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Factors Influencing the Competition between *Streptococcus oligofermentans* and *Streptococcus mutans* in Dual-Species Biofilms

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Glucose · Hydrogen peroxide · Lactic acid production · Sequence of inoculation · Sucrose

Abstract
Previous studies have shown that *Streptococcus oligofermentans* inhibits the growth of cariogenic *Streptococcus mutans* in biofilms in vitro and is considered a probiotic candidate for caries prevention. This study aimed to examine the effects of various environmental factors on the competition between *S. oligofermentans* and *S. mutans* in a dual-species biofilm model. Single or dual *S. oligofermentans* and *S. mutans* biofilms were grown in a 96-well active attachment model for 48 h. Several growth conditions were examined in the model, namely: *S. oligofermentans* was inoculated 24 h before *S. mutans* or vice versa; the growth medium was supplemented with 0.2% sucrose or 0.4% glucose; biofilms were grown under a constantly neutral pH or pH-cycling condition, which included 8 h of neutral pH and 16 h of pH 5.5. The 48-h biofilms were examined for viable cell counts and lactic acid and hydrogen peroxide production ability. When *S. oligofermentans* was inoculated first, it clearly inhibited the growth of *S. mutans* and reduced the biofilm lactic acid production by up to 8-fold through hydrogen peroxide production, independently of sugar supply and pH conditions. When *S. mutans* was inoculated first, the level of inhibition by *S. oligofermentans* varied depending on the sugar supply and pH conditions. Thus, the inhibition efficacy of *S. oligofermentans* against *S. mutans* in dual-species biofilms is influenced by environmental factors. This study provides practical information on how to maximize the efficacy of *S. oligofermentans*.

Dental plaque is an oral microbial community that accommodates dynamic interspecies interactions [Marsh, 2006]. A healthy homeostatic state is maintained by a balanced interspecies interaction within this community. Disruption of balance can result in various oral infectious

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diseases, including dental caries and periodontitis [Sbordone and Bortolaia, 2003]. Probiotic bacteria are assumed to restore homeostasis to an unbalanced complex ecosystem. Probiotics are defined by the World Health Organization as "live microorganisms that can provide benefits to human health when administered in adequate amounts, which confer a beneficial health effect on the host" [Joint FAO/WHO Working Group, 2002]. The application of probiotics has been shown to reduce the growth and production of toxic products in opportunistic pathogens, but its beneficial health effects are yet to be confirmed [Matsubara et al., 2016; Gruner et al., 2016].

Streptococcus oligofermentans has been considered a potential oral probiotic candidate for caries prevention [Liu et al., 2014; Bao et al., 2015]. It is frequently isolated from caries-free tooth surfaces [Tong et al., 2003; Zhang et al., 2010] and is capable of inhibiting the growth of the cariogenic bacterial species *Streptococcus mutans* in a biofilm, via hydrogen peroxide (HP) production [Tong et al., 2007]. The mechanism of HP production in *S. oligofermentans* has been well studied. The levels of HP produced by *S. oligofermentans* were reported to be sufficient to inhibit *S. mutans*, but not itself [Tong et al., 2007]. *S. oligofermentans* uses 3 types of enzymes to produce HP, namely, pyruvate oxidase, lactate oxidase (LOX), and L-amino acid oxidase. The ability of converting lactate to HP through the LOX enzyme is particularly interesting because lactic acid in dental biofilms is highly associated with sucrose or glucose fermentation and, consequently, with caries formation. Although the genes encoding pyruvate oxidase and LOX are rather conserved among the commensal HP-producing streptococci, thus far the function of LOX in HP production has only been confirmed in *S. oligofermentans* [Tong et al., 2007; Kreth et al., 2008]. Moreover, *S. oligofermentans* has recently been reported to be capable of inhibiting *S. mutans* under simulated cariogenic conditions using a high-throughput pH-cycling biofilm model. This evidence suggests that *S. oligofermentans* might be a suitable candidate to compete against cariogenic bacteria including *S. mutans* in vivo [Bao et al., 2015]. Despite convincing evidence, the inhibitory effect of *S. oligofermentans* in biofilms has only been observed when *S. oligofermentans* and *S. mutans* were inoculated simultaneously and in the presence of sucrose [Tong et al., 2007; Bao et al., 2015].

Previous studies have shown that interspecies competition can be affected by various environmental factors, such as the order of bacterial colonization on a surface, the type of carbohydrates present, and pH. Kreth et al. [2005] investigated the growth competition between *Streptococcus sanguinis* and *S. mutans*, and found that the sequence of inoculation determined mutual exclusion between the 2 species. However, the effect of inoculation sequence became less crucial under "nutrient-rich" or "stress" conditions. In this case, the nutrient-rich condition refers to bacterial growth medium supplemented with sucrose, whereas the stress condition refers to the adjustment of the pH of bacterial growth medium to 5.5. Similarly, Liu et al. [2012] reported that environmental factors affected the competition between *S. oligofermentans* and *S. mutans*. However, most of the results mentioned above were obtained from an agar plate-based competition assay. This assay is simple and straightforward, but does not represent the interaction of two bacterial species in biofilms. Moreover, investigations on the effect of pH on bacterial competition have revealed poor bacterial growth at constantly low pH (pH 5.5), due to which investigation of competition at this pH is impossible [Kreth et al., 2005; Liu et al., 2014]. Our previous study [Bao et al., 2015] showed that both *S. oligofermentans* and *S. mutans* grew in biofilms under pH values oscillating between 7.0 and 5.5, thus providing an additional model to study bacterial competition at low pH.

Whether environmental factors influence the inhibitory effect of *S. oligofermentans* on *S. mutans* in a biofilm remains unclear. Therefore, this study aimed to examine the competition between *S. oligofermentans* and *S. mutans* under various environmental factors in a dual-species biofilm model. These environmental factors include the sequence of inoculation, type of carbohydrates (sucrose vs. glucose), and pH (constantly neutral pH vs. pH-cycling). The pH-cycling condition includes 8 h at neutral pH and 16 h at pH 5.5, mimicking the prolonged low pH conditions, as might be present at the approximal site in an oral cavity.

Materials and Methods

Bacterial Strains and Growth Conditions

The strains used in this study were *S. mutans* UA159 and *S. oligofermentans* LMG22279. Both bacterial strains were grown anaerobically with palladium catalyst at 37°C under pH 7.0. Biofilms were grown in a modified semidefined biofilm medium (BM), prepared as described by Bao et al. [2015]. Since the biofilm pH drops as soon as they are supplied with carbohydrate, the desired pH values of the biofilms were maintained by the addition of buffer solutions. In order to maintain a neutral pH in the BM during biofilm growth, 0.76 mM K_2HPO_4 and 15 mM KH_2PO_4 were added to the medium. The phosphate buffer was replaced with 30 mM MES buffer to prepare BMs of pH 5.5. Moreover, the concentration of carbohydrate that promoted biofilm formation without causing a

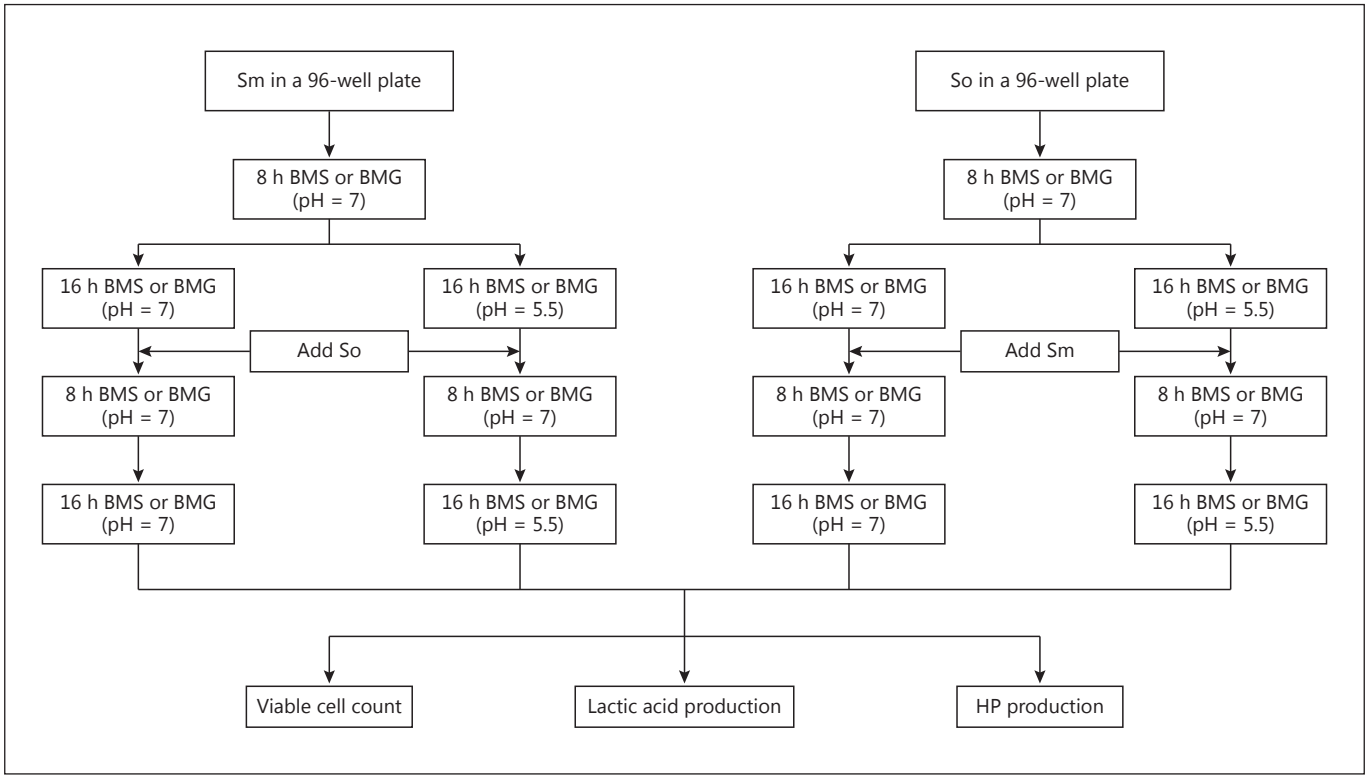

Fig. 1. Diagram of biofilm cultivation. Sm, *S. mutans*; So, *S. oligofermentans*; BMG, biofilm medium containing 22 mM glucose; BMS, biofilm medium containing 5.8 mM sucrose.

pH drop was chosen for biofilm growth. For biofilm growth, 5.8 mM (0.2%) sucrose or 22 mM (0.4%) glucose were supplemented to the BM (BMS or BMG, respectively).

Biofilm Growth

The biofilm cultivation scheme is illustrated in Figure 1. All biofilms were cultivated in a previously described active attachment model [Li et al., 2014]. The general procedure for growing a biofilm is as follows: overnight (16 h) cultures were diluted to a final $OD_{600\,nm}$ of 0.04 in fresh BMS or BMG (pH 7.0), and 200 μL of this suspension was dispensed into each well of a 96-well plate. The plate was covered with a lid containing pegs to allow the active attachment and growth of biofilms. After 8 h, the pegs (biofilms attached) were first rinsed with sterile distilled water, and half of the pegs were then inserted into BMS or BMG at pH 7.0, whereas the other half were inserted into BMS or BMG at pH 5.5. The plate was further incubated for 16 h. The biofilm media were refreshed with the same medium after another 8 and 16 h. The pegs were rinsed with sterile distilled water every time before medium refreshment. The 48-h biofilms formed on pegs were collected for viable cell counts and were examined for their ability to produce lactic acid and HP.

To investigate the impact of inoculation sequence on the competition between *S. mutans* and *S. oligofermentans*, either strain was inoculated as a single-species biofilm and grown for 24 h. The second bacterial species was added to the 24-h biofilms and grown for another 24 h. All experiments were repeated 3 times, and each experiment included 4 replicates.

Viable Cell Counts

The viability of the biofilms was examined by plate counting [Bao et al., 2015]. In brief, each individual peg with biofilms was cut off with a sterile scalpel and dispersed by sonication on ice 60 times for 1 s at an amplitude of 40 W (Vibra cell™, Sonics and Materials Inc., USA). Undiluted and serially diluted samples (100 μL) were plated on brain heart infusion agar plates and incubated anaerobically for 3 days. The colony-forming units (CFUs) from each biofilm were counted. The morphologies of *S. mutans* and *S. oligofermentans* were distinct on brain heart infusion agar plates; therefore, the CFUs of *S. mutans* and *S. oligofermentans* in the dual-species biofilm samples were counted separately based on their colony morphology. The colonies of *S. mutans* were whitish and had a textured appearance, whereas the colonies of *S. oligofermentans* were yellowish and smooth [Bao et al., 2015, supplement]. The detection limit of this viable cell count method is 100 CFUs per species per biofilm.

Lactic Acid and HP Quantification

The 48-h biofilms were assessed for their lactic acid and HP production abilities. Each biofilm was incubated with 200 μL of buffered assay medium (pH 7.0) at 37°C for 4 h. The assay medium contained most components of BM, except yeast extract and 1% glucose. After incubation, 50 μL of assay medium was immediately used for HP measurement. The remainder of the medium was stored at −20°C for subsequent lactic acid quantification. HP was quantified using an enzymatic assay [Bao et al., 2015]. In detail, 50 μL of sample was mixed with 45 μL of 2.5 mM 4-aminoantipyrine

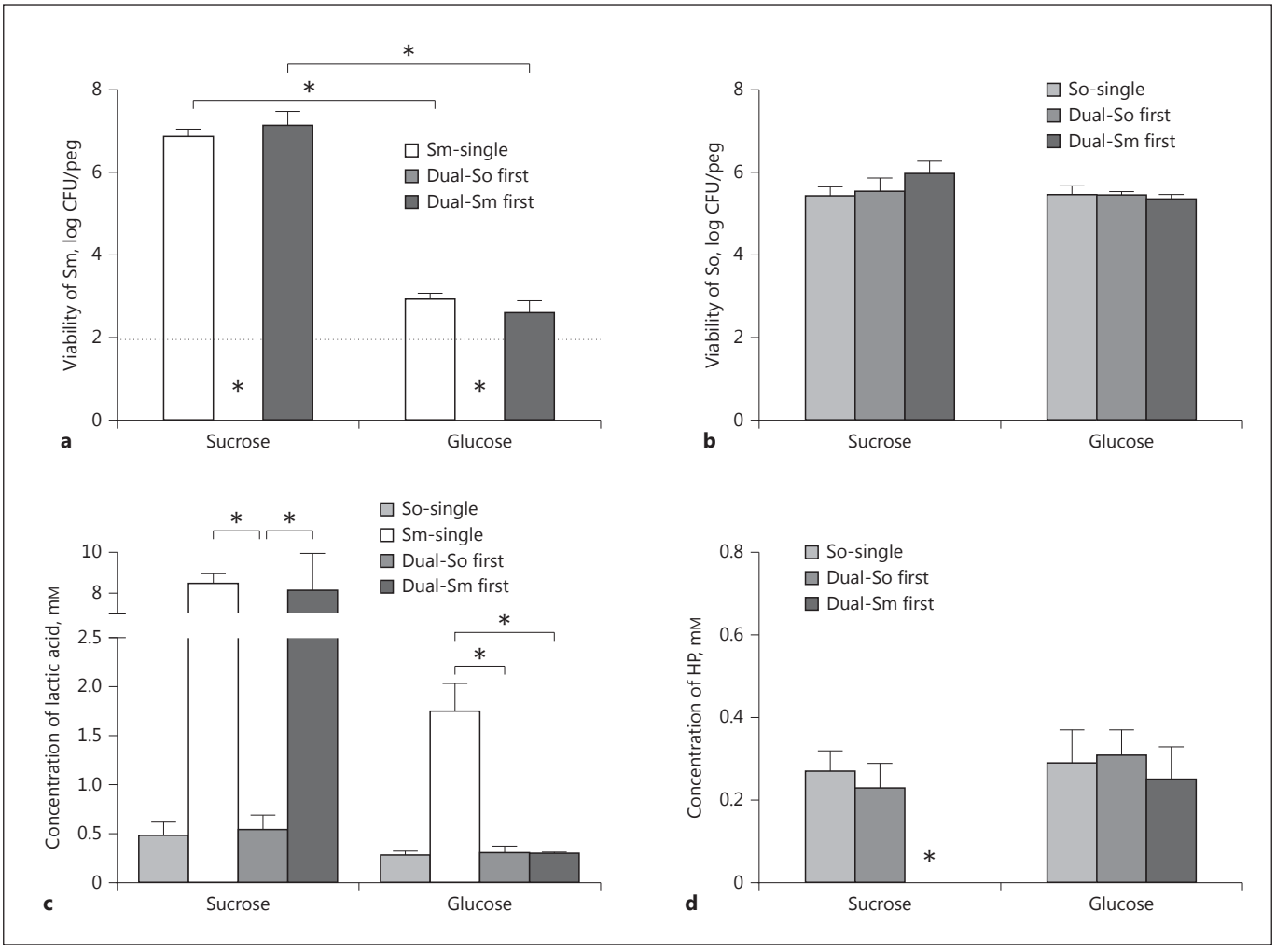

Fig. 2. Viable cell counts, lactic acid production, and hydrogen peroxide (HP) production of 48-h biofilms grown under constantly neutral pH. The numbers of viable *S. mutans* (Sm, **a**) and *S. oligofermentans* (So, **b**) in 48-h biofilms were quantified by agar plate counting. Lactic acid and HP production ability of the biofilms was examined by incubating the biofilms with 1% glucose for 4 h. The concentrations of lactic acid (**c**) and HP (**d**) in the assay medium were quantified. Sm-single, single-species *S. mutans* biofilms; So-single, single-species *S. oligofermentans* biofilms; Dual-So first, dual-species biofilms, with *S. oligofermentans* inoculated first; Dual-Sm first, dual-species biofilms with *S. mutans* inoculated first. The dotted line indicates the detection limit. * $p < 0.05$.

(4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one; Sigma) and 0.17 M phenol. After incubation for 5 min at room temperature, horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was added to the mixture at a concentration of 640 mU mL^{-1} in 0.2 M potassium phosphate buffer (pH 7.2). After another 4 min, absorbance was measured at 510 nm in a spectrophotometer (Perkin Elmer, Norwalk, CT, USA). The HP concentration of each sample was calculated from a standard curve generated using known concentrations of HP.

Lactic acid concentration was determined using an enzymatic-spectrophotometric method, which was performed by measuring the amount of NADH formed during the conversion of lactate to pyruvate in the presence of L-lactate dehydrogenase and NAD [Branda et al., 2005].

Statistical Analysis

Data were analyzed using the Statistical Package for Social Science (SPSS, version 20.0, Chicago, IL, USA). CFU counts were log transformed before statistical tests. Since the data were distributed normally, 2-way ANOVA was performed to evaluate the influence of various factors (inoculation sequence, sugar, and pH conditions) and the interactions between CFU counts, lactic acid concentrations, and HP concentrations of the biofilms. The Bonferroni method was applied as the post hoc test for comparison between groups. $p < 0.05$ was considered significant.

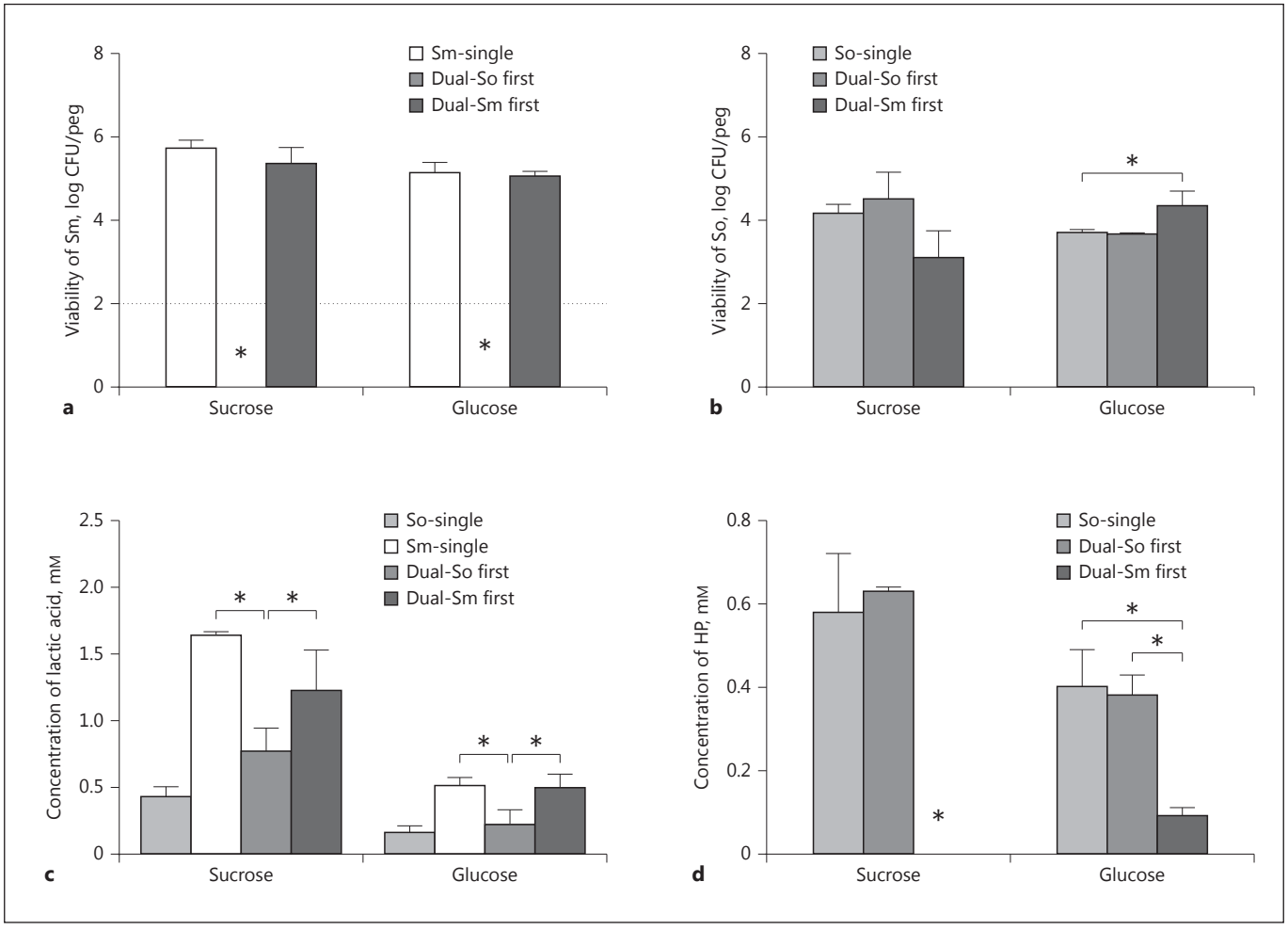

Fig. 3. Viable cell counts, lactic acid production, and hydrogen peroxide (HP) production of 48-h biofilms grown under the pH-cycling condition. The numbers of viable *S. mutans* (Sm, **a**) and *S. oligofermentans* (So, **b**) in 48-h biofilms were quantified by agar plate counting. The lactic acid and HP production ability of the biofilms was examined by incubating the biofilms with 1% glucose for 4 h. The concentrations of lactic acid (**c**) and HP (**d**) in the assay medium were quantified. Sm-single, single-species *S. mutans* biofilms; So-single, single-species *S. oligofermentans* biofilms; Dual-So first, dual-species biofilms with *S. oligofermentans* inoculated first; Dual-Sm first, dual-species biofilms with *S. mutans* inoculated first. The dotted line indicates the detection limit. * $p < 0.05$.

Results

The figures are organized based on biofilm growth conditions. Figure 2 shows the characteristics of biofilms grown under the neutral pH condition, whereas Figure 3 shows the characteristics of biofilms grown under the pH-cycling condition.

Figure 2a presents the viable cell counts of *S. mutans* in biofilms at various inoculation sequences. When *S. oligofermentans* was inoculated first (So-first), no *S. mutans* cells were detected (detection limit = 100 CFUs per sample) in dual-species biofilms. In contrast, when *S. mutans* was inoculated first (Sm-first), the number of *S. mutans* cells in dual-species biofilms was similar to that in the *S. mutans* single-species biofilms (Sm-single). A significant influence of sugar type was observed in Sm-first and Sm-single groups, where the number of *S. mutans* cells was significantly lower in the presence of glucose compared with that in the presence of sucrose. Contrary to observations with *S. mutans*, the sequence of inoculation and the sugar type did generally not affect the viable counts of *S. oligofermentans* in biofilms (Fig. 2b). The cell counts of *S. oligofermentans* were similar in both dual- and single-species *S. oligofermentans* biofilms.

In dual-species biofilms, the sequence of inoculation strongly affected lactic acid production (Fig. 2c). When *S. oligofermentans* was inoculated first, lactic acid production in dual-species biofilms was dramatically reduced compared with that in *S. mutans* single-species biofilms. However, when *S. mutans* was inoculated first, a reduction in lactic acid production was only observed in the glucose group. Figure 2d displays the HP production of various biofilms. Since *S. mutans* does not produce HP, no value was obtained. The HP production of dual-species biofilms was again determined by the sequence of inoculation. When *S. oligofermentans* was inoculated first, the HP production of the dual-species biofilms was comparable with that of *S. oligofermentans* single-species biofilms, irrespective of the sugar type supplied. However, when *S. mutans* was inoculated first, the dual-species biofilms did not produce HP when sucrose was supplied but produced a similar amount of HP as that in *S. oligofermentans* single-species biofilms when glucose was supplied.

In the presence of sucrose, the characteristics of biofilms grown under the pH-cycling condition were generally comparable with those grown under the neutral pH condition (Fig. 3). The sequence of inoculation affected viable counts of *S. mutans* in dual-species biofilms as well as lactic acid and HP production in dual-species biofilms.

However, in the presence of glucose, the characteristics of biofilms grown under the pH-cycling condition were different from those grown under the constantly neutral pH condition. These differences were mainly observed when *S. mutans* was inoculated first. Unlike under the constantly neutral pH condition, the viable counts of *S. oligofermentans* in the Sm-first glucose group were significantly higher than those in the So-single biofilms. Lactic acid production in the Sm-first glucose group was comparable with that in the Sm-single biofilms, and HP production was significantly lower than that in So-single biofilms.

HP production in So-single biofilms grown under the pH-cycling condition was generally 2- to 3-fold higher than that under the constantly neutral pH condition when sucrose was supplied.

Discussion

Dental biofilms in an oral cavity are constantly exposed to environmental challenges, including changes in sugar and periodic pH alterations [Bowden and Hamilton, 1987; Paes Leme et al., 2006]. In this study, various environmental factors were investigated for their influence on the inhibitory action of *S. oligofermentans* on *S. mutans* in a dual-species biofilm model. Our data demonstrated that inhibition of *S. oligofermentans* on *S. mutans* was mainly affected by the sequence of inoculation. The best condition for *S. oligofermentans* as a probiotic is when *S. oligofermentans* colonizes the tooth surface first. These results suggest that a professional tooth cleaning is necessary before the application of *S. oligofermentans* to achieve an optimal outcome in vivo.

The sequence of inoculation can determine mutual exclusion in dual-species biofilms [Kreth et al., 2005; Liu et al., 2012]. Results from the present study partly support the previous findings. Early colonization of *S. oligofermentans* indeed resulted in reduced cell counts of *S. mutans* in biofilms, either because of adherence of *S. oligofermentans* to the limited surface area or because of the HP produced by *S. oligofermentans*. However, early colonization of *S. mutans* did not inhibit the growth of *S. oligofermentans* under the tested conditions. The latter finding was contradictory to the result reported by Liu et al. [2012], in which *S. mutans* inhibited the growth of *S. oligofermentans* on agar plates when *S. mutans* was inoculated first. The difference in the results might be attributed to the different test models used in the 2 studies. The study by Liu et al. [2012] investigated the competition in an agar assay, whereas the current study investigated the competition in an active attachment biofilm model. The concept of active attachment in oral in vitro biofilms is supposed to be more realistic compared with that in the in vivo situation [Exterkate et al., 2010].

Although early colonization of *S. mutans* did not inhibit the growth of *S. oligofermentans*, it suppressed the HP production of *S. oligofermentans*. *S. mutans* UA159 is reported to produce 2 nonlantibiotic mutacins, namely, mutacin IV and mutacin V. These mutacins were able to inhibit the growth of other *Streptococcus* phylogenetic subgroups, except the mutans group [Hossain and Biswas, 2011]. Mutacin production was possibly insufficient to inhibit the growth of *S. oligofermentans* in biofilms but was sufficient to inhibit its HP production activity. Further studies are necessary to prove our hypothesis.

In this study, lactic acid production of the biofilms was found to be generally correlated with the number of *S. mutans* cells in the biofilms, except when *S. mutans* was the first colonizer and the biofilms were grown in glucose under neutral pH conditions. In this biofilm group, lactic acid concentration was considerably lower than that in *S. mutans* single-species biofilms, whereas the viable counts of *S. mutans* were still high and comparable with the sin-

gle-species biofilms. In this specific biofilm group, *S. oligofermentans* possibly converted the lactic acid produced by *S. mutans* into HP and hence reduced the lactic acid concentration. However, the amount of HP produced was possibly still too low to reduce the viable counts of *S. mutans*.

Dietary fermentable carbohydrates have been recognized as the primary factors responsible for biochemical and physiological changes in dental biofilms, which promote caries [Marsh, 1994]. Sucrose and glucose are both fermentable carbohydrates for most oral bacteria. Sucrose is considered to be the "arch criminal" from the dietary perspective because it serves as a substrate for extracellular polysaccharide synthesis [Bowen, 2002; Marsh, 2003], which is crucial for cell attachment and biofilm formation [Rolla, 1989; Schilling and Bowen, 1992]. Our data indicated that in terms of the competition between *S. oligofermentans* and *S. mutans*, the sugar type is secondary to the sequence of inoculation. Compared to glucose, sucrose seems to promote the growth and consequently the lactic acid production of *S. mutans* in biofilms, but does not affect *S. oligofermentans*.

pH is a known key factor for caries formation. The pH of dental biofilms fluctuates after carbohydrate consumption. Net demineralization on a tooth surface occurs when the pH of the surrounding dental biofilms remains lower than pH 5.5 for a prolonged period. Hence, several studies have attempted to investigate the influence of pH on bacterial competition [Kreth et al., 2005; Liu et al., 2014]. However, no conclusion was obtained because the bacterial cells tested in those studies did not grow at a constantly low pH (pH 5.5). Using the previously established pH-cycling model [Bao et al., 2015], the present study showed that pH has less influence on the competition between *S. oligofermentans* and *S. mutans* compared to the sequence of inoculation. Moreover, we confirmed that the HP production of *S. oligofermentans* was higher when the biofilms grew under pH-cycling conditions than under constantly neutral pH conditions when sucrose was applied, as shown in our previous study [Bao et al., 2015]. We also observed that when glucose was applied, the viable counts of *S. mutans* single-species biofilms grown under pH-cycling conditions were higher than those grown under neutral pH conditions. However, lactic acid production was lower in the biofilms grown under pH-cycling conditions than in those grown under neutral pH conditions. *S. mutans* possibly has a higher tendency to form biofilms under poor growth conditions (prolonged low pH conditions), whereas biofilm cells grown under poor conditions are metabolically less active and therefore produce less lactic acid. Additional experiments are needed to prove our hypothesis.

In summary, this study investigated the influence of 3 environmental factors, namely, inoculation sequence, sugar type, and pH changes, on the inhibition of *S. mutans* by *S. oligofermentans*. Our data suggest that under the current experimental settings, the inhibition efficacy of *S. oligofermentans* was mainly determined by the sequence of inoculation and was less affected by the sugar type and pH changes. The data provided in this study will be useful for the future application of *S. oligofermentans* as a probiotic in caries prevention.

Disclosure Statement

There are no conflicts of interest.

Author Contributions

Conceived and designed the experiments: X. Gao, J.J. de Soet, C. van Loveren, D. Deng. Performed the experiments: X. Bao, H. Liu. Analyzed the data: X. Bao, J. Yang, J.J. de Soet, D. Deng.

Contributed reagents/materials/analysis tools: X. Bao, J. Yang, D. Deng. Wrote the paper: X. Bao, J. Yang, X. Gao, J.J. de Soet, C. van Loveren, D. Deng.

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