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ARTICLES

ANTIBACTERIAL ACTIVITY OF BIFIDOBACTERIA ISOLATED FROM INFANT FAECES

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Abstract: *Antibacterial activity of bifidobacteria isolated from Mongolian infant faeces was elucidated on pathogenic intestinal bacteria for the development of a new antibacterial bifidobacteria, the permission for which was granted by the Mongolian Medical Ethics Committee Approval (MMECA). A total of forty-nine single colonies were obtained from 3 samples by using a BL medium enrichment. 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 a standard bifidobacteria, however, lower activity against Staphylococcus aureus. The antibacterial activity was probably due to the production of organic acids.*

Keywords: *Probiotic; clear zone; pathogenic bacteria; organic acid; pH;*

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 humans 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 with the production of organic acids. Makras and Vuyst found

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astrong antibacterial activity of bifidobacteria towards Gram−negative bacteria, *Salmonella* and *E. coli* strains, with the production of lactic and acetic acidsand reported the inhibition of growth of Gram−positive bacteria with the production of a bacteriocin [16]. The agar plate spot test of bifidobacteria on several pathogenic bacteria showed antibacterial activity and one of the main inhibitory reasons was reported by the production of organic acids[5]. Georgieva described the comparison of acidic and neutralized cell−free supernatants cultured by bifidobacteria, indicating that the acidic supernatant was active on several pathogenic bacteria and the neutralized supernatant also showed antibacterial activity. In brief, 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 the products of bifidobacteria fermentation with sugar. The highest amount of lactic and acetic acids was obtained from the fermentation of lactose determined by 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 the 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 faeces of Mongolian infants 0−6 months of age by 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.

MATERIALS AND METHODS

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. Anaero pack and QIAamp DNA mini kit were obtained from Mitsubishi Gas Chemical Co. Ltd., (Tokyo, Japan) and QIAGEN. V. (Hilden, Germany), respectively. Standard bifidobacteria, *Bifidobacterium adolescents, Bifidobacteriumlongum*, 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 from the stock strains at the Mongolia Institute of Veterinary Medicine. Enzymatic Bioanalysis / Food Analysis UV method kit (EBFA kit) was purchased from R−Biopharm AG (Darmstadt, Germany).

Isolation of bifidobacteria*:* Three samples of faeces of Mongolian infants aged from 0 to 6 months were collected with the permission of the Mongolian Medical Ethics Committee. 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) after which the mixture was well stirred. The sample (0.1 mL) was spread on

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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.

DNA extraction and identification of bifidobacteria*:* The GAM broth culture of 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. Precipitated bifidobacterium 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 programmed for the amplification of 16S rDNA. The tube was heated for 5 min at 94°C as 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.

Quantitative analysis of organic acids in bifidobacteria cultured solution: A typical procedure for measuring the concentration of organic acid in the incubation supernatant is 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].

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 hours at 37°C to give fermented products. After centrifugation, the cell free supernatant was discarded, and then PBS broth (0.9%, 3 mL) was added to the tube. The mixture was suspended by using a vortex shaker and then a 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 hours at 37°C, the color of the mixture 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 that showed (+) for fermentation, (-) for no fermentation, and (w) for weak fermentation, respectively.

Antibacterial activity test: A typical procedure for the antibacterial activity on *Escherichia coli* 1099 was as follows.

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INFbre 1 (20×10^9 cell/mL in2µL of MRS broth) was inoculated on an MRSagarplate and then incubated for 24 hours 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, 48, and 72 hours 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.

RESULTS AND DISCUSSION

Isolation of *B. breve* and *B. longum* bifidobacteria from infant faeces: 49 single colonies were obtained on the agar plate medium from the faeces collected from 3 Mongolian infants. From among them, 20 colonies showed Gram−negative and rod−like shapes, indicating that these strains were not bifidobacteria. The remaining 29 colonies had Gram−positive and catalase−negative properties and showed the cell morphology like maul or Y shaped short rod when observed under a microscope, 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 kinds of 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 1 shows the results of organic acid production in the supernatant by the 29 bifidobacteria

Table 1. Production of organic acids by isolated bifidobacteria[a]

Name of isolate	pH[b]	Organic acid[c]			Strain[d]
		D−Lactic acid g/L	L−Lactic acid g/L	Acetic acid g/L	
INFbre 1	3.86	0.06	6.02	6.74	*B. breve*
INFbre 2	3.89	0.28	6.65	6.80	
INFbre 3	3.90	0.32	6.49	6.86	
INFbre 4	3.90	0.12	6.04	6.34	
INFbre 5	3.91	0.32	5.20	6.25	
INFbre 6	3.93	0.32	5.33	5.82	
INFbre 7	3.95	0.28	5.10	5.97	
INFbre 8	3.97	0.32	5.17	6.28	
INFbre 9	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	

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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 hours 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 hours 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 pH values relatively lower 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.

Carbohydrate fermentation ability: Carbohydrate fermentation ability of bifidobacteria was examined by using several carbohydrates. The results are demonstrated in Table 2.

Table 2. 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	+	+	+	+	+	+	+	+	+	+	+

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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 at three levels, (+) Positive, (w) weak but positive, and (-) negative.

Lactic and acetic acids were obtained as a result of metabolism. The fermentation ability was evaluated at three levels, (+) positive, (w) weak but 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 expectribose was not fermented by the isolated *B.breve*, arabinose was fermented by *B.longum* to give organic acids. Hexoses, disaccharides, and alditols expectinositol were fermented by all tested strains belonging to both species. A polysaccharide starch and glycosides were fermented by the *B. breve* strains, but it was either not fermented, or the fermentation was weak by using the *B. longum* strains. The fermentation ability of both bifidobacteria on rhamnose, sorbose and inositol was low. Melicitose was 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.

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 1 shows the results of the agar 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 hours at 37°C, the clear zone without *E. coli* and *S. typhimurium* appeared, indicating that the bifidobacteria inhibited the multiplication of the pathogenic bacteria.

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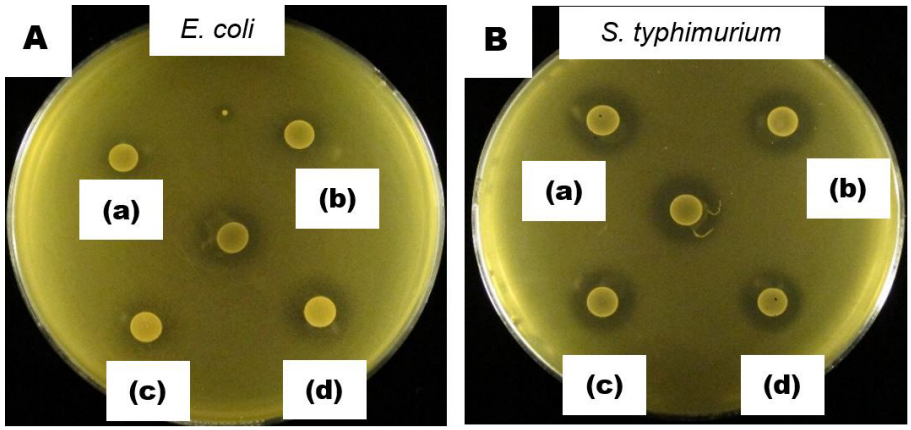

Figure 1. Antibacterial activity of isolated bifidobacteria toward Gram−negative pathogenic bacteria (A) E. coli and (B) S. typhimurium. (a) INFbre 11, (b) INFlon 22, (c) B. breve 01192, and (d) B. longum 01217. The pathogenic bacteria was overlaid on the isolated bifidobacteria agar plate, respectively, to appear clear zones.

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 shown in Table 3, the isolated *B. longum* strain INFlon 22 was found to show potent anti-

Table 3. 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.

bacterial activity toward *E. coli* and *S. typhimurium*, and the *B. breve*I NFbre 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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CONCLUSIONS

We isolated and identified several bifidobacteria from Mongolian infant faeces 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 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 the 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), but 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 investigation.

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Conflict of Interest: The authors declare no conflict of interest.

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