Archives of Oral Biology 85 (2018) 58–63

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

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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ARTICLE INFO

Keywords:
Probiotic
Lactobacillus rhamnosus GG
Bifidobacterium animalis subsp. *lactis* BB-12
Matrix metalloproteinase (MMP)
Tissue inhibitor of metalloproteinases (TIMP)
Saliva

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, & Tenovuo, 2008) or by affecting the immune

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http://dx.doi.org/10.1016/j.archoralbio.2017.10.003
Received 20 March 2017; Received in revised form 11 September 2017; Accepted 7 October 2017

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% CO_2 athmosphere 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 $CaCl_2$, 50 μM $ZnCl_2$, 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