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Short communication

Lactobacillus reuteri in bovine milk fermented decreases the oral carriage of mutans streptococci☆

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Abstract

The effect of *Lactobacillus reuteri* against one of the major cariogenic organism, *Streptococcus mutans*, was studied. Yogurt products containing *L.reuteri* showed a significant growth inhibitory effect against *S. mutans*, whilst yoghurts with lactobaccilli other than *L. reuteri* did not show such inhibition. Further, double-blind, placebo-controlled trial demonstrated that consuming yogurt with *L. reuteri* significantly reduced the oral carriage of mutans streptococci, compared with the placebo yogurt.

Keywords: *Lactobacillus reuteri*; Yogurt; Mutans streprococci; Caries prevention

1. Introduction

Lactobacillus reuteri is an obligately heterofermentative resident in the gastrointestinal tracts of humans, and it is reported to produce compounds that exhibit antagonistic activity, i.e., reuterin (Talarico et al., 1988) and reutericyclin (Ganzle et al., 2000), which are water-soluble, broad-spectrum antimicrobials, effective over a wide pH range, and resistant to proteolytic and lipolytic enzymes (el-Ziney and Debevere, 1998).

It has been documented that reuterin-producing strains of *L. reuteri* have inhibitory effects against Gram-positive bacteria, e.g., *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*, and Gram-negative bacteria, e.g., *Escherichia coli*, *Yersinia enterocolitica* and *Pseudomonas fluorescens*, in synthetic media (el-Ziney et al., 1999). Recently, it was demonstrated that reuterin was able to reduce the viability of *L. monocytogenes* and *E. coli* O157:H7 in milk and cottage cheese at refrigeration temperature (el-Ziney and Debevere, 1998).

The reduction of dental caries of children consuming milk fermented by the probiotic bacterium *L. rhamnosus* has been documented (Nase et al., 2001) Therefore, the search for effective, caries-preventing probiotic microorganisms appears to be a promising research avenue. There is, however, no data on the inhibitory effects of *L. reuteri* against oral cariogenic bacteria.

In the present study, we examined (i) the inhibitory effects of *L. reuteri* against *Streptococcus*

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mutans; (ii) the inhibitory effects of commercially available yogurt, including yogurt containing *L. reuteri*, against *S. mutans*; and (iii) the effect of *L. reuteri* containing yogurt on the oral carriage of mutans streptococci.

2. Materials and methods

2.1. Microorganisms and culture conditions

S. mutans Ingbritt provided by the Department of Oral Microbiology, Hiroshima University, were used in the study. The isolate was grown in trypticase soy broth (Difco; Japan Becton-Dickinson, Tokyo Japan) supplemented with 0.5% yeast extract (Difco) (TSBY). Cells were harvested during the exponential growth phase by centrifugation at 1000 × *g*, washed twice with PBS and resuspended in the same buffer. The cell suspensions were subjected to a low-intensity ultrasonic treatment to disperse bacterial aggregates.

L. reuteri SD2112 (ATCC55730), known as a reuterin producer and provided by Chichiyasu Dairy Products (Hiroshima, Japan), was used in the study. The isolate was grown in brain–heart infusion broth (BHI: Difco), harvested during the exponential growth phase by centrifugation at 1000 × *g*, washed twice with phosphate-buffered saline (PBS; pH 6.8) and resuspended in the same buffer. The optical densities of the bacterial suspensions were measured in a 1.0-ml cuvette with a 1 cm light path, and the suspensions were adjusted to a final concentration of 1.0×10^8 colony forming unit (CFU)/ml before use.

2.2. Inhibitory effect of L. reuteri

The assay was conducted as follows. The suspensions of *S. mutans* and of *L. reuteri* prepared as mentioned earlier were mixed in the ratios stated in Fig. 1 in sterile centrifugation tubes, and 100 μl were added to 10 ml of BHI broth and vortex mixed for 10 s, followed by incubating for 90 min at 37 °C with gentle shaking. As a control, the suspension of *S. mutans* w was mixed with the same amount of PBS. Afterwards each suspension was centrifuged at 1000 × *g*, washed twice with PBS, and plated on Mitis–Salivarius (MS) agar (Difco) supplemented with bacitracin and sucrose (Gold et al., 1973) incubated at 37 °C for 48 h to determine the CFU of *S. mutans*. The % survival rate of *S. mutans* was obtained using the following formula:

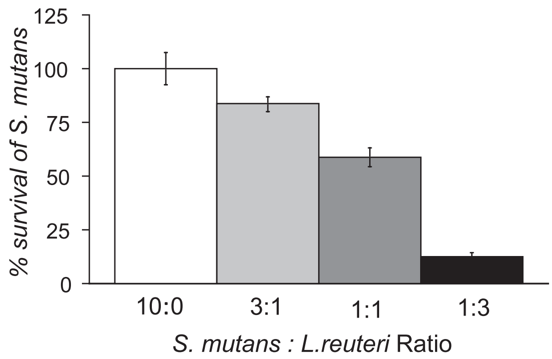

Fig. 1. Percent survival of *S. mutans* Ingbritt incubated with *L. reuteri* (*$p < 0.01$).

$$\%\ \text{survival of } S.\ mutans = \frac{\text{CFU of } S.\ mutans \text{ incubated with } L.\ reuteri}{\text{CFU of } S.\ mutans \text{ incubated with PBS}} \times 100.$$

The assays were carried out on at least two independent occasions, with quadruplicate samples on each occasion. All the numerical data obtained were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 1% level.

2.3. Inhibitory effects of commercially fermented milk products

A total of 18 commercially available fermented milk products, listed in Table 1, were used in the second part of the study. Sterilized filter paper (6 mm in diameter) was immersed in each product for 20 s. These paper discs were placed in MS agar plates inoculated with *S. mutans*, incubated at 37 °C for 24 h and the inhibitory zone recorded.

2.4. Effects of milk fermented with L. reuteri (Reuteri yogurt) and placebo fermented milk (Placebo yogurt) on oral carriage of mutans streptococci

Streptococcus thermophilus was added to a large amount of frozen stock culture of *L. reuteri*, provided

Table 1
Yoghurt used and zones of inhibition on MS agar inoculated with *Streptococcus mutans*

Products	Company		pH	Inhibitory zone (mm)
Kenko	Glico Dairy Products	Japan	4.18	n.d.
Renpo	Azehira Milk Products	Hiroshima, Japan	4.17	n.d.
Makiba	Koiwai Dairy Products	Tokyo, Japan	3.90	n.d.
Plane	Koiwai Dairy Products	Tokyo, Japan	4.37	n.d.
Hokkaido	Japan Milk Net	Tokyo, Japan	4.00	n.d.
Danone Yogurt	Calpis Ajinomoto Danone	Tokyo, Japan	4.24	n.d.
Danone Yogurt Fruits	Calpis Ajinomoto Danone	Tokyo, Japan	4.15	n.d.
Reuteri[a]	Chichiyasu Dairy	Hiroshima, Japan	4.08	6.3
Low sugar	Chichiyasu Dairy	Hiroshima, Japan	3.96	n.d.
Bifidum	Chichiyasu Dairy	Hiroshima, Japan	3.99	n.d.
Vanilla	Nippon Meat Packers	Osaka, Japan	4.37	n.d.
Tokachi	Meiji Dairies	Tokyo, Japan	3.91	n.d.
Bulgaria	Meiji Dairies	Tokyo, Japan	4.13	n.d.
LG21	Meiji Dairies	Tokyo, Japan	4.03	n.d.
Bifidas	Morinaga Milk Industry	Tokyo, Japan	4.32	n.d.
Nature	Snow Brand Milk Products	Tokyo, Japan	4.17	n.d.
Tokachi Plane	Yostuba	Tokyo, Japan	4.32	n.d.
New Jersey	Hiruzen Noukyou	Okayama, Japan	4.17	n.d.

n.d.: inhibition not detected.
[a] Reutei is a brand of Yogurt containing *L. reuteri*.

by Biogaia (Stockholm, Sweden), to prepare Reuteri Yogurt. Placebo yogurt was prepared with *Lactobacillus bulgaris* and *S. thermophilus*, which are widely employed in fermented milk products. Standard fermentation candidates were used. The pH values of Reuteri yogurt and Placebo yogurt were 4.10 and 4.00, respectively.

A cohort of equal standing in terms of oral health was selected from students of dental hygienists. In total, 40 healthy female subjects, who were 20 years old were divided into two groups. None of the subjects exhibited any active caries lesions, symptoms of either gingivitis or peroidontal disease. Subjects in the first group were given a cup (95 g) of Placebo yogurt at lunchtime (12:00–13:00) daily, for a period of 2 weeks, and then a cup of Reuteri yogurt at lunchtime a day, for another 2 weeks. Subjects in the second group were given Reuteri yogurt at lunchtime daily for 2 weeks, and then Placebo yogurt at lunchtime daily for another 2 weeks. Before and after consumption of each variety of yogurts, the levels of oral carriage of mutans streptococci were determined, as follows. Approximately 5 ml of unstimulated whole saliva was collected in a container on ice at 15:00–16:00. Then the oral carriage of mutans streptococci was determined using conventional viable counts. Briefly, each sample was serially diluted with sterile distilled water, inoculated on the MS agar, and incubated at 37 °C for 48 h, to determine the colony-forming unit of mutans streptococci in saliva for each individual.

Both the subjects and investigators were unaware of which yogurt contains *L. reuteri* throughout the study. The use of other products containing *Lactobacillus* or other pharmaceutical lactic acid bacteria was forbidden for 1 week prior to and throughout the intervention. Data were analyzed by Wilcoxon's test at 1% and 5% levels.

2.5. Degradation of hydroxyapatite (HAP)

To assess the ability of an isolate of *L. reuteri* and *S. mutans* to degrade hydroxyapatite (HAP), the assay was conducted according to our previous study (Nikawa et al., 1998) with some modification. Briefly, 50 mg of HAP beads (49.95 ± 0.08 mg) were placed on the bottom of each well of 24-multiwell tissue culture plates (Nunclon® Delta, Nunc, Kamstrup, Denmark). Fifty microliters of suspensions of lactobacilli (3.0×10^8 CFU/ml) or streptococci (3.0×10^8 CFU/ml) were inoculated into each well in which HAP beads were placed, and 2.0 ml of BHI broth (for lactobacilli) or TSBY (for streptococci) were

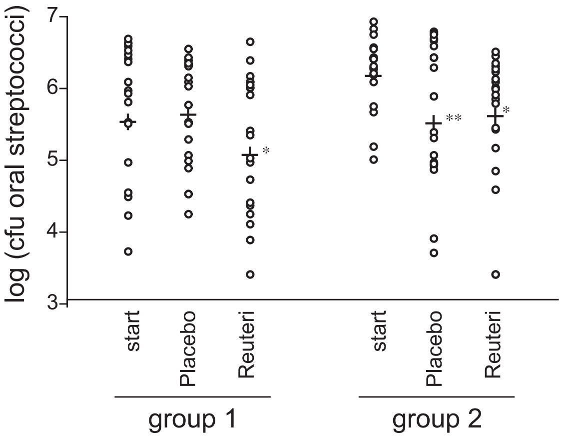

Fig. 2. Effect of eating of Yogurt (once a day for 2 weeks) on oral carriage of mutans streptococci. The eating of Reuteri yogurt significantly reduced the oral carriage of mutans streptococci in each group (*$p<0.05$; **$p<0.01$; Wilcoxon's test).

carefully added followed by incubation for 0, 3, 6, 12, 18, 24, 36, 48, 72 and 96 h at 37 °C. After each period of incubation, calcium release was spectrophotometrically measured at 575nm after addition of *o*-cresolphthalein (Wako Junyaku, Osaka, Japan) as described by Chestnutt et al. (1995). As controls, the calcium release from the HAP added in each medium without bacteria was determined. The assays were carried out on at least two independent occasions, with quadruplicate samples on each occasion. All the numerical data obtained were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 5% and 1% levels.

3. Results and discussion

Milk and other dairy products contain calcium, which is the principal mineral in bones and teeth. Developing tooth tissues are affected by factors associated with the intake and metabolism of calcium and phosphorus. Milk, on its own, is a complex colloidal fluid of organic and inorganic compounds that appears to be enamel-protective (Gedalia et al., 1991). Milk also has 'cariostatic properties when ingested simultaneously with food considered a cariogenic challenge' (Bowen and Pearson, 1993). Calcium lactate, also present in dairy products, is known to be anticariogenic (Kashket and Yaskell, 1997). It can thus be expected that milk products would be ideal vehicles for administrating enamel-protective agents particularly for children or elderly. In addition, Ishihara et al. (1985) have shown that several probiotic bacteria, like *Enterococcus faecium*, *Streptococcus equinus*, *Lactobacillus fermentum*, and *Lactubacillus salivarius* possessed growth inhibitory effects against *S. mutans* in vitro. *L. fermentum*, which is phenotypically closely related to *L. reuteri*, has been reported to possess growth inhibitory effects against *S. mutans* in vitro (Ishihara et al., 1985). Thus we expected at least an in vitro growth inhibition.

As show in Fig. 1, loss of viability of *S. mutans* was noted via incubation with *L. reuteri* in a ratio-dependent manner ($p<0.01$). In fact, at a 1:3 *S. mutans*/*L. reuteri* ratio, more than a 90% loss of viability was observed. Furthermore, the inhibitory zone against *S. mutans* was noted only with a fermented milk product containing *L. reuteri* (Table 1), whilst the remainder of products, containing lactobaccilli other than *L. reuteri*, did not show such inhibition. These results confirm the antibacterial effect of bovine milk fermented by *L.reuteri* against *S. mutans*.

Caries risk assessment is a complex issue. Researchers have been searching for the factors that would enable the prediction of development of caries. Many variables have been investigated for their association with the carious process. Risk indicators may include socioeconomic factors such as income, social factors, such as health attitudes, and oral health habits, clinical variables such as the number of filled teeth, microbiological parameters such as numbers of mutans streptococci and lactobacilli and salivary calcium content

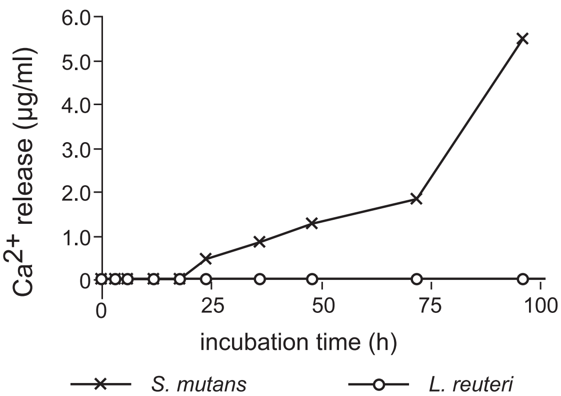

Fig. 3. Calcium release from hydroxylapatite (HAP) caused by *L. reuteri* SD2112 and *S. mutans* Ingbritt.

(Hausen et al., 1996). In order to overcome these (Mazengo et al., 1996) variables, we employed a cohort of equal standing from a dental hygiene school as volunteers for the present study.

Bjarnason and Kohler (1997) have shown that caries frequencies and salivary microorganisms correlated significantly with a subsequent 3-year increment of the number of decayed or restored teeth. It is also reported that a saliva sample positive for *S. mutans* correlates with clinical findings on caries (Splieth and Bernhardt, 1999). Hence we employed the salivary levels of mutans streptococci as the indicator of the caries risk, in the present study. The eating of Reuteri yogurt significantly reduced the oral carriage of mutans streptococci in each groups (Fig. 2: $p<0.05$ for group 1; $p<0.01$ for group 2), and such effects were not observed when Placebo yogurt was not consumed ($p>0.05$). Taken together, these results suggest that fermented milk containing *L. reuteri* should be a helpful aid to reduce caries risk.

On the other hand, some have suggested that *L. salivarius* has a cariogenic potential (Seppa et al., 1989; Jacques et al., 1980; Fitzgerald et al., 1980). Nase et al. (2001) have pointed out that *L. rhamnosus*, which is homofermentive, can ferment sucrose slowly and produce lactic acid, thus increasing the risk of dental caries and demineralization of tooth enamel. Hence, we examined the degradation of HAP by the lactobacilli used in our preliminary study. The effect of *S. mutans* was negligible within 24-h incubation, but calcium release strongly increased during the period of incubation (Fig. 3). At 96 h of incubation, calcium release from HAP caused by *S. mutans* was 5.4 μg/ml (0.135 μmol/l). Chestnutt et al. (1995) reported similar results for *S. mutans*. However, the calcium release caused by *L. reuteri* was negligible (Fig. 3).

In conclusion, our data, when taken together, suggest that bovine milk fermented by *L. reuteri* may help decrease the risk of dental caries, and this organism may be a useful probiotic organism for dairy products.

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