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Production of a Mixture of Antimicrobial Organic Acids from Lactose by Co-Culture of *Bifidobacterium longum* and *Propionibacterium freudenreichii*

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The antimicrobial activities of standard solutions of three organic acids (lactic, acetic, and propionic acids) were compared using *Micrococcus luteus*, *Pseudomonas* sp. and *Staphylococcus aureus* as test microorganisms. At the same concentrations of the undissociated form, the antimicrobial activities of acetic and propionic acids were higher than that of lactic acid, irrespective of test microorganisms. In a single cultivation of *Bifidobacterium longum*, a mixture of lactic (17 g/l) and acetic (20 g/l) acids was produced from 50 g/l lactose and its antimicrobial activities against *M. luteus*, *Pseudomonas* sp., and *S. aureus* correspond to that of 32, 19, and 25 g/l of acetic acid, respectively. To increase the total antimicrobial activity, a co-culture of *B. longum* and *Propionibacterium freudenreichii*, in which lactic acid produced once from lactose by *B. longum* was converted to acetic and propionic acids by *P. freudenreichii*, was done using TPY medium containing commercially available peptones as a nitrogen source. By the sequential conversion of lactose using the two microorganisms, the culture supernatant containing a mixture of acetic (27 g/l) and propionic (13 g/l) acids without lactic acid was produced. The antimicrobial activities of the mixture against *M. luteus*, *Pseudomonas* sp., and *S. aureus* were 35, 30, and 26 g/l as a concentration of acetic acid, respectively, higher than that obtained in the cultivation of *B. longum* alone. When the medium containing an enzymatic hydrolyzate of whey proteins with a protease was used in the co-culture of *B. longum* and *P. freudenreichii*, the culture supernatant containing the mixture of organic acids was also obtained in the same manner as the co-culture using TPY medium and the activities were 43, 29, and 29 g/l as a concentration of acetic acid for *M. luteus*, *Pseudomonas* sp. and *S. aureus*, respectively.

Key words: co-culture; antimicrobial culture; food preservative; *Bifidobacterium longum*; *Propionibacterium freudenreichii*

A wide variety of combinations of different methods such as packing, storage at low temperatures, heat and pressure treatments, addition of chemicals, and fermentation have been used for prevention of food spoilage by microorganisms and for extending the shelf life of many foods. Among food preservation methods, fermentation is considered to be one of the most favorable procedures because the products fermented by food microorganisms have been consumed safely by humans for hundreds of years and have increased quality and functions (digestibility, taste, flavor etc.) compared with the original food materials.[1-4] Just like yeasts, lactic acid bacteria are typical food microorganisms safe for humans. A large number of species of lactic acid bacteria used for the production of fermented foods such as dairy, meat, cereal, and vegetable products have been shown to be strongly antagonistic toward spoilage organisms and pathogens.[1-4] This inhibition is attributed mainly to organic acids,[1,5,6] hydrogen peroxide,[2,4] diacetyl,[2,4] and bactericidal proteins (bacteriocins).[1-4] The use of these cell-free materials offers obvious advantages over the use of whole cultures containing cells as a food preservative due to minimizing texture and flavor changes, especially in non-fermented foods. However, lactic acid, one of the inhibitory metabolites from heterofermentative as well as homofermentative lactic acid bacteria, has only a weak antimicrobial activity and was not used widely as a food preservative. Moreover, nisin, one of the antibiotic polypeptides, has a narrow spectrum and acts only on Gram-positive bacteria.[7,8] Therefore, to use the metabolites of lactic acid bacteria widely as a safe preservative it is necessary to increase the antimicrobial activity of metabolites from lactic acid bacteria.

In this work, improvement of the antimicrobial activity of a mixture of organic acids produced from sugar was studied. Thus, since *Bifidobacterium* produces lactic and acetic acids at a molar ratio of 2:3 from sugar as a carbon source[9] but the culture supernatant after removing the cells is now not always used, we selected

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Bifidobacterium longum as a safe food microorganism for producing antimicrobial metabolites. We attempted to improve the total antimicrobial activity of an organic acid mixture in fermented broth of *B. longum* by converting lactic acid to acetic and propionic acids using *Propionibacterium freudenreichii*, which is used as a starter for making Swiss-type cheese.[1,6] We investigated the evaluation of the antimicrobial activity of both standard organic acids and fermented broth from *B. longum*. In addition, we report on the production of the fermented matter having high antimicrobial activity from lactose, one of the wastes in the dairy industry, by sequential conversion in a co-culture using *B. longum* and *P. freudenreichii*.

Materials and Methods

Microorganisms. *B. longum* M61 and *P. freudenreichii* 7025 obtained from the Central Research Institute, Meiji Milk Products Co., Tokyo were used throughout this study.

Fermentation. *B. longum* and *P. freudenreichii* were cultivated at 37°C in TPY medium containing 50 g of lactose, 8 g of Trypticase Peptone (Becton Dickinson Microbiology System, USA), 3 g of Phytone Peptone (Becton Dickinson Microbiology System, USA), 5 g of yeast extract (Difco Laboratories, USA), 2 g of K_2HPO_4, 3 g of KH_2PO_4, 0.5 g of $MgCl_2 \cdot 6H_2O$, 0.5 g of L-cysteine, and 0.01 g of $FeSO_4 \cdot 7H_2O$ per liter (pH 6.5). Ten g/l of lactic acid was also used as a carbon source for a single culture of *P. freudenreichii*. The microorganisms were cultured in test tubes and inoculated into TPY medium at an initial turbidity of 0.05–0.1 at 660 nm. Cultivations were done in a fermentor (TBR-1, Chiyoda Seisakusho Co., Nagano, Japan) with a working volume of 700 ml. The pH was kept at 6.5 by addition of 4 N NaOH using a peristaltic pump coupled to a pH controller. A mixed gas of N_2 and CO_2 at a volume ratio of 9:1 was sparged at 0.3 vvm throughout the fermentation to maintain anaerobic conditions. In co-culture, *B. longum* and *P. freudenreichii* were inoculated simultaneously into TPY medium or whey medium (this medium was prepared as described below) containing 50 g of lactose. The initial turbidity (about 0.5 at 660 nm) of *P. freudenreichii* was usually adjusted to be about 5-fold as high as that of *B. longum* because of a slow growth rate of the former.

Preparation of whey medium. To increase digestibility of proteins in cheese whey powder (a gift of the Central Research Institute, Meiji Milk Products Co.), 5.5% of the whey powder was hydrolyzed with 0.025% protease (Protease A: Amano Pharmaceutical Co., Nagoya, Japan) at 50°C and pH 7.0 for 4 h. The whey hydrolyzate was used instead of Trypticase and Phytone Peptones in TPY medium. The whey medium was prepared by adding yeast extract and lactose to the whey hydrolyzate at final concentrations of 0.5% and 5%, respectively.

Measurement of antimicrobial activity. The antimicrobial activity was measured by the plate diffusion assay method using paper discs. *Micrococcus luteus* IFO 13867, *Pseudomonas* sp. 7012, and *Staphylococcus aureus* 7009 were used as the test microorganisms and cultivated at 30°C for 24 h. The latter two were obtained from the Central Research Institute, Meiji Milk Products Co. The assay agar (1%) medium for *M. luteus*, *Pseudomonas* sp., and *S. aureus* contained 10 g of Polypepton (Nippon Pharmaceutical Co.), 3 g of meat extract (Kyokuto Pharmaceutical Co.), 1.5 g of yeast extract (Oriental Yeast Industries Co.), 3 g of NaCl, and 1 g of glucose per liter (pH 6.0), 17 g of Tryptone (Eiken Chemical Co.), 3 g of Soy Peptone (Eiken Chemical Co.), 2.5 g of K_2HPO_4 and 2.5 g of glucose per liter (pH 7.3), and 17 g of Polypepton, 3 g of yeast extract, 2.5 g of K_2HPO_4, 5 g of NaCl and 2.5 g of glucose per liter (pH 7.0), respectively.

The commercially available organic acids (lactic, propionic, and acetic acids) and the supernatant obtained after centrifuging the culture broth at 17,000 × *g* for 10 min were used as sample solutions for the plate assay. Here, the pH of standard organic acid solutions was adjusted in the range of 6.5 to 3.5 by adding a 6 N HCl solution or a 6 N NaOH solution. On the other hand, the pH of supernatant solution was adjusted to 3.5 by 2 N or 4 N HCl to remove the influence of pH on the growth of test microorganisms for the plate assay. The antimicrobial activity of the supernatant was estimated by comparison with a concentration of standard acetic acid and expressed as a corresponding acetic acid concentration.

Other analytical methods. The cell concentration was measured by the turbidity at 660 nm. The number of viable cells for *B. longum* and *P. freudenreichii* was measured by the plate culture method. BL agar medium (Eiken Chemical Co., Tokyo) containing selective reagents (propionic acid, neomycin sulfate, paromomycin sulfate, and lithium chloride)[10] and YEL medium with 10 g/l of sodium lactate were used for counting the number of colonies of *B. longum* and *P. freudenreichii*, respectively. The supernatant obtained by centrifugation (17,000 × *g*, 10 min) was analyzed for measurement of lactose, lactic acid, propionic acid, and acetic acid concentrations. Lactose was measured by HPLC with a Shim-Pack CLC 101C column (Shimadzu, Kyoto) and a refractive index detector (RID-6A, Shimadzu). Distilled water was used as the mobile phase at a flow rate of 1.0 ml/min at 80°C. Lactic, propionic, and acetic acids were concurrently measured using an HPLC system for analysis of organic acids. The HPLC system was equipped with a Shim-Pack SCR 102H column (Shimadzu) and a conductivity detector (CDD-6A, Shimadzu). Three mM Bis-Tris solution containing 3 mM *p*-toluenesulfonic acid and 100 μM EDTA was used as the mobile phase at a flow rate of 0.8 ml/min at 40°C.

Results and Discussion

Antimicrobial activity of standard organic acids

The antimicrobial activities of the standard solutions

of three organic acids were compared using *M. luteus*, *Pseudomonas* sp., and *S. aureus* as test microorganisms, which were representatives of Gram-positive, Gram-negative, and pathogenic bacteria, respectively. A significant influence of the pH of sample on the measurement of antimicrobial activity was observed. For example, when *M. luteus* was used as a test organism, 20 g of lactic and acetic acid solutions had 11.0 mm and 32.9 mm diameter clear zones at pH 4.5, but no clear zone was observed at pH 5.5 for both acids. On the other hand, below pH 5.5, propionic acid inhibited the growth of *M. luteus* (data not shown). Thus, the antimicrobial efficiency of these weak acids increased as the pH was reduced, leading to the assumption that the antimicrobial properties of the weak acid are a function of these undissociated molecules. Lactic acid, with a lower pKa (3.9), was thought to be of less value as an antimicrobial reagent than acetic (pKa=4.8) and propionic (pKa=4.9) acids.[1,3] The previous reports showed that at the same pH, the order of antibacterial efficiency of the three weak organic acids produced by the food bacteria was propionic>acetic>lactic against *Salmonella* sp.[11] and *Listeria monocytogenes*.[12] The findings strengthen the earlier assumption that the antimicrobial activity and the concentration of undissociated molecules are directly related. Figure 1 shows the antimicrobial activity of the standard organic acids as a function of these undissociated concentrations. At a lower pH, the proportion of undissociated molecules of an acid is higher, and its antimicrobial efficiency greater, than at a higher pH. When the diameters of the zone of growth inhibition were plotted against the log undissociated concentrations of the organic acids, straight line responses were obtained for all of the organic acids, irrespective of test microorganisms. At the same concentration of the undissociated form, the antimicrobial activity of acetic acid against *Pseudomonas* sp. was higher than that of propionic acid, but against *M. luteus* and *S. aureus* the activities of the former were almost equal to those of the latter. On the other hand, lactic acid had the least inhibitory effect on the three test microorganisms. In fact, lactic acid had no antimicrobial activity for *S. aureus*, as shown in Fig. 1C.

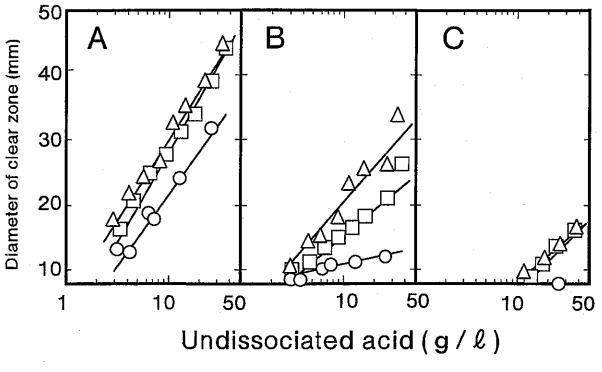

Fig. 1. Antimicrobial Activity of Standard Organic Acids.
The antimicrobial activities of acetic acid (△), propionic acid (□), and lactic acid (○) were evaluated against *M. luteus* (A), *Pseudomonas* sp. (B), and *S. aureus* (C) as test microorganisms.

The antimicrobial effect of the weak organic acids is principally produced by the undissociated molecules through the acidification of cytoplasm, destruction of the transmembrane proton motive force, and loss of active transport of nutrients through the membrane.[1,2,5,6,13] However, although the undissociated molecules generally have a more inhibitory effect, several researchers have reported that the total inhibitory action of weak acid is dependent upon the combined effects produced by undissociated molecules as well as the dissociated ions.[1,2,13] Moreover, the degree of these multifunctional effects is dependent upon time and temperature of exposure, the target microbial strains, and the composition of medium for assay as well as the kind of weak acid, its concentration and pKa, and the pH of the environment. Despite the findings reported previously, there are still unanswered questions about several changes brought about by the weak acids in microbial cells. The mechanism of growth inhibition caused by the organic acids is really complicated. Apart from the exact estimation of antimicrobial action of the weak acids, the results of Fig. 1 show that the antimicrobial activities of acetic and propionic acids were higher than that of lactic acid, suggesting that conversion of lactic acid produced by food microorganisms to acetic and propionic acids leads to the increase of the total antimicrobial activity of those cultures. We attempted the conversion of lactic acid formed once from sugar by *B. longum* to acetic and propionic acids by *P. freudenreichii*. The sequential conversion of lactose to acetic and propionic acids through lactic acid was achieved by co-culture of *B. longum* and *P. freudenreichii*, as will be described below.

Production of antimicrobial culture by single culture

Figure 2 shows the results of single cultivation of *B. longum* with the pH controlled at pH 6.5 when 50 g/l of lactose was used as a carbon source. On the basis of turbidity, the cells grew logarithmically for the first 6 h, followed by gradual growth. The number of viable cells increased initially but decreased after 12 h. The decrease in the growth rate seems to be caused by the accumulation of organic acids, just as with other lactic acid bacteria described previously.[14,15] The maximum cell concentration obtained was about 4.4 g dry cells/l at 20 h. Lactic and acetic acids increased along with the cell growth. The concentrations of lactic and acetic acids reached maximum values of 16.5 and 19.8 g/l at 24 h, respectively. The antimicrobial activity of the culture supernatant, which was expressed as the concentration of acetic acid, was almost proportional to the total concentrations of the organic acids. Its antimicrobial activity at 24 h corresponds to that of 32.0 g/l of acetic acid.

Figures 3 and 4 show the results of single cultivation of *P. freudenreichii* with the pH controlled at pH 6.5 using 50 g/l of lactose or 10 g/l of lactic acid as a carbon source, respectively. This strain of *P. freudenreichii* consumed little or no lactose but 10 g/l of lactic acid was entirely consumed by 48 h and produced acetic (6.2 g/l) and propionic (2.1 g/l) acids at 56 h. The culture supernatant obtained showed only a very slight antimicrobial activity due to the low concentrations of organic acids.

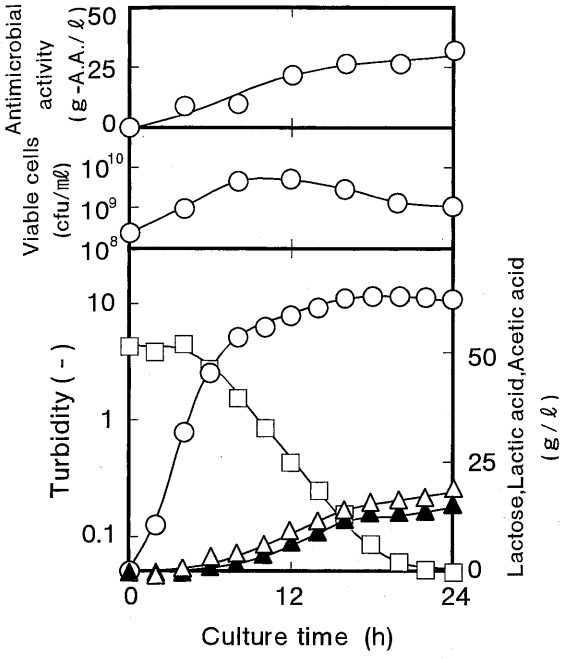

Fig. 2. Production of a Mixture of Antimicrobial Organic Acids from Lactose by *B. longum* Alone Using TPY Medium.

The antimicrobial activity was measured using *M. luteus* as a test microorganism and expressed as a corresponding acetic acid (A.A.) concentration. The number of viable cells of *B. longum* is shown using an unit of cfu/ml in the middle figure. (○) Turbidity; (□) Lactose; (▲) Lactic acid; (△) Acetic acid.

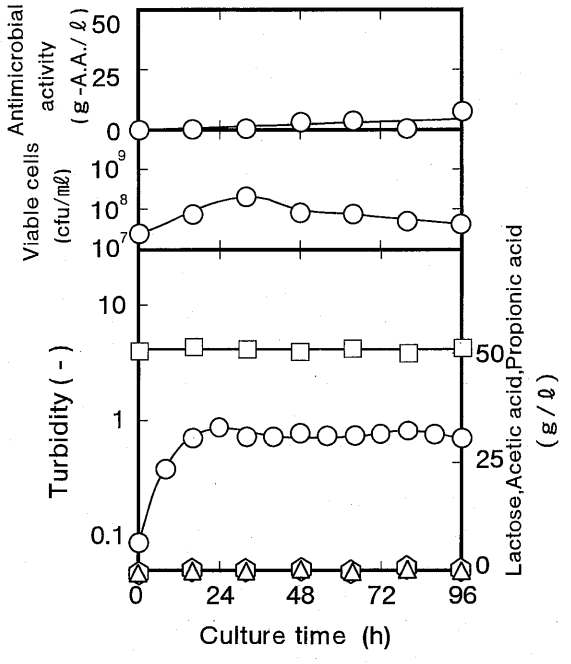

Fig. 3. Production of a Mixture of Antimicrobial Organic Acids from Lactose by *P. freudenreichii* Alone Using TPY Medium.

The antimicrobial activity was measured using *M. luteus* as a test microorganism and expressed as a corresponding acetic acid (A.A.) concentration. The number of viable cells of *P. freudenreichii* is shown using an unit of cfu/ml in the middle figure. (○) Turbidity; (□) Lactose; (△) Acetic acid; (⬡) Propionic acid.

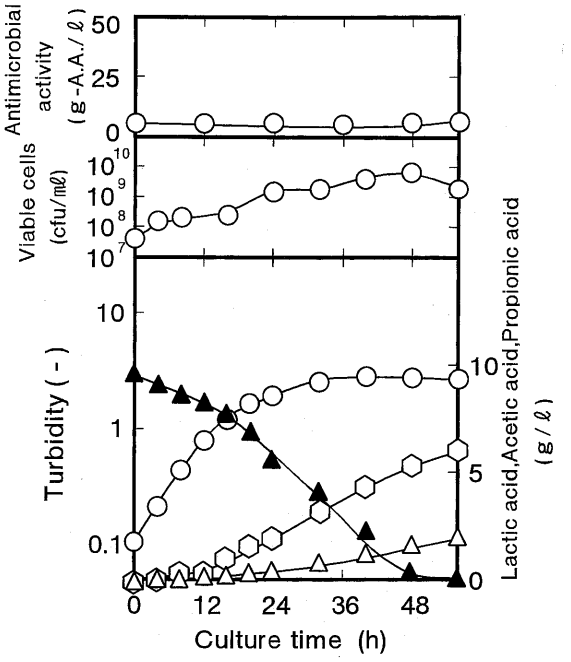

Fig. 4. Production of a Mixture of Antimicrobial Organic Acids from Lactic Acid by *P. freudenreichii* Alone Using TPY Medium.

The antimicrobial activity was measured using *M. luteus* as a test microorganism and expressed as a corresponding acetic acid (A.A.) concentration. The number of viable cells of *P. freudenreichii* is shown using an unit of cfu/ml in the middle figure. (○) Turbidity; (▲) Lactic acid; (△) Acetic acid; (⬡) Propionic acid.

In addition, the conversion rate of lactic acid to acetic and propionic acids by *P. freudenreichii* is slower than that of lactose to lactic and acetic acids by *B. longum*. The growth of *P. freudenreichii* was inhibited by lactic acid at the initial concentration of more than 30 g/l (data not shown) probably due to what is called substrate inhibition. The results show that it is difficult not only to produce acetic and propionic acids from lactose but also to obtain acetic and propionic acids at high concentrations even from lactic acid by this strain alone. Even when glucose was used as a carbon source, the growth rate of *P. freudenreichii* was so slow that the time required for complete consumption of 50 g/l of glucose was more than 96 h (data not shown). Therefore, we considered the co-culture where in the first step lactose was gradually converted to lactic and acetic acids by *B. longum* and subsequently the lactic acid was fermented to acetic and propionic acids by *P. freudenreichii*.

Increase of antimicrobial activity by sequential conversion using co-culture

The co-culture was done by simultaneous inoculation of *B. longum* and *P. freudenreichii* into TPY medium containing 50 g/l of lactose and subsequent incubation as described in Materials and Methods. Figure 5 shows the production of a mixture of acetic and propionic acids from lactose by the co-culture of *B. longum* and *P. freudenreichii*. Taking into account the low growth rate of *P. freudenreichii* described above, the initial concentration of *P. freudenreichii* was adjusted to be higher

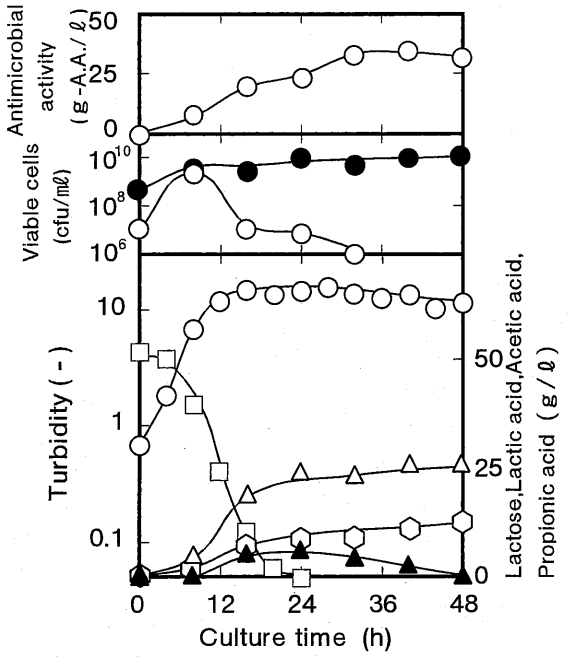

Fig. 5. Production of a Mixture of Antimicrobial Organic Acids from Lactose by Co-culture of *B. longum* and *P. freudenreichii* Using TPY Medium.

The antimicrobial activity was measured using *M. luteus* as a test microorganism and expressed as a corresponding acetic acid (A.A.) concentration. The numbers of viable cells of *B. longum* (○) and *P. freudenreichii* (●) are shown using an unit of cfu/ml in the middle figure. (○) Turbidity; (□) Lactose; (▲) Lactic acid; (△) Acetic acid; (⬡) Propionic acid.

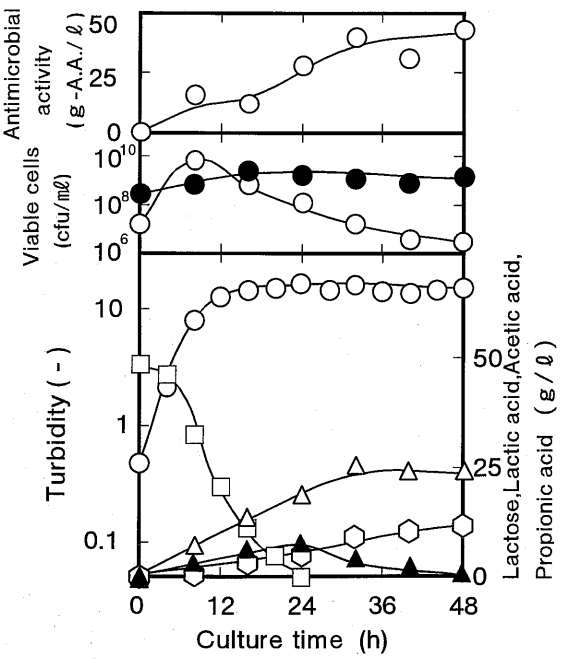

Fig. 6. Production of a Mixture of Antimicrobial Organic Acids from Lactose by Co-culture of *B. longum* and *P. freudenreichii* Using Whey Medium.

The antimicrobial activity was measured using *M. luteus* as a test microorganism and expressed as a corresponding acetic acid (A.A.) concentration. The numbers of viable cells of *B. longum* (○) and *P. freudenreichii* (●) are shown using an unit of cfu/ml in the middle figure. (○) Turbidity; (□) Lactose; (▲) Lactic acid; (△) Acetic acid; (⬡) Propionic acid.

than that of *B. longum* as described in Materials and Methods. The numbers of viable cells of the two bacteria increased for 8 h. Thereafter, the number of viable cells of *P. freudenreichii* was maintained at a level of more than 10^9 throughout the fermentation while that of *B. longum* decreased rapidly probably due to the accumulation of the organic acids. Lactic acid was produced as an intermediate and once accumulated up to a level of 6.9 g/l at 24 h. Then, the lactic acid concentration decreased at a slow rate. On the contrary the concentrations of acetic and propionic acids gradually increased and reached maximum values of 26.5 g/l and 12.6 g/l at 48 h, respectively. The antimicrobial activity of the culture supernatant against *M. luteus* was 34.8 g/l as a concentration of acetic acid at 48 h.

In view of the practical use of the cell-free culture of food microorganisms as a preservative, the cost of medium constituents should be reduced as much as possible. In this study, we used a hydrolyzate of whey proteins obtained by the hydrolysis with protease instead of commercially available peptones. The medium containing whey protein hydrolyzate (whey medium) was prepared as described in Materials and Methods. Figure 6 shows the production of a mixture of acetic and propionic acids by the co-culture of *B. longum* and *P. freudenreichii* in the whey medium. Very similar profiles to the results using TPY medium were observed with respect to the cell growth and the production of organic acids. The final concentrations of acetic and propionic acids were 24.7 g/l and 12.0 g/l, respectively, which were as high as those for the co-culture using TPY medium. The antimicrobial activity of the culture supernatant containing the organic acids against *M. luteus* was 43.2 g/l as a concentration of acetic acid at 48 h.

Comparison of antimicrobial activity of cultures

The table shows a comparison of the antimicrobial activities of culture supernatant obtained by the different culture methods. In the single culture, *B. longum* produced 16.5 g/l of lactic acid and 19.8 g/l of acetic acid from 50 g/l of lactose in TPY medium (No. 1 in Table). The antimicrobial activities of the fermentation supernatant of *B. longum* alone against *M. luteus*, *Pseudomonas* sp., and *S. aureus* were 32 g/l, 19.2 g/l, and 24.8 g/l as an acetic acid concentration, respectively. Since *M. luteus* was sensitive to lactic acid as shown in Fig. 1, high activity seems to be obtained. When *Pseudomonas* sp. was used as a test microorganism, the antimicrobial activity of the supernatant from *B. longum* culture containing lactic and acetic acids seems to be based only on the action of acetic acid because *Pseudomonas* sp. was more resistant to lactic acid than *M. luteus* (Fig. 1). However, fairly high activity against *S. aureus* was obtained, although lactic acid alone has no antimicrobial effect (Fig. 1). This high activity is probably attributable to a synergistic effect when lactic and acetic acids were used together.[1,13] *P. freudenreichii* could produce little or no acids from lactose and consequently the culture su-

Table Comparison of Antimicrobial Activities of the Culture Supernatants Obtained by Different Culture Methods

No.	Culture method	Medium	Culture time (h)	Lactic acid (g/l)	Acetic acid (g/l)	Propionic acid (g/l)	Antimicrobial activity (g-A.A./l)[a]			Remarks
							M. luteus	*Pseudomonas* sp.	*S. aureus*	
1.	Single culture	TPY	24	16.5	19.8	0	32.0	19.2	24.8	Fig. 2
2.	Single culture	TPY	96	0	0.31	0.67	8.0	—[c]	—[c]	Fig. 3
3.	Co-culture	TPY	48	0	26.5	12.6	34.8	29.8	26.4	Fig. 5
4.	Co-culture	Whey[b]	48	0	24.7	12.0	43.2	29.2	29.2	Fig. 6

Nos. 1 and 3 show the cultivation results of *B. longum* and *P. freudenreichii*, respectively.
[a] The antimicrobial activity was expressed as a corresponding concentration of standard acetic acid (A.A.).
[b] The whey medium contained the enzymatic hydrolyzate of whey proteins.
[c] The antimicrobial activity was not detected.

pernatant obtained showed only a very slight antimicrobial activity (No. 2 in Table). However, *P. freudenreichii* could convert lactic acid to acetic and propionic acids. When *P. freudenreichii* was inoculated with *B. longum* into TPY medium, a mixture of acetic and propionic acids with the higher antimicrobial activity were formed directly from lactose. The antimicrobial activities of the culture supernatant obtained from the co-culture against *M. luteus*, *Pseudomonas* sp., and *S. aureus* were 34.8 g/l, 29.8 g/l, and 26.4 g/l as an acetic acid concentration, respectively (No. 3 in Table) which were higher than those for the single culture of *B. longum* (No. 1 in Table). Although the concentrations of acetic and propionic acids obtained in the co-culture using whey medium were almost as high as those in the co-culture using TPY medium as described above, the antimicrobial activities against *M. luteus* and *S. aureus* for the former co-culture (No. 4 in Table) were higher than those for the latter co-culture. The high antimicrobial activities seems to be based on components of whey protein hydrolyzate and/or their metabolites. However, unfortunately there is still no information on the antimicrobial activity of the substances existing in the hydrolyzate of whey proteins.

Acetic, propionic, and lactic acids and their mixture are approved for use in food against different spoilage and pathogenic microorganisms, although the use might be limited due to their flavor and taste. As shown in Fig. 1, the antimicrobial activities of acetic and propionic acids were higher than that lactic acids. *B. longum* produced a mixture of lactic and acetic acids from sugar, which is potentially considered to be used as a safe food preservative. However, the mixture of organic acids has a low antimicrobial activity. In this study, by the co-culture where *P. freudenreichii* was simultaneously inoculated with *B. longum* into whey medium as well as TPY medium, lactose was directly converted to a mixture of acetic and propionic acids with a higher antimicrobial activity. The culture supernatant obtained from the co-culture showed the antimicrobial activity higher than that from the single culture of *B. longum*, regardless of the strain used as test microorganisms. The culture broth and/or its filtrate containing the mixture of acetic and propionic acids obtained in such co-cultures are expected to be used as a safe preservative, especially for meat products (ham and sausage), daily dishes (shao mai and boiled fish paste), and cakes (custard cream).

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