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Note

Amounts and species of probiotic lactic acid bacteria affect stimulation of short-chain fatty acid production in fecal batch culture

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The effects of lactate and probiotic lactic acid bacteria (LAB) on intestinal fermentation were analyzed using a fecal batch culture. Lactate was efficiently metabolized to butyrate and propionate by butyrate-utilizing bacteria in fecal fermentation. Probiotic LAB could stimulate butyrate and propionate production through their lactate production in fecal fermentation. It was considered that 10^9 cfu/g or more of probiotic LAB would be required to stimulate butyrate and propionate production in the large intestine. Due to the low production of lactate, a larger number of heterofermentative LAB than homofermentative LAB would be required for this stimulation.

Key words: probiotics, lactate, short-chain fatty acids

Numerous bacteria are harbored in the human large intestine. These bacteria are attracting attention because they have great effects on human health, such as on immune function and metabolic function [1–3]. Health-promoting effects of short-chain fatty acids (SCFAs) produced by the bacterial fermentation of undigested food material and endogenous substances have been reported [4–7]. Therefore, stimulation of SCFA production in the large intestine has been considered to be effective for promoting host health. It is well known that intake of probiotic lactic acid bacteria (LAB) improves the large intestinal microbiota. Since lactate produced by probiotic LAB is metabolized to propionate and butyrate by indigenous lactate-utilizing bacteria, intake of probiotic LAB stimulates production propionate and butyrate in the large intestine [8–11]. However, the amount of probiotic LAB required to affect SCFA production in the large intestine has not been shown as far as we know. Although there are many kinds of probiotic LAB, differences in the effects on SCFA production in the large intestine among them have not been examined. In this study, we investigated the effects of each of four probiotic LAB on fecal fermentation using a batch culture method to determine the amount of probiotic LAB required to affect SCFA production and to clarify differences in the effects on SCFA production among probiotic LAB.

First, to confirm the usefulness of the fecal batch culture method, we examined the metabolism of lactate to SCFAs in fecal culture. Fresh human feces were collected from five healthy males (21 to 25 years old) who consumed normal Japanese diets. Each fecal sample was immediately diluted with 24 volumes of phosphate-buffered saline (PBS) containing 0.5% (w/v) L-cysteine HCl monohydrate (PBS+L) adjusted to pH 7.4. After the diluted samples were stirred well, they were squeezed through four layers of surgical gauze under an N_2 atmosphere. Six milliliters of each strained fecal slurry was inoculated into a 20 mL bottle, which contained substrates resolved in 6 mL of PBS+L. Soluble starch (2 g/L; Sigma-Aldrich, St. Louis, MO, USA), pectin (0.5 g/L; Kanto Chemical, Tokyo, Japan), inulin (0.5 g/L; Wako Pure Chemical, Osaka, Japan), xylan (0.5 g/L; Sigma-Aldrich), arabinogalactan (0.5 g/L; Sigma-Aldrich), guar gum (0.5 g/L; Sigma-Aldrich), and mucin (0.5 g/L; Wako Pure Chemical) were used as fermentation substrates. In addition, sodium lactate was added to them to a final concentration of 20 mM. A culture without sodium lactate was used as a control. The bottles were filled with N_2 gas and closed with butyl rubber stoppers and aluminum clips. The experiment was carried out in triplicate. After incubation at 37°C for 48 hr, the culture slurry in each bottle was sampled to analyze its organic acid concentration and microbiota. For organic analysis, the sampled culture slurry was treated according to Ohashi *et al.* [12]. Its organic acid content was then analyzed via ion-exclusion high-performance liquid chromatography (HPLC) as described by Ushida and Sakata [13]. Using bacterial genomic DNA extracted from the culture slurry according to Godon *et al.* [14], the copy numbers of 16S rRNA genes in the *Roseburia*/*Eubacterium rectale* group, *Eubacterium hallii*, butyrate-producing bacterium SS2/1, and

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Anaerostipes caccae and *Veillonella* spp. were quantified via real-time polymerase chain reaction (PCR) with each specific primer, as described previously [12, 15, 16]. Fecal collection and culture were done between March 2014 to March 2017. This study was performed in accordance with the Helsinki Declaration as updated in Brazil in 2013. The details of this study were explained to all volunteers. Written informed consent was obtained from all volunteers before the experiment. Statistical analysis was performed using the Wilcoxon test.

After incubation, lactate was hardly detectable in any of the cultures. The acetate concentration was not significantly different between the control and lactate-added cultures, but the propionate and butyrate concentrations were significantly higher in the lactate-added culture than in the control (Table 1). These results suggested that lactate was efficiently metabolized to propionate and butyrate. However, there was no significant difference in the copy number of 16S rRNA genes in the *Roseburia*/*E. rectale* group between the lactate-added culture and the control (Table 1), even though the *Roseburia*/*E. rectale* group is one of the abundant groups of butyrate-producing bacteria in the human large intestine. On the other hand, the copy numbers of 16S rRNA genes in *E. hallii*, butyrate-producing bacterium SS2/1, and *A. caccae* and *Veillonella* spp. were significantly higher in the lactate-added culture than in the control (Table 1). *E. hallii*, butyrate-producing bacterium SS2/1, and *A. caccae* are known to be lactate-utilizing, butyrate-producing bacteria, and *Veillonella* spp. are known to be lactate-utilizing, propionate-producing bacteria [8, 9]. These lactate-utilizing bacteria appeared to contribute to the metabolism of lactate to butyrate and propionate. Thus, it was shown that lactate is metabolized to propionate and butyrate by lactate acid-utilizing bacteria in fecal culture in this study as well as in previous studies [8–11]. The fecal batch culture system used in this study is valuable method for accurate evaluation of the effects of probiotic LAB on large intestinal fermentation. Therefore, we conducted a second experiment using this fecal batch culture system to determine the number of probiotic LAB required to affect SCFA production and to clarify differences in the effects on SCFA production among probiotic LAB.

Four probiotic LAB (A, B, C, and D) that were isolated from commercially available fermented milk and stored in our laboratory were used. Probiotic LAB A, B, C, and D had previously been identified as *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, and *Levilactobacillus brevis*, respectively. They were anaerobically incubated in 4 mL of MRS broth (Becton Dickinson, Franklin Lakes, NJ, USA) using AnaeroPack (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) at 37°C for 24 hr. The culture solutions were then centrifuged at 10,000 × g for 1 min at 4°C. After collection, the bacterial cells were suspended in PBS+L. The bacterial density of 150 µL of this solution was measured using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA) at OD 650 nm and was adjusted to 2.0. With this preparation, we confirmed that the number of bacteria in the bacterial solution is approximately 10^{10} cfu/mL. The prepared bacterial solutions were further diluted 10-fold and 100-fold with PBS+L and used as inocula in the fecal cultures. Since there are interindividual differences in fecal microbiota, lactate-utilizing bacteria in particular, to standardize the differences, equal amounts of each fresh feces sample collected from three healthy females (22 years old) were mixed and subjected to fecal culture. The fecal culture was performed as described above. For the probiotic fecal culture, 100 µL of each prepared probiotic bacterium was inoculated into the fecal culture. Instead of the probiotic inoculum, as in the first experiment, PBS+L and a lactate solution were used as a negative control and positive control, respectively. After incubation, the organic acid and microbiota in the culture slurry were analyzed in the same manner as described above. However, the microbiota were analyzed in one culture randomly selected from the triplicate cultures. The difference in the concentration of each SCFA between the negative or positive control and each culture was analyzed by Dunnett's test.

Among all the cultures, the highest concentration of SCFAs was found in the positive control (Fig. 1), indicating that lactate was metabolized to SCFAs in this experiment as in the previous experiment. Inoculation with 10^{10} cfu/mL of probiotic bacteria A, B, and C resulted in significantly higher concentrations of butyrate and lower concentrations of acetate than in the negative controls. Lactate-utilizing, butyrate-producing bacteria metabolize lactate to butyrate using acetate [8]. These bacteria could be considered to contribute to butyrate production in fecal cultures inoculated with 10^{10} cfu/mL of probiotic bacteria A, B, and C. The concentrations of propionate in fecal cultures inoculated with 10^{10} cfu/mL of probiotic bacteria A and C were higher than in the negative control. This suggested that the lactate

Table 1. Effect of sodium lactate on the production of short-chain fatty acids (mM) and the copy number of bacterial 16S rRNA genes (log copy number of 16S rRNA genes/mL) in the fecal batch culture

	Control	Lactate[1]
Short-chain fatty acids		
Acetate	9.4 ± 1.7	8.3 ± 3.1
Propionate	3.0 ± 0.4	6.1 ± 2.6**
Butyrate	4.1 ± 1.0	11.5 ± 3.1**
Bacteria		
Eubacterium hallii	8.1 ± 0.5	8.6 ± 0.6**
Bacterium SS2/1	7.3 ± 0.7	8.1 ± 0.7**
Anaerostipes caccae	7.1 ± 0.6	7.5 ± 0.8**
Roseburia/*Eubacterium rectale*	8.4 ± 1.9	8.5 ± 1.9
Veillonella spp.	8.7 ± 1.1	9.1 ± 1.4**

[1]Sodium lactate was added to the fecal batch culture to a final concentration of 20 mM.
**p<0.01 (vs. Control).
Values are means ± SD (n=5).

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produced by probiotic bacteria could be metabolized not only to butyrate but also to propionate. However, the concentrations of butyrate and propionate and the copy numbers of 16S rRNA genes of *E. hallii*, bacterium SS2/1, and *A. caccae* and *Veillonella* spp. in these probiotic cultures were lower than those in the positive control (Table 2). It is clear that the smaller the amount of lactate produced by probiotic LAB, the smaller the amounts of propionate and butyrate produced from lactate. We speculate that the amount of lactate produced by probiotic LAB would not be sufficient to stimulate propionate and butyrate production as in the positive control.

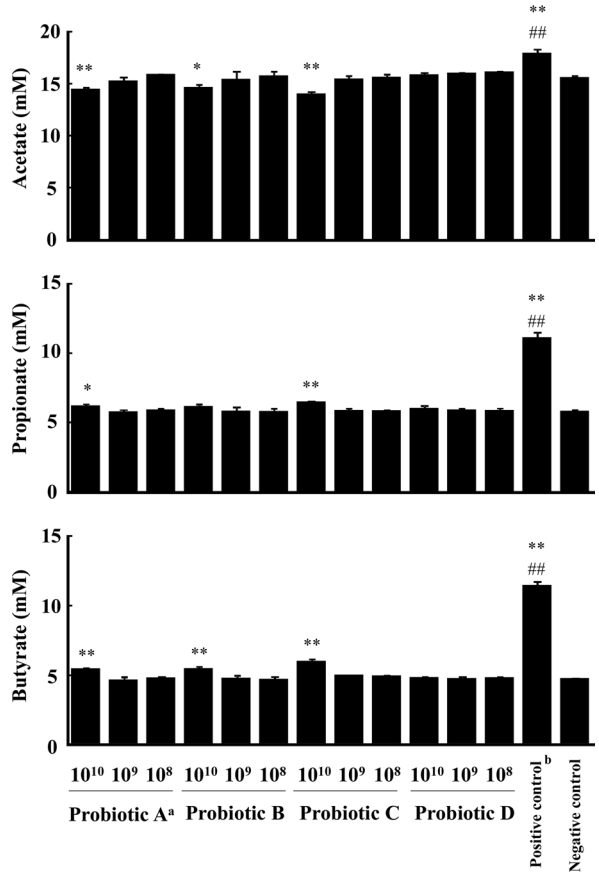

Fig. 1. Effect of probiotic LAB on the production of acetate, propionate, and butyrate in the fecal batch culture.

[a]Probiotic bacteria A, B, C, and D were identified as *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, and *Levilactobacillus brevis*, respectively. Probiotic LAB solutions (10^{10}, 10^9, and 10^8 cfu/mL) were inoculated into the fecal batch culture.

[b]Sodium lactate was added to the fecal batch culture to a final concentration of 20 mM.

*p<0.05; **p<0.01 (vs. negative control).

##The values of the positive control were significantly higher than the other values (p<0.01).

Values are means ± SD (n=3).

Inoculation with 10^9 cfu/mL or less of any of the probiotic bacterial solutions did not affect the SCFA concentration compared with the negative control. In these cultures, lactate production by probiotic LAB would be insufficient to affect SCFA production. Ingested probiotic LAB would compete with indigenous microbiota for the fermentative substrate. In this competition, a large number of probiotic LAB would be needed to produce sufficient lactate to stimulate butyrate and propionate production. In this study, which inoculated fecal cultures with 10^{10} cfu/mL of probiotic bacterial solutions, the number of probiotic LAB in the fecal cultures was calculated to be approximately 10^8 cfu/mL. This suggests that 10^8 cfu/mL probiotic LAB were required to stimulate butyrate and propionate production in this fecal fermentation. Furthermore, considering the fecal dilution ratio in this study, it was speculated that 10^9 cfu/g of probiotic LAB would be required to produce sufficient lactate to stimulate butyrate and propionate production in the large intestinal fermentation. It has been reported that 10^6/g probiotic bacteria are required to produce health benefits [17, 18]. This bacterial number differs 100 to 1,000 times from the above estimates. This may be due to the difference in the desired health benefits. *In vivo* verification may also be needed, as more complex factors affect intestinal fermentation than were investigated in this *in vitro* study. The impact of probiotic LAB on SCFA production in the large intestine likely depends on the amount of lactate produced by them, which would be influenced by the number of lactate-utilizing bacteria and the competition between the probiotic LAB and indigenous bacteria for fermentation substrates. In order to stimulate butyrate and propionate production by intake of probiotic LAB, it is necessary for as many probiotic LAB as possible to reach the large intestine alive.

On the other hand, probiotic bacterium D did not affect SCFA production. This bacterium, identified as *L. brevis*, is a heterofermentative lactic acid bacterium. The other probiotic bacteria used in this study are homofermentative LAB. The amount of lactate produced from 1 mol of glucose by heterofermentative LAB is half that by homofermentative LAB. Therefore, it was considered that heterofermentative LAB might

Table 2. Effect of 10^{10} cfu/mL of the probiotic lactic acid bacteria (LAB) solution on the copy number of bacterial 16S rRNA genes (log copy number of 16S rRNA genes/mL) in the fecal batch culture

	Probiotic LAB[a]				Positive control[b]	Negative control
	A	B	C	D		
Eubacterium hallii	8.0	7.9	8.1	8.0	8.6	7.9
Bacterium SS2/1	7.8	7.7	7.9	7.6	8.1	7.6
Anaerostipes caccae	7.9	7.6	8.0	7.6	8.4	7.8
Roseburia/Eubacterium rectale	10.1	10.1	10.1	10.0	10.3	10.1
Veillonella spp.	8.1	8.1	8.3	8.1	8.4	7.9

[a]Probiotic bacteria A, B, C, and D were identified as *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, and *Levilactobacillus brevis*, respectively.
[b]Sodium lactate was added to the fecal batch culture to a final concentration of 20 mM.

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require a greater number of bacteria than homofermentative LAB to stimulate butyrate and propionate production in large intestinal fermentation.

In conclusion, probiotic LAB could stimulate butyrate and propionate production in the large intestine through their lactate production. The impact of this stimulation would depend on the activity of the probiotic LAB. Therefore, it is desirable for as many probiotic LAB as possible to reach the large intestine alive in order to overcome the competition with indigenous microbiota for the fermentative substrate. The present study suggests that 10^9 cfu/g or more of probiotic bacteria in the large intestine would be required to stimulate butyrate and propionate production. In the case of heterofermentative LAB, a larger number of bacteria would be required than for homofermentative LAB.

REFERENCES

1. Kataoka K. 2016. The intestinal microbiota and its role in human health and disease. J Med Invest 63: 27–37. [Medline] [CrossRef]
2. Sommer F, Anderson JM, Bharti R, Raes J, Rosenstiel P. 2017. The resilience of the intestinal microbiota influences health and disease. Nat Rev Microbiol 15: 630–638. [Medline] [CrossRef]
3. Aron-Wisnewsky J, Warmbrunn MV, Nieuwdorp M, Clément K. 2021. Metabolism and metabolic disorders and the microbiome: the intestinal microbiota associated with obesity, lipid metabolism, and metabolic health-pathophysiology and therapeutic strategies. Gastroenterology 160: 573–599. [Medline] [CrossRef]
4. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. 2008. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther 27: 104–119. [Medline] [CrossRef]
5. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. 2012. Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3: 289–306. [Medline] [CrossRef]
6. Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, Terasawa K, Kashihara D, Hirano K, Tani T, Takahashi T, Miyauchi S, Shioi G, Inoue H, Tsujimoto G. 2013. The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 4: 1829. [Medline] [CrossRef]
7. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-YM, Glickman JN, Garrett WS. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341: 569–573. [Medline] [CrossRef]
8. Duncan SH, Louis P, Flint HJ. 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol 70: 5810–5817. [Medline] [CrossRef]
9. Ohashi Y, Ushida K. 2009. Health-beneficial effects of probiotics: its mode of action. Anim Sci J 80: 361–371. [Medline] [CrossRef]
10. Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, Flint HJ, Louis P. 2014. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISME J 8: 1323–1335. [Medline] [CrossRef]
11. Markowiak-Kopeć P, Śliżewska K. 2020. The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome. Nutrients 12: 1107. [Medline] [CrossRef]
12. Ohashi Y, Harada K, Tokunaga M, Ishihara N, Okubo T, Ogasawara Y, Juneja LR, Fujisawa T. 2012. Faecal fermentation of partially hydrolyzed guar gum. J Funct Foods 4: 398–402. [CrossRef]
13. Ushida K, Sakata T. 1988. Effect of pH on oligosaccharides fermentation by porcine cecal digesta. Anim Sci Technol 69: 20–27.
14. Godon JJ, Zumstein E, Dabert P, Habouzit F, Moletta R. 1997. Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl Environ Microbiol 63: 2802–2813. [Medline] [CrossRef]
15. Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97: 1166–1177. [Medline] [CrossRef]
16. Veiga P, Gallini CA, Beal C, Michaud M, Delaney ML, DuBois A, Khlebnikov A, van Hylckama Vlieg JE, Punit S, Glickman JN, Onderdonk A, Glimcher LH, Garrett WS. 2010. *Bifidobacterium animalis* subsp. *lactis* fermented milk product reduces inflammation by altering a niche for colitogenic microbes. Proc Natl Acad Sci USA 107: 18132–18137. [Medline] [CrossRef]
17. Charteris WP, Kelly PM, Morelli L, Collins JK. 1998. Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J Appl Microbiol 84: 759–768. [Medline] [CrossRef]
18. Penna FJ, Filho LA, Calçado AC, Junior HR, Nicolli JR. 2000. [Up-to-date clinical and experimental basis for the use of probiotics]. J Pediatr (Rio J) 76 Suppl 1: S209–S217 (in Portuguese). [Medline] [CrossRef]

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