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**Probiotics affect the clinical inflammatory parameters of experimental gingivitis in humans****Running title:** Probiotics affect inflammation in man

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## Abstract

**Objectives:** To determine the effects of a probiotic milk drink consumed over a period of 28 days regarding the expression of clinical inflammatory parameters of the oral gingiva during various phases of plaque-induced gingivitis.

**Material and Methods:** Twenty-eight adults with healthy gingiva took part in a prospective and clinical-controlled study. The test group was advised to consume a probiotic milk drink (Yacult®) daily during a period of 4 weeks; the control group did not receive any probiotic food or drink. After two weeks of consumption of the probiotic drink, participants were advised not to brush their teeth for 14 days. Subsequently, at baseline as well as on days 1, 3, 5, 7 and 14 the following clinical parameters were assessed: plaque index (PI), gingival index (GI), gingival crevicular fluid (GCF) volume and bleeding on probing (BOP).

**Results:** At baseline the PI was significantly higher in the test group compared to controls ( $0.44 \pm 0.50$  vs.  $0.09 \pm 0.24$  PI;  $p=0.0001$ ). The termination of oral hygiene increased clinical inflammatory parameters in both groups. At day 14 the parameters PI, GI, GCF volume and BOP were significantly higher compared to baseline values ( $p=0.0001$ ). At day 14 BOP levels ( $18.75 \pm 12.32$  vs.  $36.88 \pm 12.54$  %) and GCF volume ( $18.78 \pm 16.7$  vs.  $35.72 \pm 16.1$  PU) were significantly lower in the test group compared to the control group ( $p=0.005$ ).

**Conclusion:** The results of our study indicate that a daily consumption of a probiotic milk drink reduces the effects of plaque-induced gingival inflammation associated with a higher plaque score due to the high carbohydrate content of the probiotic milk beverage.

**Keywords:** probiotics, experimental gingivitis, gingival cervicular fluid, clinical study, inflammation

## Introduction

Probiotics are live microorganisms, which may be of health benefit if consumed in adequate amounts (FAO/WHO, 2001). The concept that probiotic bacteria can influence human health was generated by the Russian-born bacteriologist Elie Metchnikoff. He reported that Bulgarians live longer than other peoples supposedly due to their consumption of fermented milk products containing viable bacteria (*Lactobacillus bulgaricus*) (Metchnikoff, 1907).

Several studies suggested that probiotics might be also of benefit for oral health (Meurmann, 2005, Çaglar *et al.*, 2006, Meurmann & Stamatova, 2007). Several clinical studies demonstrated that a regular consumption of probiotic lactobacilli and *Bifidobacterium* decreased the number of cariogenic streptococci in saliva and dental plaque resulting in a significant lower risk of caries (Näse *et al.*, 2001, Ahola *et al.*, 2002, Çaglar *et al.*, 2006, Çaglar *et al.*, 2007, Cildir *et al.*, 2009). Several studies also revealed that probiotic *Lactobacillus* strains were reducing gingival inflammation (Krasse *et al.*, 2006, Twetman *et al.*, 2009), improving periodontal health (Shimauchi *et al.*, 2008) and decreasing the concentration of black-pigmented rods including *Porphyromonas gingivalis* in saliva and subgingival plaque (Ishikawa *et al.*, 2003, Matsuoka *et al.*, 2006). In contrast, Staab *et al.* (2009) found no changes of clinical inflammatory parameters.

These studies demonstrated that the potentially beneficial effect of probiotics regarding dental caries and inflammatory processes in the oral cavity, specifically periodontitis, are discussed controversially. Gingivitis and periodontitis are chronic inflammatory diseases of the tooth supporting tissues (periodontium) due to bacterial infection. The bacterial biofilms that are firmly attached to the surfaces of teeth cause a chronic inflammation, which may vary between a slight and reversible gingivitis and a severe irreversible periodontitis that may finally result in the loss of teeth (Philstrom *et al.*, 2005). The prevalence of gingivitis in the western populations is appr. 75%. This periodontal disease is characterized by redness, swelling and frequent bleeding of the diseased tissue. Approximately 30% of adults in the United States reveal moderate forms of periodontitis, whereas 10% of the US-population is affected by severe types of this disease (Papapanou, 1999). The experimental gingivitis model induces inflammatory reactions under highly controlled and reproducible parameters in humans. Therefore, this experimental set-up is very useful to analyze the effects of topically or systemically administered antibacterial or anti-inflammatory substances/drugs (Eberhard *et al.*, 2005).

Therefore, it was the objective of our prospective, controlled clinical study on humans to analyse if the consumption of probiotics influences the inflammation of the gingiva. Young, healthy subjects were exposed to the plaque-induced gingivitis model. The hypothesis that was set forth was that the consumption of a probiotic product may reduce the risk of gingivitis.

## Material and Methods

### Reagents

The applied product (Yacult®, Neuss, Germany) is a commercially available probiotic drink made by fermentation of a mixture of skim milk, which contains 6.5 billion live *Lactobacillus casei shirota* strains (concentration of  $10^8$  CFU/ml) per 65 ml bottle. Other ingredients are sugar (sucrose, dextrose), skim milk powder, flavor and water.

### Participants

The study was designed as a prospective, controlled and single-blinded clinical trial. Test subjects were selected according to the following inclusion criteria: (1) 20-35 years of age, (2) non-smokers, (3) no clinical signs of gingival inflammation (redness, swelling, bleeding), (4) no probing pocket depth > 3 mm at any site, (5) no approximal attachment loss > 2 mm at any site and (6) gingival index (GI) = 0 at baseline (detailed information are given below).

Exclusion criteria were: (1) systemic diseases, (2) pregnancy or breastfeeding, (3) physical or mental handicaps that may interfere with an adequate oral hygiene, (4) history of drug abuse, (5) allergies, (6) application of non-steroidal or steroidal anti-inflammatory drugs, analgesic or antibiotics within 6 weeks prior to the study, (7) untreated carious lesions, implants, crowns or maxillary orthodontic appliances in the upper right dentition, (8) unfinished dental treatment, (9) mouth breathing.

Detailed instructions were given to the participants including an information brochure with details of the study design. All subjects signed an informed consent form. Preselected participants were scheduled for a dental examination. In addition, all tooth surfaces were scaled and polished 14 days prior to initiation of the study to remove supragingival plaque, stain and calculus. Subsequently, no dental plaque (PI) or gingival inflammation (GI) was detected. The study protocol was approved by the Ethical Committee of Hanover Medical School (No. 5253).

### Experimental design

At baseline of the experimental gingivitis study, participants received toothpaste without fluoride in order to clean those teeth, which were not examined (Kinderzahngel, Weleda, Schwäbisch Gmünd, Germany). Subjects were instructed not to use other commercial toothpastes and/or mouth rinses. The clinical trial started with a 14-day “non-brushing period”. During this period, participants (test group and control group) were not allowed to perform any oral hygiene at 6 teeth of the right side of the upper jaw. Two weeks prior to baseline of the study as well as during the trial period, the test group (n=11) was advised to drink 65 ml of the probiotic drink, which comprised a total period of 4 weeks. In contrast, the control group (n=17) did not receive any probiotic drink. Subjects were

clinically examined at baseline and at day 1, 3, 5, 7 and 14. At the end of the study, a professional oral hygiene followed by a topical application of fluoride was performed.

### **Clinical readings**

The following clinical parameters were evaluated at each time of assessment in order to document the degree of inflammation of the gingiva:

- Bacterial plaque accumulation was determined using a modified Silness-Löe plaque index (PI) (Silness & Löe 1964) at buccal and oral surfaces of the selected teeth. Dental plaque was assessed visually without staining and graded by four degrees: 0 = no plaque, 1 = little accumulation of plaque only visible with magnifying glasses, 2 = moderate accumulation of plaque visible without magnification, 3 = pronounced accumulation of soft plaque filling the sulcus between the gingival margin and tooth surface. The mean PI was calculated by dividing the sum by the total number of assessed surfaces.
- The gingival index (GI) (Löe & Silness 1963) was determined by examining the selected gingival areas as follows: 0 = normal gingiva, no inflammation, discoloration, or bleeding, 1 = mild inflammation, slight color change, mild alteration of gingival surface structure, no bleeding on pressure, 2 = moderate inflammation, erythema and swelling, bleeding on pressure, 3 = severe inflammation, erythema and swelling, tendency to spontaneous bleeding, occasional ulceration. The mean GI was calculated by dividing the the sum of all scores by the total number of examined surfaces.
- The gingival crevicular fluid (GCF) was collected with a paper strip (Periopaper, Pro Flow Incorporated, Amityville, NY, USA) after gentle drying of the tooth for 10 seconds. The strip was inserted for 30 seconds into the gingival sulcus at four sites of the upper right first premolar mesio- and disto-buccally as well as mesio- and disto-orally. GCF was measured with a calibrated Periotron 6000 gingival fluid meter (Pro Flow Incorporated) and expressed in Periotron units (PU). GCF is a serum exudate that penetrates the gingival sulcus between the gingival margin and the tooth surface.
- The bleeding frequency of the gingiva was recorded at 4 sites of all selected teeth after gentle probing (BOP): mesio- and disto-buccally, mesio- and disto-orally. Inflamed gingiva starts to bleed upon probing and therefore is an indicator of inflammation. The presence or absence of bleeding was recorded after GCF collection. For probing a pressure-calibrated probe (TPS probe, Vivacare, Schaan, Liechtenstein) was used. The tip of the probe had a diameter of 0.5 mm and the probing force was set at 20 g. The BOP was calculated by dividing the total number of positive scores by the total number of probed surfaces and was expressed in percent.

All measurements were carried out under the same conditions by two calibrated investigators who were blinded regarding test or control subjects at baseline as well as on days 1, 3, 5, 7, and 14 at four sites per tooth, except for GCF that was only measured on the upper right first premolar.

### Statistical analysis

The Statistical Package for Social Sciences 17.0 for Windows (SPSS Incorporated, Chicago, IL, USA) was used for statistical analysis. Statistical unit of all tests was each individual subject, median and range values were calculated for all parameters. Differences between baseline and day 14 were calculated and expressed as box plots. The non-parametric Wilcoxon-Test was used for comparisons within groups, the Mann-Whitney-test was applied to determine significant differences between groups. A p-value of  $p \leq 0.05$  was considered to be statistically significant.

### Results

Twenty-eight participants (16 female and 12 male) aged between 20 and 33 years (mean  $24.5 \pm 3.41$  years) were examined, all of them completed the study. No adverse effects were reported by any participant of the test group which consumed the probiotic Yacult® during the 28-day-period of the study.

The data of all measurements (PI, GI, GCF and BOP at baseline as well as on days 1, 3, 5, 7 and 14) are presented in table 1 and in figures 1 to 4. At baseline, the test group showed statistically significant higher values for PI, GI, GCF ( $p=0.0001$ ) than the control group. In contrast, no significant difference for the parameter BOP could be observed between the two groups at baseline. At day 14, the participants of the test group showed significantly higher values for PI ( $p=0.004$ ) and for GI ( $p=0.024$ ), whereas BOP was significantly lower ( $p=0.002$ ) in the test group compared to the control group. No significant differences were documented for GCF.

PI scores increased significantly between baseline and day 14 (Fig. 1) from  $0.44 \pm 0.5$  PI to  $2.58 \pm 0.46$  PI in the test group and from  $0.09 \pm 0.24$  PI to  $2.29 \pm 0.59$  PI in the control group ( $p < 0.0001$ ). However, the slopes of the PI scores of both groups were almost similar and not significantly different (test group  $2.14 \pm 0.56$  PI, control group  $2.2 \pm 0.32$  PI).

The GI grades (Figure 2) significantly increased from  $0.15 \pm 0.19$  at baseline to  $1.44 \pm 0.63$  at day 14 in the test group ( $p < 0.0001$ ) and from  $0.01 \pm 0.03$  to  $1.17 \pm 0.64$  in the control group ( $p < 0.0001$ ). The differences of GI values between baseline and day 14 were not significantly different between both groups (test group  $1.29 \pm 0.42$  GI, control group  $1.16 \pm 0.42$  GI).

Initially GCF readings were significantly higher in the test group ( $23.0 \pm 17.3$  PU vs.  $10.9 \pm 11.6$  PU), but at day 14 similar values were documented (test group  $41.78 \pm 30.52$  PU, control group  $46.62 \pm 27.0$  PU). This means that the increase of GCF volume in the test group was significantly lower ( $18.78 \pm 16.7$  PU) compared to the control group ( $35.72 \pm 16.1$  PU,  $p=0.005$ ) during the experimental gingivitis period (Fig. 3).

At baseline, no significant differences of BOP readings were found between the test and control group, however at day 14 participants of the test group showed significant lower values ( $18.75 \pm 12.32$  %) than subjects of the control group ( $36.88 \pm 12.54$  %,  $p=0.002$ ). Thus, increase of BOP in the test group was significantly lower than in the control group ( $p=0.005$ , Fig. 4).

## Discussion

Our prospective clinical study demonstrated for the first time that probiotics may influence oral/periodontal health in humans. In order to investigate the effects, the experimental gingivitis model was selected. Overall, our data document that (A) a pronounced plaque formation may be associated with the daily consumption of probiotics in combination with a normal oral hygiene and (B) an anti-inflammatory effect was found during experimental gingivitis period of two week. Therefore, the tested hypothesis was confirmed.

The effects of a probiotic therapy on systemic diseases have been studied for various disorders (Broekaert & Walker, 2006). Most of the documented benefits of probiotics are associated with gastrointestinal disorders, including those caused by *Clostridium difficile* and antibiotic medication causing diarrhea (Vanderhoof *et al.*, 1999, Cremonini *et al.*, 2002, D'Souza *et al.*, 2002, Szajewska *et al.*, 2001), acute infectious diarrhea and irritable bowel syndrome (Camilleri, 2006, Quigley, 2007, Kligler & Cohrsen, 2008). Positive effects of probiotics were also observed for caries- associated risk factors (Näse *et al.*, 2001, Ahola *et al.*, 2002, Çağlar *et al.*, 2006, Cildir *et al.*, 2009) and the colonization of the oral cavity by *Candida* spp. (Hatakka *et al.*, 2007). However, there are only few studies, which investigated the influence of probiotics on gingivitis or periodontitis. Twetman *et al.* (2009) reported a reduction of clinical symptoms caused by gingivitis after the use of chewing gum containing *Lactobacillus reuteri* for two weeks. Krasse *et al.* (2006) documented the effects of probiotics on moderate to severe gingivitis in 59 patients. They showed that after the administration of the probiotic microorganism *Lactobacillus reuteri* for a 2 week period gingival inflammation was significantly reduced. In contrast to our study the clinical changes were associated with a significant drop of the plaque indices in the test group. It may be speculated that this difference is the consequence of the high sugar content of the commercial product used in our study, whereas Krasse



et al. (2006) administered solely isolated bacteria. Recently, Staab *et al.* (2009) examined the effects of an oral administration of *Lactobacillus casei Shirota* over a period of eight weeks prior to a 96-hour-period without oral hygiene. They reported no changes of the investigated clinical inflammatory parameters but a significant increase of plaque accumulation in the test group, confirming the results of our study. Our investigation showed an initial increased plaque accumulation during normal oral hygiene before baseline which was associated with significantly increased signs of gingival inflammation. It may be hypothesized that this effect is due to the increased availability of carbohydrates for the oral microorganisms by the tested probiotic milk drink, maybe followed by a shift of the composition of the oral microflora in the experimental part of our study.

In addition to potential effects on inflammation following the application of probiotics, several studies examined the effects of the composition of bacterial biofilms following the topical use of probiotics. It was demonstrated in patients, who suffered from periodontitis or gingivitis, that probiotic bacteria accumulated in microbial biofilms thus replacing or reducing pathogenic bacteria (Grudianov *et al.*, 2002, Tsubura *et al.*, 2009). Ishikawa *et al.* (2003) and Matsuoka *et al.* (2006) demonstrated that the oral application of probiotic pills containing *L. salivarius* significantly reduced the concentration of the periopathogenic bacterium *P. gingivalis* in saliva and subgingival plaque in healthy volunteers. Shimauchi *et al.* (2008) documented a reduced concentration of periodontopathogenic bacteria after the administration of probiotic *Lactobacilli* over a period of 8 weeks, which was associated with improved periodontal conditions. Although the characterization of the oral microflora was not the aim of the present study, it may be speculated that the accumulation of a non-pathogenic bacterial biofilm on the tooth surfaces was very likely causative for the reduced inflammation in the test group.

In contrast to other investigations the well-established model of experimental gingivitis was used in the present study to investigate the anti-inflammatory effects of probiotics. The termination of oral hygiene procedures led to an accumulation of bacterial deposits on the tooth surfaces and soft tissues with a subsequent increase of the bleeding frequency and gingival sulcus fluid volume. Both parameters are indicative for a local inflammatory host response. Contrary to clinical studies that investigated the effects of probiotics on inflammatory oral parameters in patients who suffer already from gingivitis or periodontitis, the experimental gingivitis model has several advantages (Löe *et al.*, 1965). Specifically, it is possible to control risk factors that may influence plaque formation or inflammatory host responses. Further, it is possible to document the association between bacterial biofilms and clinical inflammatory reactions in detail analyzing appropriate and sensitive clinical parameters (Heasman *et al.*, 1993, Eberhard *et al.*, 2002, Jepsen *et al.*, 2003). In this study, no

smokers were included. The inflammatory reactions were obviously a consequence of a lacking oral hygiene, which was indicated by several clinical parameters.

There are no data available to explain the molecular biological fundamentals for the clinical effects of probiotics in the oral cavity. Several mechanisms are considered to be responsible for the beneficial clinical effects of probiotics including an interaction with pathogenic bacteria (Michail *et al.*, 2002, Patzer, 2003), an increase of the hosts' immune response (Schultz *et al.*, 2002, Zhang *et al.*, 2006, Diaz-Ropero *et al.*, 2007, Schlee *et al.*, 2008) and a production of antimicrobial substances such as organic acids, hydrogen peroxide, and bacteriocins (Reid *et al.*, 2003). The observed clinical effects are very likely a combination of a "direct competition" between pathogenic bacteria and probiotics as well as various beneficial effects on the hosts' immune response (Kligler & Cohrsen, 2008).

Taken together, our data based on a controlled experimental setting indicate an anti-inflammatory effect of the tested probiotic milk drink. Inflammatory parameters BOP and GCF were significantly lower in test group compared to the control group after a period of 28 days of oral intake of *Lactobacillus casei shirota*. These changes were associated with an increased accumulation of bacterial biofilms that were very likely caused by the high carbohydrate content of the probiotic drink. The cariogenic potential of *Lactobacilli* (Haukioja *et al.*, 2008) and the high sugar content of the tested milk-based probiotic drink should be investigated in future long-term studies.

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Table 1: Mean values and standard deviations of GI, PI, GCF, and BOP at baseline and on day 14 as well as the differences between baseline and day 14

Parameter	baseline		day 14		$\Delta$ day 14 - baseline	
	Control	Probiotic	Control	Probiotic	Control	Probiotic
PI	0.09 $\pm$ 0.24	0.44 $\pm$ 0.50 *	2.29 $\pm$ 0.59	2.58 $\pm$ 0.46 *	2.2 $\pm$ 0.32	2.14 $\pm$ 0.56
GI	0.01 $\pm$ 0.03	0.15 $\pm$ 0.19 *	1.17 $\pm$ 0.64	1.44 $\pm$ 0.63 *	1.16 $\pm$ 0.42	1.29 $\pm$ 0.42
GCF	10.9 $\pm$ 11.6	23.0 $\pm$ 17.3 *	46.62 $\pm$ 27.0	41.78 $\pm$ 30.52	35.72 $\pm$ 16.1	18.78 $\pm$ 16.7 **
BOP	13.12 $\pm$ 9.47	10.5 $\pm$ 11.37	36.88 $\pm$ 12.54	18.75 $\pm$ 12.32 **	23.76 $\pm$ 11.9	8.25 $\pm$ 12.72 **

\* Statistically significantly higher values in the probiotic-group (Mann-Whitney-Test); significance level  $p < .05$

\*\* Statistically significantly lower values in the probiotic-group compared to control (Mann-Whitney-Test); significance level  $p < .05$

Figure 1: PI values shown as box plots at baseline, day 1, 3, 5, 7 and 14

Figure 2: GI values shown as box plots at baseline, day 1, 3, 5, 7 and 14

Figure 3: GCF values shown as box plots at baseline, day 1, 3, 5, 7 and 14

Figure 4: BOP values shown as box plots at baseline, day 1, 3, 5, 7 and 14









