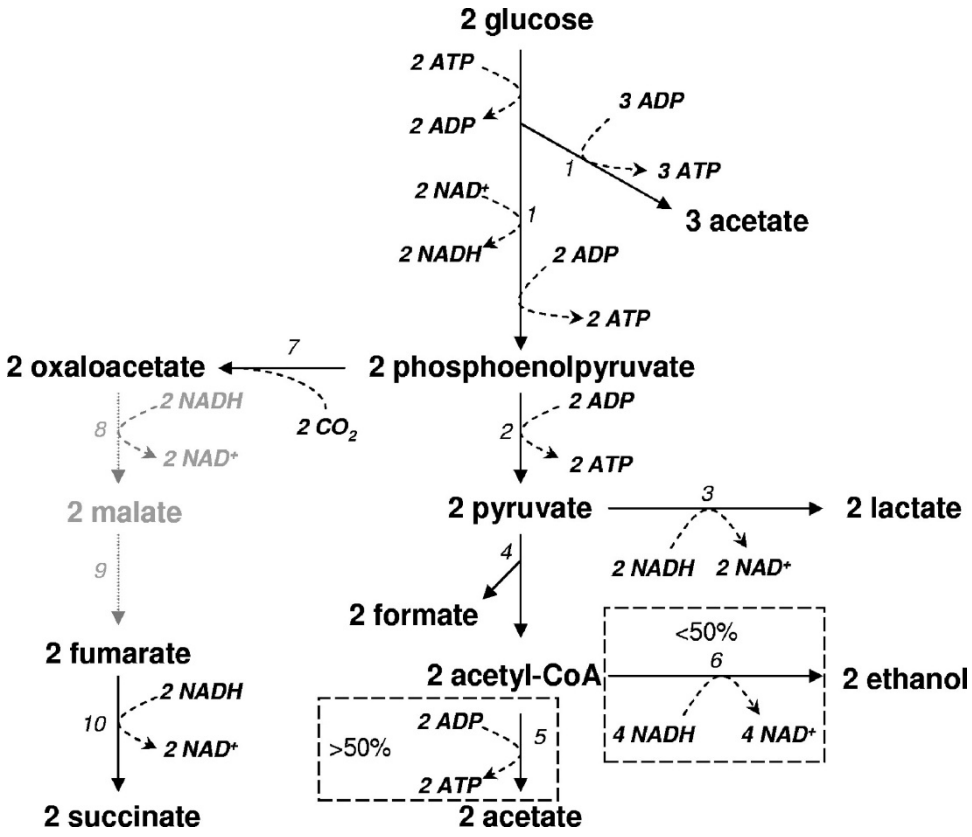


Figure 1. Sugar metabolism on bifidobacteria.[20]

1.4. The effect of the Antibacterial activity by related organic acid production and antibacterial peptide of the Bifidobacteria

Antibacterial factors of bifidobacteria depend on organic acids, and bacteriocin). The researchers found one part of the inhibitory activity that low pH

inhibits the growth of pathogenic bacteria due to organic acids caused by bifidobacteria [21]. Mani-López et al 2011, reported According to Diffusion principle, organic acids that penetrate cell cytoplasm suggest that the acidity of the bacterial cell is obstructed by acidification of pH in the cell's environment. Figure 2 is a general mechanism of organic acids. Both theories do not fully describe the regimen of organic acids. Because there are some concentrations of anions that are dissipated by any organic acids, the negative impacts cause unabated acid over the membrane that creates other adverse effects after the distribution.

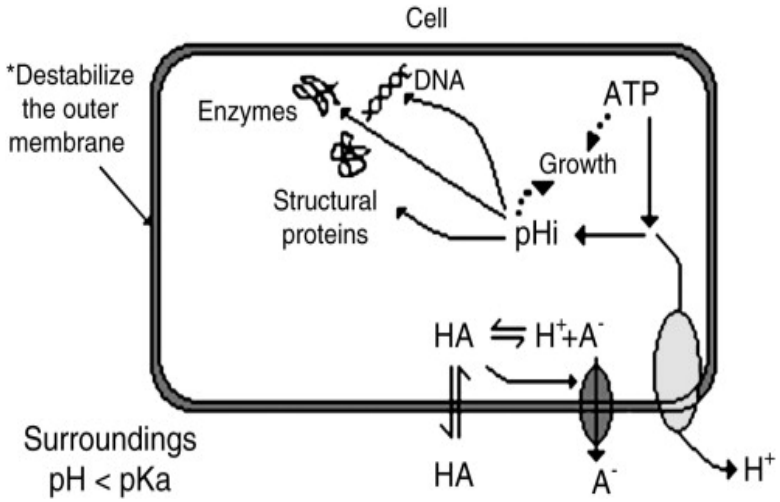


Figure 2. Function mechanism of organic acids in cytoplasm of bacterial cells.[22]

1.5. Antibacterial peptide (bacteriocin)

Among microorganisms, there are substances which are protein or peptide, and which mainly produce antibacterial substances acting bactericidally on

producer bacteria and related bacteria, and these substances are called bacteriocins. [23]

The bacteriocin that was first discovered was colicin produced by E. coli, but it became clear later that it was made by various kinds of bacteria. Among them, bacteriocin produced by lactic acid bacteria, Also, it does not cause growth inhibition like antibiotics. The most typical lactobacillus bacteriocin is nisin produced by Lactococcus lactis subsp. Lactis, a cheese starter bacterium. [23]

Bacteriocins are synthesized on ribosomal. [24] In the case of bifidobacteria, 5 strains have been shown to produce antibacterial peptides: *B. bifidum* NCFB1454[15] and *B. bifidum* NCDC 1452 [25] produces bifidocin B, and Bifidin respectively. *B. thermophilum RBL67* produces Thermophilicin B 76 [26] . *B.infantis* and *B.lactics* Bb-12 produces Bifidin I BCRC 14602 [17] and Bifilact Bb-12, in additional *B.longum*, *B.longum* Bb-46 and *B.longum* DJO10A produces Bifilong [26], Bifilact Bb-46 [24] and Lantibiotic (Bisin) respectively. “lantibiotic,” while genome analyses of *B.longum* subsp. *Logum* DJ010A revealed a complete lantibiotic gene cluster.

Bifidin isolated from *B.bifidum* is synthesized bacteriocin that was against Gram-negative and Gram positive bacteria [25]. growth, such as bacteriocin Bifidocin B, *Bacillus cereus, Enterococcus faecalis, Listeria monocytogenes, Pediococcus acidolactici, Streptococcus faecalis,* isolates from *Bifidobacterium bifidum.*

Table 2. Bacteriocins from *Bifidobacterium spp*. and their main characteristics.[26]

Bacteriocin	Species and strain	Mol. wt. (kDa)	Heat range stability	pH range stability	Production phase	Optimal production	Inhibitory spectrum	Reference
Bifidin	*B. bifidum* NCDC 1452	(–)	(100 °C—30 min)	4.8–5.5	After 48 h	pH: 4.8	Gram-positive and Gram-negative bacteria	Anand et al. (1984, 1985)
Bifidocin B	*B.bifidum*	3.3	(121 °C—15 min)	2-12	(12–18 h)	37 °C, pH 5.0–6.0	*Bacillus cereus, Enterococcus faecalis, Listeria monocytogenes*, *Pediococcus acidolactici, Streptococcus faecalis,* etc.	Yildirim and Johnson (1998);Yildirim et al. (1999)
Bifilong	*B.longum*	120	(100 °C—30 min)	2.5–5.0	(−)	(−)	Gram-positive and Gram-negative Bacteria	Kang et al. (1989)
Bifilact Bb-46	B.longum Bb-46	25-127	(121 °C—15 min)	4–7	(−)	(−)	*Staphylococcus aureus, Salmonella typhimurium, Bacillus cereus, E. coli*	Saleh and El-Sayed (2004)
Bifilact Bb-12	*B. lactis* Bb-12	25–89	Unstable for high temperatures	4–7	(−)	(−)	*Staphylococcus aureus, Salmonella typhimurium, Bacillus cereus, E. coli*	Saleh and El-Sayed (2004)
Thermophilicin B67	*B. thermophilum* RBL67	5–6	(100 °C—5 min)	2–10	24 h	pH 6 and 40 °C	*Listeria sp., Lactobacillus acidophilus*	von Ah (2006)
Bifidin I BCRC 14602	B. infantis	3	(121 °C—15 min)	4–10	18 h	(−)	*LAB strains, Staphylococcus, Bacillus, Streptococcus, Salmonella, Shigella, E. coli.*	Cheikhyoussef et al. (2009a, 2010)
Lantibiotic (Bisin)	*B.longum* DJO10A	(−)	(−)	(−)	1-8 h	Auto-induction	*Streptococcus thermophilus* ST403, *Clostridium perfringens, Staphylococcus epidermidis, Bacillus subtilis, Serratia marcescens, E. coli* DH5*a.*	Lee et al. (2001)

1.6. Bacteriocin classification and structure

Many of the bacteriocins produced by lactic acid bacteria are primary metabolites produced in conjunction with the growth of bacterial cells. Genes related to the biosynthesis, secretion, etc. of bacteriocin are encoded on chromosome or plasmid DNA. [27] As with ordinary proteins, these bacteriocins are synthesized as precursor peptides via translation on ribosomes. [24] It then undergoes dehydration of specific amino acids, intramolecular monosulfide bonds, leader peptides (processing), etc., and is secreted extracellularly as a mature bacteriocin.

Lactobacillus bacteriocins are classified into the following four classes from the viewpoint of their primary structure, molecular weight, thermal stability, antibacterial spectrum, and the like. [23]

Nisin belonging to Class I has been studied the most and its structure has been elucidated. [28] Nisin is a hydrophobic peptide composed of 34 amino acid residues and having a molecular weight of 3510 Da, particularly hydrophobicity is high on the N-terminal side, and relatively hydrophilic on the C-terminal side. [27] Nisin has an extremely characteristic structure consisting of an intramolecular bridge consisting of five thioester bonds and three dehydroamino acid residues. Intramolecular crosslinking contributes to the stabilization of the three-dimensional structure of nisin and contributes to the development of high heat resistance in

addition to being hydrophobic. The growth inhibition of microorganisms by nisin is bactericidal. Binding to the anionic lipid on the surface of the microbial cells, the proton driving force, that is, the membrane potential of the cell or the concentration gradient of hydrogen ion, causes the positively charged C-terminal region of nisin to be inserted into the membrane together with the anionic lipid, and a wedge-shaped hole is formed. It is said that intracellular ions, amino acids and ATP leak through the pores, resulting in collapse of the proton driving force, resulting in cell death. [27]

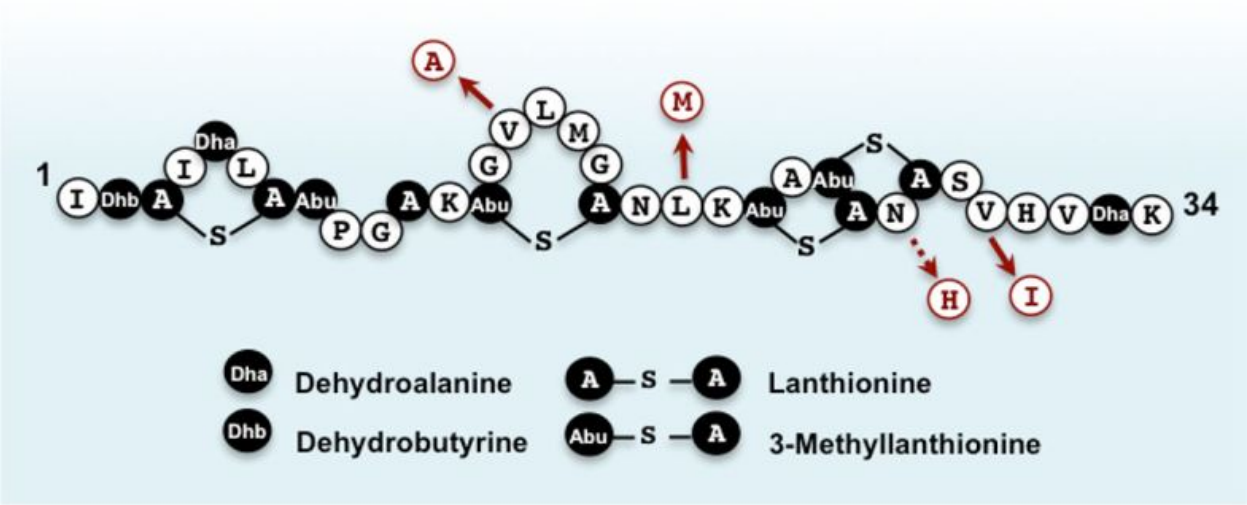


Figure 3. Structures of nisin [27]

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Chapter II

Antibacterial activity of Bifidobacteria isolated from infant faeces

2.1. Abstract

Antibacterial activity of Bifidobacteria isolated from Mongolian infant feces was elucidated on pathogenic intestinal bacteria for the development of a new antibacterial Bifidobacteria by permission of the Mongolian Medical Ethics Committee Approval (MMECA). Forty-nine single colonies were totally obtained from 3 samples by using an enrichment BL agar medium. Among them, 29 isolates had Gram−positive, catalase−negative properties, and maul−like or Y−shaped morphology, and then, 20 *Bifidobacterium breve* and 9 *Bifidobacterium longum* strains were detected by the *B. breve* and *B. longum* specific primers. Organic acids produced by the isolated Bifidobacteria in their cell free supernatants were quantitatively analyzed by a spectrophotometric absorbance at 340 nm, suggesting that D−lactic, L−lactic, and acetic acids were produced, and the pH of the supernatants was at 3.86−4.55. The isolated Bifidobacteria showed antibacterial

activity toward *Escherichia coli* and *Salmonella typhimurium* as high as that of standard Bifidobacteria, however, lower activity against *Staphylococcus aureus*. The antibacterial activity was probably due to the production of the organic acids.

2.2. Introduction

Bifidobacteria with Gram−positive and catalase−negative properties are known to have a potent antibacterial activity due to the production of organic acids such as lactic and acetic acids [1–6]. Bifidobacteria are found in the intestinal tract of human and animals [7,8] and work as probiotics for promoting health [9]. Although the antibacterial activity of Bifidobacteria is usually expressed by the produced organic acids, peptides called bacteriocin are also involved [4, 10–15].

Several papers appeared on the antibacterial activity of Bifidobacteria by the production of organic acids. Makras and Vuyst were found a strong antibacterial activity of Bifidobacteria towards Gram−negative bacteria, *Salmonella* and *E. coli* strains, by the production of lactic and acetic acids and reported the inhibition of growth of Gram−positive bacteria by the production of a bacteriocin [16]. The agar plate spot test of Bifidobacteria on several pathogenic bacteria showed the antibacterial activity and one of the main inhibitory reasons was reported by the production of organic acids [5]. Georgieva described on comparison of acidic and

neutralized cell−free supernatants cultured by Bifidobacteria, indicating that the acidic supernatant was active to several pathogenic bacteria and the neutralized supernatant also showed the antibacterial activity. Briefly, the activity against Gram-positive pathogens is mostly due to the bactericidal effect of protease sensitive bacteriocins, while the antagonistic effects towards Gram-negative pathogens could be related to the production of organic acids and hydrogen peroxide[17]. Biedrzycka reported that lactic and acetic acids were main products of Bifidobacteria fermentation with sugars. The highest amount of lactic and acetic acids was obtained from the fermentation on lactose determined by a gas chromatography analysis [18]. As mentioned above, strong antibacterial activity of Bifidobacteria was mainly caused by the production of organic acids. Recently, we showed the antibacterial activity of lactic acid bacteria (LAB), *L. hilgardii* and *L. diolivorans*, which were isolated from a traditional fermented mare's milk, airag, in Mongolia and identified by the 16S rDNA analysis [19]. Although the antibacterial activity of LAB was due to the production of organic acids, antibacterial peptides were also contained in the fermented supernatant.

In this work, we isolated and identified several Bifidobacteria from Mongolian infant feces with 0−6-month ages by the specific primers for *B. breve* and *B. longum* to select potent antibacterial strains. The antibacterial activity was

evaluated by the ability of lactic and acetic acid productions and by the inhibition of multiplication on pathogenic strains, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*. We also, determined the ability of organic acids production by carbohydrate fermentation of the isolated Bifidobacteria.

2.3. Methods and materials

2.3.1. Materials: Gifu anaerobic medium (GAM), blood liver medium (BLM), De Man Rogasa and Shape (MRS) broth, and trypticase soy broth (TSB) were purchased from Nippon Suisan Kaisha, Ltd (Tokyo, Japan) and Becton, Dickinson and Company (Erembadgem, Belgium), respectively. Anaeropack and QIAamp DNA mini kit were obtained from Mitsubishi Gas Chemical Co. Ltd., (Tokyo, Japan) and QIAGENN. V. (Hilden, Germany), respectively. Standard Bifidobacteria, *Bifidobacterium adolescents*, *Bifidobacterium longum*, and *Bifidobacterium breve*, were available from Japan Collection of Microorganism (Tsukuba, Japan). Pathogenic strain, *Escherichia coli* 1099, *Staphylococcus aureus* 1045, and *Salmonella typhimurium* 1098, was used the stock strains in Mongolia Institute of Veterinary Medicine. Enzymatic Bioanalysis / Food Analysis UV

method kit (EBFA kit) was purchased from R−biopharmAG (Darmstadt, Germany).

2.3.2. Isolation of Bifidobacteria: Three feces samples from 0−6-month aged Mongolian infants were collected by permission of the Mongolian Medical Ethics Committee. Bifidobacteria were isolated from the feces samples according to the direct plating method. Each feces sample (about 1 g) was collected in a sterile sampling tube (5 ml) and then kept at 4°C until use. The feces (0.1 g) were diluted by a trypticase soy broth (TSB) (10 mL) and then the mixture was well stirred. The sample (0.1 mL) was spread on an agar prepared with a blood liver medium (BLM) which is a medium for Bifidobacteria incubation. After the agar plate was incubated for 3 days at 37°C under anaerobic condition, several colonies appeared.

Each colony on the agar plate was recultured on a new GAM agar plate and then the new plate was anaerobically cultured for 2 days at 37°C. This procedure was repeated 3 times to give pure single colonies. The obtained single colony was examined by a Gram−staining, catalase activity, and microscope observation to obtain 29 Bifidobacteria with Gram−positive, catalase−negative characters, and maul−like or Y−shaped morphological structure.

2.3.3. DNA extraction and identification of Bifidobacteria: The GAM broth culture of the single colony as described above (1 mL) was fractionated into a micro centrifuge tube (1.5 mL) and then the tube was centrifuged for 5 min at 5000G. Bifidobacterium precipitated was collected and then DNA was extracted by using a QIAamp DNA mini kit.

Identification of the isolated Bifidobacterium was performed by a PCR using 16S rDNA analysis. Typical procedure for the PCR using specific primers, BiBRE−1 (5'−CCGGATGCTCCATCACAC−3') and BiBRE−2 (5'−ACAAAGTGCCTTGCTCCCT−3'), for *B. breve* [20] was as follows. In a PCR tube, the primer solution (0.1µL each), DNTP 1 µL, 10×Taq EX 1 µL, Takara EX Tag (0.05 µL), water (6.55µL), and template DNA 1 µL (>100 ng) was added, and the PCR tube was placed in a thermal cycler. The following was programed for the amplification of 16S rDNA. The tube was heated for 5 min at 94°Cas an initial denaturation step and then 35 cycles for 30 sec at 94°C for denaturation, 20 sec at 50°C for annealing, and 0.5 min at 72°C for elongation, respectively. Lastly, the tube was kept for 5 min at 72°C for the final extension step. The amplification product was confirmed by a Mupid electrophoresis on 1.2% agarose gel, which was then stained by ethidium bromide solution. *B. longum* strain was detected by the specific primers, BiLON−1 (5'−TTCCAGTTGATCGCATGGTC−3') and

BiLON−2 (5'−GGGAAGCCGTATCTCTACGA−3') [20], by the same procedure as above.

2.3.4. Quantitative analysis of organic acids in Bifidobacteria cultured solution: A typical procedure for the measurement of organic acid concentration in the incubation supernatants as follows. The mixture of INFbre 1 (*B. breve*) (no. 1 in Table 1) (20×10^9 cell/mL) in GAM (10 mL) broth containing 2% of glucose supplement was anaerobically incubated for 3 days at 37°C. The pH of the mixture was 3.86. The concentration of lactic and acetic acids produced in the supernatant was quantitatively determined by an EBFA−kit using a spectrophotometer measured at 340 nm according to the maker provided protocol to give 0.06 g/L of D−lactic acid, 6.02 g/L of L−lactic acid, and 6.74 g/L of acetic acid, respectively [21].

2.3.5. Carbohydrate fermentation test: A typical procedure for carbohydrate fermentation was as follows. INFbre 1 (20×10^9 cell/mL) in GAM broth (4 mL) was fermented in a 10 mL of glass tube for 24 h at 37°C to give fermented products. After centrifugation, the cell free supernatant was discarded, and then PBS broth (0.9%, 3mL) was added to the tube. The mixture was suspended by using a vortex shaker and then the small amount of the mixture (0.02 mL) was inoculated to a 10 ml of glass tube containing glucose (0.5 g) and Bromocresol purple in GAM broth

(4 mL) without dextrose. After incubation for 48 h at 37°C, the color of the mixture was changed from yellow to violet. The degree of color changing was measured by a spectrophotometer at 340 nm. The fermentation ability was compared to that of a standard Bifidobacteria *B. breve* JCM01192 and showed (+) for fermentation, (-) for no fermentation, and (w) for weakly fermentation, respectively.

2.3.6. Antibacterial activity test: A typical procedure for the antibacterial activity on *Escherichia coli* 1099 was as follows. INFbre 1 (20×10^9 cell/mL in 2μL of MRS broth) was inoculated on an MRS agar plate and then incubated for 24 h at 37°C to appear a colony at the spotted point. *E. coli* (1×10^{12} cell/mL, 20 μL) with 10 ml of TSB broth containing 0.8 % agar was overlaid at 45°C on the INFbre 1 spotted agar plate, and then the plate was aerobically incubated at 37°C. The diameter of a clear zone around the spot was measured after 24 h, 48 h, and 72 h, respectively, to show the antibacterial activity of the isolated Bifidobacterium. Commercially available *Staphylococcus aureus* 1045 and *Salmonella typhimurium* 1098, respectively, were also used as control bacteria for the antibacterial activity test and the results are shown in Table 3 and Figure 1.

2.4. Results and discussion

2.4.1. Isolation of *B. breve* and *B. longum* from infant feces: From 3 Mongolian infant feces, 49 single colonies were obtained on the agar plate medium. Among them, 20 colonies showed Gram−negative and rod−like shapes, indicating that these strains were not Bifidobacteria. Remained 29 colonies had Gram−positive and catalase−negative properties and showed the cell morphology like maul or Y shaped short rod by a microscope observation, suggesting that these 29 colonies were Bifidobacteria. These 29 single colonies were detected by using the most common Bifidobacteria specific primers of 16S rDNA, BiBRE−1 and BiBRE−2 for *B. breve* and BiLON−1 and BiLON−2 for *B.longum*, indicating that 20 *B. breve* and 9 *B. longum* strains were obtained. These two kind Bifidobacteria are known to have potent antibacterial activity. Therefore, several Bifidobacteria that produced a large amount of organic acids were selected from the 29 Bifidobacteria strains.

Table 3 shows the results of organic acid production in the supernatant by the 29 Bifidobacteria

Table 3. Production of organic acids by isolated Bifidobacteria

Name of isolate	pH[b]	Organic acid[c]			Strain[d]
		D−Lactic acid g/L	L−Lactic acid g/L	Acetic acid g/L	
INFbre1	3.86	0.06	6.02	6.74	*B. breve*
INFbre2	3.89	0.28	6.65	6.80	
INFbre3	3.90	0.32	6.49	6.86	
INFbre4	3.90	0.12	6.04	6.34	
INFbre5	3.91	0.32	5.20	6.25	
INFbre 6	3.93	0.32	5.33	5.82	
INFbre7	3.95	0.28	5.10	5.97	
INFbre8	3.97	0.32	5.17	6.28	
INFbre9	4.00	0.32	5.55	5.91	
INFbre 10	4.00	0.32	6.01	5.51	
INFbre 11	4.00	0.28	15.67	6.59	
INFbre 12	4.00	0.25	4.88	4.31	
INFbre 13	4.05	0.28	5.78	5.54	
INFbre 14	4.06	2.88	5.04	4.92	
INFbre 15	4.06	2.72	1.51	4.43	
INFbre 16	4.07	4.87	4.62	4.80	
INFbre 17	4.08	0.41	5.04	3.88	
INFbre 18	4.08	0.28	5.22	4.06	
INFbre 19	4.08	0.22	5.52	4.09	
INFbre 20	4.24	0.25	15.63	0.30	
INFlon 21	4.03	0.35	5.10	2. 64	*B.longum*
INFlon 22	4.04	0.32	6.94	6.59	
INFlon 23	4.05	0.28	5.84	5.32	
INFlon 24	4.06	0.28	4.46	4.43	
INFlon 25	4.06	0.25	5.59	4.49	
INFlon 26	4.07	0.35	5.10	5.60	
INFlon 27	4.09	0.32	3.84	2.95	
INFlon 28	4.17	0.22	5.04	5.14	
INFlon 29	4.55	0.35	5.26	0.70	

[a]Incubation of Bifidobacteria (20×10^9 cell/mL) was performed in 2% glucose containing GAM broth (100 mL) for 72 h at 37°C.
[b]pH was determined after 3 days in liquid medium by using pH meter
[c]the concentration of organic acids in the cell free supernatant were determined by a commercially available F-kit and an UV absorbance at 340 nm for D-lactic, L-lactic, and acetic acids, respectively.
[d]Bifidobacteria were identified by a PCR using Bifidobacteria specific primers.

(20×10^9 cell/mL) after incubation in GAM broth (4 mL) with 2% glucose for 72 h at 37°C. After incubation, the pH of the cell free supernatant decreased at 3.86−4.55, respectively, and the *B. breve* supernatants had relatively lower pH values than those of *B. longum*. Among the organic acids produced by the Bifidobacteria, D−lactic, L−lactic, and acetic acids were mainly produced by the metabolites of glucose. The organic acids were quantitatively analyzed by using an EBFA−kit, which gave the concentration of these organic acids measured by the absorbance at 340 nm. The production of organic acids was a good evidence for the antibacterial activity of Bifidobacteria toward Gram−negative pathogenic bacteria [18] D−Lactic acid was produced in lower proportion in the supernatant than that of L−lactic acid.

2.4.2. Carbohydrate fermentation ability: Carbohydrate fermentation ability of Bifidobacteria was examined by using several carbohydrates. The results are demonstrated in Table 4. Lactic and acetic

Table 4. Fermentation of isolated Bifidobacteria with carbohydrate[a]

Carbohydrate	Isolated *B. breve* strain									*B.longum*	
	1	2	3	4	6	8	11	12	13	22	24
Pentose											
Arabinose	-	-	-	-	-	-	-	-	-	+	+
Xylose	-	-	-	-	-	-	-	-	-	-	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Sorbose	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	+
Hexose											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+
Disaccharide											
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	w	w
Lactose	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	w	w
Melibiose	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	-
Trisaccharide											
Melicitose	-	-	-	-	-	-	-	-	-	+	+
Polysaccharide											
Starch	+	+	+	+	+	+	+	+	+	-	-
Alditol											
Mannitol	+	+	+	w	+	+	w	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-
Glycoside											
Esculin	+	+	+	-	w	w	+	+	-	-	-
Salicin	+	+	+	w	+	+	+	+	+	w	+
Amygdalin	w	+	+	+	w	w	+	+	-	-	-

[a] Fermentation ability of Bifidobacteria was evaluated by three levels, (+) Positive, (w) weakly positive, and (-) negative.

acids were obtained by the results of the metabolism. The fermentation ability was evaluated by three levels, (+) positive, (w) weakly positive, and (-) negative, and measured by the color changes of the fermented supernatant due to the concentration of organic acids determined by the absorbance at 340 nm. Bifidobacteria with relatively higher ability of acetic acid production were selected and Bifidobacteria with lower production of acetic acid, 4.31 g/L (INFBre 12) and 4.43 g/L (INFLon 24), were also selected for the comparison of the fermentation ability. Although pentoses expect ribose was not fermented by the isolated *B.breve,* arabinose was fermented by *B.longum* to give organic acids. Hexoses, disaccharides, and alditols expect inositol were fermented by all tested strains belonging to both species. A polysaccharide starch and glycosides were fermented by the *B. breve* strains, but not or lowly fermented by the *B. longum* strains. The fermentation ability of both Bifidobacteria on rhamnose, sorbose and inositol were low. Melicitose fermented only by *B.longum*. These results indicate that the isolated Bifidobacteria fermented mainly hexoses and disaccharides to produce organic acids. The INFbre 2, 3, and 12 strains were found to have strong fermentation ability. On the other hand, *B. longum* strains had relatively weak fermentation ability. Xylose was only fermented by the INFlon 24 *B. longum* strain.

2.4.3. Antibacterial activity toward *E. coli*, *S. aureus*, and S. typhimurium: Antibacterial activity of the isolated Bifidobacteria toward the pathogenic strains, *E. coli*, *S. aureus*, and *S. typhimurium*, was performed by an agar spot test. Figure 4 shows the results of the agar

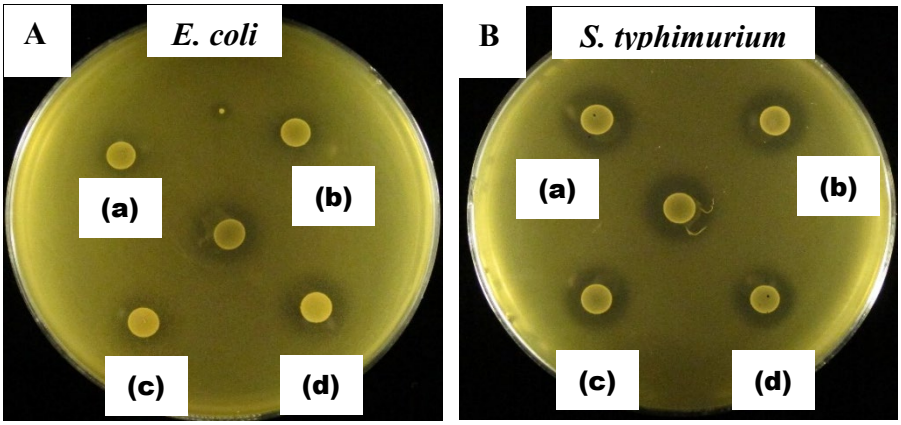

Figure 4. Antibacterial activity of isolated Bifidobacteria toward Gram−negative pathogenic bacteria (A) *E. coli* and (B) *S. typhimurium*. (a) INFbre 11, (b) INFlon 22, (c) *B. breve* 01192, and (d) *B. longum*01217. The pathogenic bacteria were overlaid on the isolated Bifidobacteria agar plate, respectively, to appear clear zones.

spot test of INFbre 11 (*B. Breve*) and INFlon 22 (*B. longum*) toward *E. coli*, and *S. typhimurium*, in which an inhibition (clear) zone was compared with that of the standard Bifidobacteria, *B. Breve* 01192 and *B. longum* 01217 strains. INFbre 11 and INFlon 22 Bifidobacteria were spotted on the agar plate, respectively, and then

pathogenic strains were overlaid on the Bifidobacteria plates. After incubation for 72 h at 37°C, the clear zone without *E. coli* and *S. typhimurium* appeared, indicating that the Bifidobacteria inhibited the multiplication of the pathogenic bacteria. The diameter of the clear zone was measured and compared with that of the standard Bifidobacteria, indicating that both isolated Bifidobacteria, INFbre 11 and INFlon 22, were found to have potent antibacterial activity on *E. coli* and *S. typhimurium* as high as that of the standard Bifidobacteria. The diameter was more than 10 mm and 17 mm for *E. coli* and *S. typhimurium*, respectively. As

Table 5. **Antibacterial activity of isolated Bifidobacteria on pathogenic bacteria**[a]

Bifidobacteria	Pathogen								
	E. coli			*S.aureus*			*S.typhimurium*		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
INFbre 11 (*B.breve*)	±	±	±	±	±	±	++	++	++
INFlon 22 (*B.longum*)	+	+	+	-	-	-	+++	+++	+++
B.breve 01192	±	±	±	±	±	±	++	++	++
B.longum 01217	+	+	+	-	-	-	+++	+++	+++

[a] Antibacterial activity was carried out by an agar spot test [4].
The evaluation was as follows. (-): no inhibition, (±): below 10 mm of the inhibited zone with unclear halo, (+): below 10 mm of the inhibition zone with clear halo, (++): below 17 mm of the inhibition zone with clear halo, and (+++): more than 17 mm of the inhibition zone with clear halo.

shown in Table 3, the isolated *B. longum* strain INFlon 22 was found to show potent antibacterial activity toward *E. coli* and *S. typhimurium*, and the *B. breve*

INFbre 11 strain showed medium or higher antibacterial activity. However, both isolated Bifidobacteria did not inhibit the multiplication of *S. aureus*, because the clear zone did not appear. The antibacterial activity of the isolated Bifidobacteria in this work was attributed to the production of organic acids such as lactic and acetic acids. Other isolated Bifidobacteria in Table 1 also showed antibacterial activity on the pathogenic strains.

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Chapter III

Potent antibacterial activity of peptides produced by Bifidobacteria in Mongolian young livestock

3.1. Abstract

We aimed high antibacterial potential probiotic found for the development of a new antibacterial peptide. Thirty-six single colonies were totally obtained 15 samples of the faeces and rumen cud collected from Mongolian young livestock animals of around the three provinces (Tuv, Khovd, Khuvsgul). The 5-type species *Bifidobacterium* identified *B.bifidum B.catenulatum*, *B.ruminantum*, *B.longum*, *B.pseudocatenulatum* and 8 type species lactic acid bacteria *Enterococcus alcedinis*, *Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae*, *Enterococcus lactis*, *Enterococcus mundti*, *Lactobacillus reuteri*, and *Lactobacillus plantarum* by 16S rDNA sequencing analysis.

Isolated from the lamb rumen *B.bifidum* (BBLA72) was produced 29.6 mmol per liters acetic acid and 19.3 mmol per liters of lactic acid, thereby releasing pH from 7.0 to 4.57. However, organic acids were produced small in

comparison with other strains, the antibacterial activity of the pH neutralized up to 7.0 cell-free supernatant was more significant than other strains.

BBLA 72 starts with early stationary phase and its antibacterial activity is the highest in 18 hours of bacterial culture. In the pH stabilization, bacterial resistance to 1-10 was active and 5, 6, and 7 did not significantly reduce the antibacterial activity.

The arbitrary unit was measured 25600 AU/ml against to *E.coli*. It is preliminary for this substance as a bacteriocin-like substance that weighs 2 kDa and 3 kDa, capable of withstanding a 100-degree heat to 30 minutes, proteinase K and trypsin enzyme.

3.2. Introduction

Bifidobacteria with Gram-positive and catalase−negative properties (Orla-Jensen., 1924[25], Scardovi et al., 1974[30]) are known to have a potent antibacterial activity due to the production of organic acids (Yusof et al., 2000[42], Asahara et al., 2001)[1]. Bifidobacteria are found from the intestinal tract of mammalian (Baivati et al, 2000[7], Matsuki et al, 2004)[22] and work as probiotics for promoting health (Mitsuoka 1990)[23]. Although the antibacterial activity of Bifidobacteria is usually expressed by the produced organic acids (Lefteris et al., 2006[20], Vlková et al.,

2009[38] and Tejero-Sarifiena et al., 2012[34]), peptides called bacteriocin are also participated (Daw et al., 1996[15], Yildrim et al., 1999[43], Lee et al., 2011[19], Toure et al., 2003[36] and Chiyekiyosef et al., 2009[10]).

Several papers appeared on the antibacterial activity of Bifidobacteria by the production of organic acids. Makras et al, 2006 were found a strong antibacterial activity of Bifidobacteria towards Gram-negative bacteria, *Salmonella* and *E. coli* strains, by the production of lactic and acetic acids and reported the inhibition of growth of Gram-positive bacteria by the production of a bacteriocin[20]. The agar spot test of Bifidobacteria on several pathogenic bacteria showed the antibacterial activity and one of the main inhibitory reasons was reported by the production of organic acids (Tejero-Sarifiena et al., 2012[34] and Bayar et al., 2018[5]). Georgieva et al., 2015 described on a comparison of acidic and neutralized cell-free supernatants cultured by Bifidobacteria, indicating that the acidic supernatant was active to several pathogenic bacteria and the neutralized supernatant also showed the antibacterial activity[17]. Biedrzycka et al., 2003 reported that acetate and lactate were the main products of Bifidobacteria fermentation with sugars[8]. The highest amount of lactate and acetate was obtained from the fermentation on lactose determined by a gas chromatography analysis.

Yildirim et al., 1999, reported the amino acid sequence of bifidocin B and characterization which is isolated from *B.bifidum* NCFSB 1454. Properties of Bifdocin B is studied which was sensitive for some enzymes but resistant to pH 2-10 and inhibited the growth of the food-borne, food-spoilage bacteria such as *Bacillus*, *Enterococcus*, *Lactobacillus*, *Listeria* and *Pediococcus*[43].

Touré et al., 2003, obtained antilisterial activity Bifidobacteria isolated from infants faeces. The purified peptide properties was hydrophilic, the limit of the heat resistance was 100 °C for 5 min and sensitive to protease[36].

Ueli von Ah., 2006, Isolated from baby faeces *Bifidobacterium thermophilum* RBL67 produced Thermophilicin B67. Its formed diameter of 13 mm, 11 mm, and 10 mm inhibited zone to Lactobacillus *acidophilus*, *listeria ivanovi* and *listeria innocua* respectively[37].

Cheikhyoussef et al., 2010 partial sequenced of Bifidin I isolated from *Bifidobacterium infantis*. Antibacterial active was after growing 30 min about 99% inhibited of cell-free supernatant per ml of all indicator strains. Bifidin I was purified by using a three-step purification procedure. Antibacterial activity of Bifidin I was after dialysis 52000 AU/ml [10].

Cheikhyoussef et al., 2009, another hand, Bifidin I also, has a potential antibacterial activity for Gram-positive bacteria such as *Staphylococcus*,

Streptococcus and *Clostridum*, and Gram-negative bacteria such as *Salmonella*, *Shigella* and *E. coli* [9]. As mentioned above, a strong antibacterial activity of Bifidobacteria was mainly caused by the production of organic acids. Recently, we showed the antibacterial activity of lactic acid bacteria (LAB), *L. hilgardii* and *L. diolivorans*, which were isolated from a traditional fermented mare's milk, airag, in Mongolia and identified by the 16S rDNA analysis[26]. Although the antibacterial activity of LAB was due to the production of organic acids, antibacterial peptides were also contained in the fermented supernatant.

In this work, we isolated and identified several Bifidobacteria from Mongolian livestock's young animals by the universal primers for *B.catenulatum*, *B.longum*, *B.pseudocatenulatum*, *B.ruminantum* to select potent antibacterial strains. The antibacterial activity was evaluated by the ability of lactic and acetic acid productions and pH by the inhibition of multiplication on pathogenic strains, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*. Also, we found antibacterial peptide producing form of *Bifidobacterium pseudocatenulatum,* antibacterial activity AU/ml determined in enzyme treatment, heat treatment, pH 1-10 resistance potential by AU/ml respectively Molecule mass of antibacterial peptide was determined by SDS-page result.

3.3. Methods and materials

3.3.1. Materials: Gifu anaerobic medium (GAM), blood liver medium (BLM), De Man Rogasa and Shape (MRS) broth, and trypticase soy broth (TSB) were purchased from Nippon Suisan Kaisha, Ltd (Tokyo, Japan) and Becton, Dickinson and Company (Erembadgem, Belgium), respectively. Anaeropack and QIAamp DNA mini kit were obtained from Mitsubishi Gas Chemical Co. Ltd., (Tokyo, Japan) and QIAGEN. V. (Hilden, Germany), respectively. Reference strains used in this study and their origins are listed (no. 6 in Table). Enzymatic Bioanalysis / Food Analysis UV method kit (EBFA kit) was purchased from R−biopharma (Darmstadt, Germany).

Table 6. **Bacterial strains used in this study**

Bacterial species	Strain
Probiotic strain	
Bifidobacterium longum	JCM-1217[a]
Pathogenic strain	
Bacillus subtilis	NBRC 13722[b]
Escherichia coli	ATCC 25922[C]
Escherichia coli	MIVM 10977[d]
Salmonella typhimurium	MIVM SN8
Staphylococcus aureus	MIVM 5695

[a]JCM (Japanese collection of microorganism)
[b]NBRC (Nite Biological Resource Center)
[c]ATCC (American Type Culture Collection)
[d]MIVM (Mongolian Institute of Veterinary Medicine)

3.3.2. Bacterial strain and culture condition

Isolation of Bifidobacteria: Twenty-two rumen cud and faeces samples from Mongolian livestock's young animals were collected. Bifidobacteria were isolated from the faeces samples according to the direct plating method. Each faeces sample (about 1 g) was collected in a sterile sampling tube (5 ml) and then kept at 4°C until use. The faeces (0.1 g) were diluted by a trypticase soy broth (TSB) (10 mL) and then the mixture was well stirred. The sample (0.1 mL) was spread on an agar prepared with a blood liver medium (BLM) which is a medium for Bifidobacteria incubation. After the agar plate was incubated for 3 days at 37°C under anaerobic condition several colonies appeared.

Each colony on the agar plate was recultured on a new GAM agar plate and then the new plate was anaerobically cultured for 2 days at 37°C (Watanabe et al., 2009). This procedure was repeated 3 times to give pure single colonies. The obtained single colony was examined by a Gram−staining, catalase activity, and microscope observation to obtain 11 Bifidobacteria with Gram-positive, catalase−negative characters, and maul−like or Y−shaped morphological structure.

Pathogenic strains were cultured in TSB or LB at 37°C for 24 hours. All strains were kept with 20% glycerol stock at -80°C.

3.3.3. DNA extraction and identification of Bifidobacteria: The GAM broth culture of the single colony as described above (1 mL) was fractionated into a microcentrifuge tube (1.5 mL) and then the tube was centrifuged for 5 min at 5000G. Bifidobacterium precipitated was collected and then DNA extracted by using QIAamp DNA mini kit.

Identification of the isolated *Bifidobacterium* was performed by a PCR using 16S rDNA analysis. Typical procedure for the PCR using universal and genus primers 9F (5'GAGTTTGATCCTGGCTCAG-3'), g-Bifid-F (5'CTCCTGGAAACGGGTGG-3') and 800R (5'-TACCAGGGTATCTAATCC-3'), g-Bifid-R (5'GGTGTTCTTCCCGATATCTACA-3') for *B.pseudocatenulatum* was as follows, respectively. In a PCR tube, the primer solution (0.1μL each), dNTP 1 μL, 10×Taq EX 1 μL, Takara EX Tag (0.05 μL), sterilized distilled water (6.55μL), and template DNA 1 μL was added, and the PCR tube was placed in a thermal cycler. The following was programed for the amplification of 16S rDNA (Matsuki et al., 2004 [22] and Oyundelger et al., 2016 [26]).

3.3.4. 16S rDNA sequence analysis: The DNA extracted from agarose gel used by PCR clean up gel extraction kit (MACHEREY-NAGEL, Düren, Germany) as recommended by the manufacturer for cycle sequencing. Extracted DNA was

used for 16S rDNA sequence analysis performed with big dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were automatically analyzed 3130 Genetic analyzers (Applied Biosystems). The resulted partial rDNA sequences were compared with sequences in BLAST of NCBI (National Center of Biotechnological Information, USA) (no. 7 in Table).

3.3.5. Quantitative analysis of organic acids in Bifidobacteria cultured solution: A typical procedure for the measurement of organic acid concentration in the incubation supernatants as follows. The mixture of La-72 (*B. bifidum*) (no. 3 in Table 1) (2.5×10^8 cell/mL) in GAM (10 mL) broth containing 2% of glucose supplement was anaerobically incubated for 3 days at 37°C. The concentration of lactic and acetic acids produced in the supernatant was quantitatively determined by an EBFA−kit using a spectrophotometer measured at 340 nm according to the maker provided protocol.

3.3.6. Antibacterial screening of bifidobacterial for pathogenic bacteria: A typical procedure for the antibacterial activity on *Escherichia coli* 10977 was as follows. La-72 1 (2.5×10^8 cell/mL in 2μL of MRS broth) was inoculated on an MRS agar plate and then incubated for 24 h at 37°C to appear a colony at the spotted point. *E. coli* (6.64×10^7 cell/mL, 20 μL) with 10 ml of TSB or LB broth containing 0.8 % agar was overlaid at 45°C on the La-72 spotted agar plate, and then the plate was

aerobically incubated at 37°C (Toure et al., 2003[36]). The diameter of a clear zone around the spot was measured after 8 h, 12 h, 18 h, 24 h and 48 h respectively, to show the antibacterial activity of the isolated *Bifidobacterium*. Commercially available *Bacillus subtilis* NBRC 13722[b], *Escherichia Coli* 10977, *Staphylococcus aureus* and *Salmonella typhimurium SN8*, respectively, were also used as control bacteria for the antibacterial activity test and the results are shown (no. 4 in Table), (fig 2).

3.3.7. Antibacterial activity test in NCFS by agar spot: Firstly, overnight *Bifidobacterium* strains was inoculated to GAM broth and incubated for 18 h under 37 ° C. Then, centrifuged at 8000 rpm for 10 min. The supernatant was neutralized to pH 7.0 by addition of 1.0 N NaOH All of them were filter-sterilized through a sterile 0.22-µm-pore-size filter (Whatman Inc, Part of the Healthcare Bio-Sciences Corp, UK).

Then, seriously diluted two-fold with sterilized physiological saline 5 µl was added dropwise to TSA or LB plate medium, and the plate was infiltrated for 1-2 hours. Thereafter, the indicator bacteria which had been pre-cultured in advance were inoculated TSA or LB soft agar medium. After solidification, the cells were cultured at 37 ° C for 24 hours to confirm the formation of blocking circles. Thus, the arbitrary unit of inhibition activity per one thousand microliters was determined as 2^n x 1000µl / 5 µl (Yamomoto et al., 2003[41], Batdorj et al., 2006[4]). The experiment was carried out three times in triplicate (no. 5 in Table), figure 5.

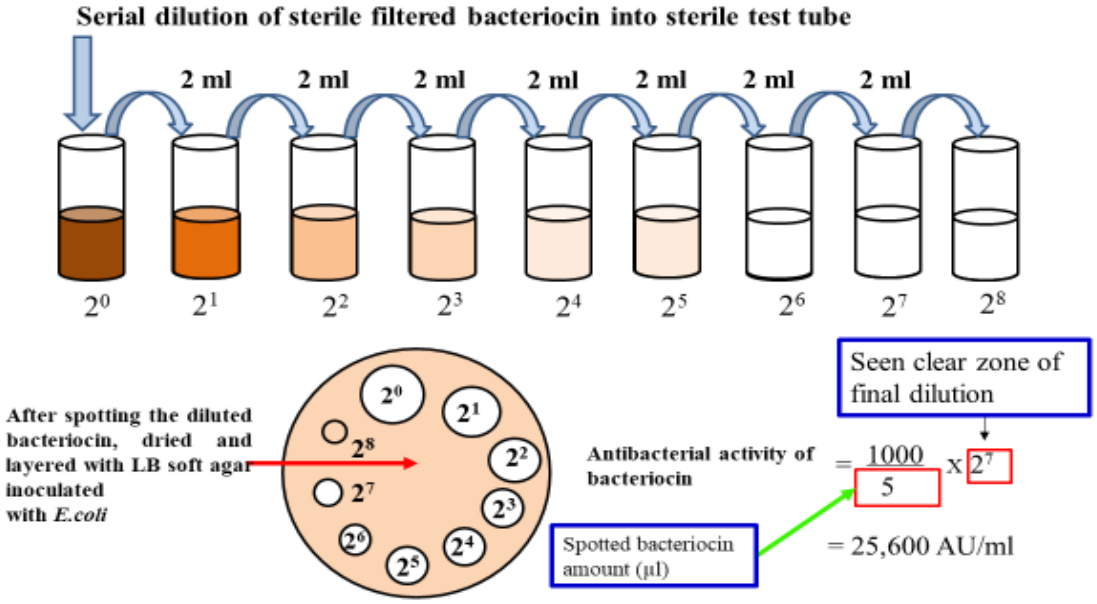

Figure 5. Agar spot test

3.3.8. Peptide production by the Bifidobacterium bifidum LA 72 in MRS medium: slightly modified by according to the (Azuma et al., 2007[3])

3.3.9. Resistance activity of enzymes, heat and pH: Two type enzymes on peptide activity were determined according to Batdorj et al., (2006)[4]. 200 µl of sterilized filter CFS was incubated with twenty microliters of the following enzymes: trypsin and proteinase K at 0.1 mg per millilitre of final concentration.

After 2 hours incubated at 37°. Control was untreated cell-free supernatant. The pH-sensitive of the active supernatant was adjusted by using 1 normal sodium hydroxide or 1 normal hydrophilic acid. The pH of the supernatant was between 1 and 10. Then after, spotted 5 µl of each two-fold diluted substance in LB agar plated. After 2 hours, with 20 µl LB soft agar medium was poured into the absorbed to agar plate medium. Then Incubated at 37° C, for 24 hours scored antibacterial activity AU/ml. Evaluation of the heat resistance of the cell-free supernatant, neutralized cell-free supernatant was incubated in at 80°, 90° and 100°C for 30, 60 and 90 min respectively, control was untreated cell-free supernatant. Thereafter, residual activity was measured by the agar spot assay (no. 9 in Table), (Fig 5).

3.3.10. Ammonium sulfate precipitation: Ammonium sulfate precipitation was according to (Cheikhyouseff et al., 2009) [11]. The precipices were resuspended in ten millilitres of sterile distilled water and dialyzed by using molecule weight cut-off 500-1000D of dialysis membrane (Biotech co., Ltd) against sterile distilled water for 24 hours. The total antimicrobial activity was determined against to indicator strain *E.coli* 10977 by agar spot method.

3.3.11. Molecular weight determination by SDS-PAGE: After dialysis substances and cell-free supernatant of molecular weight was determined an SDS-PAGE as described by using vertical gel apparatus (ATTO, Japan) with 14-20% separating gel. Antibacterial peptide preparation and a with low molecular weight marker (Precision Plus Protein, BIORAD, U.S.) were run at 30 mA for 60 minutes (Yamomoto et al., 2003 [41], Oyundelger et al., 2016 [26]). Positive control and negative control were Boum serum albumin 1mg/ml and sterilized distilled water, respectively. After electrophoresis, antibacterial activity, according to (Bhunia et al., 1987 [6] and Daba et al., 1991[14]). Fig 4.

3.4. RESULTS

3.4.1. Isolation of Bifidobacterium from young animals' intestinal tract: Thirty-six single colonies were totally obtained on agar plate from 22 samples of

Mongolian young animal livestock's around the three provinces (Tuv, Khovd, Khuvsgul) such as *Ovis aries* (5 samples), *Capra hircus* (4 samples), *Bos Taurus* (3), *Bos grannies* (4 samples), *Camelus Bactrianus* (3 samples) by using an enrichment BL agar medium. Among them, 11 isolates had Gram-positive, catalase−negative properties, and maul−like or Y−shaped morphology, and the 4-type species table 2 shows *Bifidobacterium* identified *B.bifidum*, *B.catenulatum*, *B.kashinowohense*, *B.longum*, *B.pseudocatenulatum*, *B.ramosum* and 4 type species lactic acid bacteria *Enterococcus faecalis Enterococcus faecium, Enterococcusmundti, Enterococcus hirae*, *Enterococcus lactis*, *Lactobacillus reuteri*, and *Lactobacillus plantarum* by 16S rDNA sequencing analysis.

Table 7. Properties of single colonies

isolate name	Source Animal	Catalase activity	Gram staining	Identified Species	Identified %
Ca-2	calf faeces-1	-	+	*Enterococcus faecium*	99
Ca-3	calf faeces-1	-	+	*Enterococcuss lactis*	99
Ca-4	calf faeces-1	-	+	*B.catenulatum*	96
Ca-5	calf faeces-1	-	+	*Lactobacillus reuteri*	99
Ca-7	calf faeces-2	-	+	*B.longum*	98
Ca-9	calf faeces-2	-	+	*Enterococcus faecalis*	98
Ca-10	calf faeces-2	-	+	*Enterococcus hirae*	96
Ca-11	calf faeces-3	-	+	*Eneterococcus hirae*	99
Ca-12	calf faeces-3	-	+	*B.catenulatum*	96
Ca-13	calf faeces-3	-	+	*B.catenulatum*	96
Ca-14	calf faeces-3	-	+	*Streptococcus pasteurianus*	96
Ca-17	calf faeces-4	-	+	*Lactobacillus reuter*	96
Ca-24	calf faeces-5	-	+	*Enterococcus lactis*	99
Ca-28	calf faeces-6	-	+	*Enterococcus lactis*	99
Ca-29	calf faeces-6	-	+	*Enterococcus lactis*	99
Ca-32	calf faeces-7	-	+	*B.longum*	97
Ca-33	calf faeces-7	-	+	*Enterococcus faeiceum*	99
Ca-34	calf faeces-7	-	+	*Enterococcus lactis*	99
Ca-35	calf faeces-7	-	+	*Enterococcus lactis*	97
Coa-39	coat faeces	-	+	*Lactobacillus reuteri*	97
Co-44	colt faeces-1	-	+	*B.ruminantum*	96
Co-46	colt faeces-1	-	+	*Enterococcus faecalis*	96
La-60	lamb stomach-2	-	+	*Enterococcus hirae*	96
La-61	lamb stomach-2	-	+	*B.pseudocatenulatum*	100
La-62	lamb stomach-2	-	+	*Enterococcus hirae*	100
La-63	lamb stomach-3	-	+	*B.pseudocatenulatum*	99
La-64	lamb stomach-3	-	+	*Lactobacillus plantarum*	99
La-65	lamb stomach-3	-	+	*Enterococcus hirae*	98
La-66	lamb stomach-4	-	+	*Enterococcus faecium*	98
La-69	lamb stomach-5	-	+	*B.pseudocatenulatum*	99
La-70	lamb stomach-5	-	+	*Enterococcus hirae*	99
La-71	lamb stomach-5	-	+	*Enterococcus hirae*	99
La-72	lamb stomach-5	-	+	*B.bifidum*	97
La-73	lamb stomach-5	-	+	*Enterococcus mundti*	98
Le-74	leveret-1	-	+	*Vagococcus fluvialis*	96
Le-75	leveret-2	-	+	*Enterococcus alcedinis*	99

Samples were collected from Mongolia in May and June 2018.

3.4.2. The organic acid produced by glucose fermentation test: Table 8 shows the strains were produced L, D lactic acid and acetic acid main metabolites by glucose fermentation (main carbohydrate source in GAM broth media). The concentration of the D, L-lactic acid and acetic acid measured after 3 days ranging from 1.13 mmol/L-3.5 mmol, 8.14 mmol/L-77.4 mmol/l and 26.6 mmol/L-88.7 mmol/L, respectively. The strains of the *Bifidobacterium longum* were more produced of lactic acid and acetic acid than other strains. Also, pH was most reduced than other strains. *Bifidobacterium ruminantum* glucose fermentation products were measured less than other strains and control. Acetic acid production of *B.pseudocatenulatum* and *B.longum* were measured nearest. Organic acid production of *B.bifidum* LA 72 was measured more than *B.ruminantum*. However, comparing for other strains, it was produced poor organic acids. The pH-reducing potential of the strains were about 4-5.

Table 8. Production of organic acids by isolated bifidobacteria[a]

Strain[c]	Name of isolate	Organic acid[b]			
		Final pH	D−Lactic acid mmol/L	L−Lactic acid mmol/L	Acetic acid mmol/
B. bifidum[d]	LA 72	4.57 ± 0.004	1.33 ± 0.004	17.8 ± 0.004	29.6 ± 0.004
B. catenulatum	CA 4	4.25	3.1	25.4	81
B. catenulatum	CA 12	4.14	2.9	28.2	75
B. catenulatum	CA13	4.27	2.96	26.4	54
		4.22 ± 0.05	2.98 ± 0.08	26.26 ± 0.34	70 ± 0.81
B. ruminantum[d]	CO 44	5.05 ± 0.004	1.13 ± 0.004	8.14 ± 0.02	37.73 ± 0.09
B. longum	CA 7	4.06	3.1	64.6	88.3
B. longum	CA 24	3.98	4.12	77.5	89.1
B. longum	CA 32	4.03	3.8	76.8	88.7
		4.01 ± 0.04	3.17 ± 0.07	77.4 ± 0.77	88.7 ± 0.32
B. pseudocatenulatum	LA 61	4.12	2.7	57.8	85.5
B. pseudocatenulatum	LA 63	4.15	3.1	52.3	69.1
B. pseudocatenulatum	LA 69	4.17	1.16	49.6	72.5
		4.14 ± 0.02	2.88 ± 0.04	57.56 ± 0.08	86.9 ± 0.21
B. longum (control)[d]	(JCM)	4.06 ± 0.004	3.5 ± 0.004	77.1 ± 0.004	88 ± 0.004

[a] Incubation of bifidobacterial 2×10^8 cell/mL) was performed in 2% glucose containing GAM broth (10 mL) for 72 h at 37°C.

[b] the concentration of organic acids in the cell free supernatant were determined by a commercially available F-kit and an UV absorbance at 340 nm for D-lactic, L-lactic, and acetic acids, respectively.

[c] Bifidobacteria were identified by a 16S rDNA sequencing analysis.

[d] n=3

3.4.3. Antibacterial activity by the agar spot test: Antibacterial activity of the isolated bifidobacteria toward the pathogenic strains *B.subtilis*, *E.coli*, *S.aureus* and *S.typhimurium*, was performed by an agar spot test. Table 4 shows *B.bifidum*

and species of *B.pseudocatenulatum* strains were the largest inhibited zone given than other strains against to Gram-negative intestinal pathogenic bacteria *E.coli* and *S.typhiumurium*. Also, they were given 13-15 mm inhibited growth against Gram-positive pathogenic bacteria *S. aureus*. *B.catenulatum*, *B.longum* was no inhibited effect against *B.subtilis* and *E.coli* respectively. Antibacterial inhibition effect of *B.ruminantum* was little toward *S.aureus*.

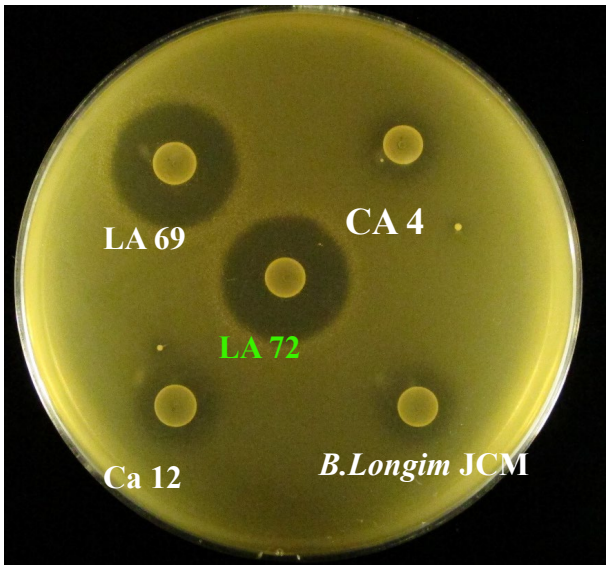

Figure 6. Peptides from BBLA72 clearly showed larger inhibited zone against *E. coli* than that of other bifidobacteria. (5 mL in the concentration of 100 mg/mL)

3.4.4. Antibacterial activity of the neutralized pH for cell-free supernatant:

Table 9 shows the effect by the neutralized pH 7 antibacterial activity AU / ml of bifidobacterial strains. Antibacterial activity of *B.bifidum* strain was 25600 AU / ml against E.coli. it was most inhibited than other *Bifidobacterium* strains. Secondly, the antibacterial activity of *B.longum* strains was inhibited 400 AU / ml

by the neutralized pH 7. They have no effected against *S.typhimurium*. also, *B.catenulatum* and *B.pseudocatenulatum* were no effected both indicator strains.

Table 9. Inhibitory effectes of cell free suternatants by neutrilized pH 7.0

Bifidobacterium spp	*Isolate name*	*E. coli*	*S.typhimurium*
B. bifidum	LA 72	25600 AU/ml	-
B. catenulatum	CA 4	-	-
B. catenulatum	CA 12	-	-
B. catenulatum	CA 13	-	-
B. longum	CA 7	400 AU/ml	-
B. longum	CA 24	400 AU/ml	-
B. longum	CA 32	400 AU/ml	-
B. pseudocatenulatum	LA 61	-	-
B. pseudocatenulatum	LA 63	-	-
B.pseudocatenulatum	LA 69	-	-
B.longum (type strain)	*(JCM1 1217)*	-	-

(-) negatively reaction

3.4.5. Peptide production by the Bifidobacterium bifidum LA 72 in MRS medium: The cells of the *Bifidobacterium bifidum* LA 72 was grown at 37° C in a flask containing supplemented by L cysteine (0.05 % v/v) MRS broth medium. Highest antibacterial activity 25600 AU/ml of the cell-free supernatant detected in stationary phase was 18 hours. In that time pH was measured 4.62 (Fig 7). In the early stationary phase, antibacterial activity and pH condition were measured 3200 AU/ml and 4.9 respectively.

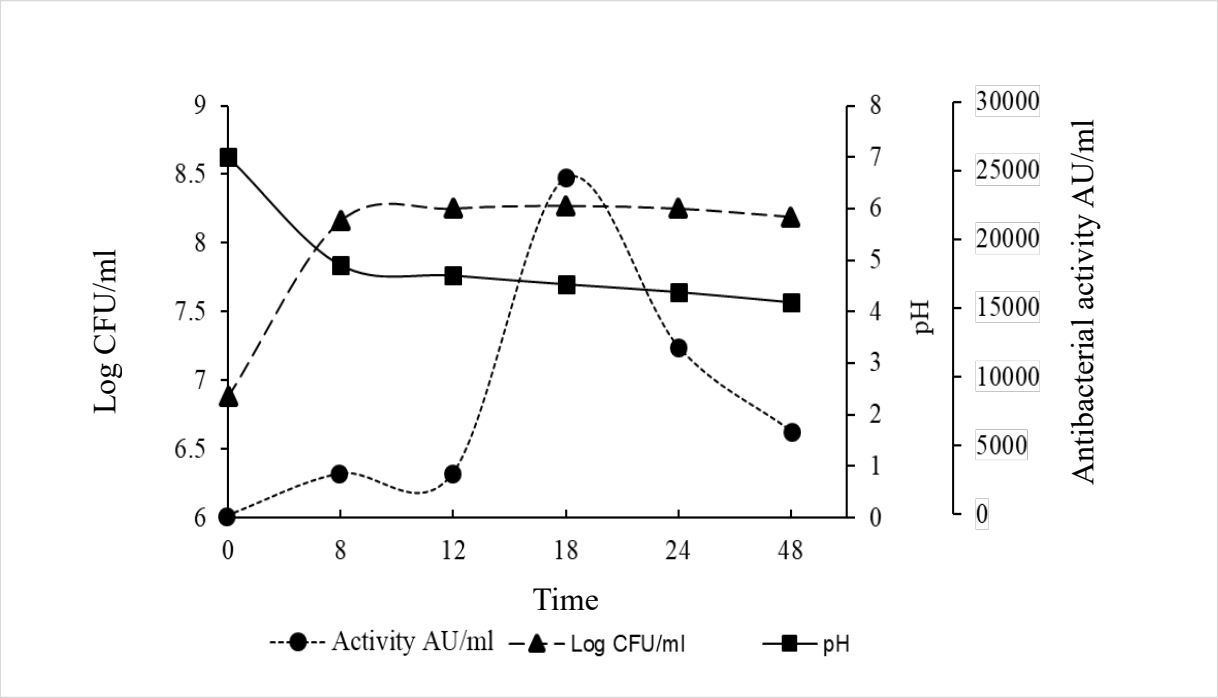

Figure 7. Peptide production phase of BBLA 72

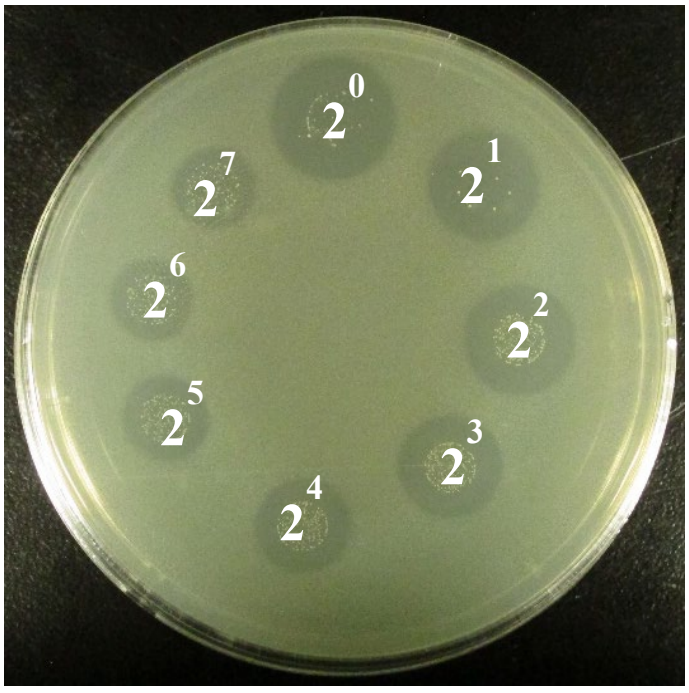

Figure 8. Agar spot test showing antibacterial activity of *Bifidobacterium bifidum* (LA 72), against *E. coli* 10977, NCFS antibacterial activity 25600 AU/ml on dried and layered with LB soft agar medium inoculated with *E. coli*.

3.4.6. Resistance activity of enzymes, heat and pH: Table 10 shows the effect of two protease activity enzymes and heat treatment antibacterial activity of cell-free supernatant of produce by BBLA72. It was sensitive to proteinase K and trypsin (table no 10 and fig 10). For heat treatment, antibacterial activity was not decreased by heating for 30 min at 100°C. Antibacterial activity decreased by 75%, 100%, respectively for treatment for 60 min at 100° C and 90 min. Control was compared with no heated supernatant.

Table 10. Effect of enzymes and heat treatment on the antibacterial peptide of *Bifidobacterium bifidum* LA 72 in the cell free supernatant

Treatment	Antibacterial activity of peptide (AU ml^{-1})
Enzymes	
Control	1600
Proteinase K	0
Trypsin	0
Heat	
Control	1600
80° C	
30 min	1600
60 min	1600
90 min	1600
90° C	
30 min	1600
60 min	1600
90 min	1200
100°C	
30 min	1600
60 min	1200
90 min	0

AU, arbitrary unit.

The cell-free supernatant active range was between pH 1-10. Most activity inhibition of the antibacterial activity was in 6, 7 and 8 in fig 9.

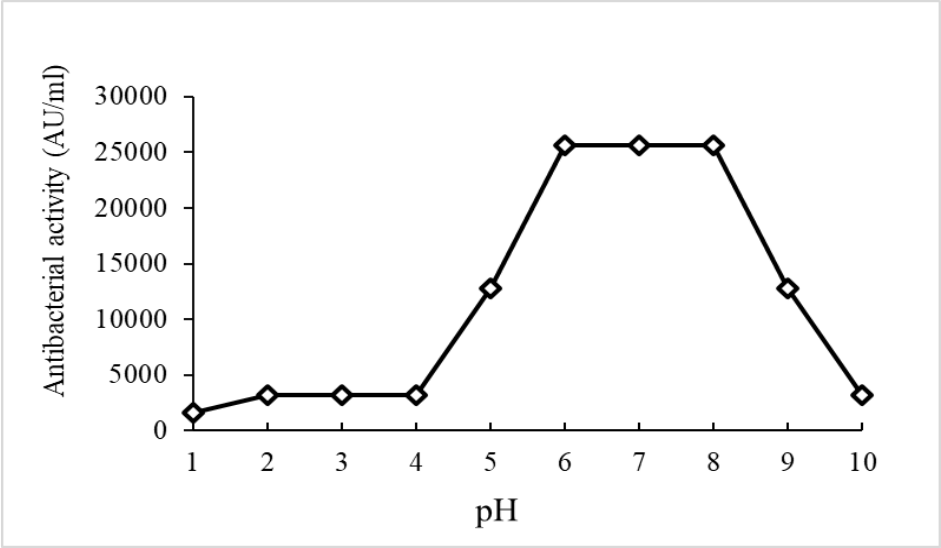

Figure 9. pH treatment of BBLA 72

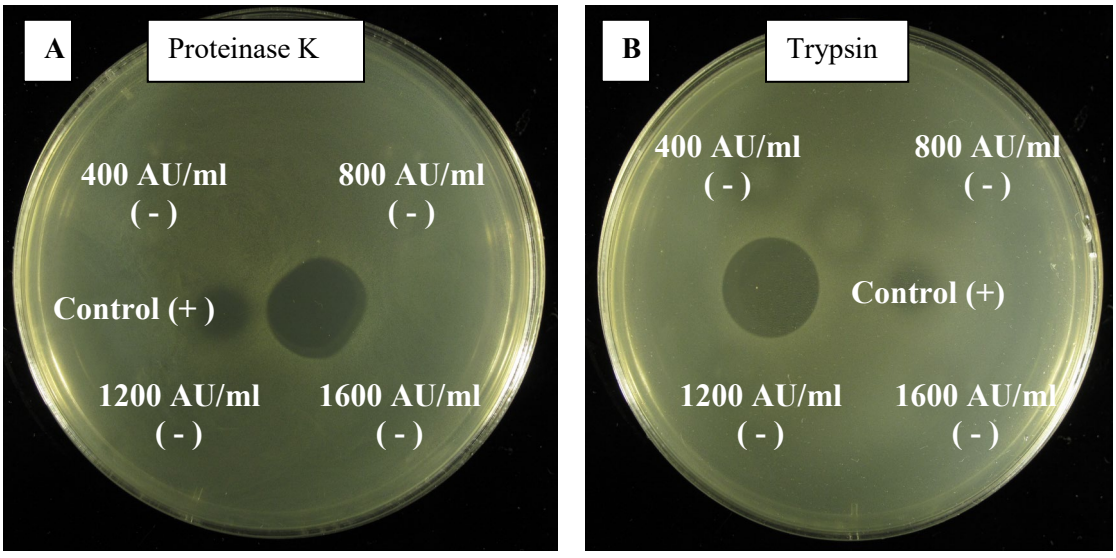

Figure 10. Enzymes treatment of BBLA 72(A). Proteinase K treatment no antibacterial activity. (B). Trypsin treatment antibacterial activity 400 AU/ml. Control was untreatment NCFS. Indicator strain was *E. coli*.

3.4.7. Molecular weight determination by the SDS page: After dialysis product, by using SDS-PAGE and in one part of gel electrophoresis by used direct antimicrobial activity detection that the molecular mass of the peptide was 2 kDa and 3 kDa. That band showed inhibition zone against *E. coli* (fig 11).

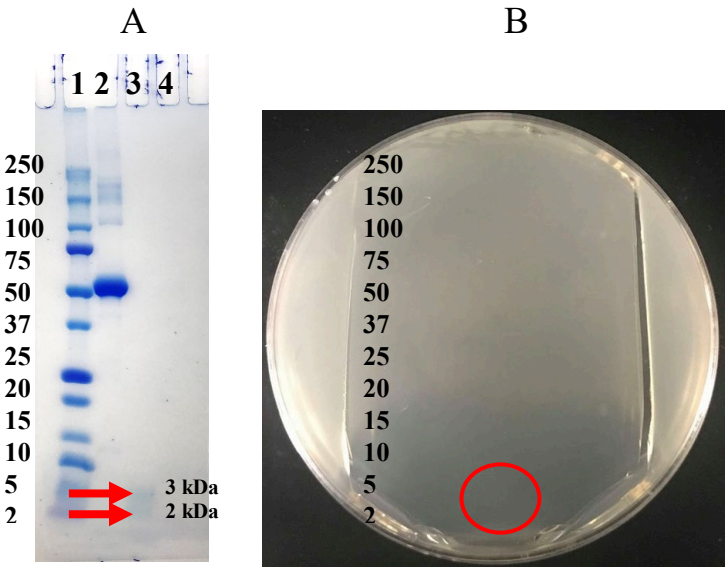

Figure 11. Tricine SDS PAGE analysis of peptide of *Bifidobacterium bifidum* (LA 72) strain A Coomassie blue stained gel, lane 1: marker of 2-250 kDa, lane 2: positive control of 1mg/ml bovium serium albumin, lane 3: BBLA 72, B. The gel was overlaid with *E. coli* to identify the band corresponding antibacterial activity.

3.4.8. Discussion

We have totally 11 isolated Gram-positive, catalase-negative properties, and maul−like or Y−shaped morphology bifidobacteria from the Mongolian young animals' livestock. In particularly, *B.bifidum* and *B.pseudocatenulatum* were obtained from lamb rumen cud. *B.longum* and *B.catenulatum* were detected in the calf faeces. *B.ruminantum* was isolated from the young camel. The results obtained in our study have demonstrated the study of ecological and physiological (Scardovi and Zani 1974[30], Matsuki et al., 2004[22]). Bifidobacteria was not detected in rabbit faeces, coat faeces and foal faeces. Almost, LAB was detected in all samples.

In theory, *Bifidobacterium* is the produce of 2 mol lactic acid 3 mol acetic acid [28]. Some researchers reported about different species of *Bifidobacterium* were produced different ratio amounts of acetic acid, lactic acid format under the same conditions [24].

In this study, D-lactic acid by glucose fermentation product of *B.bifium*, *B.catenulatum*, *B.longum*, *B.pseudocatenulatum* was produced 1.33, 2.98, 1.13, 3.17 and 2.88. L-lactic acid was produced 17.8, 26.26, 8.14, 77.4 and 57.56. Acetic acid was produced 29.6, 70, 37.73, 88.7 and 86.9 mmol/L, pH was reduced by 4.57, 4.22, 5.05, 4.01, 4.14 respectively.

Soun-Gyu Kwon et al, (2006) comparison with batch culture and cell tentative culture of *Bifidobacterium bifidum* BGN4 were produced different amount organic acids such as in the batch culture was produced acetic acid 2.4 g/L, Lactic acid 1.68 g/L after, in the cell retentive culture was 0.80 g/L of acetic acid and 0.43 g/L of lactic acid, 30 hours, respectively[18].

In this study, BBLA 72 was produced 29.6 mmol/l of acetic acid and 19.1 mmol/L of lactic acid in 2 % of glucose supplemented GAM broth media, after 72 hours.

Our isolated strain of bifidobacteria shows differently antibacterial activity in pathogenic bacteria. For example, *B.bifidum* and *B.pseudocatenulatum* have inhibited the growth of *E.coli* and *S.typhimurium* were higher than those of other cultures.

The pure cultures of our isolated bifidobacteria were deterring the growth of pathogenic indicator strains on the average of 11-20 mm in diameter, depending on the duration of the growth. According to the study, adverse effects on *E.coli* and *S.typhimurium* were higher in cultures LA 61 63, 69 and LA 72. Of these, LA 72 has averaged 2.5 times larger than the average of 24 hours of culture in 48 hours. Consequently, it can be concluded that the tendency to improve the duration of the

cultures (up to 48 hours) may have an improvement in the antibacterial activity. Then, the antibacterial activity of cell-free supernatant by adjusted to 7 pH was 25,600 AU / ml.

Toure R et al (2003) antibacterial activity bifidobacteria isolated from the infant's faeces and proteins are resistant to heat-resistance (100 ° C, 5 min) [36]. Collado et al (2005), bifidobacteria isolated from human faeces antibacterial activity toward *Helcobaciteria Pylori* and resistant to heating at 100° C, 10 min [13]. S.Tejero Sarinena et al (2012), *B.bifidum*, *B.breve*, *B.longum*, *B.infantis* antibacterial activity of the species were investigated by active organic acids, and their studies have shown that *B.breve* was more antibacterial activity against to *C.difficile* than other pathogens [34].

In this study, *B.bifidum* and *B.pseudocatenulatum* inhibited the growth of Gram-negative bacteria *E.coli* and *S.typhimurium* were higher than those of other cultures. The pure cultures of our isolated bifidobacterial were deterring the growth of pathogenic indicator strains on the average of 11-20 mm in diameter, depending on the duration of the growth. According to the study, adverse effects on *E. coli* and *S.typhimurium* were higher in cultures LA 61 63, 69 and LA 72. Of these, LA 72 has averaged 2.5 times larger than the average of 24 hours of culture in 48

hours. Consequently, it can be concluded that the tendency to improve the duration of the cultures (up to 48 hours) may have an improvement in the activity of anthocyanin therapy. Then, the pH of cells of the bifidobacterial strains of isolated *Bifidobacterium* strains up to 7, with a maximum of 25,600 AU / ml in the antibacterial determination of spot antibody. BBLA 72 was shown.

Collado et al (2005), bifidobacteria isolated from human faeces antibacterial activity toward *Helcobaciteria Pylori* and resistant to heating at 100° C, 10 min [13]. Martinez et al., (2013) summarize the information on bacteriocin of bifidobacteria . As noted, *B.bifidum* from Anand et al., (1985) investigates the presence of Gram-positive and Gram-negative bacteria antibacterial bacteria and does not mention molecular weight [21]. This bacteriocin is similar to the heat resistance of our isolated peptide, but after 48 hours protein is discharged that shows another type of peptide. BBLA 72 starts with early stationary phase and its antibacterial activity is the highest in 18 hours of bacterial culture. In the pH stabilization, bacterial resistance to 1-10 was active and 5, 6, and 7 did not significantly reduce the antibacterial activity.

Yildrim et al (1998) *B.bifidum* isolates bifidocin B, bacteria, which is about 3,3 kDa, heat resistant (121° C-15 min), with a wide range of pH resistance (2-12), optimal production phase was at 37° C, for 12 to 18 hours, and the protein is

produced by *B.bifidum* antibacterial activity against to *Bacillus cereus*, *Enterococcus ffaecalis*, *Listeria monocytogenes*, *Pediococcus acidolactici* and *Streptococcus faecilis*. Our isolated peptides are somewhat of the same nature as this bacteriocin, but they differ in molecular weight. For BBLA-72 it two proteins was antibacterial activity against to *E.coli* 10977 and that weight was 2 kDa and 3 kDa.

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CHAPTER IV

CONCLUSION

We isolated and identified several bifidobacteria from Mongolian infant feces by using the *B. breve* and *B. longum* specific primers and the antibacterial activity of the isolated bifidobacteria was investigated. The pH value of the cell free supernatant was ranged from 3.86 to 4.55, due to the production of lactic and acetic acids, which were quantitatively analyzed by the absorbance measurement at 340 nm using the commercially available EBFA−kit. The isolated INFbre 11 bifidobacterium was found to produce highest concentration of L−lactic (15.67 g/L) and acetic (6.59 g/L) acids, respectively. The INFbre 20 *B. breve* strain also produce high concentration of L−lactic acid (15.63 g/L), however, lower acetic acid (0.30 g/L). The isolated bifidobacteria, INLbre 11 and INFlon 22 gave clear zones on the *E. coli* and *S. typhimurium* agarplates, respectively, as large as that of the standard bifidobacteria, indicating that the isolated bifidobacteria had potent antibacterial activity probably due to the production of organic acids. In addition, the isolation of antibacterial peptides produced by the isolated bifidobacteria is under investigated.

Isolated from Mongolian young animals livestock strains were identified by 16S rDNA sequencing analysis, they were *B.catenulatum*, *B.bifidum*, *B.pseudocatenulatum*, *B.ruminantum* and *B.longum*. Isolated from the lamb rumen *B.bifidum* (BBLA72) was produced 29.6 mmol per liters acetate and 19.3 mmol per liters of lactate, thereby releasing pH from 7.0 to 4.57. However, organic acids were produced small in comparison with other strains, the antibacterial activity of the pH neutralized up to 7.0 cell-free supernatant was more significant than other strains. The arbitrary unit was measured 25600 AU/ml against to *E.coli*. It is preliminary for this substance as a bacteriocin-like substance that weighs 2 kDa and 3 kDa, capable of withstanding a 100-degree heat to 30 minutes, proteinase K and trypsin enzyme.

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