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Interference of *In Vitro* and *In Vivo* Growth of Several Intestinal Bacteria by *Lactococcus* Strains

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The ability of *Lactococcus* strains to inhibit the growth of intestinal bacteria was examined. In *in vitro* cocultures, we observed that among eighteen *Lactococcus* strains tested, the ability to inhibit growth of *Escherichia coli* varied, with the *L. lactis* N7 showing the greatest growth inhibition. Strain N7 (8.94×10^{10} CFU/day for 7 days) was orally administered to mice, and the viable count of strain N7 in feces appeared at a level of 10^{4-5} CFU/g. After administration, the proportion of *Bacteroidaceae* to total intestinal bacteria decreased. Lactococci may act as probiotic bacteria by inhibiting the growth of harmful bacteria.

Keywords: Probiotics, lactococci, growth inhibition, intestinal microflora

Intestinal microflora have a major influence on the ability of the host to resist various diseases. The bacterial inhabitants in the human gastrointestinal tracts consist of a wide variety of genera and species, including beneficial and harmful ones. Beneficial bacteria suppress external harmful bacteria and stimulate immune functions. If the balance between beneficial and harmful bacteria is disturbed and harmful bacteria become predominant, there is an increase in toxic, carcinogenic, or mutagenic products, which cause various diseases. The major manifestation of enteric infection is diarrhea. For example, at least 80% of traveler's diarrhea is caused by bacterial enteropathogens.

In recent years, the probiotic activities of several lactic acid bacteria (LAB) have been emphasized. Probiotics may be defined more fully as "live supplements which beneficially affect the host animal by improving its intestinal microbial balance" [3]. It is well known that probiotic LAB may hamper the virulence of pathogens through mechanisms such as competitive adhesion to intestinal cells, competition for nutrition, and production of antibacterial substances [2, 6, 9, 11, 19]. The most widely used probiotic bacteria were lactobacilli and bifidobacteria isolated from human or animal intestinal tracts.

In contrast, there have been few studies concerning the probiotic effects of *Lactococcus* strains, although they play important roles in the manufacturing of dairy products, because they are not generally considered to be natural habitants in the gastrointestinal tracts. However, several works have shown that lactococci can reach to the human or animal intestines alive [7, 14, 16, 20]. Furthermore, *Lactococcus* strains were recently shown to have some desirable properties such as adhesion to Caco-2 cells [12], improvement of lipid metabolism [17], immunomodulatory activity [13], and anti-aging activity in mice [15]. However, there are few reports concerning the effect of *Lactococcus* strains on intestinal microflora [5]. The aim of the present study was to determine whether *Lactococcus* strains are antagonistic toward enteric bacteria.

Eighteen strains of lactococci were examined for their ability to inhibit the *in vitro* growth of the enteric bacterium *Escherichia coli* MAFF 911145 by using the method of Gilliland and Speck [6]. All strains, except strain HP, inhibited the growth of *E. coli* MAFF 911145, at varying ratios (Fig. 1). *L. lactis* subsp. *lactis* biovar *diacetylactis* N7 was the most effective in inhibiting *E. coli* and thus was selected for the next experiments.

As the number of N7 cells increased, *E. coli* was more effectively inhibited (Table 1). The antagonistic action of strain N7 was better than that of other species of LAB tested, except *Enterococcus faecalis* IFO 12964. In this experiment, the inhibitory effects caused by low pH and exhaustion of nutrients were eliminated by replacing the GAM medium (Nussui Seiyaku, Tokyo, Japan) [10] at 8–10 h intervals [2]. As a result, the growth of *E. coli* was

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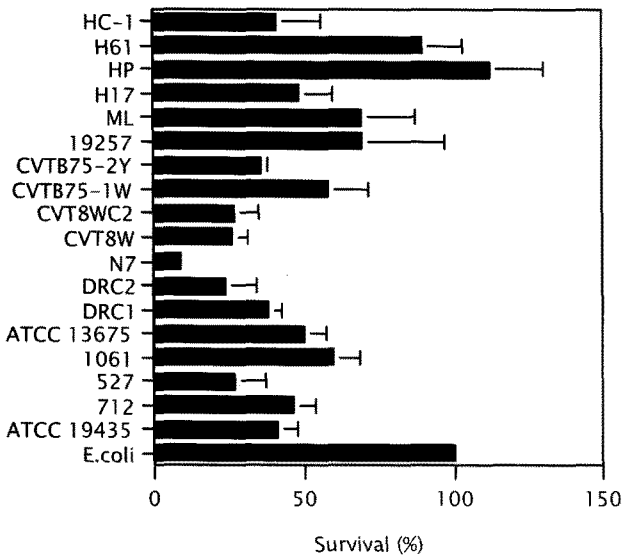

Fig. 1. Survival of *Escherichia coli* MAFF 911145 by co-culture with *Lactococcus* strains.

Results are mean values of two trials in duplicate, with SE represented by vertical bars. A tube containing 5 ml of GAM broth was inoculated with 10^3 colony-forming unit (CFU)/ml of *E. coli* and 10^7 CFU/ml of *L. lactis* strains. Controls (not inoculated with *L. lactis* strains) and co-cultures were incubated anaerobically for 24 h at 37°C. At the end of the incubation time, the viable counts of *E. coli* were enumerated on desoxycholate agar. All plates were incubated for 48 h at 37°C.

inhibited by 16%, a rate similar to that seen in the same co-culture where changes in pH were not eliminated (Fig. 1). This indicates that acidification of the culture medium by strain N7 was not the major cause of growth inhibition of *E. coli*. In this study, strain N7 was grown under anaerobic conditions; hydrogen peroxide production was unlikely to be the cause of the observed inhibition.

Table 1. Growth inhibition of *E. coli* MAFF 911145 by co-culture with *Lactococcus* strains and other lactic acid bacteria in replaced medium.

Bacteria	Survival of *E. coli* (%) Inoculative rate of lactic acid bacteria to *E. coli*	
	1	10^4
Enterococcus faecalis IFO 12964	63	6
Enterococcus faecium IFO 13712	67	21
Lactobacillus acidophilus MAFF 401301	110	79
Lactobacillus gasseri JCM 1022	76	53
Lactobacillus gasseri JCM 1131	92	60
Lactococcus lactis subsp. *lactis* biovar *diacetylactis* 8W	77	22
Lactococcus lactis subsp. *lactis* biovar *diacetylactis* N7	73	16

To eliminate the effect of a decrease in pH resulting from acid produced by lactic acid bacteria and nutrient consumption, at 8–10 h intervals all media were replaced as follows; Lactic acid bacteria was inoculated simultaneously with *E. coli* into broth. After incubation, cultures were centrifuged and pellets were resuspended in fresh medium. All values are the means for two trials.

Table 2. Inhibition of intestinal bacteria by *L. lactis* N7 in co-culture.

Test culture	Sample	CFU of pathogen[a]/ml
Escherichia coli MAFF 911145	Control	4.46×10^8
	Strain N7	7.33×10^7
Escherichia coli TISTR 527	Control	7.49×10^8
	Strain N7	1.97×10^8
Klebsiella pneumoniae NGRI G-1	Control	3.36×10^9
	Strain N7	2.90×10^8
Staphylococcus aureus IFO 15055	Control	1.15×10^9
	Strain N7	3.50×10^6
Staphylococcus aureus TISTR O29	Control	1.52×10^8
	Strain N7	6.00×10^5

[a]Determined after 24 h of incubation of associative cultures at 37°C.

All values are the means for two trials.

A tube containing 5 ml of GAM broth was inoculated with 10^3 colony-forming unit (CFU)/ml of the enteric bacteria (*E. coli*, *K. pneumoniae*, and *S. aureus*) and 10^7 CFU/ml of strain N7. Controls and co-culture were incubated anaerobically for 24 h at 37°C. At the end of the incubation time, the viable counts of *E. coli* and *K. pneumoniae* were enumerated on desoxycholate agar and those of *S. aureus* were enumerated on mannitol salt agar. All plates were incubated for 48 h at 37°C.

Strain N7 was also effective in inhibiting the growth of harmful bacteria such as *Klebsiella pneumoniae* and *Staphylococcus aureus* (Table 2). In particular, the growth of *S. aureus* was reduced by 3 log compared with controls after 24 h incubation. Control cultures of the pathogens were at pH 5.79 and the associative cultures were at pH 5.6–6.0. The decreases in pH resulting from growth of strain N7 in co-culture do not appear sufficient to be entirely responsible for the antagonistic actions exerted on the pathogens.

Inhibitory activity of culture supernatant of strain N7 toward enteric bacteria (*E. coli*, *K. pneumoniae*, and *S. aureus*) was determined by paper-disk assay [2]. No inhibition of these enteric bacteria was observed for the tested supernatant (data not shown), indicating that antimicrobial substances such as bacteriocins were not produced by strain N7. As described above, large numbers of strain N7 seemed to be more effective in inhibiting the growth of *E. coli*. Thus, the possible mechanism of growth inhibition by strain N7 may be that it acts as a competitive culture.

These results suggest that the presence of strain N7 in large quantity in the intestine might effectively suppress harmful intestinal bacteria such as *E. coli*, *Klebsiella* species, and *S. aureus*. Previous studies showed that strain N7 could survive in the mouse intestine [14]. If a certain amount of viable cells of strain N7 is present in the intestine, it might affect the microflora. In the present study, the

effect of strain N7 on the growth of enteric bacteria in mice was evaluated.

Adult female BALB/c mice (n=8), obtained from Charles River Japan Inc. (Kanagawa, Japan), were housed with the basic feed (MM-3; Funabashi Farm, Chiba, Japan). Water was provided *ad libitum*. Cells of strain N7 were administrated to mice orally as a suspension in 10% (wt/vol) nonfat milk at a single dose of 8.94×10^{10} CFU per mouse per day for 7 days. Before administration and on day 8, feces were collected. The ability of strain N7 to pass through and survive in the gastrointestinal tract was investigated by analyzing the recovery of the strain in fecal samples during oral administration to mice using *L. lactis*-specific PCR amplification [14, 18]. Generally, undigested but dead bacteria can also be detected by PCR analysis. Thus, in the present study, PCR for quantitative analysis was performed on the colony grown on M17 agar (Becton, Dickinson and Company, MD, U.S.A.) to detect live bacteria in feces. As the results, *L. lactis* was not detected in feces before administration. During administration, strain N7 appeared at a level of 10^4–10^5 CFU/g in feces from six out of eight mice tested, and was not detected in feces from two mice.

Table 3. Viable count of several intestinal bacteria in feces and proportion to total aerobic and anaerobic bacteria before and after administration of *L. lactis* N7.

	Log CFU/g feces	Population to total bacteria (%)
Enterobacteriaceae		
Before administration	7.40	3.70
After administration	7.50	2.50
Enterococcus/Streptococcus		
Before administration	6.90	0.20
After administration	6.30	0.20
Lactobacillus		
Before administration	9.40	56.0
After administration	9.20	58.2
Bifidobacterium		
Before administration	ND	
After administration	ND	
Bacteroidaceae		
Before administration	8.90	31.9
After administration	8.70	16.6
Total bacteria		
Before administration	9.60	100.0
After administration	9.50	100.0

ND, not detected.

All values are means for two trials. Each trial involved 4 mice.

Stool samples at serial dilutions with 0.85% NaCl solution were streaked on modified LBS agar (84 g LBS agar, 8 g Lab-lemco powder, 15 g sodium acetate·$3H_2O$, 3.7 ml acetic acid, 1 l distilled water) for *Lactobacillus* sp.; NBGT agar (1 l EG agar, 50 ml NBGT solution containing 2.5 g sodium taurocholate, 0.5 g neomycin sulfate, and 5 mg brilliant green) for *Bacteroides* sp.; BS agar (50 ml BS solution containing 30 g sodium propionate, 100 mg paromomycin sulfate, 400 mg lithium chloride, and 6 g neomycin sulfate) for *Bifidobacterium* sp.; DHL agar for *Enterobacteriaceae*; and TATAC agar (15 g peptone, 10 g tryptone, 10 g yeast extract, 1 g sucrose, 1 g esculin, 16 g agar, 1 l distilled water, 50 ml horse serum, 22 ml TATAC I solution containing 0.099 g sodium azide and 6.6 g sodium glutamate, 20 ml TATAC II solution containing 2 g acridine orange, 20 mg triphenyl tetrazolium chloride, 0.33 g thallous sulfate, and 6.5 mg crystal violet) for *Enterococcus/Streptococcus* sp. Trypticase Soy Blood Agar was used as a nonselective medium for aerobic bacteria. EG agar and BL agar were used as nonselective media for anaerobic bacteria. After 24–48 h incubation at 37°C, colonies were counted.

To determine viable counts of intestinal bacteria, collected feces from mice were inoculated in selective agar for each intestinal bacterium. As a result, the proportion of *Bacteroidaceae* to total intestinal bacteria decreased from 31.9% to 16.6% (Table 3). Many enteric bacteria including the genera *Bacteroides* have β-glucuronidase activity [4, 8], which has been linked to the development of cancer [1, 21]. On the other hand, administration of strain N7 did not significantly affect the growth of *Enterobacteriaceae*, *Lactobacillus* species, or *Enterococcus/Streptococcus* species. One of the reasons may be that healthy mice were used in this study. Oral administration of strain N7 might be useful in mice with unbalanced microflora. Another possible reason is that the viable count of strain N7 in the intestine might be low. Our previous study revealed that strain N7 administered to BALB/c mice could not colonize the intestine [14]. We await methods that will allow large numbers of viable strain N7 to reach the intestine, and/or allow strain N7 to adhere to intestinal cells and multiply to achieve a large cell count. For example, some compounds such as a capsule, which protects strain N7 against gastrointestinal stresses, may be useful. The survival of strain N7 may be different in humans compared with mice. The effect of strain N7 on the intestinal microflora of humans will be the subject of future studies.

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