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ORIGINAL ARTICLE

Short-term consumption of probiotic lactobacilli has no effect on acid production of supragingival plaque

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Abstract Acidogenicity and the levels of mutans streptococci (MS) in dental plaque after the use of *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus reuteri* were determined. The study had a randomised, double-blind, crossover design. Thirteen volunteers used tablets containing LGG or a combination of *L. reuteri* SD2112 and PTA 5289 for 2 weeks. At baseline and at the end of each tablet period, all available supragingival plaque was collected. Lactic acid production was determined from a fixed volume (8 μl) of fresh plaque and the rest of the plaque was used for culturing MS and lactobacilli. The retention of probiotics to the plaque was assessed using PCR techniques. No probiotic-induced changes were found in the acidogenicity of plaque. Also, MS counts remained at the original level. The number of subjects with lactobacilli in plaque increased in the *L. reuteri* group (p=0.011) but not in the LGG group. PCR analysis of plaque revealed the presence of LGG in four and *L. reuteri* in six subjects after the use of the probiotic. The use of the lactobacilli did not affect the acidogenicity or MS levels of plaque. Short-term consumption of LGG and *L. reuteri* appeared not to influence the acidogenicity of plaque.

Keywords Mutans streptococci · *Lactobacillus rhamnosus* GG · *Lactobacillus reuteri* · Acid production · Dental plaque · Probiotic

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Introduction

Probiotic bacteria have beneficial health effects especially when used to prevent or treat gastrointestinal infections [1]. They may also reduce the risk of infections in infancy [2]. From a dental point of view, acidogenic and aciduric lactobacilli are generally considered cariogenic [3] and may be regarded as a risk for dental health. Both *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus reuteri* produce acids from glucose in vitro and decrease pH, like *Streptococcus mutans* [4]. We have recently shown in vitro that four commonly used probiotics, including LGG and *L. reuteri*, interfered with *S. mutans* biofilm formation, and that the antimicrobial activity against *S. mutans* was pH-dependent [5]. The antimicrobial activity of lactobacilli is largely attributed to their ability to generate a low pH [6]. In clinical studies, LGG and *L. reuteri* were, however, not able to colonise the oral cavity permanently [7–9].

Most studies on probiotics and oral health have focused on measuring changes in mutans streptococci (MS) counts [review 10]. Even though high counts of MS do not necessarily mean an increased caries risk, decreasing MS without affecting the normal flora should make the plaque less virulent. A significant decrease in MS counts has been

demonstrated in association with the consumption of *L. reuteri* [10]. *L. reuteri* ATCC 55730, as such or combined with *L. reuteri* PTA 5289, has been given to adults in short-term clinical studies, and all studies found a decrease of MS counts [10]. LGG consumed for 3 weeks in the form of cheese or for 7 months in milk was, however, associated with minor changes in the MS counts [11, 12].

The few studies on probiotics and caries occurrence suggest that probiotics are beneficial rather than hazardous to dental health. LGG has been suggested to reduce caries occurrence in children. Näse et al. [12] examined the effect of *L. rhamnosus* GG milk used 5 days a week for 7 months on the dental health of preschool children. The results showed that LGG reduced caries occurrence. In a recent Swedish study, preschool children used milk supplemented with fluoride and *L. rhamnosus* LB21 for 21 months [13]. Caries occurrence decreased significantly, but as the authors themselves state, it is difficult to establish the role of the probiotic organism in the result.

Since the consumption of probiotics is increasing, and even infants are given probiotics, studies concerning the safety of probiotics from the oral point of view are needed. In theory, it is possible that regular use of probiotic *Lactobacillus* strains could increase the acid production potential of plaque. Our aim was to expose plaque to LGG and *L. reuteri* twice a day and study the changes in the acid production of plaque, the counts of acidogenic plaque microorganisms, MS and lactobacilli, as well as the retention of LGG and *L. reuteri* to plaque. The null hypothesis was that the consumption of neither LGG nor *L. reuteri* has any effect on the acid production of plaque.

Materials and methods

Subjects

Altogether, 28 students of the University of Turku, Finland were screened for the presence of salivary MS (Dentocult SM Strip Mutans, Orion Diagnostica, Espoo, Finland). The 13 students [mean age (SD), 25.3 (2.4) years; five male, eight female] showing salivary MS counts of $\geq 10^4$ colony-forming units (CFU)/ml were invited to participate and they volunteered for the study. All subjects had normal flow rates of paraffin-stimulated saliva (>1 ml/min). All subjects had good oral hygiene with low DMFT (decayed, missing, filled teeth) scores [mean DMFT (SD) 2.8 (2.7)].

The inclusion criteria were good general health, willingness to participate and salivary MS counts $\geq 10^4$ CFU/ml. The study was approved by the ethics committee of the Hospital District of Southwest Finland (identification code 55/2009). A written informed consent was obtained from all subjects. The ethics committee defined the stopping rules.

Test products

Commercially available tablets containing, according to the manufacturer, 200 million CFU of *L. reuteri* ATCC 55730 and PTA 5289 per tablet (Reladent^R, Biogaia AB, Sweden) were purchased from a Finnish pharmacy. In our quality control by plate culturing, we found 180 million CFU per tablet. Tablets containing *L. rhamnosus* GG (196 million CFU per tablet; plate culturing) were specially manufactured free of charge by Karl Fazer AB (Vantaa, Finland). The *L. rhamnosus* GG used in the LGG tablets was from Probiotical S.p.A., Novara, Italy. The appearance of the LGG tablet was similar to that of the *L. reuteri* tablet. A technical assistant, unaware of the study, packed the tablets in identical plastic bottles. The bottles containing adsorbent pads to control humidity were marked with a letter A or B and kept at +4°C until distribution. A 2-week supply was given to all subjects, along with the instructions to store the bottles in a refrigerator at home. The viability of the probiotics did not change during the storage at +4°C.

Study design

A 3-week run-in period chosen according to earlier studies [7–9] preceded the study. At the beginning of the run-in period, the subjects were given written instructions: products containing *L. rhamnosus* GG or *L. reuteri* (the most common trade names in Finland are Gefilus^R and RELA^R, respectively) were not allowed, but otherwise, the subjects were to retain to their normal toothbrushing and dietary habits. None of the subjects were habitual consumers of Gefilus or RELA products before the run-in period. Four of the subjects consumed yoghurt with bifidobacteria and/or *L. acidophilus* on a daily basis. They were instructed not to change their consumption habits during the study. All subjects used xylitol products (mostly chewing gum) on a daily basis. To ensure compliance, the subjects were instructed to continue with their consumption habits. All subjects brushed their teeth at least once a day. Throughout the study, the subjects used the same brand of fluoridated toothpaste provided to them free of charge. The drinking water in the city of Turku contains 0.3 mg/l fluoride. Antimicrobial mouthrinses were not allowed. None of the subjects had been on antimicrobial medication for at least 4 weeks before the study. Compliance to the given instructions was checked at each plaque-sampling visit. At these visits, the subjects were also interviewed for the use of xylitol products, other dietary habits and oral/general health. These data were used to check for compliance, possible adverse effects and other confounding factors.

The study had a double-blind, crossover design. The subjects were randomly allocated into two groups: half of the subjects used *L. rhamnosus* GG tablets first and then

L. reuteri tablets for 2 weeks twice a day, while the other half used the tablets in the reverse order. The first tablet was instructed to be taken in the morning, and the second after the evening toothbrushing. These instructions were to be followed also on those days when plaque samplings were carried out. The subjects were asked to chew the tablets thoroughly to disrupt the structure before swallowing. In between the two tablet sequences, there was a 5-week washout period. To ensure compliance, the subjects could choose the day of entrance individually.

Outcome measures, sample size and blinding

The primary outcome measure was the lactic acid production of plaque and the secondary outcome was the MS level of plaque. As an earlier study demonstrated significant *L. reuteri*-induced decrease in MS levels in a group of only ten MS-positive subjects, sample size calculation was not used here [10]. Furthermore, our 13 subjects used both test products and, therefore, paired sample analysis, sensitive for even small changes, could be used in this study.

The first author (AM), who recruited the subjects and collected all samples, as well as the technical assistants analysing the samples, was blinded to the study assignment. The author (ES) who had the code did not participate in collecting or analysing the samples nor had any contact with the subjects.

Plaque sampling

Plaque was allowed to accumulate for 24 h with no oral hygiene at baseline and at the end of each tablet period. On the morning of the plaque collection day, the subjects were instructed to use the probiotic tablet. Plaque sampling was performed in the afternoon by the first author (AM). All available supragingival plaque was collected with dental curettes. A standard volume of 8 μl of plaque was collected in a surgical spoon, transferred into a test tube containing 1 ml of cold fermentation minimal medium (FMM) [14] and the rest was transferred into 1 ml of cold saline.

Acid production of plaque

Immediately after plaque collection, the FMM-tube was vortexed for 5 s and 50 μl of 200 mM glucose was added. The tube was vortexed again for 5 s and incubated at +37°C for 30 min. The cells were separated by centrifugation (13,300×*g*, 10 min) and the supernatant was stored at −70°C until lactic acid determination (L-lactic acid kit, R-biopharm AG, Darmstadt, Germany). The method measures NADH, the amount of which is stoichiometric to the amount of L-lactic acid in the sample. The increase in NADH was determined by means of its light absorbance at 340 nm.

Microbiological analyses

The rest of the pooled plaque was dispersed by pumping the suspension back and forth with a disposable plastic pipette in saline. Then, duplicate samples (100 μl) were added into 900 μl tryptic soy broth with 10% glycerol (TSB, Difco, MI, USA) and stored at −70°C before microbial analyses. The remaining plaque suspension was used to quantify *L. rhamnosus* by quantitative PCR (qPCR) (below).

Plate culturing of microorganisms The TSB tubes were thawed and vortexed for 1 min. The content was pumped back and forth using a disposable pipette and the resulting suspension was mildly sonicated. After serial tenfold dilutions, the bacteria were plated on Mitis Salivarius (Difco, Detroit, MI, USA) agar containing bacitracin (MSB). The plates were incubated at +37°C in a 7% CO_2 atmosphere for 2 days. MS were identified as described earlier [15]. The number of MS was identified based on colony morphology and counted by means of a stereomicroscope. The identification of *S. mutans* was based on consistent findings of "rough" colony morphology, positive fermentation with sorbitol, mannitol, raffinose and melibiose and negative dextran agglutination. Identification of *S. sobrinus* was based on "smooth" colonies, positive fermentation with mannitol but negative with raffinose and melibiose and positive dextran agglutination. Total streptococci were cultured on Mitis Salivarius agar (Difco) and total facultative bacteria on blood agar (Orion Diagnostica, Espoo, Finland). The lactobacilli and total facultatives were grown for 2 days anaerobically (80% N_2, 10% CO_2, 10% H_2) at +37°C on Rogosa agar (Difco) and blood agar (Orion Diagnostica, Espoo, Finland), respectively. The results were expressed as colony-forming units. The detection limit of the plate culturing was 100 CFU per sample.

Quantitative PCR detection of L. rhamnosus DNA extraction of plaque samples suspended in 800 μl of phosphate buffered saline was performed with the method previously described by Nylund et al. [16] involving mechanical cell disruption by two rounds of bead beating as a pre-treatment step followed by an automated DNA extraction by using the KingFisher magnetic particle processor (Thermo Electron, Vantaa, Finland) with an InviMag® Stool DNA kit (Invitek GmbH, Berlin, Germany). The final elution volume of extracted DNA was 80 μl.

L. rhamnosus species-specific qPCR was performed with the 16S rRNA gene targeted forward 5′-GGTGCTTG CATCTTGATTTA-3′ and reverse 5′-CAGTTCGGCTACG TATCATT-3′ primers, which amplify a 237-bp fragment of the gene [17]. qPCR analysis was carried out in an Applied Biosystems 7300 Fast Real-Time PCR System and using SYBR Green chemistry (Power SYBR Green PCR Master

Mix, Applied Biosystems, Foster City, CA, USA) and 0.3 μM of each primer. The total volume of qPCR reaction was 25 μl, employing 1–5 μl of DNA sample or standard as a template. The thermocycling programme was +95°C for 10 min; 35 cycles of +53°C for 30 s, +72°C for 1 min and +95°C for 15 s; +72°C for 5 min, followed by cooling to +4°C.

The standards were prepared by amplifying 16S rDNA from *L. rhamnosus* ATCC 53103. The amplified fragment was purified (QIAquick PCR purification kit, Qiagen, Duesseldorf, Germany), the concentration quantified (NanoDrop ND-1000 spectrophotometer, NanoDrop® Technologies, Wilmington, DE, USA) and appropriate dilutions with a known number 16S rRNA gene copies prepared to be used as standards.

Plaque samples were assayed in duplicate in two independent runs and the results were analysed using Applied Biosystems 7300 Fast Real-Time PCR System SDS Software (version 1.4.0). Melting curve analysis was performed after the PCR to confirm the specificity of amplification. The amount of 16S rRNA gene copies of *L. rhamnosus* in plaque samples was determined by comparing the threshold cycle (Ct) values of samples to those of the standard curves. The detection limit of the method was 100 CFU per sample.

Strain-specific PCR of L. rhamnosus GG For qualitative PCR, two to three lactobacilli colonies were collected from Rogosa plates with plastic loops from the post-intervention plaque samples of those subjects who harboured lactobacilli. The colonies were collected from the plates with plastic loops, bacterial cells were washed twice with TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA pH 8) and resuspended in 50 μl of a TE buffer. The bacterial suspension was heated at +98°C for 10 min to release DNA from the cells. After cooling on ice, the suspension was centrifuged 10 min at 13,000×*g* and the supernatant was collected.

The LGG strain-specific PCR was performed as described previously by using end-point PCR with the primers forward 5′-CGCCCTTAACAGCAGTCTTCAAAT-3′ and reverse 5′-ACGCGCCCTCCGTATGCTTAAACC-3′ and agarose gel electrophoresis and ethidium bromide staining [18]. Five microlitres of the extracted DNA from a bacterial isolate was used as a template in a 25-μl reaction volume.

Species-specific PCR of L. reuteri For the gene sequence analyses, two to three lactobacilli colonies were collected from Rogosa plates with plastic loops from the plaque samples of those subjects who had consumed the *L. reuteri* tablets and who harboured lactobacilli after the intervention. The colonies were collected in 1 ml MRS medium (Scharlau Chemie A.G., Barcelona, Spain) and the tubes were stored at −70°C before PCR analyses. To identify the lactobacilli by species, a 1,420-bp long fragment of the 16S rRNA gene was PCR amplified and sequenced. One single cell clone was dissolved in 50 μl Milli-Q (MQ) and 5 μl was used in the PCR mixture together with 12.5 μl HotStarTaq Mastermix (Qiagene), 5.5 μl MQ and 10 μM of each primer 16S-LF and 16S-R in a total volume of 25 μl. The sequence of the primers 16S-LF and 16S-R was 5′-AGA GTT TGA TCC TGG CTC AG-3′and 5′-GGG CGG TGTGTACAAGGC-3′, respectively. The thermal cycling conditions were 15 min at +95°C, 35 cycles of 30 s at +94°C, 30 s at +59°C and 1.5 min at +72°C, followed by +72°C for 10 min.

The PCR products were sent to Eurofins MWG Operon (Egerberg, Germany) for purification and sequencing using primers 16S-LF and 16S-R. To search for DNA similarity, the Human Oral Microbiome Database (http://www.homd.org) was used. The analysis is species-specific, and detects both *L. reuteri* SD2112 and PTA 5289.

Statistical analysis

The *t* test for paired samples and Wilcoxon signed ranks test were used to study the differences in lactic acid and the microbiological results of plaque between baseline and at the end of each tablet period. These tests were also used to compare the baseline values to those preceding the experimental periods. Spearman's rank correlation test was used to study the associations between individual intervention-induced changes in the acidogenicity of plaque and changes in the levels of lactobacilli and MS. The

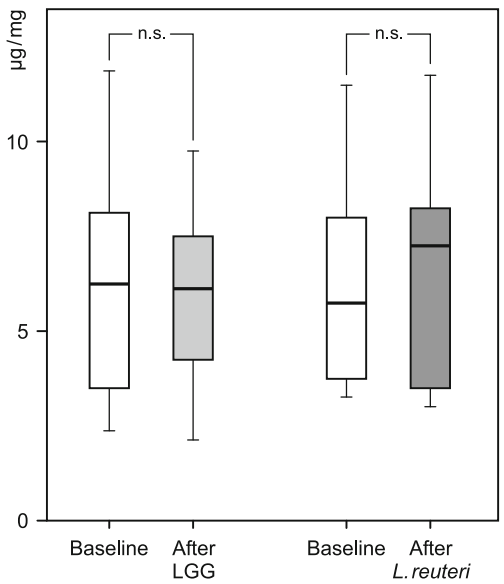

Fig. 1 Lactic acid production of plaque suspension (in micrograms per milligram plaque) at baseline and after 2-week consumption of tablets containing LGG or *L.reuteri.* The boxplots show medians, first and third quartiles and the minimum and maximum of the lactic acid concentrations in the cell-free supernatants after 30-min incubation. *n.s.* not significant

Table 1 Counts of MS, lactobacilli, total streptococci and facultatives (mean log CFU±standard deviation) in the plaques at study baselines and after the 2-week consumption of LGG and *L. reuteri* tablets

	Baseline	After LGG	*p*	Baseline	After *L. reuteri*	*p*
Mutans streptococci	4.6 (1.6)	4.4 (1.7)	0.492	4.5 (1.9)	4.8 (2.2)	0.391
Lactobacilli	0.7 (1.4)	1.4 (2.1)	0.132	1.1 (1.2)	2.4 (2.4)	0.011
Total streptococci	6.8 (0.8)	7.0 (0.7)	0.407	6.7 (0.5)	7.0 (0.9)	0.076
Facultatives	7.2 (0.9)	7.5 (0.8)	0.308	7.1 (0.7)	7.4 (0.9)	0.197

The *t* test for paired comparisons was used to test statistical significance

programme package SPSS 14.0 for Windows was used. The level of statistical significance was set at $p<0.05$.

Results

All plaque samples of the 13 subjects produced detectable amounts of lactic acid in the acid production tests. There were interindividual differences in acid production potentials of plaque, but the intraindividual differences were rather small. No differences were detected in lactic acid production after the LGG- or the *L. reuteri*-tablet periods when compared to baseline (Fig. 1). Intervention-induced changes in the acidogenicity of plaque showed no association with changes in the lactobacilli and MS levels.

At baseline, all subjects had *S. mutans* in plaque while only one had a combination of *S. mutans* and *S. sobrinus*. No study-induced changes in plaque MS counts were detected either in the LGG or *L. reuteri* group (Table 1).

At baseline, one subject presented with high counts of lactobacilli (log CFU>4), three showed low counts barely exceeding the detection limit (log CFU<4) and the rest did not present with detectable numbers of lactobacilli (Table 2). The use of LGG did not affect the lactobacilli counts (Table 1). As one subject showed high counts of lactobacilli at baseline, only the after-intervention samples were used for PCR analyses. In the quantitative, species-specific PCR available for detection of *L. rhamnosus*, seven subjects showed detectable plaque counts of *L. rhamnosus* after the use of the LGG tablets. The qualitative PCR, however, confirmed that only four of these samples contained *L. rhamnosus* GG (Table 2).

The use of *L. reuteri* tablets significantly increased the lactobacilli levels ($p=0.011$; Table 1). However, only two subjects showed an increase from low to high lactobacilli counts. In three subjects, the increase barely exceeded the detection limit. Five subjects had no lactobacilli before or after the intervention. In the 16S rRNA gene sequence analysis of lactobacilli-like colonies collected from the eight lactobacilli-positive subjects, six presented with *L. reuteri* (Table 2).

No differences were detected in the counts of total streptococci or facultatives between the baseline and after the tablet periods (Table 1). The interviews carried out at each plaque-sampling visit revealed no potential confounding factors. Also, no side effects were reported during the course of the study.

Discussion

The results of the study confirmed the null hypothesis: the consumption of neither *L. reuteri* nor LGG had any effect on the acidogenicity of plaque. Even though the number of subjects in the present study was limited, the sensitive paired comparisons used in the statistical analyses would have detected systematic increases/decreases in the variables. Furthermore, those subjects who showed increases in their MS or lactobacilli counts and/or harboured *L. reuteri* or LGG in their plaque after the intervention showed no systematic increase in the plaque acid production. Concerns have been expressed whether the habitual consumption of probiotic lactobacilli could increase the level of lactic acid bacteria in the oral cavity [19]. Since the most commonly used probiotic lactobacilli are both acidogenic and aciduric

Table 2 Distribution of the lactobacilli counts in the study subjects at baseline and after the use of LGG and *L. reuteri*. The number of subjects harbouring LGG or *L. reuteri* (PCR-positive) vs. lactobacilli (LB)-positive subjects is also shown

Probiotic	Time	Lactobacilli (plate culturing)			PCR-positive/LB-positive
		Not detected	CFU<10^4	CFU>10^4	
LGG	Baseline	9	3	1	Not analysed
	After LGG	8	3	2	4/5
L. reuteri	Baseline	9	3	1	Not analysed
	After *L.reuteri*	5	5	3	6/8

[3], the consumption of such lactobacilli could, in theory, also increase the lactic acid production of plaque. Since the consumption of probiotics is increasing, and even infants are given probiotics, studies concerning the safety of probiotics from the oral point of view are important. To our knowledge, there are no previous studies on this issue.

In earlier studies with study designs different from the one we used, consumption of *L. reuteri* and LGG have not increased the salivary levels of lactobacilli [12, 20–22]. In our study, the subjects were instructed to use the probiotic tablet in the morning of the plaque-sampling day. Thus, it is no surprise that the use of the *L. reuteri* tablets resulted in an increase in the lactobacilli counts of plaque; this was expected also for the LGG tablet use. Even though the *L. reuteri* intervention resulted in a statistically significant increase in the lactobacilli counts, most increases in the lactobacilli counts barely exceeded the detection limit.

The retention studies with LGG and *L. reuteri* suggest that they can be detected in saliva during the consumption of probiotics, but when the use stops, they show rapid clearance from the oral cavity [7–9]. In connection with the consumption of *L. reuteri* ATCC 55730 tablets, all 25 subjects harboured the probiotic in their saliva during the intervention, but it was eliminated within a few weeks [8]. LGG was found in the saliva of 70–90% of adult subjects during the consumption of yoghurt and cheese, but within a few weeks also, LGG was eliminated from the saliva [9]. In our study, the PCR analyses showed that only six subjects harboured *L. reuteri* in their supragingival plaque samples at the end of the intervention period. For LGG, this number was even lower; only four subjects had detectable counts of LGG in their plaque. Plaque may thus not be an oral reservoir for ingested probiotics.

No study-induced effect on plaque MS counts was found in the *L. reuteri* group. Most of the earlier studies have demonstrated a decrease in salivary MS counts with chair-side tests both with *L. reuteri* ATCC 55730 [20] and the combination of *L. reuteri* ATCC 55730 and PTA 5289 [21, 22]. In the above short-term studies, *L. reuteri* was administered to young healthy adults one to three times a day through straw, tablet or chewing gum [20–22]. If *L. reuteri* was used once a day in chewing gum, no decrease in MS count was detected [22]. Also in our short-term study, the subjects were young adults and the *L. reuteri* doses and daily consumption frequencies were similar to the earlier studies. Contrary to the studies above, we studied the levels of MS from plaque not from saliva. This may partly explain the discrepancy between our current results and those of earlier studies. Recently, a study by Cildir et al. demonstrated that one daily dose of *L. reuteri* fails to reduce the salivary MS counts in children [23]. More research is clearly needed on this topic. Also, in the LGG group, no study-induced effect on MS was detected. This result is supported by earlier studies which detected only small decreases [11] or trends of decreases [12] in the salivary MS counts in association with LGG consumption. Also, two recent trials on the consumption of *L. rhamnosus* LB21 in milk did not reveal any decreases in salivary or plaque MS counts [13, 24]. A confounding factor in our study could be the xylitol consumption of the subjects. It should, however, have affected both interventions in the same way. Furthermore, in earlier studies conducted in similar subjects with good oral health, habitual xylitol consumption has decreased counts of MS but not affected the counts of lactobacilli [25, 26].

In conclusion, short-term administration of probiotic lactobacilli did not affect lactic acid production or MS counts of plaque. Thus, consumption of *L. reuteri* and LGG appeared not to influence the acidogenicity of plaque.

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Conflicts of interest The authors declare that they have no conflicts of interest.

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