INTERACTIONS BETWEEN *BIFIDOBACTERIUM BIFIDUM* N4 AND *ESCHERICHIA COLI* K-12 IN THEIR MIXED CULTURES¹

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Summary The interactions between *Bifidobacterium bifidum* N4 (*B. bifidum*) and *Escherichia coli* K-12 (*E. coli*) were investigated in their mixed cultures.

Under conditions in which both bacteria grew well in their pure culture, *B. bifidum* inhibited the growth of *E. coli* even when the latter was inoculated at 10⁴-fold and preincubated for 41 hr. The inhibition in the mixed cultures appeared when the pH values were reduced below 4.6. When the lowering of pH was prevented by the addition of NaOH, no inhibition was observed. At the same initial pH of 6.5, lactic acid and acetic acid, metabolites of *B. bifidum*, had more inhibitory effect on the growth of *E. coli* than other aliphatic fatty acids.

On the other hand, in the mixed cultures with *E. coli*, *B. bifidum* grew in the absence of its essential vitamins, riboflavin and pantoic acid and, furthermore, aerobically.

Bifidobacteria are predominantly living bacteria in the digestive duct (1) and believed to be associated with human health, especially that of infants (2). There have been many attempts to increase the number of *Bifidobacteria* in the intestine (3). Their proliferation in the duct is closely related to their interactions with other intestinal microorganisms, which should be elucidated.

Interactions of *Lactobacillus acidophilus* (*L. acidophilus*), which has properties similar to *Bifidobacterium bifidum* (*B. bifidum*), with different kinds of bacteria have

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been reported. TORREY and KAHN (4) reported that *Bacterium acidophilus* (now called *L. acidophilus*) inhibited the proteolytic anaerobes and this inhibition was due to the lowering of pH values caused by the former metabolites, lactic acid and acetic acid. YOUNG *et al.* (5) also mentioned that in the interaction between *Candida albicans* and *L. acidophilus* there were parasitic and antagonistic phenomena.

In this paper we made media for the selective counting of the number of viable cells grown in the culture by a capillary tube method, originally developed by YANAGITA (6), and then the parasitic and antagonistic phenomena between *B. bifidum* N4 and *Escherichia coli* K-12 (*E. coli*) in their mixed cultures were investigated from nutritional and physiological points of view.

MATERIALS AND METHODS

Chemicals. Polypeptone (Daigo Nutrition Chemicals Co.), bacto-agar, casamino acids and yeast extract (Difco Laboratories), and sodium azide (E. Merck) were used. Other chemicals were of analytical grade. A set of pH indicator papers (Toyo Roshi Co.) were used for pH measurement.

Bacteria. *B. bifidum* N4 and *E. coli* K-12 were used throughout this experiment.

Determination of the growth of both bacteria. The viable cells of *B. bifidum* and *E. coli* in a sample were determined by the capillary tube method using the selective media. The selective media for both bacteria are shown in "RESULTS." The procedures were performed according to NAKAMURA *et al.* (7). The turbidities of cultures were measured by a Photo-Electric Spectrophotometer Type 6B (Hirama Rika Kenkyujo, Kawasaki) at 650 nm.

Preincubation. Both bacteria were preincubated in a preculture medium (8) for *B. bifidum* (arbitrarily called Medium D), which consists of 3.2 g polypeptone, 8.0 g casamino acids, 0.4 g L-cysteine, 4.8 g yeast extract, 10 g lactose, 3.2 g NaCl in 1,000 ml of distilled water adjusted to pH 6.8. *B. bifidum* was incubated at 37°C under an atmosphere of 10% CO₂: 90% N₂ (anaerobic incubation) and *E. coli* aerobically at 37°C for 24 hr.

Preparation of inoculum. Both bacteria were separately preincubated, washed three times with 5 ml of sterile physiological saline and suspended in 5 ml of the saline. After proper repetitions of ten-fold dilutions with the saline, a 0.1 ml aliquot was inoculated into the media described below to make the final volume 7 ml.

Medium for testing the interactions between *B. bifidum* and *E. coli*. For this purpose two standard media were used. One was Medium D and another a complete synthetic medium, modified György medium used by NAKAMURA *et al.* (9) (arbitrarily called GM-medium). The composition of GM-medium is as follows: 2.5 g K₂HPO₄; 35 g lactose; 25 g CH₃COONa; 5.0 g vitamin-free casamino acids; 0.2 g alanine, L-cysteine and tryptophan; 0.1 g asparagine; 0.01 g xanthine, adenine, guanine and uracil; 0.2 mg thiamine · HCl and riboflavin; 1.2 mg pyridoxine; 0.6 mg

nicotinic acid; 12.5 μ g PABA (*p*-aminobenzoic acid), folic acid and biotin; 50 μ g pantetheine; 200 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 10 mg NaCl; 6 mg MnSO_4 in 1,000 ml of distilled water at pH 6.8.

RESULTS

Selective media for counting the number of viable cells of B. bifidum and E. coli

Medium D, a preculture medium for *B. bifidum*, was added with 0.002% BCG (bromocresol green) and 0.15% agar, and modified as below to produce the media for the capillary tube method.

From the data in Table 1, 0.02% NaN_3 was added to the medium for counting the number of viable cells of *B. bifidum*. Coexistence of *E. coli* had no influence on the production of the colonies as in Table 2.

Table 1. Effect of sodium azide on the number of colonies of *B. bifidum* and *E. coli*.

NaN_3 (%)	<i>B. bifidum</i> (10^5 diln.) Mean $\pm$ SD	<i>E. coli</i> (10^0 diln.) Mean
0	183 $\pm$ 8.7	++*
0.004	193 $\pm$ 9.5	+
0.008	212 $\pm$ 6.6	0
0.012	189 $\pm$ 8.0	0
0.016	195 $\pm$ 17.0	0
0.020	202 $\pm$ 11.0	0

The experiments were carried out in triplicate. The data are given as the number of colonies produced in 0.5 ml of the counting medium, (++) or (+) represents that the growth of *E. coli* was detectable, but the number of colonies was not determined due to the occurrence of cracks.

Table 2. Number of colonies produced by *B. bifidum* in the presence of *E. coli* using the selective medium for *B. bifidum*.

Test strain		Number of colonies
<i>B. bifidum</i> (diln.)	<i>E. coli</i> (diln.)	Mean $\pm$ SD
10^5	10^0	130 $\pm$ 6.7
10^5	10^1	152 $\pm$ 2.7
10^5	10^2	155 $\pm$ 15.9
10^5	10^3	159 $\pm$ 16.8
10^5	10^4	152 $\pm$ 3.2
10^5	10^5	155 $\pm$ 18.0
10^5	10^6	140 $\pm$ 13.3
10^5	*	160 $\pm$ 3.5

Both 0.1 ml of 10^5 -fold diluted culture of *B. bifidum* and appropriately diluted culture of *E. coli* were added to selective medium for *B. bifidum*. The experiments were carried out in triplicate. The data were shown as in Table 1. The symbol (*) represents that saline was replaced with diluted culture of *E. coli*.

As reported by NAKAMURA *et al.* (10), no colonies of *B. bifidum* were detected by removing lactose from the medium. From the data in Table 3, 1.5% KNO₃ (6) was added to the medium to prevent the appearance of any cracks produced by *E. coli*. Coexistence of *B. bifidum* did not affect the production of the colonies as in Table 4.

Table 3. Effect of potassium nitrate on the suppression of cracks produced by *E. coli*.

KNO ₃ (%)	<i>E. coli</i> (10 ⁵ diln.)	
	Mean $\pm$ SD	Occurrence of cracks
0	240 $\pm$ 35.6	+ + *
0.1	258 $\pm$ 5.5	— *
0.2	264 $\pm$ 14.0	— *
0.5	244 $\pm$ 9.0	— *
1.0	259 $\pm$ 20.8	— *
1.5	251 $\pm$ 14.6	— *
2.0	244 $\pm$ 31.8	— *

The experiments were carried out in triplicate. The data are given as the number of colonies produced by *E. coli* in 0.5 ml of the lactose-free counting medium. The symbols (+ + *) and (— *) represent the presence and absence of cracks in the counting medium, respectively.

Table 4. Number of colonies produced by *E. coli* in the presence of *B. bifidum* using the selective medium for *E. coli*.

Test strain		Number of colonies
<i>E. coli</i> (diln.)	<i>B. bifidum</i> (diln.)	Mean $\pm$ SD
10 ⁵	10 ⁰	214 $\pm$ 20.5
10 ⁵	10 ¹	193 $\pm$ 19.9
10 ⁵	10 ²	173 $\pm$ 6.7
10 ⁵	10 ³	178 $\pm$ 9.5
10 ⁵	10 ⁴	184 $\pm$ 20.7
10 ⁵	10 ⁵	184 $\pm$ 9.3
10 ⁵	10 ⁶	188 $\pm$ 4.2
10 ⁵	*	187 $\pm$ 2.1

Both 0.1 ml of 10⁵-fold diluted culture of *E. coli* and appropriately diluted *B. bifidum* were added to selective medium for *E. coli*. The experiments were carried out in triplicate. The data were shown as in Table 3. The symbol (*) represents that saline was replaced with diluted culture of *B. bifidum*.

Interactions between both bacteria under the conditions in which they grew well in their pure culture

In order to grow well in their pure culture, they were incubated anaerobically at 37°C in Medium D. Figure 1 shows the growth and pH values in their pure culture.

Fig. 1. Growth of *B. bifidum* and *E. coli* in an anaerobic pure culture and the pH values in their culture. An aliquot (0.1 ml) of suspension containing *B. bifidum* or *E. coli* (about 10^6 viable cells/ml) precultured according to the procedures described in this text, was inoculated into 6.9 ml of Medium D and incubated anaerobically for 92 hr at 37°C. Symbols (—●—) and (—○—) represent the viable cells in 1 ml of each culture of *B. bifidum* and *E. coli*, respectively. Triangles (—▲—, —△—) similarly represent the pH values of the culture.

The generation time of *E. coli* was shorter than that of *B. bifidum*, and the pH value of the culture of *B. bifidum* (3.8) became lower than that of *E. coli* (4.6). In their mixed culture under the same conditions (Fig. 2), the pH value of the culture approached 4.0 after 30 hr, when the growth of *E. coli* was depressed rapidly. Additionally, even when the number of viable cells of *E. coli* inoculated was 10^4 -fold that of *B. bifidum*, and when *B. bifidum* was inoculated into the culture in which *E. coli* had been incubated for 41 hr, the growth of *E. coli* was similarly inhibited (not shown in the Figure).

As described above, the inhibition of the growth of *E. coli* was closely associated with the pH values of the cultures. Therefore, we attempted to prevent the pH values of the cultures from lowering by the addition of an appropriate quantity of 2N NaOH just after samplings. As shown in Fig. 3, no inhibition was observed. On the contrary, as shown in Table 5, some acids had an inhibitory effect on the growth of *E. coli* even at the initial pH of 6.5. Acetic acid and lactic acid, the metabolites of *B. bifidum* showed a relatively potent inhibition, and PABA, a bacterial vitamin, completely inhibited the growth of *E. coli* at a higher concentration (60 mM).

Fig. 2. Growth of *B. bifidum* and *E. coli* in their anaerobic mixed culture and the pH values in their culture. Aliquots (0.1 ml) of bacterial suspension (about 10^6 viable cells/ml) were inoculated into 6.8 ml of Medium D. The following procedures were the same as in Fig. 1. Arrows (∇) indicate that the number of viable cells/ml of the culture are under the indicated values. Viable cells/ml (—●—, *B. bifidum*; ---○---, *E. coli*). pH values (---△---).

Fig. 3. Effect of prevention of pH values of the culture from lowering on the growth of both bacteria in the anaerobic mixed culture. Aliquots (0.1 ml) of bacterial suspension (about 10^6 viable cells/ml) were inoculated into 6.8 ml of Medium D and incubated for 95 hr at 37°C. During incubation, an appropriate quantity of 2N NaOH was added to the culture to prevent pH values from lowering. Arrows represent the times when NaOH was added. Viable cells/ml (—●—, *B. bifidum*; ---○---, *E. coli*). pH values (---△---).

Interactions between both bacteria under conditions in which B. bifidum did not grow in the pure culture

B. bifidum required riboflavin and pantetheine in anaerobic incubation and did not grow in aerobic incubation even in complete GM-medium (Table 6). On the

Table 5. Effect of acids on the growth of *E. coli*.

Acid	(pK _{a1})	Growth response OD _{650 nm} incubation time (hr)			
		10	20	35	51
Hydrochloric	—	0.72	0.83	0.87	0.89
Tartaric	2.93	0.05	0.58	1.05	1.12
Fumaric	3.03	0.11	1.06	1.29	1.32
Citric	3.09	0.62	1.42	1.47	1.54
Formic	3.75	0.08	0.91	1.00	1.13
Lactic	3.87	0.35	0.46	0.47	0.54
Succinic	4.21	0.24	0.63	0.70	0.71
p-Aminobenzoic	4.65	0.01	0.01	0.02	0.03
Acetic	4.76	0.02	0.36	0.55	0.61
Butyric	4.82	0.02	0.25	0.61	0.63
Propionic	4.87	0.01	0.16	0.60	0.63

An aliquot (0.1 ml) of the suspension of *E. coli* (10⁶ viable cells/ml) was inoculated into the 7 ml of Medium D (pH 6.5) containing 60 mM of test compound and incubated anaerobically for 51 hr at 37°C. The OD values at 650 nm were corrected for those of the blank.

Table 6. Requirement of vitamins for the growth of *B. bifidum* and *E. coli* and effect of anaerobic condition on it.

Medium	Growth response OD _{650 nm}			
	<i>B. bifidum</i>		<i>E. coli</i>	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Complete	0.02	1.10	0.45	0.36
(-) Thiamine	0.02	0.94	0.47	0.27
(-) Riboflavin	0.04	0.13	0.56	0.32
(-) Pyridoxine	0.03	0.89	0.53	0.30
(-) Nicotinic acid	0.03	0.69	0.55	0.34
(-) PABA	0.04	0.79	0.48	0.29
(-) Folic acid	0.05	0.57	0.57	0.36
(-) Biotin	0.02	0.57	0.50	0.38
(-) Pantetheine	0.02	0.06	0.50	0.20

An aliquot (0.1 ml) of the suspension of *B. bifidum* or *E. coli* (10⁶ viable cells/ml) was inoculated into 7 ml of a vitamin deficient GM-medium and incubated aerobically or anaerobically for 100 hr at 37°C. The OD values at 650 nm were corrected for those of blank.

other hand, *E. coli* grew aerobically better than anaerobically and had no requirement for vitamins. We then investigated whether *B. bifidum* could grow or not in a mixed culture with *E. coli* under conditions in which the former did not grow in the pure culture. The growth of *B. bifidum* in riboflavin-deficient medium was enhanced significantly in the presence of *E. coli*, while such an enhancement disappeared after the decrease of viable cells of *E. coli* (Fig. 4). On the other hand, the growth of *B. bifidum* in the pantetheine-deficient medium was slightly stimulated by *E. coli* and a decrease of *E. coli* was not observed (Fig. 5).

B. bifidum did not survive aerobically in the pure culture but grew fully in the presence of *E. coli*. On the other hand, the growth of *E. coli* was inhibited completely after the pH value of the culture reached 4.6 (Fig. 6).

Fig. 4. Growth of bacteria under anaerobic incubation with riboflavin-deficient GM-medium. Aliquots (0.1 ml) of bacterial suspension (about 10^6 viable cells/ml) were inoculated into 6.8 ml of GM-medium and incubated for 120 hr at 37°C. Viable cells/ml in pure culture (—●—, *B. bifidum*; —○—, *E. coli*) and in mixed culture (---●---, *B. bifidum*; ---○---, *E. coli*). pH values in mixed culture (---△---).

Fig. 5. Growth of bacteria under anaerobic incubation with pantetheine-deficient GM-medium. The procedures were the same in Fig. 4. Viable cells/ml in pure culture (—●—, *B. bifidum*; —○—, *E. coli*) and in mixed culture (---●---, *B. bifidum*; ---○---, *E. coli*). pH values in mixed culture (---△---).

Fig. 6. Growth of bacteria under aerobic incubation with pyridoxine-deficient GM-medium. The procedures were the same as in Fig. 4. Viable cells/ml in pure culture (—●—, *B. bifidum*; —○—, *E. coli*) in mixed culture (---●---, *B. bifidum*; ---○---, *E. coli*). pH values in mixed culture (---△---).

DISCUSSION

NAKAMURA *et al.* (7) developed a capillary tube method for counting *B. bifidum* viable cells by the use of a modified Medium D. This medium became selective for the bacteria by the addition of sodium azide and was not influenced by the presence of *E. coli* in our experiments (Tables 1 and 2). On the contrary, by the addition of potassium nitrate and the subtraction of lactose, the medium became suitable for counting of viable cells of *E. coli* (Tables 3 and 4).

B. bifidum is anaerobic and has a high requirement for nutrients. On the other hand, *E. coli* is aerobic and has a low requirement for nutrients. In their mixed culture, parasitism was remarkable under the conditions where *B. bifidum* was not able to grow by itself. Firstly, anaerobic condition, in which oxygen molecules toxic for the growth of *B. bifidum* exist, was changed to anaerobic by the better growth of *E. coli*. Secondly riboflavin and pantetheine, essential vitamins for the growth of *B. bifidum*, were probably provided in the medium by *E. coli*, though the supply of pantetheine is insufficient. It seems to be due to the contamination with biotin in vitamin-free casamino acids that *B. bifidum* did not require the vitamin for the growth.

B. bifidum is acid-producing while *E. coli* is not resistant to acids. When the former grew well in the mixed culture, the growth of *E. coli* was inhibited markedly probably because of the lowering of the pH values of the medium (Figs. 2, 3, 4, 5 and 6). The additional toxic effect of acetic and lactic acids by themselves (Table 5) would not be negligible though the mechanism is not clear.

There is a possibility that parasitism and antagonism between *Bifidobacteria* and *E. coli* similar to those observed *in vitro* might be present in the intestine in the formation of intestinal flora.

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