Keywords
probiotics; salivary IgA; mutans streptococci; Lactobacillus reuteri.

Abstract
We investigated whether ingestion of probiotic bacteria could influence salivary IgA levels, specific anti-mutans streptococci IgA levels and specific antibodies towards the ingested probiotic bacterium. The study was a randomised, double-blind, placebo-controlled trial, where the test group (n=11) received twice daily chewing of gum containing Lactobacillus reuteri (2 x 10^9 CFU per dose) and the control group (n=12) received placebo. Resting saliva was collected before and after 12 weeks of treatment and 4 weeks after end of treatment. Total salivary IgA concentrations were measured by ELISA. Specific IgA reactivity was determined using a whole-cell ELISA. Results were expressed as % IgA per protein in saliva. The level of total IgA% per protein increased significantly between pretreatment levels (13.5%) and follow-up treatment levels (14.4%) within the test group only (P < 0.05). No changes were seen in the control group during the trial. The level of probiotic-reactive antibodies decreased significantly between pre- and post-treatment samples (from 12.2% to 9.0%, P < 0.05) in the test group. Similarly, the level of specific mutans streptococci antibodies decreased significantly between pre- and post-treatment samples (P < 0.05) in the test group only (for Streptococcus mutans from 20.1% to 15.0%; for Streptococcus sobrinus from 7.4% to 5.3%). Ingestion of probiotic bacteria might influence the adaptive immune response of the host.

Introduction
Probiotic bacteria have created increased awareness within oral health care as a complementary way to prevent diseases caused by the oral microbial flora (Meurman & Stamatova, 2007; Teughels et al., 2007; Twetman & Stecksén-Blicks, 2008). Proposed mechanisms of action for beneficiary effects of intake of probiotics include that probiotic bacteria compete for space and nutrients as well as release of antagonistic substances with pathogens to establish a healthy and diverse flora (Meurman & Stamatova, 2007). The stimulating effect on the immune response by probiotic bacteria has also been suggested as another mechanism in some studies (Surono et al., 2011; Yan & Polk, 2011). Probiotics may also affect levels of inflammatory mediators in crevicular fluid (Twetman et al., 2009).

Several studies have demonstrated that lactobacilli-derived probiotic strains (i.e. Lactobacillus reuteri ATCC PTA 5289, L. reuteri ATCC 55730, Lactobacillus rhamnosus GG ATCC 53103 and L. rhamnosus LC 705) during short-term (10–21 days) intake reduced the counts of mutans streptococci in saliva and plaque (Ahola et al., 2002; Nikawa et al., 2004; Caglar et al., 2008), but no effect could be seen with one strain (L. rhamnosus LB21; Lexner et al., 2010). Only one study using probiotic bacteria has demonstrated a clinical carries-preventive effect where Nåse et al. (2001) showed that drinking milk supplemented with L. rhamnosus GG for 7 months could be caries preventive among 3- to 4-year-old children.

Immune-stimulating effects of probiotic bacteria have been reviewed by Kelly et al. (2005), and it is documented that intake of probiotic lactobacilli will enhance production of
IgA (Corthésy et al., 2007). Relevant to the oral ecosystem, high salivary IgA titres against mutans streptococci antigens have been demonstrated to be caries protective in humans (Smith & Mattos-Graner, 2008) and recently also in a rat model (Shi et al., 2012). Of particular interest for immune regulation affecting the mucosal immunity and caries prevention, an increase in salivary IgA, but not serum IgA, has been demonstrated after 90 days ingestion of a probiotic strain Enterococcus faecium among under-nourished children (Surono et al., 2011).

Therefore, the hypothesis in this study was that ingestion of a probiotic strain for prevention of oral diseases can have immunomodulating effects measurable in saliva. The aim of this study was to investigate whether the introduction of L. reuteri ATCC PTA 5289 through the use of probiotic chewing gum can elicit an increased immune response towards mutans streptococci as well as towards the ingested probiotic bacterium.

Materials and methods

Patients and study design

The study was a randomised, double-blind, placebo-controlled trial. Exclusion criteria were use of antibiotics, oral antiseptics or periodontal treatment 6 months prior to the trial. Detailed description of methods and results on clinical outcome, plaque formation, plaque and salivary bacteria from the current investigation has been reported by Sinkiewicz et al. (2010). The research protocol was approved by Ethics Committee at Lund University, Sweden.

Twenty-four healthy volunteers aged 18 years or more randomised into test or control subjects. One subject in the test group was excluded due to the use of antibiotics during the trial period (Sinkiewicz et al., 2010). The antibody response in saliva towards mutans streptococci and towards the probiotic strain L. reuteri ATCC PTA 5289 was measured before and after chewing gum containing the probiotic strain.

Probiotic bacteria

Chewing gum active product containing L. reuteri (an equal mix of ATCC PTA 5289 and ATCC 55730, at a total of 2 × 10^8 CFU per dose; BioGaia AB, Sweden) was chewed 10 min directly after dental hygiene procedures in the morning and in the evening. The placebo contained no bacteria, but was identical to the active chewing gum in terms of taste, shape, texture and composition.

Clinical trial outline

Patients were randomised in two groups; test group (n = 11) received active product and the control group (n = 12) received placebo. At baseline, resting saliva samples were collected (1 mL) from each participant in a tube (without any oral movements) and diluted 10 times by adding 8 mL 0.15% NaCl and 1 mL glycerol, mixed and frozen to −80 °C.

The study/placebo products were taken daily for 12 weeks after which the subjects were re-analysed as at baseline. After completion of the study, subjects were invited to return 4 weeks following the last intake of the product for saliva sampling to determine washout of L. reuteri ATCC PTA 5289.

Sampling was carried out systematically in the morning. Resting saliva samples were collected before and after 12 weeks of treatment and 4 weeks after end of treatment. At follow-up, saliva from one individual was not collected.

Lactobacillus reuteri reuteri in saliva

The level of L. reuteri ATCC PTA 5289 in saliva was enumerated using Man–Rogosa–Sharpe (MRS) agar plates. To confirm the identity of L. reuteri ATCC PTA 5289, a PCR method described by Magnusson et al. (2003) was employed.

Salivary IgA and protein determinations

Salivary IgA concentrations were measured by enzyme-linked immunosorbent assay (ELISA) previously described in detail by Sonesson et al. (2011). The wells of MicroWell™ plates (MaxiSorp™ surface, Nunc-Immuno™ Plate; Thermo Fisher Scientific, Denmark) were coated overnight using 100 µL alpha-chain-specific goat anti-human IgA (Sigma 10884; Sigma Chemical Co., St. Louis, MO) in a coating buffer (0.05 M carbonate-specific goat anti-human IgA (Sigma 10884; Sigma Chemical Co., St. Louis, MO) in a coating buffer (0.05 M carbonate-specific goat anti-human IgA (Sigma 10884; Sigma Chemical Co., St. Louis, MO) in a coating buffer (0.05 M carbonate-specific goat anti-human IgA (Sigma 10884; Sigma Chemical Co., St. Louis, MO) in a coating buffer (0.05 M carbonate-buffer, pH 9.6). Each well was washed three times with 200 µL 0.05% PBS Tween 20 (PBST) and blocked with 100 µL PBST containing 1% BSA (Sigma Chemical Co.) for 1 h at 37 °C. After washing, 100 µL of standards (Colostrum standard; Sigma Chemical Co.) and diluted saliva samples in PBST was added and incubated for 1 h at 37 °C.

After washing as above, 100 µL alpha-chain-specific goat anti-human IgA conjugated with alkaline phosphatase in PBST (Sigma A 9669; Sigma Chemical Co.) was added and incubated for 1 h in 37 °C. After washing, 200 µL per well of phosphatase substrate (Sigma P5869; Sigma Chemical Co.) in diethanolamine buffer (pH 9.8) was incubated for 30 min at 37 °C. The colour reaction was stopped after 30 min by adding 3 M NaOH.

Absorbance was read in a microplate reader (ELx800™; BioTek Instruments, Inc., Winooski, VT) at 405 nm. Saliva samples in duplicates were plotted against a colostrum standard curve and multiplied with the dilution factor. Results are presented as mg 100 mL⁻¹.

The total protein concentration in the saliva samples was determined using the Bio-Rad Laboratories protein assay (Richmond, CA). Levels of IgA were also expressed as percent per protein.

Specific IgA reactivity using a bacterial whole-cell ELISA

This method was developed for the one probiotic strain L. reuteri ATCC PTA 5289 that adhered well into the microtitre plates. Laboratory strains of Streptococcus...
mutans NCTC 10449 and Streptococcus sobrinus B13 were cultured in Todd-Hewitt broth (5% CO₂ in N₂) at 37 °C 18–24 h. Lactobacillus reuteri strain ATCC PTA 5289 was cultured in MRS broth for 18–24 h.

After harvesting and washing (× 3) in PBS, the optical density (OD) was set to 0.25 for mutans streptococci and for L. reuteri ATCC PTA 5289 to OD 0.50 in carbonate buffer (pH 9.6). ELISA plates (MaxiSorp, NUNC) were coated with 100 μL of the bacteria in 0.05 M carbonate buffer (pH 9.6) and incubated at +4 °C overnight. After aspiration and washing with PBST × 3 (washing step), 100 μL of blocking agent, 1% BSA in PBST, was added and plates were incubated at 37 °C for 1 h. After washing step as above, 100 μL PBST (blank) and diluted saliva (1 : 10) were added and the plate was incubated 1 h at 37 °C.

Washing step was performed as above, 100 μL of goat anti-human alpha-chain IgA conjugated with alkaline phosphatase 1 : 10 000 (Sigma A-9669) was added and incubated 1.5 h at 37 °C. After incubation, 1% BSA in PBST was added and plates were incubated at 37 °C for 1 h. After washing step as above, 100 μL of blocking agent, 1% BSA in PBST, was added and plates were incubated at 37 °C for 1 h. After washing step as above, 100 μL PBST (blank) and diluted saliva (1 : 10) were added and the plate was incubated 1 h at 37 °C.

After harvesting and washing (× 3) in PBS, the optical density (OD) was set to 0.25 for mutans streptococci and for L. reuteri ATCC PTA 5289 to OD 0.50 in carbonate buffer (pH 9.6). ELISA plates (MaxiSorp, NUNC) were coated with 100 μL of the bacteria in 0.05 M carbonate buffer (pH 9.6) and incubated at +4 °C overnight. After aspiration and washing with PBST × 3 (washing step), 100 μL of blocking agent, 1% BSA in PBST, was added and plates were incubated at 37 °C for 1 h. After washing step as above, 100 μL PBST (blank) and diluted saliva (1 : 10) were added and the plate was incubated 1 h at 37 °C.

Washing step was performed as above, 100 μL of goat anti-human alpha-chain IgA conjugated with alkaline phosphatase 1 : 10 000 (Sigma A-9669) was added and incubated 1.5 h at 37 °C. After incubation, 1% BSA in PBST was added and plates were incubated at 37 °C for 1 h. After washing step as above, 100 μL PBST (blank) and diluted saliva (1 : 10) were added and the plate was incubated 1 h at 37 °C.

Washing step was performed as above, 100 μL of goat anti-human alpha-chain IgA conjugated with alkaline phosphatase 1 : 10 000 (Sigma A-9669) was added and incubated 1.5 h at 37 °C. After incubation, 1% BSA in PBST was added and plates were incubated at 37 °C for 1 h. After washing step as above, 100 μL PBST (blank) and diluted saliva (1 : 10) were added and the plate was incubated 1 h at 37 °C.
consistently seen longitudinally within the test group only. Therefore, the conclusive interpretation must be that ingestion of probiotic bacteria has an effect on salivary IgA concentration, but not to an extent that reveals a difference between test and control groups.

The large variation among subjects and their relatively low number might explain lack of significant effect between groups. The salivary IgA levels are highly dependent on salivary secretion rate (Ericson et al., 1982). So are salivary protein levels (Wu et al., 2008). To diminish the effect of fluctuation of salivary IgA concentrations related to differences in flow rate among the subjects, the level of total and

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg mL⁻¹)</td>
<td>Post–Pre Follow-up–Pre Follow-up–Post</td>
<td>Post–Pre Follow-up–Pre Follow-up–Post</td>
</tr>
<tr>
<td>Total IgA (mg mL⁻¹)</td>
<td>3.9 (8.5) 1.0 (3.0) −2.8 (6.8)</td>
<td>1.1 (3.4) 1.9 (4.5) 1.4 (6.6)</td>
</tr>
<tr>
<td>%IgA/protein</td>
<td>−0.1 (3.8) −1.1 (4.6) −0.9 (4.8)</td>
<td>1.8 (3.5) 1.8 (2.5)† 0.6 (2.8)</td>
</tr>
<tr>
<td>L. reuteri, Abs/%IgA/protein (×1000)</td>
<td>−0.7 (3.7) −1.3 (6.4) −0.6 (4.6)</td>
<td>−3.2 (3.4)† −2.5 (3.0)† 0.8 (3.8)</td>
</tr>
<tr>
<td>10449, Abs/%IgA/protein (×1000)</td>
<td>−0.7 (4.9) 1.1 (8.8) 1.8 (9.0)</td>
<td>−5.1 (7.2)† −3.0 (4.1)† 1.5 (5.8)</td>
</tr>
<tr>
<td>B13, Abs/%IgA/protein (×1000)</td>
<td>−0.7 (2.8) −0.3 (3.4)§ 0.4 (2.2)</td>
<td>−2.1 (1.6)† −1.8 (1.5)†§ 0.2 (1.4)</td>
</tr>
</tbody>
</table>

*SD. †Statistically significant (Student’s paired t-test, \( P < 0.05 \)). ‡Statistically significant (Wilcoxon’s signed-rank test, \( P < 0.05 \)) within test group. §Statistically significant (Wilcoxon’s rank sum test, \( P < 0.05 \)) between control and test group. Bold numbers indicate statistical significant values in the test group.
specific antibodies was expressed in relation to total salivary protein level.

The results of this study indicate that ingestion of probiotic bacteria induces an increase in total IgA in saliva. This might be interpreted as an immunostimulatory effect of probiotic bacteria, which has been suggested (Kelly et al., 2005; Corthésy et al., 2007). However, such an effect has been shown only among under-nourished children (Surono et al., 2011). Such increase in salivary IgA among under-nourished, but not in normal weight children, could be an effect not relating to a specific immunostimulation by the probiotic bacteria per se, but rather an effect of normalisation of the immune system along with better nourishment status among the children, similar to what was seen among malnourished mice (Maldonado Galdeano et al., 2011).

A similar lack of effect on salivary IgA levels was reported among athletes (Cox et al., 2010). The use of probiotics to stimulate a specific response to a cholera vaccine (Painneau et al., 2008) actually resulted in an increase in the salivary IgA titre against the vaccine, but the total IgA was not measured in the study. A recent review (Maidens et al., 2013) indicates at most a modest stimulatory effect of probiotics on the IgA response, when used concomitant with vaccines. Alas, the mechanisms by which probiotics influence the immune response are poorly understood. It is, however, suggested that the adjuvant effects might be mediated by components of the cell wall—like lipoteichoic acid and polysaccharide–peptidoglycan complexes (de Vrese et al., 2005). In the current study, the total IgA levels increased while ingesting probiotic bacteria and tended to decrease to baseline values after washout, indicating an immunostimulatory effect.

This study infers that the specific immune response to all target organisms seemed to decrease. The decrease in L. reuteri ATCC PTA 5289-specific IgA antibodies in collected saliva among the test subjects during the treatment phase might be attributed to antibody absorption onto bacteria in saliva during the collection procedure. Thus, the numerous (a dose 10^8 bacteria twice daily) probiotic bacteria present in saliva could act as specific immunosorbents covered with specific antibodies that eventually were spun down, during centrifugation of saliva samples. The antibodies would then be present in lower concentration in the supernatant that was analysed. Given a certain cross-reactivity with the tested mutants streptococcal strains (i.e. due common surface antigens), an adsorption onto L. reuteri ATCC PTA 5289 in saliva could also explain the decrease in the salivary-specific immune response towards the mutants streptococci. One would, however, anticipate that the measured specific IgA reactivity would return quickly to pretreatment levels once the L. reuteri ATCC PTA 5289 strains disappeared from the oral cavity. This was not seen, but merely suggested by a trend of the specific IgA response to return to baseline values (Fig. 4). It is also conceivable that the probiotic strains induced a cross-tolerance towards the mutants streptococci (Edelman & Kasper, 2008) indicated by that the specific IgA responses to all strains followed the same trend (Figs 4–6).

The presence of anti-L. reuteri ATCC PTA 5289 IgA in saliva in baseline saliva samples in both test and control group could be due to presence of cross-reactive antibodies or to previous exposure to the test bacteria as 3 of 23 individuals harboured L. reuteri from start. A possible cross-reactivity with other common oral bacteria is plausible, as the reactivity towards S. mutans and S. sobrinus followed the pattern of that against the probiotic strain L. reuteri ATCC PTA 5289.

In conclusion, the results from this article indicate that ingestion of probiotic bacteria might influence the immune response of the host. Further investigations are necessary to verify and understand the mechanisms on the effect of probiotics bacteria on salivary immunology.

Acknowledgements

We thank Jayanthi Stjernswärd for language revision and Mikael Aström for statistical consultation.

References


