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The influence of a probiotic milk drink on the development of gingivitis: a pilot study

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Abstract

Aim: The aim of this study was to determine the effect of a probiotic milk drink on gingival health and the development of experimental gingivitis.

Material and Methods: Fifty volunteer students took part in a parallel-designed non-blinded study. The test group drank a probiotic drink once a day; the control group did not receive any product to drink. After 8 weeks, individual mechanical plaque control was interrupted for 96 h. Papilla bleeding index, interproximal plaque and Turesky plaque index (PI) were recorded at baseline, after 8 weeks and again 96 h later. At the same time points, gingival crevicular fluid had been collected for analysis of polymorphonuclear elastase, myeloperoxidase (MPO) and matrix metalloproteinase-3 (MMP-3).

Results: Interproximal PI and papillary bleeding were not different between the groups. In the test group, elastase activity and MMP-3 amount were significantly lower after the intake of the probiotic milk drink ($p < 0.001$ and 0.016). There was a significant increase of MPO activity in the control group; both groups were different at the end of the study ($p = 0.014$).

Conclusions: The data suggest a beneficial effect of the probiotic milk drink on gingival inflammation.

Key words: gingival crevicular fluid; gingivitis; probiotics

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The WHO definition of probiotics is ''live microorganisms which when administered in adequate amounts confer a health benefit on the host'' (WHO 2002). Probiotics are available in different foods and dietary supplements. The most used and studied probiotics are lactic acid bacteria, in particular *Lactobacillus* spp., and *Bifidobacterium* spp.

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.
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Probiotics may prevent paediatric antibiotic-associated diarrhoea (Johnston et al. 2007) and *Clostridium difficile*-induced antibiotic-associated diarrhoea (Vanderhoof et al. 1999, Cremoniniet et al. 2002, D'Souza et al. 2002). Moreover, they are used in the treatment of infectious diarrhoea in children (Szajewska & Mrukowicz 2001, Huang et al. 2002, Van Niel et al. 2002), prevention and treatment of paediatric atopic dermatitis (Lee et al. 2008) and irritable bowel syndrome (Camilleri 2006, Quigley 2007). Application in liver disease and pancreatitis is under discussion but more clinical trials are needed (Jonkers & Stockbrügger 2007). Kligler & Cohrssen (2008) summarize that significant adverse effects after the use of probiotics are rare.

There is evidence that certain bacteria activate Peyer's patch T cells to drive the mucosal immune system via Toll-like receptors on antigen-presenting cells also at other sites of the human body such as the oral cavity. Promoting T helper type 1 (Th1) cytokine responses and down-regulating Th2 may influence distant mucosal sites (Clancy & Pang 2007). Immunomodulation through the consumption of probiotics has received more attention. Specific strains of probiotics have been shown to be able to stimulate and to regulate several aspects of natural and acquired immune responses (Gill & Prasad 2008). Indirect effects on the local immune defense system, e.g. in the oral cavity comprising saliva, the mucosa and the gingival crevicular fluid, are conceivable (Meurman 2005).

The gingival crevicular fluid is an exsudate (Lamster 1997). Its amount and composition is related to the degree of inflammation (Golub & Kleinberg 1976). Here, various inflammatory and anti-inflammatory mediators play an important role (Kim et al. 2007). Mediators such as polymorphonuclear (PMN) elastase, several matrix metalloproteinases (MMP-3) and myeloperoxidase (MPO) originate from PMNs. PMN elastase is a non-oxidative marker of inflammation and is increased during gingivitis (Gonzáles et al. 2001); MPO is an oxidative marker. MMP-3, e.g. the stromelysin MMPs, are responsible for the degradation of extracellular matrix components (Woessner 1991). The activities are increased during inflammation.

Increasing antibiotic resistance is one reason to search for other possibilities in the treatment of infections also in the oral cavity. One alternative to antibiotics may be the use of probiotics. Although only one study focusing on the relationship between the use of probiotics and gingivitis and salivary variables by Krasse et al. (2006) is known, Shimauchi et al. (2008) described a beneficial effect of probiotics in the treatment of periodontitis patients. The aim of our study was to verify whether there is any effect on gingival health during an experimental gingivitis study after the intake of a probiotic milk drink for a longer time period when immunomodulatory effects may be assumed.

Material and Methods

Subjects and clinical examination

Fifty male and female healthy volunteers (mean age: 24.4 ± 1.9 years), students of dentistry and medicine at the Medical Faculty of the University of Leipzig; participated in the study. The study was approved by the Ethics Committee of the Medical Faculty of the University of Leipzig, the participants signed an informed consent.

Subjects were excluded if they received antibiotics or anti-inflammatory drugs during the 6 months before the study or if they were pregnant or nursing. Only subjects without periodontitis were considered.

Taking into account smoking behaviour and gender, the subjects were randomly assigned to the study and control group, respectively. A randomization table was used. For 8 weeks, the study group drank 65 ml of a probiotic milk drink (Yakult®, Homsha Co., Tokyo, Japan) daily, which contained *Lactobacillus casei* strain Shirota. No placebo was allocated and no influence on personal oral hygiene procedures was exerted. The subjects were clinically examined at baseline, after 8 weeks and 4 days after a period of experimental gingivitis without any mechanical or chemical plaque control immediately after the test period. The interproximal plaque index (API, Lange et al. 1977), plaque index (PI, Turesky et al. 1970) and the papilla bleeding index (PBI, Saxer & Mühlemann 1975) were recorded. The examiner had been calibrated by measurements in duplicate at randomly chosen teeth in patients who were not included in the study. Calibration was accepted when the results were identical on $>85\%$ of occasions.

At two teeth, gingival crevicular fluid was collected with paper points for quantitative biochemical analysis of PMN elastase (NE), MPO and MMP-3. The sampled paper points were supplemented with 100 μl isotonic sodium chloride and stored at -20°C.

Biochemical analysis

Biochemical analysis was performed at the Institute of Medical Microbiology at the University of Jena. After thawing and dilution of the samples with 500 μl isotonic sodium chloride, the activities of PMN elastase and MPO as well as the amount of MMP-3 were determined.

Human neutrophil granulocyte elastase activity was measured with a microplate assay using the chromogenic substrate, *N*-methoxysuccinyl-Ala-Ala-pro-Val-*p*Na (Sigma-Aldrich, Taufkirchen, Germany). The substrate was dissolved in dimethylsulphoxide (DMSO) to 10 mM, and the working solution was made up to 1 mM by dilution with 0.05 M Tris, pH 7.5. In brief, 10 μl of the substrate working solution was added to each 90 μl of the eluate of the specimen. Absorbency at 405 nm was measured immediately and also after incubation at 37°C for 30 min. in a microplate reader. One unit was calculated as the amount of enzyme that hydrolyses 1 nmol of substrate in 1 min.

MPO activity was assayed after mixing 40 μl of the eluate of the specimen with 160 μl of the substrate containing 0.8 mM *O*-dianisidine (Sigma-Aldrich) and 0.1 mM H_2O_2. The change in absorbency was measured at 450 nm for 30 min. The results were expressed in units, where one unit of MPO activity is defined as that which degrades 1 μM of H_2O_2/min. (Davies & Edwards 1989).

The amount of MMP-3 was analysed by enzyme linked immunosorbent assay (ELISA) using the Human MMP3 Cytosets™ kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions at a wavelength of 450 nm. The kit includes the specific coating antibody, detection antibody, a standard and a streptavidine–HRP conjugate, and calibration curves were generated.

Data analysis

The statistical package for social sciences 11.5 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis. Non-parametric tests were used for within-group (Friedman/Wilcoxon test) and between-group (Mann–Whitney *U*-test) testing. The subject was the unit of analysis in all the statistical tests performed. A *p*-value of $\leqslant 0.05$ was considered to be statistically significant. The Bonferroni adjustment for multiple testing was applied. Significant results ($p<0.017$) after adjustment are marked with an asterisk in the table.

Results

All 50 subjects completed the study; no volunteer had to be excluded. The demographical data considering the number of volunteers per group, age, gender and number of smokers are given in Table 1. Here, an equal distribution in the groups can be seen.

The slight decrease of the interproximal PI that occurred in the control group during the 8 weeks after baseline examination was non-significant. The interproximal PI increased in both groups during the experimental gingivitis period ($p<0.001$). No significant differences were found between the groups at any examination; the results are given in Table 2. Testing the results of the PI given in Table 3 within the groups, a significant increase in PI was seen from the first to the second and the last examination in both groups (each $p<0.001$). Significant differences also existed between both groups after the experimental gingivitis period ($p=0.001$). The test group had higher plaque accumulation at the buccal and oral surfaces of the teeth.

Papilla bleeding was not significantly different between the groups at any examination. The results are shown in Table 4. Looking at the within-group changes, no significant difference was found between the baseline and the beginning of the experimental gingivitis in the control group. In the test group, no significant difference occurred between the beginning and the end of experimental gingivitis. All other within-group comparisons resulted in significant differences ($p < 0.001$–0.003).

Polymorphonuclear elastase activity changed significantly in both groups during the whole study period. In the test group, a non-significant decrease of elastase activity occurred during the 8-week period of drinking the probiotic milk drink, resulting in significantly lower results than the control group at the beginning of the experimental gingivitis period ($p < 0.001$). There was a significant increase of elastase activity in both groups after experimental gingivitis ($p = 0.001$). The results are given in Table 5.

Only in the control group was there a slight increase of MPO activity during the experimental gingivitis. At the end of the experimental gingivitis period, the control group had a significantly higher MPO activity than the test group ($p = 0.014$). The results and the statistical analysis are shown in Table 6.

The amount of MMP-3 was not detectable in a few samples of gingival crevicular fluid due to laboratory difficulties; one of the ELISA plates fell down. Because of a limited volume of gingival crevicular fluid the laboratory

Table 1. Demographic data

	Test group ($n = 25$)	Control group ($n = 25$)
Age (years)	24.48 ± 1.85	24.24 ± 1.92
Gender		
Male	12	13
Female	13	12
Smoker	10	12

Table 2. Interproximal plaque index (API) for all teeth

	Test group ($n = 25$)		Control group ($n = 25$)		*U*-test
	mean	standard deviation	mean	standard deviation	*p*
Baseline	39.2	19.1	38.9	6.9	0.846
Beginning of experimental gingivitis (1)	27.8	14.9	31.3	14.4	0.419
End of experimental gingivitis (2)	93.1	8.8	96.6	8.0	0.093
Friedman test *p*	<0.001		<0.001		
Wilcoxon test *p* baseline – 1	0.160		0.021		
Wilcoxon test *p* 1 – 2	<0.001*		<0.001*		
Wilcoxon test *p* baseline – 2	<0.001*		<0.001*		

*Significant after Bonferroni adjustment.

Table 3. Plaque index (PI) for all teeth

	Test group ($n = 25$)		Control group ($n = 25$)		*U*-test
	mean	standard deviation	mean	standard deviation	*p*
Baseline	0.76	0.23	0.68	0.23	0.217
Beginning of experimental gingivitis (1)	0.98	0.32	0.82	0.34	0.053
End of experimental gingivitis (2)	2.52	0.61	2.14	0.30	0.001
Friedman test *p*	<0.001		<0.001		
Wilcoxon test *p* baseline – 1	0.001*		0.044		
Wilcoxon test *p* 1 – 2	<0.001*		<0.001*		
Wilcoxon test *p* baseline – 2	<0.001*		<0.001*		

*Significant after Bonferroni's adjustment.

Table 4. Papilla bleeding index (PBI) for all teeth

	Test group ($n = 25$)		Control group ($n = 25$)		*U*-test
	mean	standard deviation	mean	standard deviation	*p*
Baseline	0.67	0.30	0.80	0.27	0.127
Beginning of experimental gingivitis (1)	0.99	0.34	0.89	0.36	0.308
End of experimental gingivitis (2)	1.17	0.57	1.12	0.36	0.985
Friedman test *p*	<0.001		<0.001		
Wilcoxon test *p* baseline – 1	0.001*		0.061		
Wilcoxon test *p* 1 – 2	0.071		0.003		
Wilcoxon test *p* baseline – 2	<0.001*		<0.001*		

*Significant after Bonferroni adjustment.

Table 5. Elastase activity (NE, μU) in the gingival crevicular fluid

	Test group ($n = 25$)		Control group ($n = 25$)		*U*-test
	mean	standard deviation	mean	standard deviation	*p*
Baseline	0.0043	0.0061	0.0054	0.0083	0.676
Beginning of experimental gingivitis (1)	0.0011	0.0035	0.0144	0.0219	<0.001
End of experimental gingivitis (2)	0.0190	0.0117	0.0430	0.0387	0.064
Friedman test *p*	<0.001		<0.001		
Wilcoxon test *p* baseline – 1	0.036		0.061		
Wilcoxon test *p* 1 – 2	<0.001*		0.001*		
Wilcoxon test *p* baseline – 2	<0.001*		<0.001*		

*Significant after Bonferroni's adjustment

Table 6. Myeloperoxidase activity (MPO, μU) in the gingival crevicular fluid

	Test group ($n = 25$)		Control group ($n = 25$)		*U*-test
	mean	standard deviation	mean	standard deviation	*p*
Baseline	2.66	4.86	3.83	4.07	0.426
Beginning of experimental gingivitis (1)	4.43	5.18	4.92	5.06	0.655
End of experimental gingivitis (2)	3.58	5.27	8.50	8.00	0.014
Friedman test *p*	0.326		0.024		
Wilcoxon test *p* baseline – 1	0.339		0.563		
Wilcoxon test *p* 1 – 2	0.427		0.036		
Wilcoxon test *p* baseline – 2	0.382		0.015*		

*Significant after Bonferroni's adjustment.

tests could not be repeated in those cases. The amount of MMP-3 was significantly reduced in the test group after drinking the probiotic milk drink ($p < 0.001$) and increased after the experimental gingivitis period ($p < 0.001$). The test and control groups were significantly different at baseline, with very high results in the test group at baseline ($p = 0.001$) as well as after 8 weeks, with a lower amount in the test group ($p = 0.016$). The results are presented in Table 7.

Discussion

Probiotics can activate and modulate the immune system (Kato et al. 1983). Current knowledge of the mechanisms of probiotic action mostly originates from gastrointestinal studies. Recently, a review on probiotics and oral healthcare has been published (Teughels et al. 2008). However, the influence of probiotics on oral health needs additional research. In this context, the aim of our study was to elucidate the influence of the ingestion of a probiotic milk drink on the expression of plaque-induced gingivitis and gingival health in general.

Only one study using a formulation with *Lactobacillus reuteri* and a placebo for testing the influence of probiotics on gingivitis and gum bleeding can be found in the literature (Krasse et al. 2006). Here, two markers of PMNs were analysed. Recently, a study of periodontal conditions after the use of probiotic-containing tablets has been published (Shimauchi et al. 2008).

Polymorphonuclear elastase has been widely studied as an increasing variable during gingivitis (Gonzáles et al. 2001). It is positively correlated with the gingival crevicular fluid volume (Jin et al. 2003). Also, MPO activity as the other marker derived from PMNs is positively correlated with the gingival index (Kowolik & Grant 1983). Karhuvaara et al. (1990) demonstrated a correlation between the actual purified MPO and the acute gingival inflammation in periodontal pockets. A significant decrease of peroxidase activity in the gingival crevicular fluid occurs after anti-inflammatory treatment of gingivitis with hyaluronan (Jentsch et al. 2003). MMPs, e.g. the stromelysin MMP-3, are responsible for the degradation of extracellular matrix components (Woessner 1991). MMP-3, a 72 kDa gelatinase, additionally plays a role in the activation of pro-MMP1, pro-MMP-8 and pro-MMP-9 (Ogata et al. 1992) and fibroblast type collagenase (Unemori et al. 1991). It is possible to differentiate between healthy and periodontally diseased sites by MMP-3 (Haerian et al. 1995). Increased activity of the 72 kDa gelatinase MMP-3 due to advanced glycation end products has been described; MMP-3 is also up-regulated by the epidermal growth factor in gingival fibroblasts (Cury et al. 2007, Nah et al. 2007). Recently, a new function of MMP-3 as a *trans*-regulator of connective tissue growth factor has been described (Eguchi et al. 2008).

In the present study, a probiotic milk drink with *Lactobacillus casei* Shirota at a concentration of 100 billion per 100 ml was tested. The nutrient data in 65 ml of this drink are 50 kcal, 0.9 g protein, 11.2 g carbohydrates and <0.1 g fat.

In the study by Krasse et al. (2006), the PI decreased in the study groups but no significant change occurred in the placebo group. In contrast to these results, we found an increase of the PI in both groups. There is no doubt that after 4 days of refraining from mechanical and chemical plaque control, plaque accumulates. This increase was more pronounced in the test group, resulting in a significant difference between the two groups at the end of the study. Here the intake of additional carbohydrates may influence this result. No volunteer received any instruction to modify oral hygiene procedures.

Probiotics may have immunomodulatory effects in the gingival region. No increase of papillary bleeding due to more plaque was found in the test group after the experimental gingivitis. Furthermore, elastase activity and the amount of MMP-3 were decreased after the 8-week period of intake of the probiotic milk drink in the test group. A slight increase of elastase activity was measured in the control group. MPO activity was also not elevated after the experimental gingivitis in the test group

Table 7. Amount of the matrix metalloproteinase (MMP-3, μU/site) in the gingival crevicular fluid

	Test group			Control group			*U*-test
	n	mean	standard deviation	*n*	mean	standard deviation	*p*
Baseline	22	218.30	155.96	20	82.73	85.39	0.001
Beginning of experimental gingivitis (1)	24	16.33	13.26	22	20.85	11.48	0.016
End of experimental gingivitis (2)	23	27.03	8.59	23	30.21	11.00	0.223
Friedman test *p*		<0.001			0.623		
Comparison baseline – 1		218.30	155.96		82.73	85.39	
		13.69	5.43		21.49	11.61	
Wilcoxon test *p*	22	<0.001*		20	0.019		
Comparison 1 – 2		13.84	5.35		21.09	11.70	
		27.03	8.59		30.66	10.99	
Wilcoxon test *p*	23	<0.001*		21	0.033		
Comparison baseline – 2		218.30	155.96		75.75	81.65	
		27.59	8.36		29.99	11.19	
Wilcoxon test *p*	22	<0.001*		19	0.136		

*Significant after Bonferroni's adjustment.

but it was in the control group. Our results indicate a decreasing effect on both neutrophil-derived markers, confirming other studies (Shibolet et al. 2002, Peran et al. 2007) that found a reduced colonic MPO activity in a model of rat colitis. In contrast, Ferencík et al. (1999) described elastase and MPO activity elevation after the ingestion of waffles containing lyophilized *Enterococcus faecium* M-74 and discussed the results as an immunostimulatory effect on neutrophil phagocytosis.

The increased results of elastase activity as well as the amount of MMP-3 at the end of the experimental gingivitis period could suggest only a temporary effect of the probiotics in relation to the more important role of plaque accumulation. It has to be considered that results for the amount of MMP-3 could not be obtained from all volunteers.

In our study we included healthy volunteers without periodontitis. Plaque-induced gingivitis was not a criterion for exclusion, as shown by the volunteers with a PBI of 0.67 and 0.80 at the beginning of the study. An individually different gingival inflammatory response to plaque must be taken into account (Trombelli et al. 2008); this response can be modified by the activation of immune cells by soluble β-1,3/1,6-glucans (Preus et al. 2008). There was an approximately equal proportion of smokers in both groups (10 *versus* 12 of 25 volunteers) to avoid an effect of smoking, e.g. on elastase activity, which was described earlier (Söder et al. 2002). In contrast, Persson et al. (1999) did not find significant differences between the elastase activity of smokers and non-smokers; however, large inter-individual variations have been registered. The subjects did not undergo any professional prophylaxis at the beginning of the study, and no microbiological analysis was performed. Thus, an influence of different microbiota in both groups cannot be totally excluded. It is known that periodontopathogens such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* stimulate the release of factors influencing collagen degradation, e.g. MMP-3 (Zhou & Windsor 2006, 2007, Bodet et al. 2007). Because the amount of MMP-3 decreased, this influence may be of minor importance.

Direct effects on bacteria or microbiota of the oral cavity were of a short duration of up to 2 weeks, although high levels of *Streptococus mutans* and salivary yeasts were reduced (Ahola et al. 2002). Considering this and our own results, from our point of view, the effect of the probiotic milk drink could be immunomodulatory.

It has to be considered that this is an explanation of the results of a pilot study and based especially on the results of the gingival crevicular fluid. Further studies are strictly necessary.

Within the limits of this pilot study (e.g. no blinding, number of volunteers), one may conclude that the results of our study reveal a beneficial effect of a probiotic milk drink on the periodontal health in non-immunocompromised subjects. Probiotics may have za reversible immunomodulating effect on plaque-induced inflammation of the gingiva.

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Clinical Relevance

Scientific rationale for the study: The study compares the effect of an 8-week intake of a probiotic milk drink on gingival inflammation and experimental gingivitis.

Principal findings: Despite a higher plaque challenge in the test group, no significant difference from the control group was seen in papillary bleeding. The probiotic milk drink reduced PMN elastase and MMP-3 in the GCF, and MPO was lower and significantly different from the control group after experimental gingivitis.

Practical implications: The supplementary use of the probiotic milk drink may reduce the inflammatory reaction to plaque challenge without compromising the defence function of gingival crevicular fluid.