The Journal of Microbiology (2011) Vol. 49, No. 2, pp. 193-199
DOI 10.1007/s12275-011-0252-9

Inhibitory Effect of *Lactobacillus reuteri* on Periodontopathic and Cariogenic Bacteria

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(Received July 7, 2010 / Accepted December 1, 2010)

The interaction between *Lactobacillus reuteri*, a probiotic bacterium, and oral pathogenic bacteria have not been studied adequately. This study examined the effects of *L. reuteri* on the proliferation of periodontopathic bacteria including *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Tannerella forsythia*, and on the formation of *Streptococcus mutans* biofilms. Human-derived *L. reuteri* strains (KCTC 3594 and KCTC 3678) and rat-derived *L. reuteri* KCTC 3679 were used. All strains exhibited significant inhibitory effects on the growth of periodontopathic bacteria and the formation of *S. mutans* biofilms. These antibacterial activities of *L. reuteri* were attributed to the production of organic acids, hydrogen peroxide, and a bacteriocin-like compound. Reuterin, an antimicrobial factor, was produced only by *L. reuteri* KCTC 3594. In addition, *L. reuteri* inhibited the production of methyl mercaptan by *F. nucleatum* and *P. gingivalis*. Overall, these results suggest that *L. reuteri* may be useful as a probiotic agent for improving oral health.

***Keywords*:** *Lactobacillus reuteri*, periodontopathic bacteria, *Streptococcus mutans*, inhibitory effect, biofilms

Dental plaque is a biofilm composed of more than 700 different oral bacterial species and their products, and is the main etiological agent of oral infectious diseases, such as periodontitis and dental caries (Paster *et al*., 2001, 2006; Aas *et al*., 2005). Gram-negative bacteria have been shown to be responsible for the initiation and progression of periodontal disease. Among these, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, and *Fusobacterium nucleatum* are considered the major putative etiological agents of periodontal diseases (Haffajee and Socransky, 1994; Slots and Ting, 1999; Haffajee *et al*., 2006). Socransky *et al*. (1998) described 5 major bacterial complexes present in subgingival niches; red, orange, green, yellow, and purple. *A. actinomycetemcomitans* is an etiologic agent of aggressive periodontitis (Mandell, 1984; Meyer and Fives-Taylor, 1997), whereas *P. gingivalis*, *T. forsythia*, and *T. denticola* as the red complex species are strongly correlated with the severity of chronic periodontitis (Kasuga *et al*., 2000). *F. nucleatum*, a member of the orange complex (Socransky *et al*., 1998), interacts with many members of oral biofilms, leading to a significant increase in tissue damage during active periodontal disease (van Winkelhoff *et al*., 2002). Mutans streptococci, considered to be major cariogenic bacteria, encompass *Streptococcus mutans*, *S. sobrinus*, *S. downei*, *S. rattus*, *S. cricetus*, *S. ferus*, and *S. macacae* (Kawamura *et al*., 1995). Of these, *S. mutans* is strongly associated with human dental caries (Loesche, 1986).

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Oral malodor or bad breath is also a major oral health problem for many individuals, and is caused mainly by volatile sulfur compounds (VSC) consisting principally of hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) (Tonzetich, 1971). These VSC are believed to be produced mainly by periodontal pathogens (Persson *et al*., 1990; De Boever and Loeshe, 1995). One study showed that the increase in CH_3SH to H_2S ratio in human gingival crevicular sites is associated with the deeper pockets or bleeding pockets (Coli and Tonzetich, 1992). Lancero *et al*. (1996) reported that exposure to CH_3SH reduces the intracellular pH and inhibits cell migration in periodontal ligament cells. This suggests that CH_3SH may not only be responsible for oral malodor but may also contribute to the pathogenesis of periodontal disease.

Lactic acid bacteria (LAB), such as *Lactobacillus* species, have protective effects against a variety of pathogenic infections in the gastrointestinal systems of humans and animals (Gilliland *et al*., 1985; Gill *et al*., 2001). These strains produce a wide range of antimicrobial agents, and have been studied extensively with regard to their possible utility in the field of probiotics. Probiotics may be a useful alternative for the treatment and prevention of a variety of infectious diseases caused by oral, enteric, and urogenital pathogens (Shornikova *et al*., 1997; Çaglar *et al*., 2006; Abad and Safdar, 2009). *Lactobacillus reuteri*, an established probiotic agent, is used widely in viral diarrhea diseases (Saavedra, 2000; Szajewska and Mrukowicz, 2005). *L. reuteri*, an obligatory heterofermentative resident in the gastrointestinal tract of humans, produces an antimicrobial compound, reuterin (Talarico *et al*., 1988), which is water soluble, effective over a wide pH range, and resistant to pro-

teolytic and lipolytic enzymes (El-Ziney *et al*., 1999). Reuterin-producing *L. reuteri* strains have inhibitory effects against many members of Gram-positive and Gram-negative bacteria (Talarico *et al*., 1988; Cleusix *et al*., 2007) including enteric pathogens (Spinler *et al*., 2008). These findings suggest that *L. reuteri* has potential as a useful probiotic agent.

The interaction between probiotic bacteria and a major cariogenic pathogen *S. mutans* have also been studied. *Lactobacillus rhamnosus*, *L. fermentum*, *L. salivarius*, *L. reuteri*, and *Weissella cibaria* exhibited growth inhibitory effects on *S. mutans* (Ishihara *et al*., 1985; Ahola *et al*., 2002; Nikawa *et al*., 2004; Kang *et al*., 2006a). However, there are no data on the inhibitory effects of *L. reuteri* on oral pathogens associated with periodontal disease.

The present study examined the effects of *L. reuteri* on the growth and the VSC production of periodontopathic bacteria. In addition, the inhibitory effects of *L. reuteri* on the biofilm formation of *S. mutans* were evaluated.

Materials and Methods

Bacterial strains and growth conditions

L. reuteri KCTC 3594 and KCTC 3678 were isolated from the intestines and feces of humans, respectively. *L. reuteri* KCTC 3679 was isolated from rat. *L. reuteri* strains were grown in De Man, Rogosa, Sharpe broth (MRS broth; Difco, USA). *S. mutans* Ingbritt was grown in brain heart infusion broth (BHI broth; Difco). These bacteria were incubated at 37°C for 16 h under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2). *A. actinomycetemcomitans* ATCC 33384 was cultivated using trypticase soy broth (TS broth; Difco) supplemented with yeast extract (1 mg/ml; Difco) and horse serum (10%). *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 10953 were grown in Brucella broth supplemented with yeast extract (1 mg/ml), hemin (10 μg/ml; Sigma, USA) and menadione (5 μg/ml; Sigma) (Kang *et al*., 2008). *T. forsythia* ATCC 43037 was grown in Brucella broth (Difco) supplemented with yeast extract (0.5 mg/ml), hemin (5 μg/ml), menadione (0.5 μg/ml), N-acetylmuramic acid (10 μg/ml; Sigma), and fetal bovine serum (10%). These bacteria were incubated anaerobically at 37°C for 48 h. Bacterial cultures in the logarithmic growth phase were used in all experiments.

Effect of *L. reuteri* on the growth of periodontopathic bacteria and *S. mutans*

Each *L. reuteri* strain was incubated for 24 h at 37°C under anaerobic conditions. After pelleting the bacterial cells (4,000×g, 20 min, 4°C), the supernatants were obtained, filter-sterilized (0.22 μm pore size; Millipore, USA), vacuum-dried and resuspended in each growth medium of the tested organisms to the starting volume. The effects of the *L. reuteri* culture supernatant on the growth of periodontopathic bacteria and *S. mutans* were tested. Logarithmic-phase cultures of the bacteria were added to the culture medium containing the *L. reuteri* culture supernatants in the wells of microtiter plates, and their growth was assessed after a period of incubation. A one tenth volume of the tested organisms cultures were inoculated with 0.1 ml of the *L. reuteri* culture supernatants. The inoculum size of the tested bacteria was determined by measuring the optical density at 600 nm (OD_{600}) and extrapolating the CFU/ml using preset standard curves. The final inoculum concentrations of the periodontopathic bacteria and *S. mutans* were 1×10^6 and 5×10^5 CFU/ml, respectively. The controls consisted of cells grown in the medium only. After incubation under anaerobic conditions at 37°C for 24-72 h, the level of microbial growth over time was measured using a microplate reader (Molecular Devices, USA) at 600 nm.

Effect of *L. reuteri* on VSC production

The ability of *L. reuteri* to inhibit VSC production was also investigated using *F. nucleatum* and *P. gingivalis*. *F. nucleatum* or *P. gingivalis*, was added into the culture medium containing the *L. reuteri* culture supernatants in a sterile tube sealed with a silicon plug. One milliliter of the *L. reuteri* culture supernatants was mixed with 1 ml of the test bacteria cultures. The inoculum of the test bacteria was 5×10^6 CFU/ml. After incubation under anaerobic conditions at 37°C for 48 h, the vapor present above the culture was removed using a gas-tight syringe. Subsequent to aspirating 1 ml of air with a syringe, 0.5 ml of air was injected into a gas chromatograph. The level of VSC was analyzed using a portable gas chromatograph (Oral Chroma, Abilit, Japan), as described elsewhere (Kang *et al*., 2006b).

Effect of *L. reuteri* on biofilm accumulation of *S. mutans in vitro*

Beaker-wire tests were performed to determine the effects of *L. reuteri* strains on *in vitro* plaque accumulation, as described previously (Kang *et al*., 2006a). Briefly, equal amounts (1×10^6 CFU/ml) of *S. mutans* and each of the *L. reuteri* strains were incubated in beakers containing a test medium [a mixture of equal volume of BHI and MRS with 5% sucrose, 0.5% yeast extract and 0.1 M of MES (2-[N-Morpholino] ethanesulfonic acid monohydrate, pH 6.5) at 37°C. The test medium inoculated with *S. mutans* alone was used as the control. Three stainless steel wires (Remanium, Germany) were hung on the lid and immersed in each of the beakers, and incubated under slow agitation at 37°C for 24 h. The wires were weighed and the wet weights of plaque accumulation on the wires were determined by subtracting the wire weight from the total weights.

Acid and hydrogen peroxide (H_2O_2) production

The amount of acid produced by *L. reuteri* was determined indirectly by measuring the pH of the supernatant of the culture with a pH meter (Corning Inc., USA). Each *L. reuteri* strain was tested for their ability to generate H_2O_2 using a modification of the method described by Eschenbach *et al.* (1989). *L. reuteri* was plated onto MRS agar containing 0.25 mg/ml of TMB (3,3′,5,5′-tetramethylbenzidine; Sigma) and 0.01 mg/ml of peroxidase (Sigma), and was incubated anaerobically for 48 h. Subsequently, *L. reuteri* was exposed to the ambient air. Colonies showing a blue color were considered H_2O_2 producers.

Quantification of reuterin

Reuterin was prepared as described elsewhere (Slininger *et al*., 1983; Doleyres *et al*., 2005). Briefly, *L. reuteri* was incubated at 37°C for 24 h under anaerobic conditions. The cells were harvested by centrifugation at 5,000×g for 15 min at 20°C, washed with 50 mM sodium phosphate buffer (pH 7.4) and resuspended to a concentration of 1.5×10^{10} CFU/ml in 300 mM glycerol solution. The cells were incubated at 37°C for 3 h under anaerobic conditions to examine the production of reuterin as a function of time. Simultaneously, samples were taken immediately after resuspension in glycerol, and the number of viable *L. reuteri* cells was obtained. The reuterin-containing supernatants were collected by centrifugation (5,000×g, 15 min), filter-sterilized and stored at 4°C until use. The reuterin samples were analyzed colorimetrically as described elsewhere (Lüthi-Peng *et al*., 2002) with some modifications. Briefly, 1 ml of the reuterin samples was

mixed with 0.75 ml of 10 mM tryptophan dissolved in 0.05 M HCl followed by addition of 3 ml of 12 M HCl and incubation at 37°C for 20 min. The optical density of each reaction was measured at 560 nm. Acrolein (Sigma) was used as the calibration standard in 50 mM sodium phosphate buffer. The amount of reuterin was normalized to the initial CFU/ml of the culture.

Antimicrobial characterization

Cell-free supernatants of *L. reuteri* were prepared and tested against *S. mutans* to determine the cumulative inhibitory activity, reflecting the activity of the organic acids, H_2O_2 or a bacteriocin-like compound (BLC). The inhibitory effects of organic acids on growth were determined after mixing sterilized supernatants with proteinase K (0.1

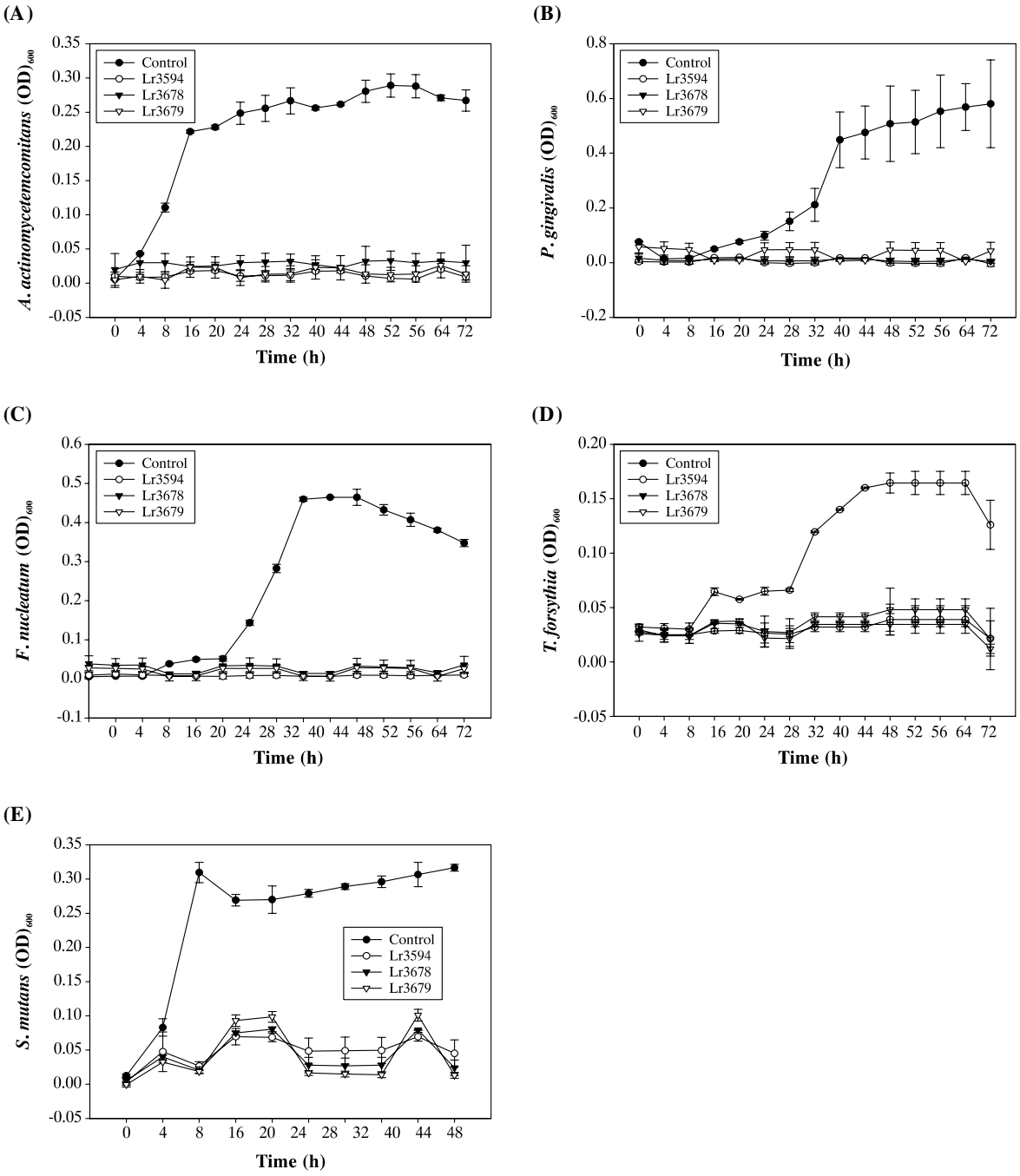

Fig. 1. Effects of the *L. reuteri* culture supernatants on the growth of periodontopathic bacteria (A-D) and *S. mutans* (E) over time. The growth of bacteria was determined by measuring the optical density of the cultures at 600 nm. The data is expressed as the mean±SD of a representative experiment performed in triplicate. Lr3594, *L. reuteri* KCTC 3594; Lr3678, *L. reuteri* KCTC 3678; Lr3679, *L. reuteri* KCTC 3679.

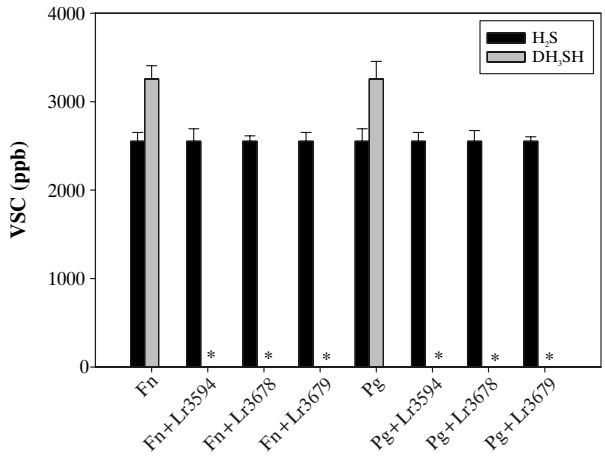

Fig. 2. Effects of the *L. reuteri* culture supernatants on VSC production by *F. nucleatum* and *P. gingivalis.* The level of VSC was analyzed using a portable gas chromatograph. The data is expressed as the Mean±SD of a representative experiment performed in triplicate. *=$p<0.05$, *L. reuteri* culture supernatant treated vs. control. Fn, *F. nucleatum*; Pg, *P. gingivalis*; Lr3594, *L. reuteri* KCTC 3594; Lr3678, *L. reuteri* KCTC 3678; Lr3679, *L. reuteri* KCTC 3679.

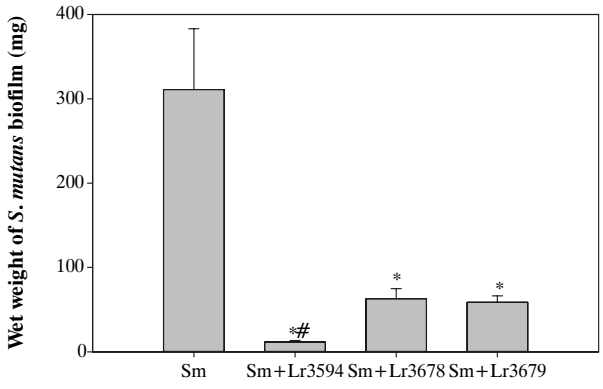

Fig. 3. Effects of *L. reuteri* on the formation of *S. mutans* biofilm. Equal amounts (1×10^6 CFU/ml) of the *L. reuteri* strains and *S. mutans* were cultured in beakers containing a test medium. The level of *in vitro* plaque accumulation on the wires immersed in the cultures was measured. The data is expressed as the Mean±SD of a representative experiment performed in triplicate. *=$p<0.05$, *L. reuteri* treated vs. control. #=$p<0.05$, the inhibitory effect of *L. reuteri* KCTC 3594 on plaque accumulation was significantly greater than other strains. Sm, *S. mutans*; Lr3594, *L. reuteri* KCTC 3594; Lr3678, *L. reuteri* KCTC 3678; Lr3679, *L. reuteri* KCTC 3679.

mg/ml; Sigma) and catalase (0.5 mg/ml; Sigma). The H_2O_2-dependent activity was evaluated using neutralized, proteinase K-treated supernatants and the BLC-dependent activity was analyzed using catalase. The culture medium was used as the control. A one-tenth volume of an overnight culture of *S. mutans* (5×10^5 CFU/ml) was added to 0.1 ml of the test supernatant. After 24 h incubation, OD_{600} of the cell suspension was measured and the inhibition percentage was calculated.

Statistical analysis

Each experiment was carried out in triplicate. Statistical analysis was performed using SPSS version 13.0 (Statistical packages for Social Science version 13.0; SPSS Inc., USA). A Mann-Whitney test and Kruskal-Wallis test were used to determine the statistically significant differences, and a Mann-Whitney test was used to compare the groups as a post hoc test. A p value <0.05 was considered significant.

Results

Inhibitory effect of *L. reuteri* against periodontopathic bacteria and *S. mutans*

The antibacterial activities of *L. reuteri* against periodontopathic bacteria and *S. mutans* were evaluated. All three *L. reuteri* strains inhibited the growth of all the bacteria tested, over the 72 h time period (Fig. 1; $p<0.05$). All three inhibited the growth of the periodontopathic bacteria and *S. mutans* by more than 90%.

Inhibitory effects of *L. reuteri* on the VSC production

The VSC concentration was estimated to determine the capacities of *L. reuteri* strains to inhibit VSC production of *F. nucleatum* and *P. gingivalis*. All three *L. reuteri* strains inhibited the production of CH_3SH by *F. nucleatum* and *P. gingivalis* (Fig. 2). A control assay containing only *F. nucleatum* and *P. gingivalis* revealed a very strong odor and produced very high concentrations of VSC above the maximum detection level (H_2S, 2553 ppb; CH_3SH, 3256 ppb). All three *L. reuteri* strains completely inhibited (100%) the production of CH_3SH ($p<0.05$); however, none of the strains inhibited H_2S production.

Inhibitory effect of *L. reuteri* on the formation of *S. mutans* biofilms

Beaker-wire tests were performed to determine the effects of *L. reuteri* strains on *in vitro* plaque accumulation by *S. mutans* (Fig. 3). All three *L. reuteri* strains inhibited the formation

Table 1. Production of antimicrobial substances of *L. reuteri*

	L. reuteri KCTC strains		
	3594	3678	3679
Source	Human intestine	Human feces	Rat
H_2O_2 production	Positive	Positive	Positive
pH of medium at the end of growth	4.35	4.34	4.35
Reuterin production (mM)[a]	2.51	0.00	0.00

[a] Reuterin was produced from each *L. reuteri* strain in 300 mM glycerol and analyzed using a quantitative method. Each concentration was determined by a colorimetric method performed in triplicate and is represented as the mean.

Table 2. Antimicrobial activity of organic acid, hydrogen peroxide (H_2O_2) and a bacteriocin-like compound (BLC) in cultured supernatants of *L. reuteri* against *S. mutans*

L. reuteri KCTC strains	Inhibition (%)[a] by		
	Organic acid[b]	H_2O_2[c]	BLC[d]
3594	100±0.00	52.19±9.73*	61.59±2.71*
3678	100±0.00	54.31±5.02*	59.98±1.85*
3679	100±0.00	46.52±4.35*	55.34±0.14*

[a] OD_{600} of the cell suspension was measured and the percentage inhibition was calculated using the following formula: Inhibition (%)=OD_{600} (control - tested supernatant)/OD_{600} (control)×100. The values are reported as the mean±SD of three independent experiments.
[b] Inhibition of growth by organic acid was measured after treatment of the sterilized supernatants with proteinase K (0.1 mg/ml) and catalase (0.5 mg/ml).
[c] H_2O_2-dependent activity was evaluated using the neutralized and proteinase K-treated supernatants.
[d] Inhibition of growth by the BLC-dependent activity was evaluated using the neutralized and catalase-treated supernatants.
* =$p<0.05$, inhibition by H_2O_2 or BLC vs. organic acid.

of artificial *S. mutans* biofilms ($p<0.05$).

Characterization of antimicrobial substances

L. reuteri strains produced antimicrobial substances including H_2O_2, organic acid and reuterin (Table 1). Among these, reuterin was produced only by *L. reuteri* KCTC 3594. The antimicrobial activities of *L. reuteri* were modified by pH, catalase and proteinase K (Table 2). The inhibitory effects of *L. reuteri* on the growth of *S. mutans* were not affected by a proteinase K and catalase treatment. The acid product of *L. reuteri* inhibited the growth of *S. mutans* completely. The antimicrobial activity of *L. reuteri* was reduced significantly after neutralization ($p<0.05$). However, all three neutralized *L. reuteri* strains were still able to inhibit the growth of *S. mutans*. The H_2O_2 or BLC of *L. reuteri* exerted at least 46% growth inhibition of *S. mutans*, with no significant differences observed among *L. reuteri* strains ($p>0.05$).

Discussion

Dental caries and periodontitis are two of the most common infectious diseases in humans. Recently, there has been increasing interest in a probiotic approach for the management of these oral infections. Thus far, a number of clinical trials have been carried out to elucidate the possible impact of probiotics on oral health. Several authors have recently suggested that some strains including *L. rhamnosus*, *L. salivarius*, *L. reuteri*, and *W. cibaria* are potential candidates for probiotics for oral diseases (Ahola *et al*., 2002; Nikawa *et al*., 2004; Kõll-Klais *et al*., 2005; Çaglar *et al*., 2006; Kang *et al*., 2006a). Kõll-Klais *et al.* (2005) reported that oral lactobacilli flora suppressed the growth of periodontal pathogens. The study reported here is the first on the inhibitory effects of *L. reuteri* on periodontopathic bacteria. It showed that *L. reuteri* strains strongly inhibited the proliferation of periodontopathic bacteria. In addition, it found that the *L. reuteri* strains significantly inhibited the proliferation and biofilm formation of *S. mutans.* This concurs with the study by Nikawa *et al*. (2004), who reported that yogurt produced using *L. reuteri* could reduce the growth of *S. mutans*.

Of the VSC, both H_2S and CH_3SH are predominant in mouth air with CH_3SH being quite toxic (Scully *et al*., 1997). CH_3SH may not only be responsible for the oral malodor but may also contribute to the pathogenesis of periodontal disease. Periodontal microflora including *F. nucleatum* and *P. gingivalis* can produce large amounts of CH_3SH and H_2S from methionine and cysteine (Persson *et al*., 1990). Recently, Burton *et al*. (2005) reported that *Streptococcus salivarius* had an inhibitory effect on the production of VSC by competing with the species responsible for higher levels of VSC for colonization sites. The present group also reported that *W. cibaria* isolates from the oral cavity inhibited the production of H_2S and CH_3SH by *F. nucleatum* (Kang *et al*., 2006b). The possible mechanism for the decrease in VSC is that the H_2O_2 generated by *W. cibaria* inhibits the proliferation of *F. nucelatum*.

In the present study, all three *L. reuteri* strains inhibited CH_3SH production significantly. The production of CH_3SH by *F. nucleatum* and *P. gingivalis* were suppressed completely by *L. reuteri*, showing that all three *L. reuteri* strains generated a substantial quantity of H_2O_2 sufficient to inhibit the production of CH_3SH from *F. nucleatum* and *P. gingivalis.* On the other hand, Kleinberg and Westbay (1992) suggested that the production of malodorous molecules was inhibited by an acidic environment. Therefore, both H_2O_2 production and acidity may be responsible for suppressing CH_3SH production.

LAB produce lactic acid and some other organic acids that can exhibit antimicrobial activity (Taniguchi *et al*., 1998). The present study revealed that three *L. reuteri* strains were excellent producers of antimicrobial agents, inhibiting periodontal and caries pathogens. The nature of their antimicrobial activity remains unclear. The antimicrobial activity of the *L. reuteri* culture supernatants was not lost after a catalase and proteinase K treatment. In addition, after adjusting the supernatants to pH 7.0, there was a significant decrease in antimicrobial activity. This suggests that the antimicrobial activity of *L. reuteri* might be attributed to acids. However, *L. reuteri* still showed strong growth inhibition. Therefore, organic acids are not the only antimicrobial substances produced by these *L. reuteri* strains. This suggests the presence of other antimicrobial substances, such as H_2O_2 or BLC. The present study corroborates many other studies, which showed that LAB can produce organic acids, H_2O_2 and bacteriocins (Eschenbach *et al*., 1989; Reid *et al*., 2004).

Many authors reported that *L. reuteri* produced reuterin (Slininger *et al*., 1983; Talarico *et al*., 1988; El-Ziney *et al*., 1999; Cleusix *et al.*, 2007). Our present study showed that *L. reuteri* KCTC 3594 produced the highest quantity of reuterin. Interestingly, *L. reuteri* KCTC 3594 had the highest inhibitory effect on plaque accumulation by *S. mutans* among the *L. reuteri* strains. Although only *L. reuteri* strain KCTC 3594 produced reuterin, the three *L. reuteri* strains had similar ability to inhibit the proliferation of oral pathogens. This suggests that the inhibitory effect is not solely due to the reuterin activity.

In conclusion, *L. reuteri* can inhibit the growth of periodontopathic bacteria and *S. mutans* as well as the production of CH_3SH by *F. nucleatum* and *P. gingivalis.* These results suggest that *L. reuteri* may help decrease the risk of oral infectious disease including dental caries and periodontal disease. Moreover, this organism may be useful as a probiotic for improving oral health.

Acknowledgements

This study was supported by a grant (CRI09010-1) of the Chonnam National University Hospital Research Institute of Clinical Medicine.

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