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ORIGINAL ARTICLE

Modulation of the host response by probiotic *Lactobacillus brevis* CD2 in experimental gingivitis

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OBJECTIVE: Probiotic *Lactobacillus brevis* CD2 (CD2) exerts anti-inflammatory properties by preventing nitric oxide synthesis. It is hypothesized that oral application of CD2 can inhibit naturally occurring gingival inflammation.

MATERIALS AND METHODS: Thirty-four healthy adults were randomized to receive *L. brevis* CD2 lozenges or placebo, three times daily for 14 days. The subjects refrained from oral hygiene, the extent of which was determined at various time points.

RESULTS: In both groups, bleeding on probing scores increased continuously throughout the study except on day 3. In the placebo group, scores increased significantly from 9.50 at baseline to 14.75 and 14.81 on days 10 and 14, respectively ($P < 0.05$). No significant change from baseline was observed in the CD2 group. However, scores were consistently higher with placebo, and significant intergroup differences were observed on day 10. Plaque and gingival indices increased from baseline in both treatment groups, but no intergroup differences were observed. Measurements of immune markers in gingival crevicular fluid revealed increased production of nitric oxide in the placebo group ($P < 0.05$). Prostaglandin E_2 production decreased over time in both groups.

CONCLUSION: *Lactobacillus brevis* CD2 may delay gingivitis development in this model by downregulating an inflammatory cascade.

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Keywords: probiotics; gingivitis; *Lactobacillus brevis*; gingival crevicular fluid

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Introduction

The relationship between dental plaque and periodontal inflammation has been extensively studied in humans as well as in non-human models. One of the most commonly used models to study the relationship between gingivitis and plaque accumulation is referred to as 'experimental gingivitis' (Löe *et al*, 1965, 1967; Theilade *et al*, 1966). Recent experiments and extensive reviews suggest that the susceptibility to plaque-induced gingivitis differs significantly among subjects without significant compositional differences in plaque. Such individual differences in the inflammatory responses to dental plaque have been described in experimental gingivitis (Lie *et al*, 1995; Trombelli *et al*, 2004), suggesting that host-related factors play important roles in determining the severity and the course of a gingival inflammatory response to plaque accumulation (Scapoli *et al*, 2005, 2007). Based on such findings, efforts have been made in animal and human models to understand whether host factor modulation can be an additional strategy to prevent or attenuate the gingival inflammatory responses to plaque accumulation as an adjunct to conventional mechanical and chemical removal of plaque (Müller *et al*, 2006; Paquette *et al*, 2006).

Salvi and Lang (2005) performed a comprehensive analysis on various biochemical agents that can modulate host response in the management of periodontal diseases. Six therapeutic approaches with different mechanisms of actions were discussed, which included modulation of arachidonic acid metabolites via non-steroidal anti-inflammatory drugs, lipoxin as an endogenous modulator of inflammation, modulation of matrix metalloproteinases, modulation of bone remodelling, modulation of host cell receptors and modulation of nitric oxide (NO) formation. As for inhibition of NO synthesis, the use of mercapatoalkylguanidines and poly(ADP-ribose) polymerase inhibitors was discussed.

The topic of probiotics has drawn large interest from the medical and dental communities for its potential human application to manage certain conditions. Specific

probiotic strains are efficacious in infectious conditions such as pouchitis (Gionchetti *et al*, 2003; Mimura *et al*, 2004), antibiotic-associated diarrhoea and antibiotic-induced *Clostridium difficile* diarrhoea (Vanderhoof *et al*, 1999; D'Souza *et al*, 2002), and infectious diarrhoea (Van Niel *et al*, 2002). Furthermore, probiotic studies have shown that it is possible to modify and regulate several aspects of natural and acquired immune responses, including downregulating the inflammatory cascades through various proposed mechanisms (Gill and Prasad, 2008). Several human studies with probiotic products have shown some clinical benefits in various oral inflammatory conditions, including established gingivitis (Krasse *et al*, 2006; Shimauchi *et al*, 2008; Staab *et al*, 2009; Iniesta *et al*, 2012; Karuppaiah *et al*, 2013). However, few studies in dental literature have examined the clinical benefit of probiotics. One study showed improvements in clinical parameters in both test and control groups. However, there was no significant difference between the two groups (Shimauchi *et al*, 2008). On the other hand, a conflicting study failed to show any clinical benefit in plaque index (PI), gingival index (GI) and bleeding index, but showed significant modification in inflammatory markers (Staab *et al*, 2009). The most frequently hypothesized mechanism of action of probiotics to benefit the subjects with established gingivitis was competitive inhibition, which was demonstrated by an increase in probiotic strains in dental plaque and a decrease in potentially pathogenic species (Kõll-Klais *et al*, 2005; Iniesta *et al*, 2012; Vuotto *et al*, 2014).

Lactobacillus brevis CD2 has been studied in various conditions, such as recurrent aphthous ulcers in Bechet's disease (Tasli *et al*, 2006; Niscola *et al*, 2012), periodontitis (Riccia *et al*, 2007; Maekawa and Hajishengallis, 2014), *Helicobacter pylori*–associated gastritis (Linsalata *et al*, 2004) and paediatric gingivitis (Ierardo *et al*, 2010). Rather than acting against pathogenic bacteria, the strain has been selected for its unique capacity to modify the host inflammatory response by downregulating the inducible nitric oxide synthase (iNOS) pathway to attenuate the production of NO (Riccia *et al*, 2007), a potent inflammatory mediator.

The purpose of this study was to evaluate the preventive or delaying effect of *L. brevis* CD2 in an experimental gingivitis model.

Materials and methods

This 2-week investigation was a single-centre, randomized, parallel-group, double-blind, placebo-controlled trial. Its design and protocol were reviewed and approved by the Institutional Review Board at Gangneung-Wonju National University Dental Hospital (IRB 2009-8) as conforming to the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki.

Study participants

The appropriate sample size of 32 was calculated on the basis that the type I error for a two-sided test is 0.05, the power is 0.80, differences and within-group standard deviation for gingivitis score are both 0.2, and the ratio of control to experimental patients is 1:1. Based on this, a total of 34 subjects (eight women and 26 men) participated in this study (Figure 1). Participants were recruited from Gangneung-Wonju National University. Inclusion criteria were as follows: (i) at least 19 years of age, (ii) non-smoker, (iii) systemically healthy, (iv) free of periodontitis and free of gross caries, (v) presence of 28 fully erupted permanent teeth and (vi) willingness to adhere to the study protocol. Exclusion criteria were as follows: (i) pregnant or lactating subjects, (ii) long-term medication and/or debilitating systemic disease, (iii) non-plaque-induced gingival disease, (iv) known allergies to the investigational materials, (v) use of antibiotics within 4 months preceding the investigation and (vi) the presence of an orthodontic appliance. Subjects included in the investigation were provided with oral and written explanations of the study objectives, procedures, risks and benefits. Following this, signed informed consents were obtained from all participating subjects. Subjects fulfilling the inclusion and exclusion criteria were subsequently randomized equally into two groups by paired matching. Subjects were placed into experimental (n = 17) and control (n = 17) groups, based on PI, GI and bleeding on probing (BOP) scores at the screening session. Randomization of study subjects was managed by one of the study authors, an oral epidemiologist.

Subjects were withdrawn during the study if any of the following conditions were met: (i) changes in the participant's medical status or medications, (ii) non-compliance to the study protocol, (iii) development of serious dental infection or attachment loss, (iv) voluntarily withdrawal from the study and (v) development of a serious adverse event.

Investigational products

All investigational and placebo products were provided by VSL Pharmaceuticals Inc. (Townson, MD, USA) and were identical in physical appearance and colour. The investigational product and the placebo were prepackaged by the provider according to a randomization code and dispensed accordingly. *L. brevis* CD2 lozenges contained no less than 1 billion (10^9) viable cells of *L. brevis* CD2 as the active ingredient. Placebo lozenges contained a mixture of excipients in the active formulation. The daily dose of the trial medication was three lozenges per day: one lozenge to be taken every 8 h preferably in between meals. Lozenges were kept in the mouth and allowed to slowly dissolve. No drinking was allowed for at least 30 min after lozenge use. Treatment compliance was monitored during each visit by verifying the tablets remaining in the participant's bottle and reviewing the subject's diary.

Study design

This study design represented a modification of the experimental gingivitis in healthy volunteers study as originally described by Löe *et al* (1965). At the screening visit, informed consent was obtained, medical history, height, weight and vital signs were collected, and a full-mouth clinical examination was performed to assess GI (Löe and Silness, 1963), PI (Silness and Loe, 1964), probing depths and BOP scores (yes/no) at each of six sites per tooth for 28 teeth, resulting in 168 measurements per subject (Figure 2).

Oral hygiene phase. Dental prophylaxis was performed 2 weeks prior to the experimental phase start (day 0) and consisted of scaling, polishing and thorough oral hygiene instruction. Identical toothpaste and toothbrushes were distributed to all subjects, and panoramic X-rays were obtained.

Experimental phase. Following the 2-week oral hygiene phase, study subjects returned for baseline measurements (day 0). From that point forward, subjects abstained from brushing, flossing and using toothpicks, mouth rinse and chewing gum throughout the 2-week experimental phase. On day 0, all subjects received either the investigational product *L. brevis* CD2 (CD2 group) or placebo in a blinded manner.

Clinical examination. Subjects were examined on days 0 (baseline), 3, 7, 10 and 14. All clinical examinations were performed in a blinded manner by a single examiner, a periodontist. The examiner was calibrated prior to study commencement and received training of study procedures and documentation of acceptable intrameasurement liability (kappa values were 0.65 for PI and 0.53 for GI). Loss of attachment and BOP were evaluated on days 0, 3, 7, 10 and 14. PI and GI were recorded on days 0, 7 and 14.

Recovery phase. After the completion of the experimental phase, subjects received a professional dental prophylaxis and were instructed to resume home dental care (i.e. toothbrushing and flossing).

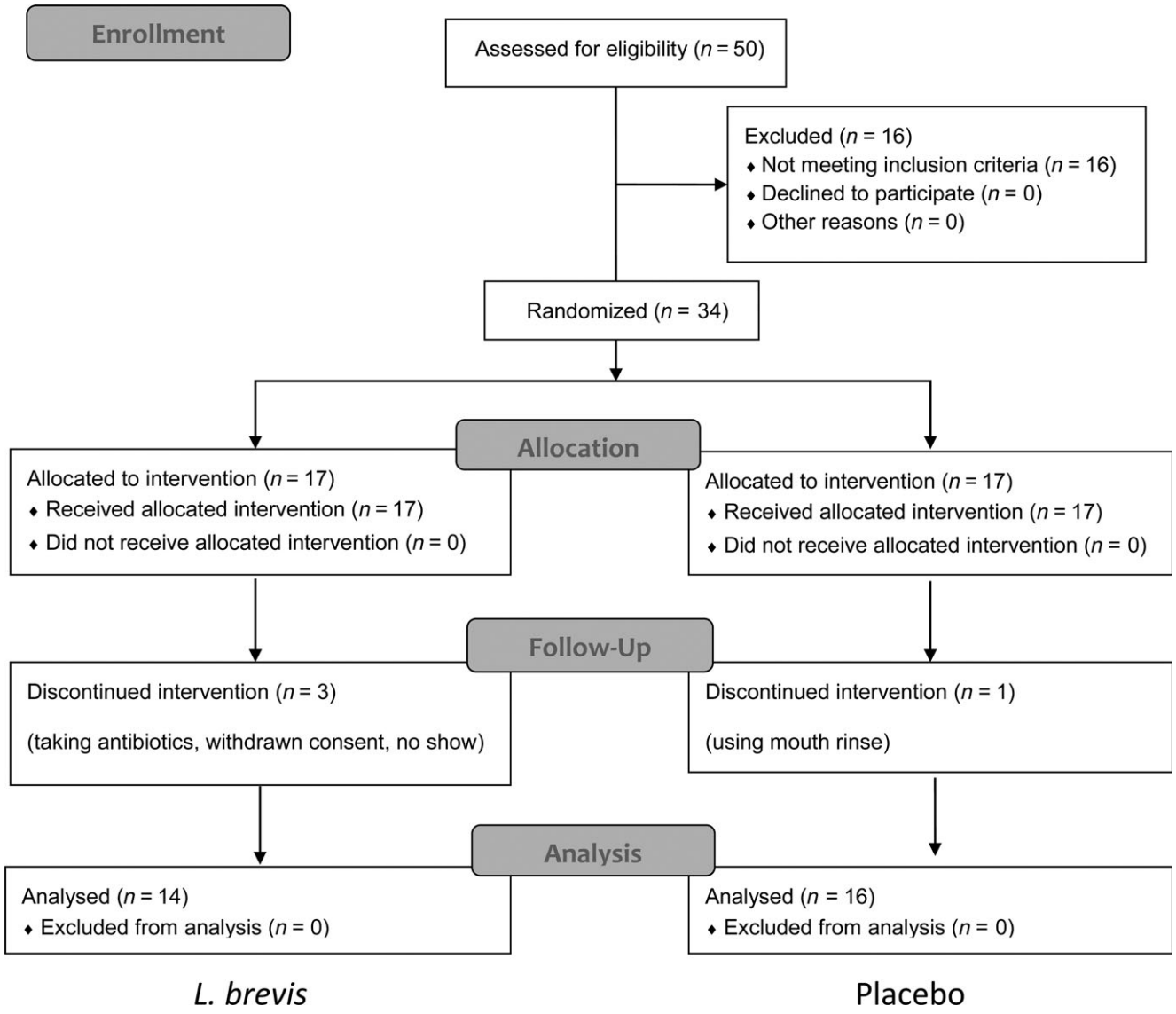

Figure 1 Study design

Gingival crevicular fluid sampling and processing

Gingival crevicular fluid (GCF) samples were collected on days 0 (baseline), 3, 7, 10 and 14 and were analysed to determine the levels of matrix metalloproteinase-8 (MMP-8), prostaglandin E_2 (PGE_2) and nitrite/nitrate. GCF was collected from first and second premolars in the following order: maxillary right, mandibular left, maxillary left and mandibular right. A total of four strips of GCF samples were collected at each visit for each subject. Prior to sampling, the teeth were isolated with cotton rolls and dried using a gentle airstream. Periopaper (Oralflow, Smithtown, NY, USA) was advanced 1 mm into the gingival crevice and left for 30 s. Periopaper with visible contamination, such as blood, was discarded. The volume of fluid absorbed was rapidly measured using Periotron 8000 (Harco Electronics Limited, Winnipeg, MB, Canada). Periopapers were placed in microtest tubes containing 800 μl phosphate-buffered saline and stored at -80°C until analysis.

Measurement of NO levels. GCF samples were filtered using a Microcon® centrifugal filter cartridge (10 000 nominal molecular weight; Millipore Corp., Billerica, MA, USA) prior to NO measurements. After GCF samples were treated with nitrate reductase to convert nitrate to nitrite, total nitrite level was determined based on the Griess reaction (R&D Systems, Minneapolis, MN, USA). The concentration of NO (μmol l^{-1}) was measured in the diluted samples with the respective dilution factor according to the manufacturer's instructions.

Measurement of PGE_2 and MMP-8 levels. PGE_2 and MMP-8 levels in GCF samples were determined using enzyme immunoassay (Cayman Chemical Co, Ann Arbor, MI, USA) and enzyme-linked immunosorbent assay (R&D Systems) kits according to the manufacturers' instructions. All samples, controls and standards were assayed in duplicate. GCF concentrations of PGE_2 (pg ml^{-1}) and MMP-8 (ng ml^{-1}) were measured in the diluted samples with the respective dilution factor based on volume of collected GCF.

Statistical analysis

The change in clinical variables scores was compared from baseline to follow-up during each study period. Limited statistical analyses were performed because this study was designed as a pilot study. Nonparametric Wilcoxon matched pair tests were used to compare non-normally distributed data. Comparisons of differences between the CD2 and placebo groups were also analysed by nonparametric Mann–Whitney *U*-test. Baseline characteristics and clinical parameters were presented from simple

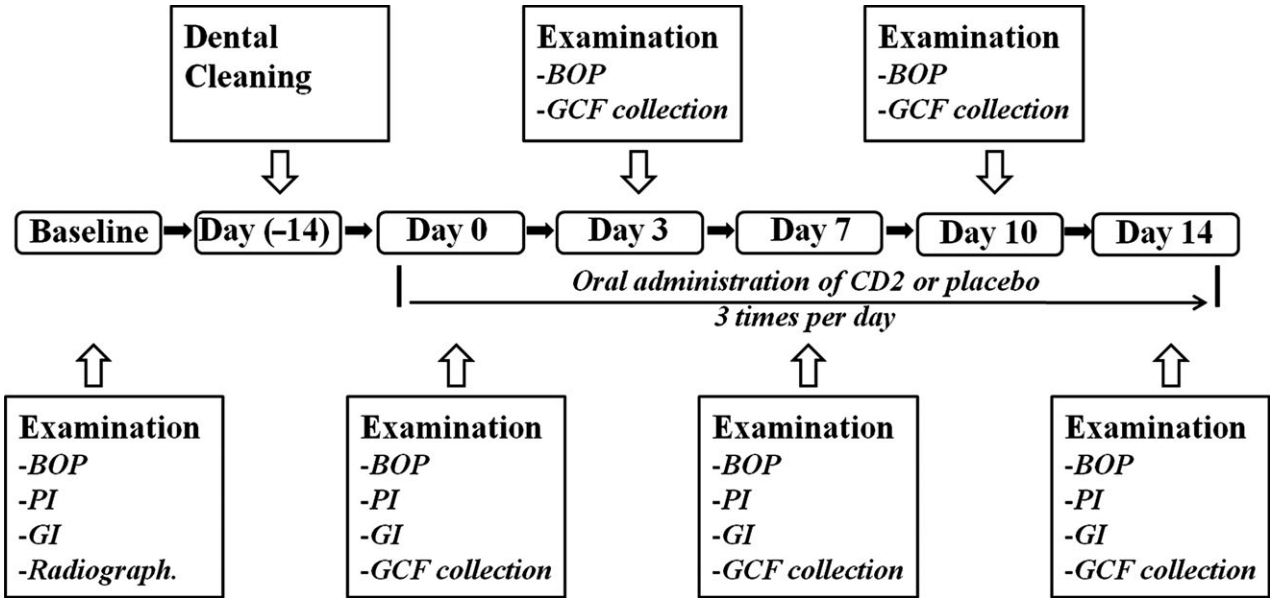

Figure 2 Experimental protocol

descriptive statistics. The relationship between clinical variables at each time period for each treatment group was assessed using Spearman's rank correlation. A *P*-value of ≤ 0.05 was accepted as statistically significant for all analyses. Statistical analysis was performed with Statistical Package for Social Sciences version 18.0 for Windows (SPSS, Chicago, IL, USA).

Results

Clinical findings

Of 34 randomized patients, three patients in the CD2 group and one patient in the placebo group were excluded due to non-compliance with protocols (Figure 1). The total number of patients included in the final statistical analysis was 16 patients in the placebo group and 14 patients in the CD2 group. Patient demographics, including age, age range, gender and clinical characteristics, are presented in Table 1 for each study group.

In both groups, BOP scores increased continuously throughout the study period, with the exception of day 3. On day 3, BOP scores were lower than baseline for both groups; however, this did not reach statistical significance. In the CD2 group, within-group comparisons revealed that BOP values were not significantly altered ($P > 0.05$ for all comparisons) throughout the experimental gingivitis period compared to baseline (Table 2). However, in the placebo group, mean BOP increased significantly from 9.50 at baseline, to 14.75 on day 10 ($P = 0.013$) and 14.81 on day 14 ($P = 0.016$). In the placebo group, BOP increased significantly from day 10 until day 14 compared to baseline ($P < 0.05$). Comparing BOP on day 10, 8 of 14 (57%) participants in the CD2 group and 12 of 16 (75%) in the placebo group had elevated scores. On day 14, 10 of 14 (71%) participants in the CD2 group and 13 of 16 (81%) in the placebo group showed increasing BOP levels. BOP scores of the placebo group were consistently higher than in the CD2 group throughout the study, and the intergroup difference became significant on day 10. However, no significant difference was found on day 14.

PI results are shown in Table 3. Within-group analysis revealed a significant increase in PI between day 0 and day 7 in both groups ($P < 0.001$ in the placebo group; $P = 0.001$ in the CD2 group). PI increased significantly in both groups between days 7 and 14. There was no intergroup difference for PI at any given time point. Mean GI increased significantly from day 0 to follow-up day 7 in both groups ($P = 0.005$ in the placebo group; $P = 0.041$ in the CD2 group). Similarly, on day 14, significant increases in mean GI were observed in both groups

Table 1 Demographic variables and clinical parameters of subjects at baseline

	Demographic variables			*Clinical parameters (mean ± s.d.)*		
Group	*Average age (years)*	*Age range (years)*	N *(male/female)*	*BOP*	*GI*	*PI*
L. brevis CD2	22.1	19–28	14 (9/5)	16.71 ± 7.76	0.24 ± 0.11	0.31 ± 0.26
Placebo	21.6	19–29	16 (11/5)	16.88 ± 9.75	0.24 ± 0.17	0.35 ± 0.23

BOP, bleeding on probing; GI, gingival index; PI, plaque index.
BOP was expressed as total score per person.

Table 2 Descriptive statistics and comparisons of bleeding on probing

	L. brevis *CD2* (N = *14*)		*Placebo* (N = *16*)		
Time	*BOP*	P*	*BOP*	P*	P†
Day 0	8.93 ± 7.53	–	9.50 ± 6.69	–	0.900
Day 3	5.79 ± 4.93	0.107	7.63 ± 4.57	0.244	0.179
Day 7	9.93 ± 4.74	0.649	10.06 ± 4.32	0.270	0.967
Day 10	10.71 ± 3.91	0.330	14.75 ± 4.90	0.013	0.016
Day 14	13.43 ± 4.27	0.079	14.81 ± 4.92	0.016	0.194

Results are expressed as mean ± s.d., and BOP scores are expressed as total score per person.
*P-value by Wilcoxon signed ranked t-test; compared to baseline.
†P-value by Mann–Whitney U-test; comparison between groups.

($P = 0.001$ in the placebo group; $P = 0.008$ in the CD2 group). GI levels in the placebo and CD2 groups were not significantly altered between days 7 and 14. There were no intergroup differences for GI at any time point (Table 3).

Biochemical findings

NO levels. In the placebo group, mean NO measured from the ratio of nitrite/nitrate increased significantly from 7274.2 on day 0 to 10226.7 on day 3 ($P = 0.04$). On day 14, mean NO scores were 10651.5 and were significantly elevated compared with baseline NO levels ($P = 0.049$). In the CD2 group, NO levels remained unchanged throughout the experimental period (Table 4). Furthermore, no differences in NO were observed between the placebo and CD2 at any time point.

MMP-8 levels. MMP-8 measurements did not change significantly in either group throughout the experimental period (Table 4). Furthermore, no intergroup differences in MMP-8 level were observed at any time point.

PGE_2 levels. Compared with baseline, PGE_2 levels on days 7 and 14 were significantly decreased in both groups (Table 4). No intergroup differences in PGE_2 levels were observed at any time point.

Correlations between the clinical variables determined for the CD2 and placebo groups were calculated separately. A statistically significant positive correlation was found between NO levels and GI in the CD2 group (Table 5). No correlations were observed for the placebo group at day 14 (data not shown). There was no correlation found between PI and GI in either group at any time point of measurement.

Discussion

The effect of probiotic supplementation on systemic health has been described in numerous *in vivo* and *in vitro* studies, however, mostly for gastrointestinal conditions. Diverse mechanisms of action have been proposed to explain the positive health effects of probiotics, including replacement of potentially harmful bacteria and/or modulation of host immune responses (Rijkers *et al*, 2010). The interest in probiotic application for oral conditions is relatively new, and the oral cavity has only recently been suggested as a relevant target for probiotic application. The potential benefits of probiotics for periodontal inflammation have been assessed in several studies that have demonstrated various outcomes. In an animal model, Teughels *et al* (2007) demonstrated that repeated application of *Streptococcus sanguinis*, *Streptococcus salivarius* and *Streptococcus mitis* following scaling and root planing successfully reduced and maintained low levels of anaerobic species and black-pigmented bacteria while, at the same time, reduced BOP scores. This study was the first to assess the role of commensal oral streptococci to modulate periodontal pocket recolonization. Shimauchi *et al* (2008) conducted a randomized, double-blinded, placebo-controlled trial to study the effect of *Lactobacillus salivarius* WB21 on periodontal conditions. The clinical parameters did not reveal significant differences between the probiotic and placebo groups at the end of the 8-week study period. Interestingly, the study showed that the level of lactobacilli in saliva and supragingival and subgingival plaque decreased compared to baseline in both groups, while salivary lactoferrin levels were only decreased in the smoking group. Such findings suggest that the limited clinical benefit observed may be attributed to an immunomodulatory effect rather than pathogenic micro-organism replacement. Additional probiotic studies failed to show improvements in clinical parameters, but were able to demonstrate the modulation of inflammatory cytokines and markers, suggesting that modulation of the host response can be achieved with probiotic application (Krasse *et al*, 2006; Riccia *et al*, 2007; Staab *et al*, 2009; Twetman *et al*, 2009). In the light of the current knowledge that host factors can influence the onset and severity of plaque-induced

Table 3 Descriptive statistics and comparisons of plaque index and gingival index

	Plaque index				*Gingival index*			
Time	L. brevis *CD2* (N = *14*)	P	*Placebo* (N = *16*)	P	L. brevis *CD2* (N = *14*)	P	*Placebo* (n = *16*)	P
Day 0	0.00 ± 0.00	–	0.00 ± 0.00	–	0.09 ± 0.12	–	0.06 ± 0.08	–
Day 7	1.22 ± 0.45	0.001	1.17 ± 0.23	<0.001	0.22 ± 0.15	0.041	0.19 ± 0.14	0.005
Day 14	1.44 ± 0.27	0.001	1.41 ± 0.31	<0.001	0.25 ± 0.11	0.008	0.26 ± 0.10	0.001

Results are expressed as mean ± s.d., and plaque index and gingival index are expressed as total score per person.
*P-value by Wilcoxon signed ranked t-test; compared to baseline.

Table 4 Descriptive statistics and comparisons of gingival crevicular fluid

	NO ($\mu mol\ ml^{-1}$)				*MMP-8* ($ng\ ml^{-1}$)				PGE_2 ($pg\ ml^{-1}$)			
Time	L. brevis *CD2* (N = *14*)	P	*Placebo* (N = *16*)	P	L. brevis *CD2* (N = *14*)	P	*Placebo* (N = *16*)	P	L. brevis *CD2* (N = *14*)	P	*Placebo* (N = *16*)	P
Day 0	7869.8 ± 3374.1	–	7274.2 ± 3335.1	–	3040.0 ± 5431.1	–	1726.1 ± 1730.3	–	6.43 ± 5.62	–	9.88 ± 13.60	–
Day 3	12046.7 ± 10146.1	0.12	10226.7 ± 6694.0	0.04	955.3 ± 635.1	0.55	956.3 ± 1069.8	0.17	3.90 ± 3.57	0.06	6.29 ± 10.50	0.27
Day 7	11240.3 ± 6929.4	0.07	12708.1 ± 20279.8	0.61	1387.2 ± 1323.1	0.39	2296.3 ± 3717.5	0.67	3.43 ± 3.74	0.02	2.08 ± 2.97	<0.01
Day 10	11694.3 ± 6427.2	0.06	10707.5 ± 11107.0	0.30	1577.3 ± 2655.6	0.17	1478.3 ± 1673.5	0.71	5.22 ± 9.36	0.11	3.82 ± 5.86	0.06
Day 14	9120.2 ± 4094.5	0.51	10651.5 ± 6027.3	0.05	2688.6 ± 2373.9	0.33	2495.6 ± 2656.1	0.30	3.41 ± 4.80	<0.01	2.42 ± 4.08	0.01

Results are expressed as mean ± s.d.
P-value by Wilcoxon signed ranked *t*-test, compared to baseline.

Table 5 Correlation analysis in *L. brevis* CD2 group at day 14 by Spearman's rho

	BOP	*PI*	*GI*	*PGE-2*	*NO*	*MMP-8*
BOP	–					
PI	0.222	–				
GI	−0.113	0.172	–			
PGE-2	−0.294	0.038	−0.102	–		
NO	−0.093	0.218	0.607*	0.054	–	
MMP-8	0.138	−0.055	−0.080	0.045	0.288	–

*$P < 0.05$.

gingivitis, probiotics as a modality to modulate the host response to plaque can be considered a plausible option in the prevention and treatment of gingivitis.

There are several challenges facing a gingivitis clinical trial with probiotics: (i) selecting a strain based on valid scientific rationale for its safety and postulated efficacy, (ii) selecting a strain that is not acidogenic and potentially cariogenic and (iii) designing a clinical trial to minimize bias and confounding factors that often introduce various type I and type II errors. Many previous studies and publications produced inconsistent results that can be attributed to differences in the strains used and the study designs that allow attention bias of individual (Hawthorne effect) to create various noise in the data and interpretation.

The present study aimed to evaluate whether *L. brevis* CD2 can delay or prevent the development and/or progression of gingivitis in a 2-week experimental gingivitis model. The strain was selected based on previously validated efficacy and safety data. *L. brevis* CD2 has been shown to decrease the clinical signs and symptoms of inflammation in chronic periodontitis patients (Riccia *et al*, 2007) and paediatric gingivitis patients (Ierardo *et al*, 2010). This strain contains high levels of enzymes, such as arginine deiminase (AD) and sphingomyelinase (Marzio *et al*, 2001). AD is lacking in human cells and competes with arginase and nitric oxide synthase (NOS) for the substrate, arginine. Through substrate competition, AD downregulates the production of NO, which is a known potent inflammatory mediator. Increased production of NO has been associated with various inflammatory diseases. Localized elevation in iNOS and reactive nitrogen products has also been demonstrated in humans and animals with periodontal inflammation. In an animal model, Paquette *et al* (2006) demonstrated that an inhibitor of iNOS that reduced the production of NO was able to attenuate gingivitis development.

In addition to a potential anti-inflammatory effect, the alkaline-producing capacity of *L. brevis* CD2 may be considered its great advantage when considering oral application. The final product of AD is ammonia, which can in turn prevent decalcification by providing a buffering capacity to rising acidity in the oral cavity (Nascimento *et al*, 2009). These characteristics make *L. brevis* CD2 an ideal candidate to consider in oral probiotic application to control the development and progress of gingivitis.

Several parameters have been implemented to clinically assess the plaque-induced inflammatory response in man.

BOP has the strongest correlation with gingival inflammation (Greenstein, 1984; Buduneli and Kinane, 2011).

In the CD2 group of the present study, BOP did not increase during the experimental period even though plaque accumulation was significant. This demonstrates that *L. brevis* CD2 is capable of delaying the development of gingivitis and its mechanism may be related to host factor modulation rather than antiplaque effects. This hypothesis can be further supported by the fact that compared to baseline, NO levels in GCF did not increase in the CD2 group throughout the whole period, while the placebo group showed a significant increase in NO on days 3 and 14. In contrast to our observations in the CD2 group, BOP findings in the placebo group increased significantly on day 10 compared to baseline, and significant increases continued until day 14. Intergroup differences reach significance on day 10 but not on day 14. The reduced protective effect of *L. brevis* CD2 on day 14 can be attributed to the potential phase shift occurring in the placebo group from early phase to established phase and consequential attenuation of acute inflammatory responses. In addition, it is possible to hypothesize that the application of *L. brevis* CD2 has a delaying effect on the development of plaque-induced gingivitis rather than a total preventive effect, and its protective host modulation is surpassed by the gradually increased amount of plaque on day 14.

Even though NO levels increased significantly in the early phase of the experimental period, other inflammatory markers, such as MMP-8 and PGE_2, did not show distinct differences between the placebo and CD2 groups. Interestingly, PGE_2 decreased significantly in both groups at the end of experimental period compared to the respective baseline values at day 0. Neither MMP-8 nor PGE_2 demonstrated any correlation to the clinical parameters measured in this study. This is somewhat consistent with recent findings reported by Offenbacher *et al* (2010) and emphasizes the need to investigate valid biomarkers in gingivitis models.

In spite of a small sample size, the present study demonstrated that the use of *L. brevis* CD2 in a clinical model of experimental gingivitis prevented the increase in NO production and, consequently, delayed the development of gingivitis measured by BOP.

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Conflicts of interest

Arthur C. Ouwehand is an employee of Danisco, a company that manufactures and sells probiotics. The other authors declare that there are no conflicts of interest in this study.

Author contributions

JKL, SJK, ACO, and DSM contributed to the design of the study, interpretation of the results, and writing of the manuscript. JKL and DSM carried out the clinical examination. SHK carried out the biochemical experiment and analyzed their data.

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