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Effect of probiotic lozenges on inflammatory reactions and oral biofilm during experimental gingivitis

HADAR HALLSTRÖM[1], SUSANN LINDGREN[1], TÜLAY YUCEL-LINDBERG[2], GUNNAR DAHLÉN[3], STEFAN RENVERT[4] & SVANTE TWETMAN[1,5]

[1]*Maxillofacial unit, Halland Hospital, Halmstad, Sweden,* [2]*Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden,* [3]*Department of Odontology, Oral microbiology, Sahlgenska Academy at Gothenburg University, Gothenburg, Sweden,* [4]*Kristianstad University, Kristianstad, Sweden, and* [5]*Department of Odontology, Section for Cariology, Endodontics, Pediatric Dentistry and Clinical Genetics, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark*

Abstract

Aim. Probiotic bacteria have been introduced for prevention and treatment of periodontal diseases. The aim was to assess if daily oral administration of probiotic bacteria could influence the inflammatory response and the composition of supragingival plaque in an experimental gingivitis model. ***Materials and methods.*** Eighteen healthy female adults volunteered after informed consent. A double-blind randomized placebo-controlled cross-over design was used. The buccal surface of first molars was used as experimental sites. A mouth-guard covering the first premolar to second molar was used when brushing, preventing accidental cleaning during 3 weeks of plaque accumulation. Lozenges containing *L. reuteri* (ATCC55730 and ATCC PTA5289) or placebo were taken twice a day. During the run-in and washout periods, professional tooth cleaning was performed 5 days/week. At baseline and follow-up, plaque index, gingival index and bleeding on probing were recorded. Samples of gingival crevicular fluid (GCF) were analysed for concentration of seven inflammatory mediators. Bacterial samples were processed with checkerboard DNA/DNA-hybridization. ***Results.*** All subjects presented a local plaque accumulation and developed manifest gingivitis at the test sites during the intervention periods. The volume of GCF increased in both groups but was statistically significant only in the placebo group ($p < 0.05$). The concentrations of IL1-β and IL-18 increased significantly ($p < 0.05$), while IL-8 and MIP1-β decreased ($p < 0.05$). No differences were displayed between test and placebo. Likewise, the microbial composition did not differ between the groups. ***Conclusion.*** Daily intake of probiotic lozenges did not seem to significantly affect the plaque accumulation, inflammatory reaction or the composition of the biofilm during experimental gingivitis.

Key Words: *bacteriotherapy, cytokines, gingival crevicular fluid, lactobacilli, plaque*

Introduction

Probiotics are defined as 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' [1]. A regular daily intake of probiotic bacteria has been suggested to beneficially affect some gastrointestinal diseases [2] and, recently, probiotics have been reviewed for treatment of gingival and periodontal conditions [3]. The conceptual thinking is that a harmless effector strain is implanted in the host's microflora to maintain or restore a natural microbiome by interference and/or inhibition of other micro-organisms and especially pathogens. Furthermore, a systemic modulation of immunological parameters is suggested [3]. Studies *in-vitro* have demonstrated that probiotic lactobacilli can inhibit or hamper growth of pathogens associated with periodontal disease [3]. In clinical settings of pilot character, probiotic supplements have been associated with significantly improved gingival and periodontal conditions [4–9] and altered inflammatory markers in gingival crevicular fluid [10]. To our knowledge, the probiotic concept has not yet been applied to the classical experimental gingivitis

Correspondence: Dr Hadar Hallström, Maxillofacial Unit, Halland Hospital Halmstad, SE-30185 Halmstad, Sweden.
E-mail: hadar.p.hallstrom@regionhalland.se

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research tool introduced by Löe et al. [11] more than 50 years ago. This would allow a more detailed analysis of the possible influence of bacteriotherapy with so-called beneficial bacteria on gingival response to supragingival biofilm accumulation. The aim of the present study was to evaluate whether daily oral administration of probiotic bacteria could influence the composition of the supragingival plaque, clinical parameters and levels of inflammatory mediators in an experimental gingivitis model. The null hypothesis was that neither the microbiological profile in supragingival samples nor the concentrations of selected cyto/chemokines in gingival crevicular fluid would differ between the test and the placebo group.

Materials and methods

Subjects

Eighteen healthy non-smoking female adults with a mean age of 38 years volunteered after informed consent. The inclusion criteria were absence of gingival inflammation (Löe and Silness index [12] being 'zero') and no history of periodontal disease (showing no marginal bone-loss on bite-wing radiographs). Exclusion criteria were (i) pregnancy or breast-feeding, (ii) poorly controlled diabetes mellitus (HbA1c $\geq$ 6.5), (iii) intake of antibiotic/anti-inflammatory drugs within 3 months and (iv) prescribed medication with a known effect on gingival growth. A power calculation with $\alpha = 0.05$ and $\beta = 0.20$ indicated that 18 subjects were needed for each regime to detect a clinically relevant ($\approx$ 40%) difference in the clinical variables. Before the study, the subjects were asked to report their food intake in detail during a full week and their protocols were checked for any possible probiotic content. The participants were thereafter shown pictures of dairy products and groceries containing probiotic bacteria and were asked to refrain from all such products during the study period.

Study design

A double-blind randomized placebo-controlled cross-over design was used. The experimental periods were 3 weeks separated by run-in and washout periods of 2 weeks. The randomization was carried out with the aid of the computerized Excel randomization tool. All study subjects, the laboratory technician and involved clinicians were blinded for the group allocation. The protocol was ethically approved by the regional ethical committee.

A customized acrylic mouth-guard (stent) covering first premolar to second molar were constructed and applied during tooth brushing. In this way, the participants refrained from cleaning four of their lateral teeth during the experimental periods. The test persons were carefully instructed to maintain their usual oral hygiene routines throughout the study. Thus, the non-experimental teeth were kept and cared for as usual while the experimental teeth were not cleaned at all during the experimental period. The buccal surface of a first molar was used as a sampling site. Lozenges containing two strains of *L. reuteri* (ATCC55730 and ATCC PTA5289; 1×10^8 CFU of each strain) or placebo were taken twice a day during the experimental periods. The participants were instructed to actively suck on the tablet. During the run-in and washout periods, professional tooth cleaning was performed 5 days a week. At baseline (day 0) and follow-up (day 21), plaque index (PI), gingival index (GI) and bleeding on probing (BOP) were recorded. Plaque was registered using the modified Quigley & Hein index [13]. The gingival condition was graded with the Löe & Silness gingival index [12]. Bleeding on probing was registered dichotomous as bleeding or not, 30 s after measuring the pocket depth. Samples of gingival crevicular fluid (GCF) were collected from the mid-buccal and the mesial papilla with the aid of two separate perio-paper strips inserted in the gingival sulcus for 20 s after gentle drying with air. Samples were not pooled. The volume was recorded using a Periotrone 8000 (ProFlow, Amenityville, NY) and expressed as µL. The strips were thereafter stored frozen at 70°C until further analysis. Supragingival plaque was collected from the selected first molar with aid of a sterile wooden tooth pick and immediately transferred to plastic Eppendorf tubes and stored frozen. All the clinical registrations were made by one trained and calibrated examiner (SL).

Laboratory assays

The concentration of IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α and MIP-1β was determined in GCF samples using the commercial Bio-Plex Cytokine Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions and expressed as pg/mL. The plaque samples were analysed using the checkerboard DNA-DNA hybridization method [14,15] with respect to the 18 bacterial strains listed in Table I. The obtained chemiluminescent signals were transformed to a score between 0 and 5 according to Papapanou et al. [14]. Score 1 ($< 10^4$ CFU) was selected as the cut-off level to contrast between colonized/non-colonized sites.

Statistical methods

All data were analysed using the IBM-SPSS software (version 19.0, Chicago, IL). The allocation of test and placebo was not unveiled until all analyses were performed. Descriptive statistics including means and standard deviations were calculated for all variables. Differences between baseline and follow-up were compared within the groups (follow-up vs

Table I. Gingival crevicular fluid (GCF; mean μL, SD) and concentration of selected cytokines (mean pg/mL, SD) before (baseline) and after (follow-up) 3 weeks of experimental gingivitis and daily intake of probiotic lozenges containing two strains of *L. reuteri* (test) or placebo ($n = 18$).

Variable	Group	Baseline	Follow-up	$p^{\star}$
GCF (μL)	placebo	0.08 (0.14)	0.22 (0.24)	< 0.05
	test	0.13 (0.22)	0.25 (0.30)	NS
TNF-α	placebo	0.47 (0.30)	0.66 (1.03)	NS
	test	0.72 (0.81)	1.45 (4.14)	NS
IL-1β	placebo	31.2 (27.7)	60.5 (65.4)	< 0.05
	test	27.6 (22.4)	76.6 (70.2)	< 0.05
IL-6	placebo	1.69 (1.67)	1.58 (2.45)	NS
	test	3.77 (8.56)	5.15 (16.2)	NS
IL-8	placebo	81.9 (65.3)	33.4 (27.5)	< 0.05
	test	80.9 (57.7)	36.8 (34.0)	< 0.05
IL-10	placebo	0.29 (0.20)	0.38 (0.26)	NS
	test	0.36 (0.30)	0.43 (0.46)	NS
IL-18	placebo	34.0 (47.9)	116.2 (112.1)	< 0.05
	test	42.3 (59.8)	98.6 (105.7)	< 0.05
MIP-1β	placebo	20.5 (15.3)	5.5 (2.3)	< 0.05
	test	16.3 (10.8)	7.8 (11.3)	< 0.05

*Wilcoxon signed rank test.

baseline) with the aid of the Wilcoxon paired signed rank test. Differences between the groups and in distribution of bacterial scores were calculated with non-parametric tests and chi-square tests, respectively. A p-value < 0.05 was considered statistically significant.

Results

Clinical findings

All the subjects fulfilled the study protocol. There were no significant differences in PI, GI or BOP between the two baselines or between test and the placebo group at baseline. All subjects exhibited a local accumulation of supragingival plaque and all but one developed a clinically manifest gingivitis at the selected test sites during the intervention periods (Table II). No significant differences in PI, GI or BOP were displayed between the probiotic test lozenges and placebo controls. During the washout period, clinically healthy conditions were re-established. No side- or adverse effects were reported during the course of the study.

Table II. Distribution of plaque index (PI), gingival index (GI) and bleeding on probing (BOP) before (baseline) and after (follow-up) 3 weeks of experimental gingivitis and daily intake of probiotic lozenges containing two strains of *L. reuteri* (test) or placebo in 18 female adults. The figures denote the number of subjects.

Variable	Group	Baseline	Follow-up	p^{*}
PI (≥ 1)	placebo	3	18	< 0.05
	test	4	18	< 0.05
GI (≥ 2)	placebo	0	15	< 0.05
	test	0	14	< 0.05
BOP (yes)	placebo	4	18	< 0.05
	test	3	17	< 0.05

*Significantly different from baseline, chi-square test.

Cyto/chemokines in GCF

The results from the biomarkers in GCF are summarized in Table I. The volume of GCF increased in both groups during the experimental periods, but was significant ($p < 0.05$) only in the placebo group. The mean concentrations of TNF-α, IL-6 and IL-10 were not significantly altered between baseline and follow-up, whereas the cytokines IL-1β and IL-18 significantly ($p < 0.05$) increased at follow-up both in the test and placebo groups. Conversely, the mean concentrations of the chemokines IL-8 and MIP-1β were significantly ($p < 0.05$) lower at follow-up compared to baseline.

Microbiological findings

The microbial profile of the supragingval plaque at baseline and after 21 days is shown in Table III. An increasing amount of bacteria was noted in the supragingival plaque samples. *S. oralis* and *A. naeslundii* were the most prevalent species both at baseline and follow-up. *T. forsythia*, *S. mutans* and *L. fermentum* were hardly identified in any of the samples. No major

Table III. Distribution of subjects with low (< 10^4 CFU) and high (> 10^5 CFU) levels of selected bacterial counts before (baseline) and after (follow-up) 3 weeks of experimental gingivitis and daily intake of probiotic lozenges containing two strains of *L. reuteri* (test) or placebo. The numbers denote the number of subjects.

		Baseline		Follow-up	
Strain	Origin	test	placebo	test	placebo
Porphyromonas gingivalis	FDC381	13/4	8/7	11/3	12/3
Prevotella intermedia	ATCC25611	11/3	13/3	12/3	9/6
Porphyromonas endodontis	OMGS1205	15/1	12/6	13/4*	7/8
Tannerella forsythia	ATCC43037	15/0	16/0	15/1	13/0
A. actinomycetemcomitans	FDC Y4	11/3	11/2	7/7	4/4
Fusobacterium nucleatum	ATCC10953	8/3	7/4	3/15*	4/12*
Treponema denticola	OMGS3271	13/4	9/4	17/1	17/0*
Parvimonas micra	OMGS2852	12/3	9/3	13/1	14/2
Campylobacter rectus	ATCC33238	18/0	15/0	12/4*	15/1
Streptococcus intermedia	ATCC27335	16/0	16/1	12/1*	11/1
Streptococcus oralis	ATCC35037	8/8	4/11	4/13*	4/9
Streptococcus sanguinis	ATCC10566	12/4	17/1	15/0	17/1
Streptococcus mutans	ATCC25175	18/0	18/0	18/0	17/1
Veillonella parvula	ATCC10790	18/0	17/0	13/3*	15/2*
Actinomyces naeslundii	ATCC15987	9/8	6/5	7/7	7/7
Filifactor alocis	ATCC35896	11/1	10/1	10/0	11/0
Lactobacillus reuteri	ATCC55730	6/5	7/6	5/9	5/9
Lactobacillus fermentum	ATCC14931	17/0	16/0	18/0	18/0

FDC, Forsyth Dental collection, Boston, USA; ATCC, American Type Culture Collection; OMGS, Oral Microbiology, Gothenburg, Sweden.
*Statistically different compared to baseline, Wilcoxon signed rank test, $p < 0.05$.

differences were obtained between the groups concerning the microbial composition of the oral biofilm. The counts of *F. nucleatum* and *V. parvula* increased significantly in both groups during intervention ($p < 0.05$), while *S. oralis* increased only in the probiotic group. Most subjects harboured *L. reuteri* at baseline in both groups, but neither the number of subjects nor the bacterial counts changed markedly during the intervention.

Discussion

In this double-blind randomized placebo controlled cross-over study, we tested the influence of daily administered probiotic lozenges on inflammatory reactions in the gingiva as well as the gross microbiota of the biofilm. The rationale was the previous reports from different research groups having indicated that probiotic supplements can beneficially alter the biofilm of the host and reduce the grade of gingival inflammation [3–9]. The cross-over design ensured the equality of subjects in the test and placebo groups. The experimental gingivitis model was chosen since it has been a frequently used tool to study gingival inflammation as a response to an increasing plaque accumulation. In the original study by Löe et al. [11] the subjects were healthy young individuals. Later studies have shown that there is a lot of modulating factors to the plaque challenge such as puberty, menstrual cycle, pregnancy, medication, systemic diseases and a biological variation, manifested as high and low responders to biofilm challenges [16]. The present study group was recruited to be as homogenous as possible, but still, individual differences in plaque accumulation and gingival reactions were evident. To ensure maximal compliance and motivation during intervention and in order to minimize bias and drop outs the study objects were recruited among the staff at the maxillo-facial unit at Halland Hospital Halmstad. Furthermore, by using one sex only, potential gender-related differences were eliminated. As the main results failed to demonstrate any protective effect of lactobacilli-administration on the inflammatory pattern or the microbial composition of the supragingival biofilm, the null hypothesis could not be rejected. Our clinical results were mainly in agreement with a previous study by Staab et al. [6] in which mechanical plaque control was interrupted for 96 h after daily intake of a lactobacilli-supplemented milk drink for 8 weeks. In that study, the amount of interproximal plaque and papillary bleeding did not differ in the test group compared with a control group,

whereas they had significant reduction in the level of some inflammatory cytokines in the GCF. Consequently, the authors concluded a beneficial effect of the probiotic milk on gingival inflammation [6]. However, it is important to stress that our findings do not rule out the possibility that probiotics may have a reducing effect on an already established inflammation in the oral cavity, as demonstrated in the previous studies with *L. reuteri* [4,8,10]. Consequently, further clinical studies are needed to elucidate if probiotic bacteria can be of value to combat gingival or periodontal conditions as well as peri-implant mucositis alone or as an adjunct to conventional scaling and root planing.

By using the partial mouth-guard method and well-informed and motivated subjects, we secured a good compliance. Our mouth-guard prevented tooth cleaning of the selected sites effectively and caused a higher degree of PI and GI after 3 weeks when compared with a similar approach covering palatal tooth surfaces of maxillary teeth [17]. The compliance with the study protocol was considered as excellent based on regular contacts with the volunteers concerning the dietary restrictions and lozenges intake.

Our findings concerning the inflammatory mediators in GCF were mainly in accordance with those of Offenbacher et al. [18] during experimental gingivitis. The concentration of the selected pro-inflammatory cytokines TNF-α and IL-1β increased with increasing gingival inflammation as expected, albeit statistically significant only for the latter. A small but non-significant increase of the anti-inflammatory cytokine IL-10 was also noted at follow-up. Interestingly, the concentrations of the chemokines IL-8 and MIP-1β, known to attract inflammatory cells and especially neutrophils, decreased significantly in GCF at the follow-up. One explanation for this may be that the acute phase of gingival inflammation was passed after 21 days resulting in corresponding down-regulation of chemokine levels. However, Offenbacher et al. [18] registered decreased concentrations of IL-8 and MIP-1β already after 7 days. Another reason could be that the actual output was unchanged since the decreased concentration in GCF was paralleled with a significant increase in GCF flow. The levels of IL-18 were increased, which is in accordance with the results reporting its expression at sites of chronic inflammation [19]. IL-6 can act both pro-inflammatory and anti-inflammatory and is one of the most important mediators of the acute phase response. In the current study, however, no major alterations were seen under the present conditions.

The checkerboard DNA-DNA hybridization technique has been widely used to comprehensively examine the types and numbers of bacteria in supragingival plaque in healthy subject and in patients with periodontitis [20]. The technique is rapid and sensitive, although non-specific target binding and the risk of cross-reactions (false positive signals) may be a problem [21]. For example, the relatively high prevalence and counts of *L. reuteri* in both groups before and after the experimental periods (12 out of 18 subjects) could be due to cross-hybridization with other lactobacilli as well as other oral streptococci. Thus, the data on lactobacilli should, therefore, be interpreted with some caution. It should also be stressed that the whole genome in the probe was not identical to the *L. reuteri* strains incorporated in the test tablets and it was, therefore, not possible to get any information on its specific recovery in the biofilm. The general pre- and post-intervention microbial composition was in general agreement with precious findings with this technique [22], mainly reflecting a healthy flora. However, the general increase of anaerobic bacteria confirmed the picture of experimental gingivitis. Interestingly, *S. mutans*, *C. rectus*, *L. fermentum*, *T. forsythia* and *S. intermedia* were virtually absent among the participants and especially at day 0 as well as the common species associated with periodontal disease. This was also confirmed by conventional cultivation on selective media not reported here. Thus, there seems to be a need to further investigate the possible impact of probiotic supplements on truly diseased patients with an ecologically stressed biofilm, dominated by proteolytic bacteria.

Conclusions

Daily intake of probiotic lozenges containing two strains of *L. reuteri* did not seem to significantly affect the plaque accumulation, gingival inflammatory reaction or the composition of the supragingival plaque during conditions of experimental gingivitis.

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