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Probiotic intervention influences the salivary levels of Matrix Metalloproteinase (MMP)-9 and Tissue Inhibitor of metalloproteinases (TIMP)-1 in healthy adults

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ABSTRACT

Objective: To study the effect of orally administered *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus rhamnosus* GG on the salivary levels of Matrix Metalloproteinases (MMP)-8, MMP-9 and of Tissue Inhibitor of Metalloproteinases (TIMP)-1 in healthy adults. Furthermore, the correlations between MMP-8, MMP-9 and TIMP-1 and plaque and gingival indices, salivary mutans streptococci and lactobacilli counts, and stimulated saliva secretion rate were analysed.

Design: The salivary samples originated from a randomized controlled trial where healthy student volunteers consumed probiotic or placebo lozenges twice a day for four weeks. The saliva samples were collected and clinical parameters measured at the baseline and at the end of the original study. For this study, the salivary levels of MMP-8, MMP-9 and TIMP-1 were analysed with immunofluorometric assay (IFMA) and enzyme-linked immunosorbent assay (ELISA).

Results: In the probiotic group ($n = 29$), salivary MMP-9 levels increased ($p < 0.01$) and TIMP-1 levels decreased ($p < 0.01$) significantly during the intervention. Furthermore, MMP-9/TIMP-1 ratio differed significantly from the baseline level ($p < 0.01$). These changes were not observed in the control group ($n = 31$). In the whole data, salivary MMP-9 and gingival index correlated ($r = 0.260$, $p < 0.05$ at baseline and $r = 0.354$, $p < 0.01$ at the end of the study). Intergroup differences or correlations with other clinical parameters were not found. Probiotic consumption did not affect the saliva flow rate.

Conclusions: Increased MMP-9 and decreased TIMP-1 levels in saliva may indicate that probiotics have immunomodulatory effects in the oral cavity. Furthermore, increased salivary MMP-9 levels may be an indication of the defensive potential of matrix metalloproteinases.

1. Introduction

By definition, probiotics are live micro-organisms, which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2001). Some of these microbes may colonise the oral cavity temporarily (Caglar, Topcuoglu, Cildir, Sandalli, & Kulekci, 2009; Saxelin et al., 2010; Taipale, Pienihäkkinen, Salminen, Jokela, & Söderling, 2012; Yli-Knuuttila, Snäll, Kari, & Meurman, 2006). Probiotics seem to have a promising role in enhancing periodontal health while the role in dental caries is contradictory (Cagetti et al., 2013; Gruner, Paris, & Schwendicke, 2016; Stecksén-Blicks et al., 2009; Twetman, Derawi et al., 2009; Twetman, Larsen, Fiehn, Stecksén-

Blicks, & Twetman, 2009; Taipale, Pienihäkkinen, Alanen, Jokela, & Söderling, 2013; Yanine et al., 2013). In addition, probiotic therapy has been connected to increased salivary flow in elderly people (Hatakka et al., 2007). Current knowledge on mechanisms how probiotics affect the oral health is in part controversial and the effects seem to be dependent on studied population, bacterial strain and product (Cagetti et al., 2013; Gruner et al., 2016; Martin Cabezas, Davideau, Tenenbaum, & Huck, 2016). Nevertheless, probiotics affect most likely directly by influencing the oral microbiota (Caglar, Kuscu, Cildir, Kuvvetli, & Sandalli, 2008; Iniesta et al., 2012; Jäsberg, Söderling, Endo, Beighton, & Haukioja, 2016), interacting with salivary proteins (Haukioja, Loimaranta, & Tenovu, 2008) or by affecting the immune

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defence (Devine & Marsh, 2009; Twetman, Derawi et al., 2009; Twetman, Larsen et al., 2009).

Matrix metalloproteinases (MMPs) are a family of enzymes that degrade extracellular matrix and basement membrane. They are involved in physiological processes including tissue remodelling and wound healing (Sorsa, Tjäderhane, & Salo, 2004) as well as inflammation and innate immunity (Parks, Wilson, & López Boado, 2004). MMPs activity is controlled by changes in the balance of expression and synthesis of MMPs and their major inhibitors, tissue inhibitors of metalloproteinases (TIMPs). The catalysis of MMPs is controlled through activation of their proenzymes and the inhibition by TIMPs. MMP-8 degrades type 1 collagen, the main collagenous component in periodontal tissues, MMP-9 is related to host's defensive mechanisms (Sorsa et al., 2016).

In oral environment, MMPs are present in saliva, dental plaque, gingival crevicular fluid (GCF), and carious dentin. The main source of salivary MMPs is GCF, where polymorphonuclear leucocytes (and monocytes/macrophages) have released them (Sorsa et al., 2016). TIMPs are primarily produced by B-cells and the source in saliva is GCF (Verstappen & Von den Hoff, 2006). In periodontal diseases, salivary MMP-8 and MMP-9 levels are elevated (Ebersole, Nagarajan, Akers, & Miller, 2015; Rathnayake et al., 2013), and TIMP-1 levels decreased (Soell, Elkaim, & Tenenbaum, 2002). Tissue destruction reflects the relative over expression of MMPs in relation to TIMPs (Gürsoy et al., 2010; Reynolds, 1996). The salivary levels of MMP-8 may be elevated also in subjects with manifested caries lesions (Hedenbjörk Lager et al., 2015). In addition, salivary MMP-8 level correlates with saliva secretion rate (Hedenbjörk Lager et al., 2015). However, salivary mutans streptococci and lactobacilli levels were not associated with MMP-8 in head and neck cancer patients receiving radiotherapy (Vuotila et al., 2002). Still, an association between specific bacterial species and salivary MMP-levels cannot be excluded (Kuula et al., 2009). Finally, due to their ability to process non-matrix bioactive substrates such as anti-inflammatory cytokines, chemokines, growth factors, and serum components, MMPs can mediate anti-inflammatory or defensive immune responses. The defensive role of MMP-8 and MMP-9 has emerged in knockout-mice-model studies (Hernández et al., 2011; Kuula et al., 2009).

One of the immunomodulatory mechanisms of action of probiotics can be their effect on expression of MMP's. Probiotics seem to influence the amount of MMP's in GCF. In a study with gingivitis patients, probiotic intervention with *L. casei* Shirota resulted in decreased elastase activity and MMP-3 level (Staab, Eick, Knöfler, & Jentsch, 2009). In a study with patients with chronic periodontitis, probiotic therapy with *L. reuteri* connected to traditional periodontal treatment resulted in decreased levels of MMP-8 and increased levels of TIMP-1 when compared to periodontal treatment only (Ince et al., 2015). Probiotics have also decreased the levels of MMP-9 in lung tissue of experimental animals in an experimental asthma model (Wu, Chen, Lee, Ko, & Lue, 2016). However, *in vitro* results suggest that probiotic *L. bulgarii* is unable to activate proMMP-9 into the active form of the enzyme (Stamatova et al., 2007).

How probiotic intervention affects the salivary levels of MMPs or their inhibitors in healthy individuals is not known. In this study, our aim was to investigate the effect of probiotic *L. rhamnosus* GG and *Bifidobacterium animalis* sp. *lactis* BB-12 on salivary MMP-8, MMP-9 and TIMP-1 levels in healthy subjects. Salivary MMP-8, MMP-9 and TIMP-1 levels were also related to the salivary mutans streptococci (MS) and lactobacilli (LB) counts, plaque and gingival indices (PI and GI) as well as the salivary secretion rate in probiotic and control groups.

2. Materials and methods

2.1. Test subjects, probiotic intervention, sample collection

Stimulated whole saliva samples originally from a randomized,

controlled, double-blinded trial were analysed. The methods in study design, test products, participants and sample collection methods are described in detail earlier (Toiviainen et al., 2015) and summarized below.

After screening of 77 volunteers, 62 students at the University of Turku were included in the study. The inclusion criteria were good general health, willingness to participate and salivary MS level $\geq 10^3$ colony forming units (CFU)/ml. The written informed consent was obtained from all subjects. The study was approved by the ethical committee of the Hospital District of Southwest Finland. Subjects were randomly divided into two groups. Group 1 (28 females and 3 males) had mean age (SD) of 24.6 (2.7) years and group 2 (27 females and 4 males) had mean age (SD) 24.0 (3.0). None of the subjects smoked and all had good oral hygiene. Two subjects in group 1 (probiotic group) dropped out of the study leaving 29 subjects for final analysis in the probiotic group. All 31 subjects in group 2 (control group) completed the study.

During the 4-week run-in period subjects were instructed not to use commercial products containing *L. rhamnosus* GG and *B. lactis* BB-12 and to continue their dietary and tooth brushing habits. Additionally, during the run-in period subjects were instructed to use four pieces of mild-tasting, non-commercial chewing gum manufactured for the study by Karl Fazer AB (Vantaa, Finland) daily. After run-in period subjects were randomly divided into two groups; group 1 used test lozenges containing probiotic LGG and BB-12, while group 2 used lozenges without probiotics, 4 pieces per day. The lozenges were also manufactured by Karl Fazer AB (Vantaa, Finland) and contained 50% of xylitol and 46% of sorbitol. The recommended number of lozenges resulted in daily amount of approx. 2×10^9 cells of LGG and BB-12.

Salivary samples were collected with paraffin stimulation after determination of the plaque and gingival indices at the baseline and after 4-week test period. A sample of 4 ml of saliva was collected in the test tube on ice and the collection time was recorded. Before the sample collection visit, subjects were instructed to refrain from dental hygiene for 24 h and on the morning of the sample collection day subjects were instructed not to use run-in chewing gum/test tablet.

For salivary mutans streptococci and lactobacilli, after serial tenfold dilutions, samples were plated on Mitis salivarius agar (Becton Dickinson and Company, Sparks, MD, USA) containing bacitracin (MSB) for mutans streptococci and on Rogosa agar (Becton Dickinson and Company) for lactobacilli. MSB plates were incubated in 7% CO₂ atmosphere and Rogosa plates anaerobically. After 2 days' incubation at +37 °C, colonies were counted and results were expressed as CFUs per millilitre.

Periodontal probe was used to determine the Silness-Löe plaque index (Silness & Loe, 1964) and the Löe-Silness gingival index (Loe & Silness, 1963).

Plaque and gingival indices as well as the saliva secretion rates of the test subjects in the baseline and after 4-week test period are presented in Table 1. The mean values of plaque and gingival indices have been reported earlier (Toiviainen et al., 2015).

2.1.1. Salivary analysis of MMP-8, MMP-9 and TIMP-1

MMP-8 levels were detected by time-resolved immunofluorometric assay (IFMA) as described earlier (Gursoy et al., 2010; Hanemaaijer et al., 1997). Briefly, the MMP-8 specific monoclonal antibodies 8708 and 8706 (Medix Biochemica, Kauniainen, Finland) were used as a capture antibody and a tracer antibody. The tracer antibody was labelled with europium-chelate and the assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM CaCl₂, 50 µM ZnCl₂, 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/l diethylenetriaminepentaacetic acid. Salivary samples were diluted in assay buffer and incubated for 1 h, followed by another 1 h of incubation with tracer antibody. Enhancement solution was added and after 5 min incubation fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The detection limit for this assay was

Table 1

Plaque and gingival indices (PI and GI, Median [minimum, maximum]) as well as saliva secretion rates (ml/min) of the participants at the baseline and after 4-week test period. Data regarding PI and GI is from (Toivaiainen et al., 2015).

	Plaque Index			Gingival Index			Saliva flow rate		
	control	LGG + BB-12	p-value ^b	control	LGG + BB-12	p-value ^b	control	LGG + BB-12	p-value ^b
Baseline	0.88 [0.11, 2.00]	1.00 [0.46, 1.79]	0.112	0.67 [0.04, 2.00]	0.71 [0.21, 1.17]	0.929	0.67 [0.25, 3.46]	0.58 [0.21, 1.33]	0.4
4 weeks	0.79 [0.04, 2.10]	0.94 [0.30, 1.71]	0.176	0.63 [0.08, 1.67]	0.56 [0.08, 1.00]	0.571	0.63 [0.25, 2.46]	0.54 [0.25, 1.33]	0.343
P-value ^a	0.064	0.016		0.094	0.013		0.103	0.119	

^a Paired samples *t*-test between baseline and 4 week values.

^b Independent samples *t*-test between groups.

0.08 ng/ml.

MMP-9 and TIMP-1 levels in saliva were assessed with enzyme-linked immunosorbent assay using commercial ELISA kits as described earlier (Buduneli et al., 2011; Gursay et al., 2010): Quantikine ELISA for MMP-9 (R & D Systems, Minneapolis, MN, USA) and Amersham ELISA for TIMP-1 (Human, Biotrak, ELISA system (GE Healthcare, Amersham, Buckinghamshire, UK)). Assays were performed according to the manufacturers' protocol. The detection limit was 0.156 ng/ml for MMP-9 (Quantikine ELISA) and 1.25 ng/ml for TIMP-1 (Amersham ELISA).

2.1.2. Statistical analysis

Statistical package for social sciences for Windows (SPSS versions 22 and 23, Chicago, IL, USA) was used for statistical analysis. Since the data from saliva analysis were not normally distributed, non-parametric tests were used. The differences of MMP and TIMP levels between test and control groups were studied with Mann-Whitney *U* test. Wilcoxon signed ranks test was used to study the differences in the parameters between baseline and the end of the test period inside the group.

Independent samples student's *t*-test was used to analyse the differences in salivary flow rate, PI and GI between groups, and paired sample *t*-test for differences between baseline and after probiotic use in salivary flow rate. Pearson correlation was used to evaluate the connection between clinical data and salivary markers. At the baseline, there was a significant positive correlation between MMP-9 and GI in the control group ($r = 0.493$, $p < 0.01$) but not in the probiotic group ($r = 0.023$, $p = 0.906$). Therefore, we analysed the correlation between GI change and the change in measured MMP or TIMP-1 levels during the intervention.

The level of statistical significance was set at $p < 0.05$.

3. Results

Salivary levels of MMP-8, MMP-9 and TIMP-1 as well as MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios are presented in Fig. 1. In the probiotic group, MMP-9 level increased ($p < 0.01$) and TIMP-1 level decreased ($p < 0.01$) significantly during the test period. In addition, the molar ratio of MMP-9/TIMP-1 at four weeks differed significantly from baseline level in the probiotic group ($p < 0.01$). Probiotic use did not influence the salivary levels of MMP-8. There were no differences in any of the variables (MMP-8, MMP-9, and TIMP-1 or the molar ratios of MMPs and TIMP-1) between the probiotic and the control group, neither at the baseline nor at the end of the study.

When all samples were analysed, there was a weak but statistically significant correlation between GI and MMP-9 ($r = 0.260$, $p < 0.05$ at baseline and $r = 0.354$, $p < 0.01$ at the end of the study). Salivary MMP-8 level did not correlate with GI or PI. Salivary MMP-8, MMP-9 and TIMP-1 levels and salivary MS and LB counts did not correlate.

The change in molar ratio of MMP-8/TIMP-1 during the four-week intervention correlated significantly with the change in GI ($p = 0.03$) in the control group, but not in the probiotic group. There was no correlation in the change of the other measured parameters (MMP-8, MMP-9, MMP-9/TIMP-1 molar ratio) with GI, PI, salivary MS and LB counts, or salivary secretion rate.

Salivary secretion rates are presented in Table 1. There was no difference in the secretion rates between the groups. No connection was found between salivary secretion level and other parameters (PI, GI, the levels of SM or LB, or salivary MMP-8, MMP-9 and TIMP-1 levels).

4. Discussion

To our knowledge, the effect of probiotic intervention on salivary MMP-8 or MMP-9 levels has not been studied before. We observed a significant increase in the salivary MMP-9 levels and in the salivary MMP-9/TIMP-1 molar ratio, as well as a decrease in TIMP-1 level in the probiotic group after the test period, although intergroup differences in salivary MMP-8, MMP-9 and TIMP-1 levels were not seen.

In contrast to our results, earlier studies suggest decreased MMP levels and increased TIMP-1 levels as a response to the probiotic therapy. Ince (2015) et al. studied probiotics as an additional therapy to the traditional periodontal treatment. The use of probiotics resulted in significantly lower PI and GI, decreased MMP-8 levels and increased TIMP-1 levels in GCF when compared to the control group with traditional periodontal treatment only. Staab (2009) et al. studied experimental gingivitis and observed significantly lower MMP-3 levels in GCF in probiotic group than in controls, although there was no difference in clinical parameters. Wu (2014) et al. showed decreased levels of MMP-9 in lung tissue of experimental animals receiving probiotics compared to controls in an experimental asthma model. It is likely that at least a part of the differences between our results and earlier studies can be explained with different study populations: in earlier studies, diseased subjects or tissue have been investigated, while in our study the volunteers' gingival health was good. Even if matrix metalloproteinases are related to the tissue destruction related in periodontal disease and dental caries, different proteinases have different roles (Saarinen et al., 2016). In addition, both MMP-8 and MMP-9 deficient mice models as well as human studies have revealed that physiological MMP-8 and MMP-9 levels, which modify decisively extracellular matrix and immune responses, exert also defensive effects in addition to the auxiliary destructive effect (Hernández et al., 2011; Kuula et al., 2009).

Increased salivary MMP-9 levels and MMP-9/TIMP-1 molar ratio demonstrated in this study may reflect an enhanced defensive potential of the mucosal immune defence achieved by the use of probiotics. Probiotic bacteria have immunomodulatory effects (Klaenhammer, Kleerebezem, Kopp, & Rescigno, 2012), but results regarding the oral cavity are contradictory. For example, increased salivary IgA levels after probiotic intervention have been observed in a part of the studies, while in others IgA levels have been unchanged (Ericson, Hamberg, Bratthall, Sinkiewicz Enggren, & Ljunggren, 2013; Gueimonde et al., 2016; Jørgensen et al., 2016; Kotani et al., 2010; Suroño et al., 2011). *L. reuteri* seems to have a positive effect on inflammatory markers in GCF (Flichy Fernández et al., 2015; Szkaradkiewicz, Stopa, & Karpinski, 2014; Twetman, Derawi et al., 2009). In addition, LGG inhibits the formation of neutrophil extracellular traps (NETs) and capacity to oxidative burst (Vong, Lorentz, Assa, Glogauer, & Sherman, 2014). Neutrophils are, on the other hand, via GCF the main source of salivary MMPs.

The salivary samples used in this study are from subjects who had a

Fig. 1. Salivary levels of MMP-8, MMP-9, and TIMP-1 as well as MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios. p-values of Wilcoxon signed-ranks test..

very good periodontal health and most of them had hardly any dental plaque, even after refraining from dental hygiene for 24 h before the sample collection. The main source of salivary MMPs and TIMP's is GCF, especially when periodontal inflammation is present (Sorsa et al., 2004). Participants of this study had GIs indicating normal healthy gingiva or mild inflammation with no bleeding. Furthermore, the

change in salivary MMP-8/TIMP-1 ratio correlated with GI change only in the control group, but not in the probiotic group. The gingival health was very good in the probiotic group after the intervention, as indicated by the reduced GI. Thus, the level of inflammatory marker MMP-8 was likely so low that the correlation with GI simply could not be seen. In other words, healthy gingiva lacks the inflammatory marker MMP-8.

The salivary MMP-8 and MMP-9 levels vary in healthy subjects depending e.g. on age and oral health status (Nedzi Góra, Kostrzewa Janicka, & Górka, 2014; Rathnayake et al., 2013), but the salivary levels of MMP-8, MMP-9 and TIMP-1 measured in this study represent well the salivary MMP and TIMP-1 levels in healthy, young individuals.

The subjects participating this study were healthy. They had no periodontal disease or no manifested caries lesions, but a high salivary mutans streptococci count was one of the inclusion criteria. We found no correlation between salivary MMP-8, MMP-9, and TIMP-1 counts and salivary MS and LB counts. Importantly, probiotic intervention did not affect the salivary levels of MMP-8. Thus, high salivary MS counts or the use of LGG or Bb-12 are unlikely to interfere with the use MMP-8-chairside immunodiagnostic kit, which is based on correlation between salivary MMP-8 counts and manifested caries lesions (Hedenbjörk Lager et al., 2015; Heikkinen et al., 2016), in caries diagnostics.

Salivary MMP-8 levels have earlier been shown to correlate with saliva secretion rate (Hedenbjörk Lager et al., 2015), but we did not find any correlation between salivary secretion rate and salivary MMP-8, MMP-9 or TIMP-1 levels or other clinical parameters in this study. In addition, salivary secretion rate has been improved in elderly with hyposalivation after probiotic use (Hatakka et al., 2007), although this effect has not been observed in other studies (Gueimonde et al., 2016). Our results are in line with those reported by Gueimonde et al., (2016). Participants in our study were healthy, and most of them had normal saliva flow rates.

To conclude, the use of probiotics related to increased salivary MMP-9 levels. This may be an indication of a positive immunomodulatory effect of probiotics in the oral environment.

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Ethical approval

The study was approved by the ethical committee of the Hospital District of Southwest Finland. Reference number: ETMK 22/180/2012

Conflicts of interests

Dr Timo Sorsa is an inventor of US-Patents 5652223, 5736341, 5866432 and 6143476.

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