Inhibitory Effects of Human-Derived *Bifidobacterium* on Pathogenic *Escherichia coli* Serotype O-111

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Abstract The inhibitory effects of seven human-derived *Bifidobacterium* strains on pathogenic *Escherichia coli* serotype O-111 were examined in co-cultivation. All of the *Bifidobacterium* strains tested inhibited the growth of *E. coli* and limited the increase in the ammonia concentration in the co-cultures. Also, in each instance, the pH of the culture medium decreased due to their production of acetic and lactic acids. From the results of pH-controlled fermentation, the growth inhibition of *E. coli* was attributed mainly to the decrease in the pH of the culture medium. The possibility that some unidentified inhibitory substances derived from *Bifidobacterium* strains were present was little thought. In mono-cultures of *Bifidobacterium* strains, the ammonia concentration in the culture medium decreased to a level below that initially present in the medium prior to inoculation. Enzymatic assays showed that the activity of an enzyme involved in ammonia production (L-asparagine deaminase) was much weaker in each of the *Bifidobacterium* strains than in *E. coli*, whereas the activities of enzymes involved in ammonia assimilation (glutamine synthetase, glutamate synthase and glutamate dehydrogenase) were much higher in bifidobacteria. The decrease in the ammonia concentration in the co-culture appears to be related not only to the number of colony-forming units of bifidobacteria but also to the differences among the bacteria examined in enzymatic activities which are concerned with utilization and production of ammonia.

Key words: bifidobacteria; *Escherichia coli*; O-111; inhibition ammonia

Bifidobacteria comprise a major group in the human and animal intestinal flora along with bacteroides and eubacteria. This genus is divided into 28 species, including seven species isolated from the human intestine: *B. breve* and *B. infantis*, isolated mainly from babies and *B. adolescentis*, *B. longum*, *B. bifidum*, *B. catenulatum* and *B. pseudo-catenulatum*, isolated mainly from adults (6). In the large intestine, bifidobacteria produce acetic and lactic acids and is thought to inhibit the proliferation of putrefactive bacteria such as escherichiae, clostridia and eubacteria, and inhibit the synthesis of harmful products (reviewed in 5). Recently,

reports were presented which attributed these inhibitory effects of bifidobacteria to both the decrease in the pH of the culture medium and the produced acetic and/or lactic acids themselves (4), or to some unidentified inhibitory substance derived from bifidobacteria (3).

Here, the inhibitory effects of seven human-derived *Bifidobacterium* strains on pathogenic *E. coli* serotype O-111 were examined in co-cultivation by measuring the number of bacterial colony-forming units (CFUs), the pH and the ammonia concentration of the culture medium. Ammonia is known to be one of the harmful substances pro-

duced by intestinal bacteria. Assays of several bacterial enzymes concerned with changes in the ammonia concentration were also performed.

MATERIALS AND METHODS

Bacterial strains. *B. adolescentis* ATCC 15703^T, *B. catenulatum* ATCC 27675, *B. bifidum* ATCC 15696, *B. breve* ATCC 15700^T, *B. infantis* ATCC 15697^T and *B. longum* ATCC 15707^T were purchased from the American Type Culture Collection. *B. pseudocatenulatum* M101-4 was a gift from Prof. T. Mitsuoka (8). *Escherichia coli* serotype O-111 was obtained from the Institute of Medical Science, The University of Tokyo. Hereafter, each strain is represented by its species name.

Culture conditions. Cultivation was performed as previously described (1) except in pH-controlled fermentation. pH-controlled cultivation was performed using a jar fermenter (Mini Jar Fermenter Model MB-C, Iwashiyama A.D.M. Co., Ltd., Shinagawa, Japan) with continuous stirring at 90 rpm and supplying with CO₂ gas continuously. CFUs were counted as previously described (1).

Preparation of cell suspensions and cell-free extracts. Cell suspensions and cell-free extracts were prepared as previously described (1).

Assays. Concentrations of acetic and lactic acids in the culture were assayed using F-Kits (Boehringer Mannheim).

Ammonia and protein concentrations were assayed as previously described (1).

Urease and deaminases for 20 L-amino acids were assayed at pH 7.0 as previously described (1). One unit of urease or deaminase activity was defined as the amount that produces one micromole of ammonia per minute. Specific activities are shown as the activity per one OD₆₆₀ unit per milliliter of cell suspension.

Glutamate dehydrogenase (GDH), glutamate synthase (GOGAT), and Glutamine synthetase (GS) were assayed as previously described (1). The reaction pH was 7.5 for GDH and GOGAT assays, and 6.5 for GS assay. One unit of GDH or GOGAT activity was defined as the amount that oxidizes one micromole of NADPH per minute. One unit of GS activity was defined as the amount

that synthesizes one micromole of γ -glutamyl-hydroxamate per minute. Specific activities of these three enzymes are shown as the activity per milligram of protein in the cell-free extract.

Measurement of pH. pH in the culture media was measured using a model F-13 pH meter (Horioka, Japan).

Note. All experiments were performed in duplicate and means of duplicates are presented in figures and tables.

RESULTS

Co-Cultivation with Seven Human-Derived Bifidobacterium Strains and E. coli Serotype O-111

To examine the inhibitory effects of *Bifidobacterium* on the growth of *E. coli*, co-cultivation was performed for 24 hr and samples were taken at 4-hr intervals for measurement of pH, bacterial CFUs and ammonia concentration. The inoculation ratio of each *Bifidobacterium* strain to *E. coli* O-111 was mixed up to about 1 : 1. Mono-cultivation of these strains was also performed as the control. The results are shown in Fig. 1 and Table 1. In all cases, the growth curves of *E. coli* in co-cultivation with bifidobacteria (Fig. 1 from A to G) showed different patterns when compared with that obtained in mono-cultivation (Fig. 1H), whereas the growth curves of the *Bifidobacterium* strains were similar to those in the mono-cultures (Fig. 1 from A to G). With each *Bifidobacterium* strain tested, the CFU of *E. coli* after incubation for 24 hr decreased to a level below 30% of that observed in mono-culture (Table 1). The ammonia concentration was lower than that in mono-culture in each case with the exception of *B. catenulatum* (Fig. 1B, Table 1). These inhibitory effects, especially the decrease in CFU of *E. coli*, seemed to depend on the lowering of the pH of the culture medium. *E. coli* cannot grow below 4.5 (2), and the survival ratio was higher when the pH of the co-culture was above 4.5, as with *B. catenulatum* and *B. infantis* (Table 1). The decrease in the ammonia concentration seemed to depend not only on the lowering of pH and/or the resulting decrease in CFU of *E. coli* but also on the increase in CFU of *Bifidobacterium* because the ex-

Fig. 1. Growth of *E. coli* and (A) *B. adolescentis*, (B) *B. catenulatum*, (C) *B. pseudocatenulatum*, (D) *B. bifidum*, (E) *B. breve*, (F) *B. infantis* and (G) *B. longum* in co-cultures, and (H) *E. coli* in mono-culture as a control.

From (A) to (G), data of each *Bifidobacterium* strain in mono-culture are also shown. CFUs of each *Bifidobacterium* strain in co-culture ($\bullet$) and in mono-culture ($\circ$), CFU of *E. coli* ($\blacktriangle$), pH of co-culture and *E. coli* mono-culture ($\blacksquare$) and of *Bifidobacterium* mono-culture ($\square$), and ammonia concentration in co-culture and *E. coli* mono-culture ($\blacksquare$) and in *Bifidobacterium* mono-culture ($\square$) were measured at the times indicated.

Table 1. Decrease in CFU of *E. coli* and ammonia concentration in co-culture medium with *E. coli* and *Bifidobacterium* after 24 hr of incubation^a

Strain cultured with <i>E. coli</i>	pH	CFU ^b (%)	Ammonia ^c (%)
<i>B. adolescentis</i>	4.12	0.00	71.01
<i>B. catenulatum</i>	4.71	31.45	100.00
<i>B. pseudocatenulatum</i>	4.22	0.00	81.27
<i>B. bifidum</i>	4.37	3.83	91.77
<i>B. breve</i>	4.19	0.11	72.84
<i>B. infantis</i>	4.52	22.17	77.67
<i>B. longum</i>	4.09	0.19	64.67
No strain	5.15	100 ^b	100 ^c

^a The data used here were the values shown in Fig. 1.

^b % of the CFU of *E. coli* after 24 hr of cultivation in the co-culture when the CFU of *E. coli* in the mono-culture was taken as 100%.

^c % of the increase in the ammonia concentration after 24 hr of cultivation in the co-culture when the increase in the ammonia concentration in the *E. coli* mono-culture was taken as 100%.

tent of inhibition of ammonia production was higher with *B. infantis*, in which case the pH was above 4.5 and the CFU of *B. infantis* was above 10^9 CFU/ml (Fig. 1F), than it was with *B. pseudocatenulatum* or *B. bifidum*, in which cases the pH was below 4.5 but the CFU of each *Bifidobacterium* strain was below 10^9 CFU/ml (Fig. 1C, D).

pH-Controlled Co-Cultivation of *B. longum* and *E. coli*

As mentioned above, the growth inhibition of *E. coli* seemed to be due to the decrease in the pH of the culture medium. However, the potential influence of the produced acetic and/or lactic acids themselves or of some unidentified inhibitory substances derived from bifidobacteria could not be excluded. In order to confirm that the decrease in CFU of *E. coli* in co-cultures results from the lowering of pH, co-cultivation of *B. longum* and *E. coli* was performed under pH-controlled conditions. *B. longum* was chosen for its strong ability to lower the pH of the culture medium. The results are shown in Fig. 2. Comparing the results obtained under pH-controlled (Fig. 2A, C) and pH-uncontrolled (Fig. 2B, D) conditions, the ammonia concentration in the culture medium was similar (Fig. 2A, B) and the molar ratios of the

Table 2. Activities of ammonia-assimilating enzymes

Strain	GS [U/mg]	GOGAT [mU/mg]	GDH [U/mg]
<i>B. adolescentis</i>	4.71	38.64	1.42
<i>B. catenulatum</i>	0.63	64.64	0.47
<i>B. pseudocatenulatum</i>	3.20	30.46	0.72
<i>B. bifidum</i>	1.84	51.27	1.16
<i>B. breve</i>	0.66	77.27	5.22
<i>B. infantis</i>	1.33	66.13	1.04
<i>B. longum</i>	8.24	40.12	2.93
<i>E. coli</i>	0.03	2.09	0.30

acetate and lactate concentrations after 24 hr of cultivation were 1.09 and 0.74, respectively, by defining the values under pH-uncontrolled condition as 1.0 (Fig. 2C, D), whereas the growth curve patterns of both *E. coli* and *B. longum* were completely different (Fig. 2A, B). The CFU of *E. coli* was always higher than that of *B. longum* under pH-controlled conditions and, conversely, the CFU of *B. longum* was always higher than that of *E. coli* under pH-uncontrolled conditions. The CFU of *B. longum* reached a maximum of 5×10^8 CFU/ml in both instances.

Activities of Enzymes Which Affect the Ammonia Concentration

It was shown that in mono-cultures of *Bifidobacterium* the ammonia concentration of the culture medium decreased to a level below that present in the medium prior to inoculation (Fig. 1, from A to G). Since in the mono-cultivations of *B. catenulatum* (Fig. 1B) and *B. infantis* (Fig. 1F) the decrease in ammonia was smaller than that with *B. breve* (Fig. 1E) and *B. longum* (Fig. 1G) although the CFU values were similar, the activities of enzymes which assimilate or produce ammonia were considered to be important. Therefore, the activities of enzymes known to assimilate (Table 2) and produce (Table 3) ammonia were examined. In Table 2, the activities of GS and GDH differed according to the magnitude of the decrease of the ammonia concentration in the culture medium (Fig. 1, from A to G). The contribution of GOGAT activity was rather unclear. In Table 3, the activities of ammonia-producing enzymes were rather

Fig. 2. (A, B) Growth of *E. coli* and *B. longum* in co-cultures under (A) pH-controlled and (B) pH-uncontrolled conditions. Marks used are the same as in Fig. 1. (C, D) End product formation in co-cultures under (C) pH-controlled and (D) pH-uncontrolled conditions. Acetic acid (■) and lactic acid (▨) concentrations were measured at the times indicated.

Table 3. Distribution of ammonia-producing enzymes^a

Strain	Deaminase [mU/OD unit ml]					
	Asn	Asp	Gln	Ile	Leu	Lys
<i>B. adolescentis</i>	3.53	—	16.64	—	—	1.32
<i>B. catenulatum</i>	1.25	—	17.15	—	—	1.22
<i>B. pseudocatenulatum</i>	4.03	—	20.16	—	—	1.37
<i>B. bifidum</i>	—	—	15.38	—	—	—
<i>B. breve</i>	—	—	13.19	—	—	—
<i>B. infantis</i>	—	—	13.50	1.19	1.44	1.48
<i>B. longum</i>	—	—	16.63	1.00	—	—
<i>E. coli</i>	99.05	2.23	15.48	—	—	2.80

^a Though urease and deaminases for 20 L-amino acids were assayed as ammonia-producing enzymes, enzymes whose activities were not detected in any strain are omitted.

similar among *Bifidobacterium* species, but a greater number of enzymes were active in *B. catenulatum* and *B. infantis* than in *B. breve* and *B. longum*. *E. coli* was found to have much lower activities of the ammonia-assimilating enzymes (Table 2) and higher activity of an enzyme involved in ammonia production (L-asparagine deaminase) (Table 3)

compared with the *Bifidobacterium* strains tested.

DISCUSSION

In this study, the inhibitory effects of seven human-derived *Bifidobacterium* species on a pathogenic strain of *E. coli* was examined. Our results demonstrate that at least in the case of *B. longum*

the growth inhibition of *E. coli* was attributed mainly to the lowering of pH and, to some extent, to the produced acetic and lactic acids themselves. The findings that support this conclusion are the following. First, the molar ratio of the lactate concentration under pH-controlled and -uncontrolled conditions after 24 hr of cultivation was 0.74, taking the value under the pH-uncontrolled condition as 1.0, and it might be involved in the difference in inhibition. Second, and more fundamentally, as the pH of the medium decreases, the Δ pH across the cytoplasmic membrane becomes larger, and as a result the cells might tend to accumulate acetic and lactic acids to a greater extent. So the effects of these acids in bacterial cells cannot be excluded. Third, the production of some unidentified inhibitory substance by *Bifidobacterium* seems unlikely, unless such a substance is produced only after the pH of the culture medium decreased, since the maximum CFU of *B. longum* was similar under pH-controlled and -uncontrolled conditions.

Ammonia induces hepatic encephalitis and coma in patients with cirrhosis or other liver diseases when it exists at a high concentration in the human body (7). Bifidobacteria have been thought to play a role in reducing the levels of harmful substances including ammonia in the gastrointestinal tract by inhibition of intraintestinal ammonia-producing bacteria (5). From our results, the decrease in the ammonia concentration in the culture medium is thought to depend neither on the lowering of pH nor on the decrease in CFU of *E. coli* (Fig. 2A, B). Rather, it appears to depend on the CFU of each *Bifidobacterium* strain

(Fig. 1) and the differences observed among the bacteria tested in the activities of enzymes which assimilate (Table 2) and produce (Table 3) ammonia. Though the participation of GOGAT was not suggested, this enzyme might be unimportant since it does not directly consume ammonia while GS and GDH do.

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