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Effect of *Leuconostoc* spp. on the Formation of *Streptococcus mutans* Biofilm

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Insoluble glucans synthesized by *Streptococcus mutans* enhance the pathogenicity of oral biofilm by promoting the adherence and accumulation of cariogenic bacteria on the surface of the tooth. The objective of this study was to investigate the effect of *Leuconostoc* spp. on the *in vitro* formation of *S. mutans* biofilm. Three strains, *Leuconostoc gelidum* ATCC 49366, *Leuconostoc mesenteroides* ssp. *cremoris* ATCC 19254 and *Leuconostoc mesenteroides* ssp. *mesenteroides* ATCC 8293, were used in this study. They exhibited profound inhibitory effects on the formation of *S. mutans* biofilm and on the proliferation of *S. mutans*. The water-soluble polymers produced from sucrose were most strongly produced by *L. gelidum*, followed by *L. mesenteroides* ssp. *cremoris* and *L. mesenteroides* ssp. *mesenteroides*. The mean wet weights of the artificial biofilm of *S. mutans* were also significantly reduced as a result of the addition of the water-soluble polymers obtained from *Leuconostoc* cultures. According to the results of thin-layer chromatographic analysis, the hydrolysates of the water-soluble polymers produced by *Leuconostoc* were identical to those of dextran T-2000, forming predominately α-(1-6) glucose linkages. These results indicate that dextran-producing *Leuconostoc* strains are able to inhibit the formation of *S. mutans* biofilm *in vitro*.

Keywords: biofilm, dextran, *Leuconostoc*, *Streptococcus mutans*

Oral biofilm is commonly described as a dental plaque that is composed of bacterial populations and insoluble glucans. An insoluble glucan (mutan) that is primarily synthesized by *Streptococcus mutans* contains as much as 90% α-(1-3) glucose linkages (Wiater *et al.*, 1999). It possesses a marked ability to promote the adherence and accumulation of cariogenic bacteria on the tooth surface, thus increasing the pathogenicity of oral biofilm (Schilling and Bowen, 1992). *S. mutans* strains produce three distinct glucosyltransferases (GTFs; EC 2.4.1.5), GTF-I, GTF-SI, and GTF-S, which are encoded by *gtfB*, *gtfC*, and *gtfD*, respectively (Loesche, 1986; Shiroza *et al.*, 1987; Hanada and Kuramitsu, 1988; Hanada and Kuramitsu, 1989). GTF-I is an enzyme responsible for the formation of insoluble glucans. *S. mutans* has been described as the most important bacteria related to the etiology of dental caries, a biofilm-induced oral disease.

Lactic acid bacteria (LAB) that function as probiotics are well known for their beneficial effects on humans and animals (Marteau and Rambaud, 1993; Naidu *et al.*, 1999; Ljungh and Wadstrom, 2006). However, many authors have suggested that some LAB strains exhibit cariogenic activity (Harper and Loesche, 1984; Bradshaw and Marsh, 1998; Matsumoto *et al.*, 2005). We recently reported that dextran-producing LAB strains isolated from healthy oral cavities inhibited the formation of oral biofilm (Kang *et al.*, 2006a). We suggested that the dextran produced by the LAB isolates inhibited the synthesis of water-insoluble glucans by *S. mutans* via the conversion of GTF activity from the production of water-insoluble glucan to the production of water-soluble glucan.

The genus *Leuconostoc* are a heterofermentative type of LAB that are commonly used as the starter bacteria in some dairy fermentation processes. Some strains of *Leuconostoc* spp. such as *Leuconostoc mesenteroides* are used as the starter cultures for making cheese and butter. In addition, several strains of *Leuconostoc* spp. including *L. mesenteroides* (Hechard *et al.*, 1992) and *Leuconostoc gelidum* (Hastings and Stiles, 1991) produce bacteriocins. Several strains of *Leuconostoc* spp. are known to possess the ability to produce extracellular polysaccharides, such as dextran, when grown in the presence of sucrose (Lawford *et al.*, 1979).

The effective inhibition of insoluble glucan formation may constitute a pivotal approach to the prevention of biofilm-induced oral diseases. The effects of *Leuconostoc* spp. on oral biofilm have not yet been reported. Therefore, the objective of this study was to examine the effect of *Leuconostoc* spp. and *Leuconostoc*-derived dextrans on the formation of *S. mutans* biofilm under various conditions *in vitro*.

Materials and Methods

Bacterial culture and growth conditions

L. gelidum ATCC 49366, *L. mesenteroides* ssp. *cremoris* ATCC 19254 and *L. mensenteroides* ssp. *mesenteroides* ATCC 8293 were selected and grown in De Man, Rogosa, Sharpe (MRS, Difco, USA) at 30°C or 37°C for 16 h. *S. mutans* Ingbritt was grown in Brain Heart Infusion broth (BHI broth, Difco) at 37°C for 16 h.

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***S. mutans* biofilm formation by *Leuconostoc* spp. under various conditions**

Beaker-wire tests were initially performed as previously described (Kang *et al.*, 2006a) in order to determine the effects of *Leuconostoc* strains on the formation of *S. mutans* biofilm. Briefly, equal amounts (1×10^6 CFU/ml) of *S. mutans* and each of the *Leuconostoc* strains were incubated in beakers containing a test medium [a mixture of equal volume of BHI and MRS with sucrose, yeast extract (Difco) and 0.1 M of MES (2-[N-Morpholino] ethanesulfonic acid monohydrate; pH 6.5)] at 30°C and 37°C. A test medium inoculated with *S. mutans* alone was used as a control. Three stainless steel wires (Dentaurum, 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 wet weights of plaque accumulation on the wires were determined by subtracting the wire weight. Beakers for viable cell counting were prepared without wires. Each culture incubated at 30°C and 37°C for 8, 16, and 24 h was serially diluted and plated on MRS agar for the *Leuconostoc* strains and BHI agar for the *S. mutans* in order to determine the effects of *Leuconostoc* on the proliferation of *S. mutans*.

Beaker-wire tests were then performed using the culture supernatants in order to determine the effects by the culture supernatants of *Leuconostoc* strains, which were prepared as follows. Each *Leuconostoc* strain was permitted to grow in MRS broth containing sucrose for 24 h at 30°C and 37°C, and was then centrifuged. Their culture supernatants were then neutralized by 10 M NaOH, and heated at 100°C for 3 min in order to kill the remaining bacteria. The prepared culture supernatants were mixed in the beaker with an equal volume of 2-fold concentrated BHI broth containing sucrose. *S. mutans* was inoculated and incubated for 24 h at 37°C, and then each wire was weighed. Each culture was also serially diluted and plated on BHI agar to determine the effects of the culture supernatant of *Leuconostoc* strains on the proliferation of *S. mutans*.

Effect of dextrans on the S. mutans biofilm formation

Additional beaker-wire tests were carried out as described previously (Kang *et al.*, 2006a) in order to determine the effects of water-soluble polymers from *Leuconostoc* spp. In brief, the water-soluble polymers precipitated from the culture supernatants by ethanol (up to 67%, v/v) were washed, dried and weighed. Each polymer obtained from the *Leuconostoc* strains or commercial dextran T-2000 (molecular weight 2,000,000; Sigma, USA) was added at various concentrations (*Leuconostoc* polymer=1 mg/ml, 5 mg/ml and 10 mg/ml; commercial dextran=0.01 mg/ml, 0.1 mg/ml and 1 mg/ml) into BHI medium containing sucrose. *S. mutans* was inoculated, incubated, and each wire was weighed.

Effect of temperature on dextran production by Leuconostoc spp.

The *Leuconostoc* strains were cultured in MRS broth containing 5% sucrose for 24 h at 30°C and 37°C, respectively, in order to determine the effects of temperature on the water-soluble polymer production by *Leuconostoc* spp. Each bacterial culture was then centrifuged at 4,000×g for 20 min. The polymer precipitated from the culture supernatants by ethanol was washed, dried and weighed.

Thin-layer chromatographic analysis

Each polymer (1%, w/v) dissolved in a 20 mM sodium acetate buffer (pH 5.2) was incubated with *Penicillium* dextranase (1.5 U/ml; Sigma) at 37°C for 20 min in order to analyze the hydrolysates of water-soluble polymers from *Leuconostoc* strains. Dextran T-2000 was used as a positive control. The reaction digests were analyzed by thin-layer chromatography (TLC) with two ascents of 2:5:1.5 (v/v/v) nitromethane:1-propanol:water. The TLC plate was dipped in a mixture of 0.3% (w/v) α-naphthol and 5% (v/v) H_2SO_4 in methanol, and heated at 120°C for 10 min (Tanriseven and Robyt, 1993).

Statistics

Statistical analysis was carried out using a Kruskal-Wallis test for all experiments to identify statistically significant differences and Mann-Whitney test was carried out between groups as a *post hoc* test.

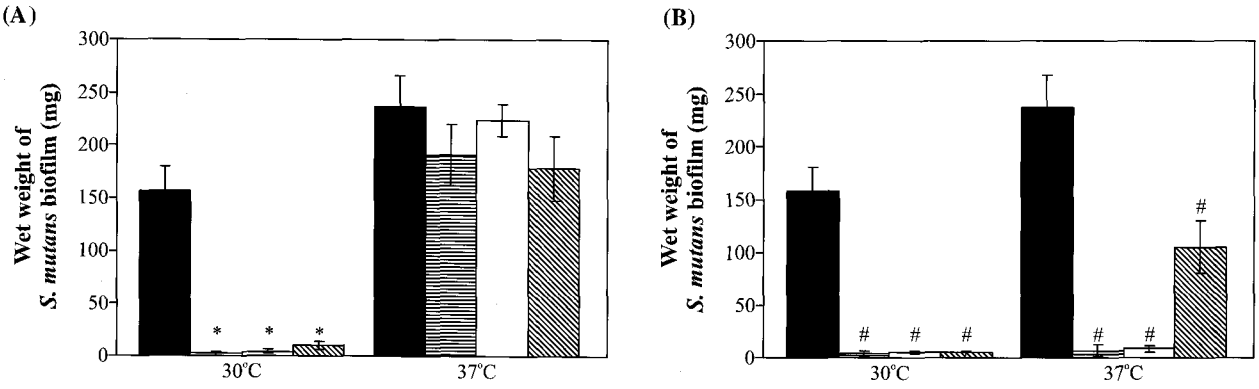

Fig. 1. Effects of cultures (A) and culture supernatants (B) of *Leuconostoc* spp. on the formation of *S. mutans* biofilm at 30°C and 37°C conditions. ■, control; ▤, *L. gelidum*; □, *L. mesenteroides* ssp. *cremoris*; ▨, *L. mesenteroides* ssp. *mesenteroides*. **P*<0.05, *Leuconostoc* cultures *versus* control; #*P*<0.05, culture supernatants of *Leuconostoc versus* control. Values are Means±S.D. of nine determinations (n=9).

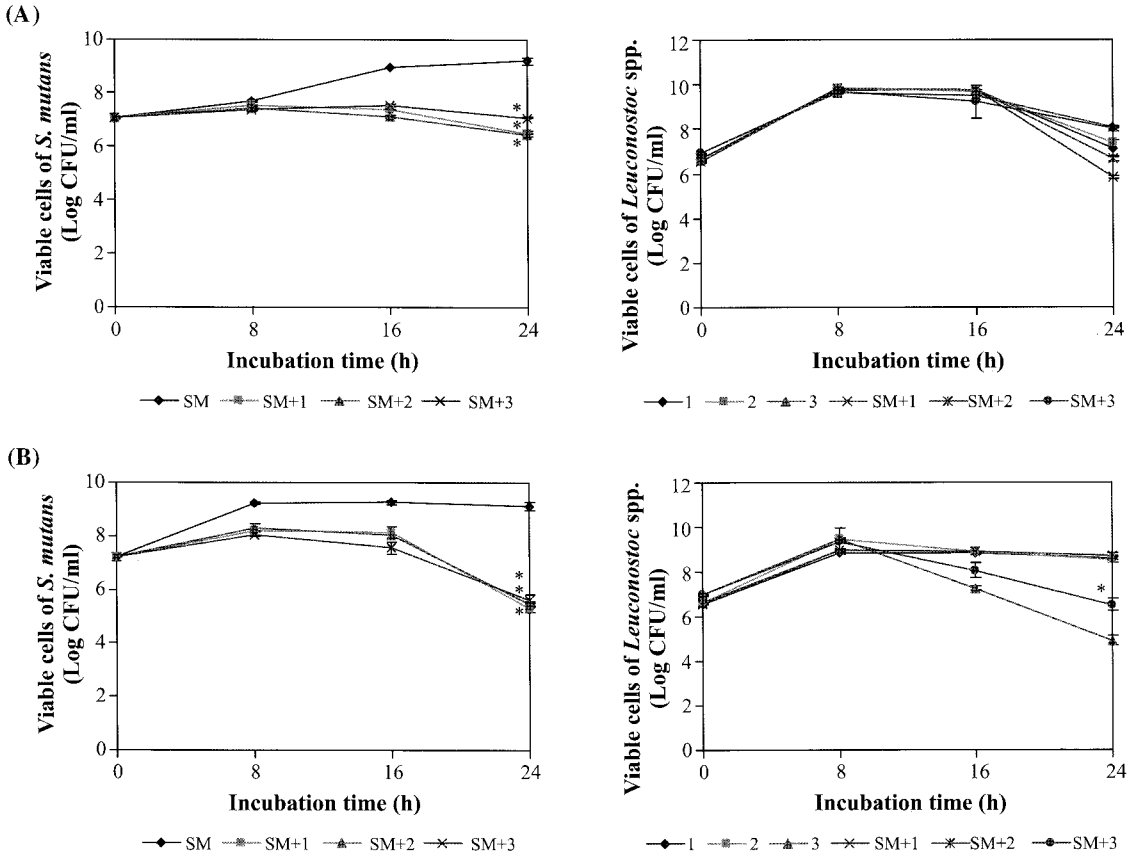

Fig. 2. Viable cells of *S. mutans* and *Leuconostoc* spp. in the mixed cultures over time at 30°C (A) and 37°C (B) conditions. SM, *S. mutans*; 1, *L. gelidum*; 2, *L. mesenteroides* ssp. *cremoris*; 3, *L. mesenteroides* ssp. *mesenteroides*. *$P<0.05$ for coculture *vs* monoculture. Values are Means±S.D. of three determinations ($n=3$).

Table 1. The viable cells of *S. mutans* in the mixed cultures of the culture supernatants of *Leuconostoc* spp. at different temperature conditions

Bacteria	Viable cells of *S. mutans* (CFU/ml)	
	30°C	37°C
S. mutans	$2.13\times10^9\pm2.64\times10^8$	$2.97\times10^9\pm6.50\times10^8$
S. mutans+CS1	$2.80\times10^9\pm6.81\times10^8$	$5.07\times10^9\pm5.19\times10^8$
S. mutans+CS2	$2.75\times10^9\pm2.50\times10^8$	$6.13\times10^9\pm3.13\times10^8$
S. mutans+CS3	$1.31\times10^9\pm9.90\times10^7$	$4.11\times10^9\pm4.72\times10^8$

Values are Means±S.D. of three determinations ($n=3$).
CS1, culture supernatant of *L. gelidum*; CS2, culture supernatant of *L. mesenteroides* ssp. *cremoris*; CS3, culture supernatant of *L. mesenteroides* ssp. *mesenteroides*

Results

Effect of Leuconostoc spp. on the biofilm formation and proliferation of S. mutans

When *Leuconostoc* strains, *L. gelidum*, *L. mesenteroides* ssp. *cremoris*, and *L. mensenteroides* ssp. *mesenteroides*, were cultured with *S. mutans* at 30°C, the *S. mutans* biofilm weights were significantly lowered ($P<0.05$), compared with the control (155.1±23.2 mg) with no differences between *L. gelidum* (2.0±1.7 mg) and *L. mesenteroides* ssp. *cremoris* (4.1±2.0 mg) or between *L. mesenteroides* ssp. *cremoris* and *L. mensenteroides* ssp. *mesenteroides* (10.6±3.8 mg) ($P>0.05$). There was significant difference between *L. gelidum* and *L. mesenteroides* ssp. *mesenteroides*. On the other hand, there were no statistically significant differences of biofilm weights on the wires in the control and mixed cultures at 37°C ($P>0.05$) (Fig. 1A). However, the wet weights of the artificial biofilm on the wires were significantly ($P<0.05$) reduced at both temperature conditions upon the addition of the culture supernatants of the *Leuconostoc* strains (Fig. 1B).

The proliferation of *S. mutans* decreased by 2-log cycles following incubation at 30°C for 24 h in the groups to which *Leuconostoc* strains had been added ($2.6\times10^6\pm4.0\times10^5$ CFU/ml, $P<0.05$), as compared to the control group ($1.5\times10^9\pm4.0\times10^8$ CFU/ml), where the growth of the *Leuconostoc* strains was not affected by *S. mutans* (Fig. 2A). The concen-

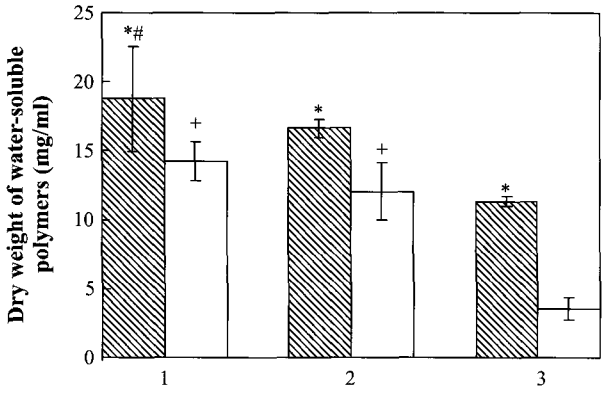

Fig. 3. Formation of water-soluble polymers by *Leuconostoc* spp. at 30°C (▨) and 37°C (□) conditions. 1, *L. gelidum*; 2, *L. mesenteroides* ssp. *cremoris*; 3, *L. mesenteroides* ssp. *mesenteroides*. **P*<0.05, 30°C vs 37°C; #*P*<0.05, *L. gelidum versus L. mesenteroides* ssp. *mesenteroides*; +*P*<0.05, *L. gelidum* or *L. mesenteroides* ssp. *cremoris versus L. mesenteroides* ssp. *mesenteroides*. Values are Means±S.D. of three determinations (n=3).

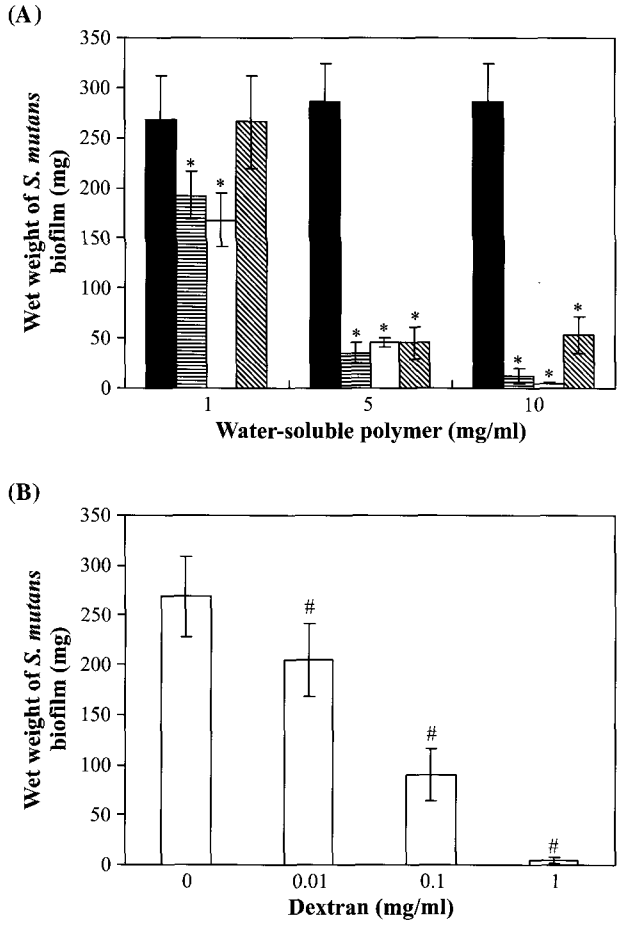

Fig. 4. Effects of water-soluble polymers of *Leuconostoc* spp. (A) or dextran T-2000 (B) on the formation of *S. mutans* biofilm. ■, control; ▤, *L. gelidum*; □, *L. mesenteroides* ssp. *cremoris*; ▨, *L. mesenteroides* ssp. *mesenteroides*. **P*<0.05, water-soluble polymer *versus* control; #*P*<0.05, dextran *versus* control. Values are Means ±S.D. of nine determinations (n=9).

trations of *S. mutans* decreased by 3-log cycles after incubation at 37°C for 24 h in the groups to which *Leuconostoc* strains had been added ($2.2\times10^5\pm9.5\times10^4$ CFU/ml, P<0.05), as compared to the control group ($1.3\times10^9\pm4.4\times10^8$ CFU/ml). On the other hand, the growth of *Leuconostoc* strains was not affected by *S. mutans*, except in the case of *L. mesenteroides* spp. *mesenteroides*. The growth of *L. mesenteroides* spp. *mesenteroides* was significantly enhanced by *S. mutans* (P<0.05) (Fig. 2B). However, the culture supernatants of three *Leuconostoc* strains exerted no apparent inhibitory effects on the growth of *S. mutans* (P>0.05) (Table 1).

Inhibitory effects of dextran against S. mutans biofilm formation

Leuconostoc strains grown in MRS broth containing 5% sucrose produced substantial quantities of water-soluble polymers as shown in Fig. 3(A) greater amount of the polymer by *Leuconostoc* strains was produced at 30°C than at 37°C (P<0.05). The water-soluble polymers by *L. gelidum* were the most strongly produced, followed by *L. mesenteroides* ssp. *cremoris* and *L. mesenteroides* ssp. *mesenteroides*.

The addition of water-soluble polymers obtained from *Leuconostoc* strains reduced *S. mutans* biofilm formation, which was dependent on the concentration of the *Leuconostoc* polymer (P<0.05) (Fig. 4A). Dextran T-2000, a kind of commercial dextran, also concentration-dependently reduced the formation of artificial biofilm (Fig. 4B).

Analysis of water-soluble polymers produced by Leuconostoc spp.

The water-soluble polymers produced by the *Leuconostoc* strains were hydrolyzed with *Penicillium* dextranase and the

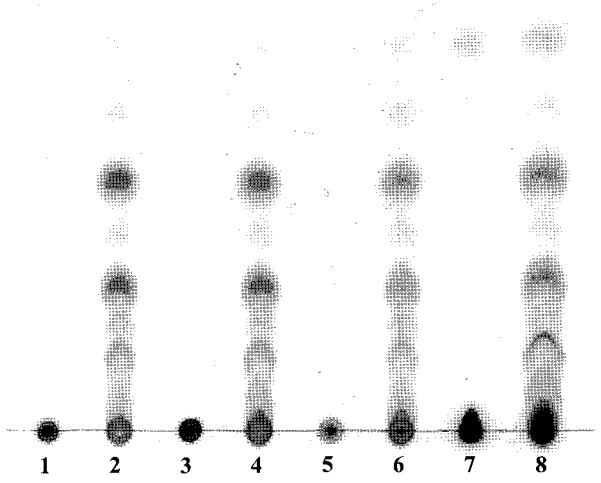

Fig. 5. Thin-layer chromatogram of dextranase hydrolysates of water-soluble polymers produced by *Leuconostoc* spp. Lane 1, water-soluble polymer produced by *L. gelidum*; 2, hydrolysates of water-soluble polymer produced by *L. gelidum*; 3, water-soluble polymer produced by *L. mesenteroides* ssp. *cremoris*; 4, hydrolysates of water-soluble polymer produced by *L. mesenteroides* ssp. *cremoris*; 5, water-soluble polymer produced by *L. mesenteroides* ssp. *mesenteroides*; 6, hydrolysates of water-soluble polymer produced by *L. mesenteroides* ssp. *mesenteroides*; 7, dextran T-2000; 8, dextranase hydrolysates of dextran T-2000.

resulting hydrolysates were compared with those of dextran T-2000 (Fig. 5). The hydrolysates of the *Leuconostoc* polymers were identical to those of dextran T-2000, predominantly forming α-(1-6) glucose linkages. Therefore, the water-soluble polymers produced by these strains were determined to be dextran.

Discussion

The term dextran describes a high molecular weight glucan that consists of α-D-glucolyranosyl units predominantly polymerized in α-(1-6) glucose linkages (Cerning, 1990). The *S. mutans* glucan is a major component of the sucrose-dependent accumulation of cariogenic bacteria on the surfaces of the teeth and subsequently leads to the formation of human dental caries (Colby and Russell, 1997). It has been reported that oxidized dextran specifically inhibited the GTF of *S. mutans* (Inoue and Smith, 1980). Dextran by *Leuconostoc* has been widely applied in the pharmaceutical and food industry (Persson and Grande, 2006; Lacaze *et al.*, 2007).

Some strains of *Leuconostoc* spp. are used in the production of dairy products as well as in the production of bacteriocins (Hechard *et al.*, 1992). The use of some dairy bacterial strains as probiotics for oral health have also been described (Comelli *et al.*, 2002). Many *Leuconostoc* strains possess the ability to produce extracellular dextran when grown in the presence of sucrose. However, there has been no report describing the effects of *Leuconostoc* dextran on *S. mutans* biofilm formation.

In this study, *Leuconostoc* strains profoundly inhibited the proliferation of *S. mutans* at both 30°C and 37°C. On the other hand, the culture supernatants of *Leuconostoc* strains reduced biofilm formation by *S. mutans*, but failed to inhibit the proliferation of *S. mutans*. Therefore, we suggested that different materials from *Leuconostoc* might be responsible for the inhibition of biofilm formation or the growth of *S. mutans*.

Koga *et al.* (1983) reported that exogenously-supplied glucans of various molecular weights stimulate GTF activity and also tend to function as primers for additional glucan synthesis. In the present study, the addition of dextrans obtained from *Leuconostoc* strains into test media reduced the formation of *S. mutans* biofilm, as did the addition of commercial dextran. This result was supported by the report by Montville *et al.* (1977) that insoluble glucan synthesis by *S. mutans* GTF could be shifted to soluble glucan synthesis via the addition of dextrans. Takada *et al.* (1985) also reported that a water-soluble glucan having a branched α-(1-6) glucan from an *S. mutans* mutant inhibited the formation of water-insoluble glucan. Moreover, water-insoluble glucan synthesis by the GTF from *S. mutans* was significantly repressed by the addition of cyclodextran (Kobayashi *et al.*, 1995). Therefore, it is conceivable that the dextrans generated by the *Leuconostoc* strains inhibit the formation of the water-insoluble glucan by *S. mutans* via the conversion of GTF activity from the production of water-insoluble glucans to the production of water-soluble glucans. This result was supported by our previous report (Kang *et al.*, 2006a) that dextran obtained from *Weissella cibaria* isolates inhibited *S. mutans* biofilm formation.

The degree of branching [α-(1-2), α-(1-3), or α-(1-4) glucose linkages] and molecular weight affect the water solubility of the dextran (Lawford *et al.*, 1979). Kim *et al.* (2003) reported that temperature had very little effect on the size of dextran but had a significant effect on the degree of branching. In this study, the increase in temperature resulted in a reduction of the amount of water-soluble dextran produced by *Leuconostoc* strains, which may be due to the increase in the degree of branching in the dextrans.

Some authors have recently reported that probiotics may offer an attractive alternative to chemicals and may have a beneficial effect on oral health (Caglar *et al.*, 2005; Anderson and Shi, 2006). We previously suggested that *Weissella cibaria* isolates from human saliva may be used as *a* probiotic due to its inhibitory effect on biofilm formation and volatile sulfur compound formation, and not being associated with any pathogenicity (Kang *et al.*, 2006a, 2006b). *Leuconostoc* spp. are Gram-positive facultative anaerobic cocci or coccobacilli, that are not part of the usual human flora but found commonly in dairy products and kimchi (Cheigh and Park, 1994). On the other hand, the idea of the development of *Leuconostoc* strains as a probiotic was discouraged due to some reported human infections (Handwerger *et al.*, 1990; Montejo *et al.*, 2000; Albanese *et al.*, 2006). Therefore, in this study, we do not predict *in vivo* effects but suggest that the dextran from *Leuconostoc* strains can be used as a prebiotic for oral health.

Dextran synthesis from *Leuconostoc* strains has been well studied (Lawford *et al.*, 1979; Rodrigues *et al.*, 2005). However, the direct effect of *Leuconostoc* spp. on *S. mutans* biofilm formation has not been investigated. In the present study, *Leuconostoc* strains showed promising results in the reduction of *S. mutans* insoluble glucan formation. In conclusion, we demonstrated that *Leuconostoc* strains are able to inhibit the formation of *S. mutans* biofilm *in vitro*. The development of the dextran from *Leuconstoc* spp. as a prebiotic for oral health will be the focus of further study in our laboratory.

Acknowledgement

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