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The effects of *Bifidobacterium bifidum* OFR9, a strain resistant to antituberculosis and antileprosy agents, on fecal flora in mice

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Since *Bifidobacterium bifidum*, one of the strains of medical preparations used for human intestinal disorders, is sensitive to rifampicin (RFP) and fluoroquinolones, its therapeutic effect cannot be guaranteed when it is administered concomitantly with these antibiotics. To develop new strains of *B. bifidum* that are resistant to these drugs, *B. bifidum* RFR61, which is highly resistant to RFP, was selected by the N-methyl-N′-nitrosoguanidine (MNNG) mutation method. Then, *B. bifidum* OFR9 was selected in vitro from *B. bifidum* RFR61 by serial passage to increasing concentrations of ofloxacin (OFLX) on a solid medium. The minimal inhibition concentrations (MIC) of RFP and fluoroquinolones for *B. bifidum* OFR9 were >256 μg/ml and 16–256 μg/ml, respectively. We investigated the effects of *B. bifidum* OFR9 on the fecal bacterial flora of mice administered with both antibiotics and *B. bifidum* OFR9. The results showed that the concurrent use of *B. bifidum* OFR9 and antibiotics prevented the decrease of bifidobacteria, and quickly restored the flora to normal as compared with the use of antibiotic or parent strain therapy alone. The survival of *Shigella* organisms in mouse feces rapidly decreased, and were removed within two days as a result of the oral administration of *B. bifidum* OFR9.

Key Words——antibiotic therapy; *Bifidobacterium bifidum*; fluoroquinolones; normal flora; ofloxacin; resistance; rifampicin

Intestinal bacteria that develop as a result of the consumption of fermented dairy food can affect the health of humans by producing both beneficial and noxious products. Disturbances of the intestinal flora have been observed in connection with the acidity of gastric juices, disorders of peristalsis, cancer or surgical operations on the stomach or small intestine, and liver or kidney disease (Modler et al., 1990; Sasaki et al., 1987). According to Rasic and Kurmann's reports, intestinal bifidobacteria inhibit the growth of harmful bacteria such as clostridia, *Shigella*, and *Escherichia coli*. However, bifidobacteria are sensitive to penicillin, tetracyclin, neomycin, and novobiocin and, as they can be easily eliminated by erythromycin, spiramycin, or chloramphenicol (which are used for the remedy of other putrefactive infections), gastrointestinal diseases can be caused by disturbing the normal flora (Rasic, 1983). In clinical trials, the relationship between antibiotics and lactic acid bacteria is very important. In particular, antituberculosis or antileprosy agents taken by patients over a long period can cause the disruption of normal flora followed by malabsorption, maldigestion, and intestinal disease (Committee on Treatment of International Union against Tuberculosis and Lung Disease, 1988). Thus, as Gordon reports (1957), antituberculosis and antileprosy agents should be administrated with viable bacterial cell preparations. Additionally, it is necessary to develop new strains of *Bifidobacterium bifidum* which are resistant to these chemotherapeutic agents from the parent strain and include them in commercially available preparations for intestinal disorders. Rifampicin (RFP)-resistant mutant *B. bifidum* RFR61 was screened by treating the parent strain of *B. bifidum* with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). The fluoroquinolone-resistant *B. bifidum* OFR9 was selected using the spontaneous multistep mutation of *B. bifidum* RFR61 (Adelberg et al., 1965; John, 1975; Schwartz and Stadtman, 1970). This article describes the biochemical characteristics of *B. bifidum* OFR9 and its growth inhibitory activity against intestinal putrefactive bacteria as well as the maintenance of resistance in vivo.

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Materials and Methods

Bacterial strains. The *B. bifidum* strain was a gift from Il-Dong Pharmaceutical, Seoul, Korea. *Shigella dysenteriae* ATCC 9752 and *E. coli* MB4-01 (Choi et al., 1994) were laboratory stock, and were used for the study of growth inhibition of intestinal pathogenic bacteria.

Medium. BL agar (Eiken Chemical, Tokyo, Japan) and BL broth [(g/l): 3.0 meat extract, 10.0 proteose peptone, 5.0 peptone, 3.0 soybean peptone, 5.0 yeast extract, 5.0 liver extract, 10.0 glucose, 0.5 soluble starch, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 NaCl, 0.007 $MnSO_4$, 1.0 polysorbate 80, and 0.5 L-cysteine · HCl · H_2O] were used for the subculture and liquid culture of *B. bifidum*, respectively, and bifidobacteria-selective solid plate (Modler et al., 1990) was used for the selection of bifidobacteria. Brain Heart Infusion (Difco, Detroit, MI, U.S.A.) was used for *S. dysenteriae* ATCC 9752.

Growth conditions. *B. bifidum* strains were incubated anaerobically by using Gas Pak Plus (BBL, Baltimore, MD, U.S.A.) and an anaerobic indicator in an Anaerobox with an atmosphere of 10% carbon dioxide, 80% nitrogen, and 10% hydrogen. Grains of the catalyst reacted with residual oxygen in the chamber.

Determination of minimal inhibitory concentration. The minimal inhibitory concentrations (MICs) of the antituberculosis and antileprosy agents, such as RFP, kanamycin, ethambutol, pyrazinamide, cycloserine, ofloxacin (OFLX), norfloxacin (NFLX), ciprofloxacin (CPFX), and sparfloxacin (SPFX), against the *B. bifidum* were determined by the agar dilution method (NCCLS, 1993).

Isolation of RFP-resistant mutants (James et al., 1983). An overnight culture of the *B. bifidum* parent type at 39°C was transferred into a new broth and cultured for 5 h to reach the mid-log phase. Bacteria were collected by centrifugation at 6,000×*g* for 5 min, and washed with 50 mM potassium phosphate buffer (pH 6.8) followed by suspension with the same buffer. Freshly-made MNNG was added to each suspension at either 50 or 100 μg/ml. For the negative controls, samples not containing MNNG were also made. To bring about mutation, the bacteria were cultured for 30, 60, and 120 min at 39°C. The cells were collected by centrifugation at 6,000×*g* for 5 min and washed with 50 mM potassium phosphate buffer (pH 6.8). They were then suspended in BL-broth. Resuspended bacterial cells were transferred to a BL-medium containing 10 μg/ml RFP, poured onto a Petri dish and grown for 3 days under anaerobic conditions to check the formation of mutants.

Isolation of double-resistant mutants. RFP-resistant mutant *B. bifidum* RFR61 in the mid-log phase was collected like the parent bacterium. BL-broth (100 μl) (10^{10} CFU/ml) resuspended bacterial cells was spread on a solid plate containing 10 μg/ml RFP and two-fold concentrated OFLX, and subsequently grown for 36–48 h at 39°C. Four cycles of the experiment were undertaken.

Measurement of lactic acid. The overnight cultures of the parent type and mutants were seeded in a new BL-broth with 2.5% and incubated for 24 h at 39°C. The lactic acid in a five-time diluted supernatant, taken after centrifugation at 6,000×*g* for 5 min, was titrated with 0.1 N NaOH preceded by the addition of two drops of 1% phenolphthalein.

Growth inhibitory effects of culture supernatant of bifidobacteria in vitro. The supernatant of the broth, in which either the parent type or mutants were grown at 39°C, was taken after centrifugation. One-quarter and one-half volume aliquots of culture supernatant were added to fresh BL-broths giving pHs of 5.72 and 4.92, respectively. This was done to make a medium for a growth inhibition test of *S. dysenteriae*. A 2.5% *S. dysenteriae* culture was inoculated and incubated in the above broth. On each alternate hour for 12 h, 10 ml of broth was taken and the absorbance was measured at 600 nm to check the growth of *S. dysenteriae*. For the control, the growth of *S. dysenteriae* in BL-broth (pH 7.0) containing no culture supernatant of *B. bifidum* was measured. To check whether the growth inhibition of *S. dysenteriae* was caused by the reduction of pH, the growth rate in the control BL-broth (pH 5.72, 4.92) was also determined.

In vivo effects of B. bifidum parent and resistant strains.

Experimental mice: ICR mice aged 42 days and weighing about 30±2 g, were used. All animals were kept in polystyrene cages in an air-conditioned room at 20±2°C, 50±5% (humidity) with a 12 h light-dark cycle. They were fed, ad libitum, a standard solid stock diet (Samyang, Korea) and had free access to water.

Experimental design: Table 1 shows the administration scheme for the bifidobacteria, *Shigella* and antibiotics. For the maintenance test of bifidobacteria in mice fecal flora, mice in groups of five were administered RFP and OFLX orally each at a dose of 50 mg/kg/day and *B. bifidum* parent or OFR9 at a dose of 4×10^8 CFU/day in 0.1 ml of anaerobic dilution broth. Fecal examinations were carried out just before administration every day during the period of treatment and every other day after the end of treatment. To investigate the in vivo effect of *B. bifidum* OFR9 against *Shigella* infection, the mice were treated with 2×10^3 CFU/day of *S. dysenteriae* and 4×10^8 CFU/day of *B. bifidum* OFR9. Feces were examined every day for 8 days.

Table 1. Scheme for oral administration with bifidobacteria, *Shigella*, and antibiotics in mice

Group	Administration time (day)							
	1	2	3	4	5	6	7	8
CON								
HA	+	+	+	+	+	+	+	+
HA+PB	+	+	+	+	+	+	+	+
HA+RB	+	+	+	+	+	+	+	+
Sh	+	+	+	+				
Sh+RB	+	+	+	+				
Sh, RB	+	+	+	+	+			

CON, not treated; HA, RFP, OFLX 50 mg/kg/day, respectively, and no bifidobacteria; HA+PB, RFP, OFLX 50 mg/kg/day, respectively, and *B. bifidum* parent (4×10^8 CFU/day); HA+RB, RFP, OFLX 50 mg/kg/day, respectively, and *B. bifidum* OFR9 (4×10^8 CFU/day); Sh, *Shigella dysenteriae* ATCC 9752 (2×10^3 CFU/day); Sh+RB, *S. dysenteriae* (2×10^3 CFU/day) and *B. bifidum* OFR9 (4×10^8 CFU/day); Sh, RB, *S. dysenteriae* (2×10^3 CFU/day) for first four days and *B. bifidum* OFR9 (4×10^8 CFU/day) on the fifth day; +, treated day.

Microbiological methods: After being treated with bacteria, 0.05–0.1 g of feces was put into a tube containing 0.9 ml of prechilled, autoclaved anaerobic dilution broth. The above sample was diluted 10^{-1} and homogenized thoroughly. The homogenized samples were diluted serially 10 times and 50 μl of serial dilutant 3 was chosen, and subjected to aliquoting and spreading with conradirod. The anaerobic plates were incubated for 48 h at 37–38°C in a Gas Pak jar.

Data analysis: The number of live bacteria was calculated from the number of colonies grown on the plate. All results are given as the mean±SD. The difference between mean values was tested for statistical significance using Student's *t*-test, and an analysis of variance was used for the animal studies.

Results

Minimal inhibition concentration

The MICs of 15 antituberculosis and antileprosy agents against *B. bifidum* parent type, RFR61 and OFR9 are presented in Tables 2 and 3. Parent type *B. bifidum* was very sensitive to RFP at 1 μg/ml, having various MICs against fluoroquinolones at 0.12–16 μg/ml.

Isolation of resistant strain

The RFP-resistant strain obtained by MNNG treatment was named *B. bifidum* RFR61. It was subjected to select OFLX-resistant strain *B. bifidum* OFR9 using spontaneous multistep mutation. The MIC of *B. bifidum* RFR61 against RFP increased by up to 256 μg/ml, and that of *B. bifidum* OFR9 against RFP was maintained at 256 μg/ml. When the MIC of *B. bifidum* OFR9 against the fluoroquinolone species was determined, the MIC increased by 64 μg/ml against OFLX and NFLX and 32 μg/ml against CFLX and SPFX.

Table 2. Antimicrobial activity of antituberculosis agents against *Bifidobacterium bifidum* strains.

Drug	MIC (μg/ml)		
	Parent	RFR61	OFR9
Rifampicin	1	>256	>256
Kanamycin	128	256	256
Isoniazid	>256	>256	>256
Ethambutol	>256	>256	>256
Pyrazinamide	>256	>256	>256
D-Cycloserine	64	64	64

Table 3. Antimicrobial activity of fluoroquinolones against *Bifidobacterium bifidum* strains.

Drug	MIC (μg/ml)		
	Parent	RFR61	OFR9
Ofloxacin	16	16	64
Pefloxacin	8	8	128
Norfloxacin	1	1	64
Ciprofloxacin	1	1	32
Sparfloxacin	0.12	0.12	32
Rufloxacin	8	8	64
Lomefloxacin	16	16	256
Levofloxacin	4	4	64
Tosufloxacin	1	1	16

Table 4. The amounts of lactic acid produced by *Bifidobacterium bifidum* strains.

Strain	Concentration (mg/ml)
Parent	10.4 (100%)
RFR61	9.9 (95.3%)
OFR9	10.1 (96.6%)

Measurement of lactic acid production

Lactic acid production of the mutant strain *B. bifidum* OFR9 was similar to, though a little lower than, that of *B. bifidum* wild type (Table 4).

Growth inhibitory effects of culture supernatant of bifidobacteria in vitro

No growth of *S. dysenteriae* ATCC 9752 was detected in media containing the culture supernatatnt of bifidobacteria with pH 4.92. The growth of *S. dysenteriae* in the pH 5.72 culture supernatant media was reduced 400–600-fold compared to that in the pH 7.0 control media, and reduced 100-fold compared to that

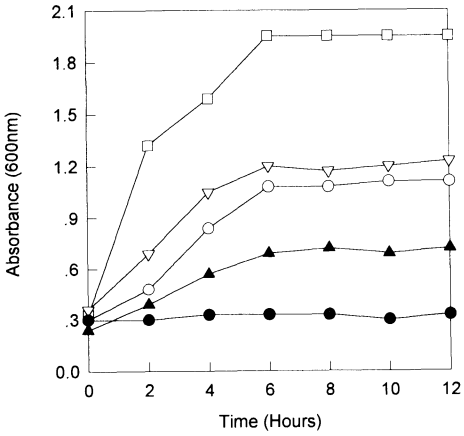

Fig. 1. Growth inhibition of *Shigella dysenteriae* ATCC 9752 by the culture supernatant of *Bifidobacterium bifidum* OFR9.

□, BL-broth (pH 7.0); ▽, BL-broth (pH 5.72); ▲, BL-broth (pH 4.92); ○, culture supernatant (1 vol) : BL-broth (3 vol), pH 5.72; ●, culture supernatant (1 vol) : BL-broth (1 vol), pH 4.92.

in media of pH 5.72 not containing culture supernatant (Fig. 1).

In vivo effects of B. bifidum parent and resistant strains

B. bifidum OFR9 administered to mice in combination with RFP and OFLX: The number of normal mouse bifidobacteria per gram of feces was about 1.3×10^7 CFU/g. When 50 μg/ml RFP and OFLX was administered, the amount of bifidobacteria decreased dramatically from day 4 and reached 6.3×10^2 CFU/g on day 9. The amount of bifidobacteria increased from the last day of RFP and OFLX administration and then finally recovered to the normal amount on day 15. In the case of the combined administration of RFR, OFLX and *B. bifidum* parent, the bacteria decreased maximally to 1.0×10^4 CFU/g on day 7, followed by a gradual increase after stopping administration. It then reached the normal amount on day 14. When RFP, OFLX, and *B. bifidum* OFR9 were administered to the mice, no decrease in bifidobacteria was detected. In contrast to the case of the *B. bifidum* parent, the bifidobacteria increased to 7.9×10^7 CFU/g on day 7. Two days after stopping administration, the number of bifidobacteria returned to normal (Fig. 2). Yamashita et al. (1985) reported that when ampicillin was administered to mice with Biofermin-R, multiresistant *Streptococcus* preparations, bifidobacteria recovered earlier and reached normal more quickly than in the case of ampicillin treatment alone.

Growth inhibitory effects of *B. bifidum* OFR9 against infection by *Shigella*: The in vivo inhibitory effects of *B. bifidum* OFR9 against the virulent intestinal strain of *Shigella* are shown in Fig. 3. When mice were administered with *S. dysenteriae* ATCC 9752 for only 2 days, the total number of the bacteria in the mice

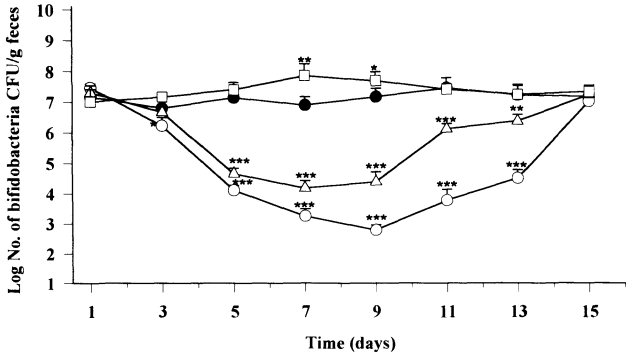

Fig. 2. Maintenance of bifidobacteria in mice fecal flora by oral administration of bifidobacteria and antibiotics (mean±SD (n=4); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

●, control, not treated; ○, RFP, OFLX 50 mg/kg/day, respectively, and no bifidobacteria; △, RFP, OFLX 50 mg/kg/day, respectively, and *Bifidobacterium bifidum* parent (4×10^8 CFU/day); □, RFP, OFLX 50 mg/kg/day, respectively, and *B. bifidum* OFR9 (4×10^8 CFU/day).

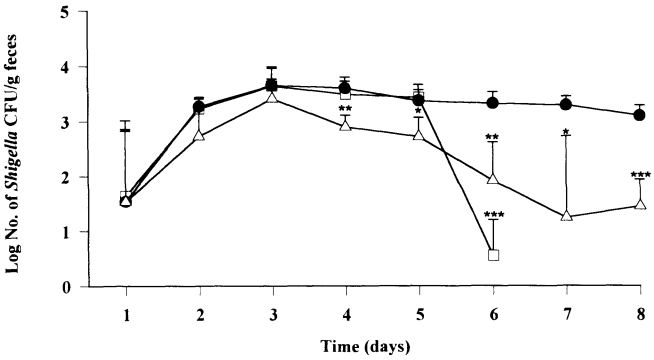

Fig. 3. Inhibition of *Shigella* in mice fecal flora by oral administration of *Bifidobacterium bifidum* OFR9 (mean±SD (n=4); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

●, *Shigella dysenteriae* ATCC 9752 (2×10^3 CFU/day); △, *S. dysenteriae* (2×10^3 CFU/day) and *B. bifidum* OFR9 (4×10^8 CFU/day); □, *S. dysenteria* (2×10^3 CFU/day) for first four days and *B. bifidum* OFR9 (4×10^8 CFU/day) on the fifth day.

feces increased from 3.5×10 CFU/g feces (before treatment with the bacteria) to 5.0×10^3 CFU/g feces and remained at 1.3×10^3 CFU/g feces even 4 days after stopping the administration of bacteria. However, the number of *Shigella* decreased significantly from the fourth day after coadministration with the resistant strain, *B. bifidum* OFR9. For example, the number of *Shigella* decreased gradually and then reached the normal amount on day 7. In the case of a one-day administration with *B. bifidum* OFR9 preceded by four consecutive days of treatment with *S. dysenteriae*, the number of *Shigella* in the feces decreased dramatically within 1 day after administration with *B. bifidum* OFR9, and the *Shigella* was not detected on days 2 and 3 after stopping the administration of *B. bifidum* OFR9.

Discussion

The MICs of 15 kinds of antituberculosis and antileprosy agents against the *B. bifidum* parent strain were measured, and a high sensitivity to RFP was detected as the MICs were 1 μg/ml. The sensitivity of *B. bifidum* to fluoroquinolones, which have been widely used recently, was also high, showing MICs of 0.12–16 μg/ml. Therefore, in the case of prolonged oral administration of antituberculosis agents to treat patients, the administration of *B. bifidum* preparations would not be effective. Therefore, the development of a resistant strain of *B. bifidum* against RFP and fluoroquinolones is needed.

The RFP-resistant strain *B. bifidum* was selected after treatment of the *B. bifidum* parent type with MNNG. Subsequently, OFLX-resistant *B. bifidum* OFR9 was made using the spontaneous multistep mutation method. The MIC of RFP against *B. bifidum* OFR9 was maintained at over 256 μg/ml. The MICs of fluoroquinolones increased 4–267 times at 32–64 μg/ml. This phenomenon was also observed in the case of other lactic acid bacteria. *Bacillus coagulans* (*Lactobacillus sporogenes*) (Kim et al., 1989) and *Streptococcus faecalis* (Choi et al., 1993), of which the resistant strains were selected using the same method for *B. bifidum*. The MIC of RFP against *B. coagulans* (*L. sporogenes*) grew to 256 μg/ml and that of OFLX increased to 32 μg/ml. The MICs of RFP and OFLX against *S. faecalis* increased by 256 and 128 μg/ml, respectively. The MICs of RFP and OFLX against multi-resistant mutant *B. bifidum* OFR9 rose to 256 μg/ml (RFP) and 64 μg/ml (OFLX), which indicates that the possibility of reverse mutation is rare because of the maintenance of resistance (data not shown). On the basis of the MIC change, the medicinal effects of *B. bifidum* on intestinal disorders should be expected after treatment by either antituberculosis or antileprosy agents, including the two kinds of antibiotics mentioned above. If the resistant strain inactivates RFP and OFLX, the original effects of the antibiotics will not be obtained. There was no possibility of the inactivation of RFP and OFLX in vitro with the stability test (data not shown).

Other biochemical characteristics such as lactic acid production and growth inhibitory effects against the putrefactive microflora of *B. bifidum* OFR9 were detected and compared with those of the parent type. The parent type and OFR9 produced as much as 10.4 and 10.1 mg/ml lactic acid, respectively, so a change in lactic acid productivity was not detected. The indirect growth inhibitory effect of bifidobacteria might be achieved mainly by a reduction in pH owing to lactic acid production. The in vitro growth of *S. dysenteriae* in culture supernatant media with a pH of 5.72 was reduced 400 to 600-fold compared to that in the control media with a pH of 7.0, and reduced only 100-fold compared to that of media with a pH of 5.72 not containing culture supernatant. So the culture supernatant of *B. bifidum* OFR9 was thought to contain other factors directly inhibiting the growth of bacteria. The uncharacterized antibacterial substance was identified from the culture supernatant of *B. bifidum* OFR9 (data not shown). Some antibacterial substances produced by bifidobacteria and lactic acid bacteria have already been reported. Bifilong, an antibacterial substance produced by *Bifidobacterium longum*, was reported by Kang et al. (1989) and lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*, was reported by Barefoot and Klaenhammer (1983).

The change in the total number of bifidobacteria was detected using mice. When RFP and OFLX were administered to mice with *B. bifidum* OFR9, the total number of bifidobacteria was similar to that of the fecal flora of mice, which is in contrast to the results of the parent type. Therefore, *B. bifidum* OFR9 was also resistant to RFP and OFLX in vivo. The in vivo growth inhibitory effects of the multiple-drug-resistant strain *B. bifidum* OFR9 against pathological bacteria, *S. dysenteriae*, were evaluated. The number of increased *Shigella* in mice feces due to the oral administration of *S. dysenteriae* culture was suppressed significantly when *B. bifidum* OFR9 was administered to the mice. For example, when *B. bifidum* OFR9 was administered concurrently with *S. dysenteriae*, the number of *Shigella* in the feces decreased significantly (50–20% of the number of *Shigella* when only administered *S. dysenteriae*) from day 3 to day 8. When administration with *B. bifidum* ORF9 was preceded by the treatment of *S. dysenteriae* for four consecutive days, a significant decrease in the number of *Shigella* in the feces was also observed. The suppression of the number of fecal *Shigella* is thought to be due to the proliferation of administered *B. bifidum*. When a pathogenic *E. coli* was administered instead of *S. dysenteriae*, a similar phenomena was also observed (data not shown). Therefore, we believe that the administration of *B. bifidum* can suppress almost all of the putrefactive bacteria residing in the intestinal tract. Ozawa (1985) has also reported that *Enterococcus faecalis* suppressed the proliferation of putrefactive organisms such as *Salmonella* and yeast in the intestinal tract. Yamashita et al. (1985) has again reported that the administration of *Streptococcus* preparation suppressed the proliferation of putrefactive bacteria. In conclusion, *B. bifidum* OFR9 is a good lactic acid bacterium having the original biochemical characteristics of bifidobacteria as well as high resistance to antituberculosis and antileprosy agents.

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References

Adelberg, E. A., Mandell, M., and Chen, G. C. C. (1965) Optimal conditions for mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K12. *Biochem. Biophys. Res. Commun.*, **18**, 788–795.

Barefoot, S. F. and Klaenhammer, T. R. (1983) Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.*, **45**, 1808–1815.

Choi, E. C., Kim, S. H., Kwan, A. R., Lee, M. J., Oh, J. J., and Kim, B. K. (1993) Development of *Streptococcus faecalis* strains resistant to rifampicin. *Yakhak Hoeji*, **37**, 350–357.

Choi, K. H., Kim, D. J., Shim, J. S., Choi, M. J., Park, N. J., Kim, B. O., Yu, Y. H., and Choi, E. C. (1994) In vitro and in vivo activity of DWC-751, a new cephalosporin. *J. Antimicrob. Chemother.*, **33**, 1233–1237.

Committee on Treatment of International Union against Tuberculosis and Lung Disease (1988) Antituberculosis regimens of chemotherapy. *Bull. Int. Union Tuberc. Lung Dis.*, **63**, 60–67.

Gordon, D., Macrae, J., and Wheater, D. M. (1957) *Lactobacillus* preparation for use with antibiotics I–II–III. *Lancet*, **1**, 899–903.

James, H. T., Richard, W. M., and Gwynn, R. C. (1983) Rapid selection of organisms with increasing resistance on subinhibitory concentration of norfloxacin in agar. *Antimicrob. Agents Chemother.*, **26**, 188–189.

John, B. J. (1975) *Genetics*, Houghton Mifflin Co., Boston, p. 352.

Kang, K. H., Shin, H. J., Park, Y. H., and Lee, T. S. (1989) Studies on antibacterial substances produced by lactic acid bacteria: Purification and some properties of antibacterial substance "bifilong" produced by *Bifidobacterium longum*. *Korean J. Dairy Sci.*, **11**, 204–216.

Kim, H. S., Choi, S. H., Choi, E. C., Lee, J. C., and Kim, T. H. (1989) Development of *Bacillus coagulans* (*Lactobacillus sporogenes*) resistant to rifampicin, an antituberculosis agent. *Korean J. Microbiol.*, **27**, 155–161.

Modler, H. W., Mckellar, R. C., and Yaguch, M. V. (1990) Bifidobacteria and bifidogenic factors. *Can. Inst. Food Sci. Technol. J.*, **23**, 29–41.

National Committee for Clinical Laboratory Standards (NCCLS) (1993) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard M7-A3, 3rd ed., NCCLS, Villanova, Philadelphia.

Ozawa, K. (1985) Growth of *Enterococcus faecalis* (*Streptococcus faecalis*) BIO-4R in the intestinal tract of animals and humans. *Bifidobacteria Microflora*, **4**, 15–22.

Rasic, J. L. (1983) The role of dairy foods containing bifidobacteria and acidophilus bacteria in nutrition and health. *N. Eur. Dairy J.*, **48**, 80–85.

Sasaki, T., Maeda, Y., and Namioka, S. (1987) Immunopotentiation of the mucosa of the small intestine of weaning piglets by peptidoglycan. *Jpn. J. Vet. Sci.*, **49**, 235–239.

Schwartz, A. C. and Stadtman, T. C. (1970) Small colonies of *Clostridium sticklandii* resulting from nitrosoguanidine treatment and exhibiting defect in catabolic enzymes. *J. Bacteriol.*, **104**, 1242–1246.

Yamashita, M., Hatano, M., Nakamura, H., Murakami, R., Matsuo, M., Matsuo, T., and Kumon, Y. (1985) Effects of Biofermin-R administered in combination with antibiotics on the fecal flora. *Bifidobacteria Microflora*, **4**, 23–30.