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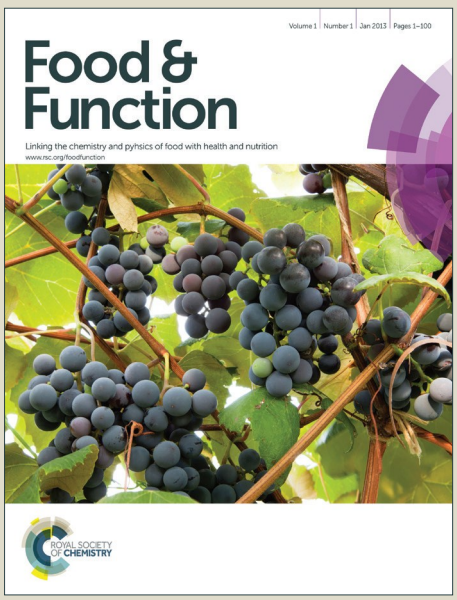

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Title: Supplementation of xylitol-containing chewing gum with probiotics: a double blind, randomised pilot study focusing on saliva flow and saliva properties

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All authors disclose any conflict of interest.

Abstract

The aim of this study was to investigate the impact of daily chewing, for 12 weeks, of 2 different probiotic gums compared with placebo, on saliva flow rate, saliva IgA levels and pH. The intervention study included 54 adult volunteers with hyposalivation in a double-blind, randomised and placebo-controlled design with three parallel groups. Volunteers were randomly assigned to 3 different groups: A (n=19) were given placebo chewing gum, B (n=17) received *Bifidobacterium animalis* ssp. *lactis* Bb12 (ATCC 27536) and C (n=18) *Lactobacillus rhamnosus* LGG (ATCC 53103), plus *Bifidobacterium longum* 46 (DSM 14583) and *Bifidobacterium longum* 2C (DSM 14579) gums, during 3 months. Two volunteers from group B left the study for personal reasons leaving 19, 15 and 18 volunteers, respectively, for analyses. Clinical examinations, personal interviews, sialometries and saliva sampling were conducted at baseline and after 1, 2, 3 and 4 months. No statistically significant differences were found between probiotic and placebo groups for any of the parameters analysed. No side effects of probiotic or placebo chewing gums were observed. Chewing gum, with and without probiotics, had a positive impact on salivary flow rate and saliva pH and IgA levels.

Key words: probiotics, xylitol, salivation, *Bifidobacterium*, *Lactobacillus, chewing-gum*.

Introduction

The World Health Organization defines probiotic bacteria as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host.[1] The most commonly used probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium*,[2-4] genera that are commonly found in the oral cavity.[5] In addition, specific probiotics may have direct antimicrobial effects mediated through production of organic acids, biosurfactans and other antimicrobial substances.[6,7] The strongest evidence for the clinical effectiveness of probiotics has been in their use for prevention and treatment of acute gastroenteritis and viral diarrhoea[8] and alleviation of symptoms of lactose intolerance.[9]

With regard to oral applications some studies have reported on the use of specific probiotic strains to reduce the colonization by oral pathogens and to balance oral microbiota.[10-14] Most of the studies on potential oral probiotics have focused on caries prevention, specifically in the reduction of *Streptococcus mutans* levels.[15-20] Some studies also suggest that *Lactobacillus* strains may be useful in reducing gingival inflammation and the number of black-pigmented rods as *Porphyromonas gingivalis* in saliva and subgingival plaque.[21-23] Probiotic bacteria have also been shown to affect the composition of salivary pellicle and streptococcal adhesion *in vitro* and reduce the prevalence of oral Candida in the elderly[24-27] and to increase salivary immunoglobulin A (sIgA) secretion contributing in the improvement of mucosal immunity and resistance against infection.[28,29] Moreover, probiotics have also been shown to inhibit the production of volatile sulphur compounds, which have been identified as causes of halitosis.[30]

Reduced salivary flow and feeling of dry mouth is a significant problem associated with caries risk, periodontal diseases and mucosal susceptibility to injury.[31] A decreased salivary secretion may also be uncomfortable because it is usually accompanied by difficulties during speaking and food swallowing, unpleasant taste and/or burning sensation in the mouth. It may also increase the susceptibility to caries, and may indirectly favour mucosal infections.[32] Furthermore, a previously published study suggested an increased salivary flow rate after consumption of a probiotic-containing cheese,[26] although other authors did not observed any effect on salivary flow on a tablet containing probiotics.[14] However, in these studies salivary flow was not the primary outcome and the delivery matrixes (cheese and tablets) may not be the better suited vehicles for improving salivation. Moreover, these studies did not aim at increasing salivary flow in persons suffering hyposalivation and the potential impact of probiotics in this population has not been explored. Thus, our hypothesis was that chewing gum containing specific probiotics may benefit oral health by increasing salivary flow rate and by modulating mucosal immunity.

The aim of the present study was to investigate whether chewing specific probiotic-containing gums, twice per day for 12 weeks, would affect saliva flow rate, saliva IgA levels and/or saliva pH in hyposalivating adults. In addition, the effect on common hyposalivation symptoms, including dry mouth sensation, burning sensation, swallowing difficulty, speaking difficulty, chewing difficulty, changes in voice, alterations of taste, halitosis or dry lips, was assessed by means of a validated questionnaire. To this end two different probiotic xylitol-containing chewing gums were tested. The first one contained *Bifidobacterium animalis* ssp. *lactis* Bb12, one of the most widely used probiotic bifidobacterial strains available in the market, with good stability in different food matrixes and different beneficial health effects.[33] The second

probiotic gum was supplemented with a mix of strains containing *Lactobacillus rhamnosus* GG, a strain with reported properties on the prevention of gastrointestinal inflections and tooth decay in children,[19,34] and *Bifidobacterium longum* strains 2C and 46, selected on the basis of their ability to stabilise gut function and gut barrier in adults and elderly.[35]

Material and methods

Volunteers

Between March and August 2010 personal interviews and sialometries were conducted to a total of 167 healthy adult volunteers assisting for a dental check-up to the Dentistry School of the University of Oviedo (Asturias, Spain). Inclusion criteria were: I) basal salivary flow rate 0.25 ml/min or less, II) low stimulated salivary flow rate 0.8ml/min or less. Exclusion criteria were: I) pregnancy, II) history of antibiotics within 30 days prior to baseline examination, III) probiotics consumption within 30 days prior to baseline examination, IV) lactation, V) alcohol abuse or drug abuse, VI) being participating in another clinical study or unwillingness to follow the study protocol. 54 volunteers fulfilled these criteria and were recruited for the intervention study. Hyposalivation in these volunteers was confirmed with three independent sialometries before the intervention phase of the study. Gender, age, and smoking status were recorded for each subject at these initial interviews: mean age of 49.75 years old, 8 men/46 women, 9 smokers/4 social-smokers/41 non-smokers (ex-smokers included).

The study was ethically approved by the Regional Committee on Clinical Research of the Asturias Region (Study nº81/10). All volunteers gave written informed consent to participate in the study.

Study design

This pilot prospective study was a double-blind, placebo-controlled, multi-arm parallel randomized trial with three experimental groups over a 4 months period. Volunteers were randomly assigned to one of the study groups with the aid of a computer-generated randomization table (Figure 1). Subjects in group A (n=19) were given placebo chewing gum during 3 months, group B (n=17) received chewing gum with *B. animalis* ssp. *lactis* Bb12 (ATCC 27536) at a dose of 2.87(+/-0.03) x 10^8 CFU/2 gums and group C (n=18) chewing-gum containing equal amounts of *L. rhamnosus* LGG (ATCC 53103), *B. longum* 46 (DSM 14583), and *B longum* 2C (DSM 14579), at a total dose of 3.35 (+/-0.06) x 10^8 CFU/2 gums.

All chewing gums contained xylitol (64%), chewing gum mass (32%) and spear mint aroma, talcum powder, lecithin and glycerol (4%). The gums were specially made for the study by Fazer Oy (Finland). The placebo chewing gum was identical in size and composition but without the addition of probiotic strains. The gums were packed in identical white plastic pots with snap-cap coded as A, B or C and each participant was given one pot containing 64 gums, every intervention month. The participants were instructed to chew one chewing gum pastille for 30 minutes twice daily, in the morning and in the evening. The subjects were told to continue with their conventional lifestyle, diet and oral hygiene but not to consume any probiotic/prebiotic products during the study period.

Follow-up registrations, sialiometries (primary outcome), interviews and samplings were conducted at baseline and after 1, 2, 3 and 4 months. Any changes in health and any use of prescription or over-the-counter medicines were reported each month, also the amount of chewing gums left in each month pot was recorded as a compliance

measure. Group assignment codes were not broken until the final completion and analysis of the study data.

Clinical examinations and saliva sampling

The oral and dental status of the participants was examined in each visit by the same dentist. The number of cavitated carious lesions, presence of periodontal disease or gingivitis and presence of candidiasis were recorded. Information on each subject's health and medication was also recorded. At each visit a questionnaire was made to the volunteer about symptoms related with low saliva flow rate (dry mouth feeling, oral burning feeling, chewing difficulty, swallowing difficulty, speaking difficulty, changes in voice, taste alterations, halitosis, dry lips, dry extra-oral musosas). Each symptom was classified in the questionnaire's scale as 0 (no), 1 (rarely), 2(sometimes), 3(frequently) and 4 (almost always). Sialometry was done each visit in order to know saliva flow rate. Unstimulated saliva was collected and measured by drainage technique.[36] In brief, the participant placed a 100 ml sterile glass resting slightly on the lower lip letting the saliva flow into the recipient during 5 minutes, after which the accumulated saliva in the mouth should be spitted into the sterile container. The subject position was sitting with the head slightly tilted forward in order to help the saliva fall into the recipient. The quantity of saliva was measured immediately and frozen at -20 C. After resting 5 minutes stimulated saliva production was measured, to this end stimulation was done soaking a cotton stick in fresh lemon (citric acid) and placing it in the papillae mouths of the major salivary glands. The salivary flow rate was calculated as ml of saliva per minute. Patients were asked not to consume any food or drink 2 h before the saliva collection.

Saliva analyses

The unstimulated saliva samples were melted and transferred to a new sterile 2ml tubes, centrifuged for 10 minutes at 14000 rpm to pellet cells and 60µl of the supernatant were taken for IgA analysis (Salivary secretory IgA indirect enzyme immunoassay kit, Salimetrics, State College, PA, USA). The rest of the supernatant was used for pH measurement (Meter Toledo Seven Easy TM pH meter S20) and the pellet was resuspended in 200 µL PBS and at -70 C until further analyses. sIgA and pH determinations were performed at baseline and the end of intervention.

Bacterial DNA of the saliva samples was extracted by using the "ZR Fungal/Bacterial DNA MiniprepTM" (Zimo research Corporation, Orange, CA, USA). PCR assays for *L. rhamnosus GG* with specific primers for this strain and *B. longum* and *B. lactis* with species-specific primers for these species were carried out as previously described.[2,37] All reactions were performed on MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems, Foster City,CA) in a 7300 Real Time PCR System (Applied Biosystems) using the SYBR Green Master Mix (Applied Biosystems). Standard curves were made with the known weight of genomic DNA purified from pure cultures of the strains which were grown overnight in GAM (Nissui Pharmaceuticals, Tokyo, Japan) medium under anaerobic conditions. Samples were analysed by duplicate in at least two independent PCR runs. Results were expressed as positive or negative according to the presence or absence of each specific microorganism.

Viability of probiotics in chewing gums was followed during nine months of storage time. At each sample two gums were homogenized in 15ml cold phosphate buffered saline (PBS; pH 7.2) by Ultrarrax® T25 (IKA-Werke GmbH, Staufen, Germany) homogenizer. Samples were diluted in PBS and plated on GAM agar (Nissui) and bacteria were enumerated after two days anaerobic incubation at 37ºC.

Statistical analyses

One way ANOVA was used to assess differences between groups at baseline. All other analyses were carried out using mixed model repeated measures analyses. Comparisons among experimental groups were carried out by using time, group and time-group interaction as independent variables. The Pearson's chi-squared test was used for assessing statistical differences among groups for the categorical variables obtained in the symptoms' questionnaire. To evaluate the effect of storage upon sIgA concentration sampling time, storage time and sampling time-storage time were used as independent variables. SAS for windows version 19.0 (SPSS, Inc., Chicago, IL, USA).

Results

Volunteers drop-outs and compliance

54 out of the 167 volunteers (32.3%) presented hyposalivation and were recruited for the intervention phase of the study. Two of the volunteers were withdrawn from the study, one subject became seriously ill and the other one left the study due to family problems. Therefore the drop-out rate was 3.7%. No significant differences between groups were found in relation with chewing gum intake during the intervention period. However, significant differences were found when comparing the intakes in the three different intervention months, in the 3rd and last month of intervention the volunteers reduced the chewing gum consumption significantly (9.93 gums less) in comparison with the two previous months (data not shown).

To further assess the adequacy of the daily dose of probiotics the stability of the bacteria in chewing gum during storage time was assessed (Figure 2). Gum B was very stable during storage; after 4 months storage the number of probiotics decreased by only 0.19

log_{10} units and after 9 months of storage by 0.45 log_{10} units when compared to the freshly prepared gum. Gum C was stable for about 4 months, at that time there was decrease of 0.45 log_{10} units, but in later time-points (7.5 and 9 months) the decrease reached 1.65 log_{10} units. However, the major part of the participants finished chewing gums intake within 4 months storage time, when the number of respective probiotics still showed a good stability.

Salivary flow rate

The unstimulated (basal) and stimulated flow rates in relation with the intervention group are presented in Figure 3. Statistically significant differences were not found among the study groups at any time point. The values of stimulated flow rate were significantly higher ($p<0.05$) in group A than in group C at all-time points, including baseline (Figure 3B). However differences between group A and B were not statistically significant nor were the differences between group B and C. These differences remained the same all through the study.

The lack of statistically significant differences among the intervention groups allowed us to assess the effect of chewing gum in the overall population by combining the results of each group. Gum chewing increased unstimulated (basal) saliva flow rate (Figure 4); this increase was progressively becoming significant ($p<0.05$) after 2 months of intervention (time point 3), continuing being significant at time points 4 (after 3 months of intervention) and 5 (after one month wash-out) when compared with baseline.

Salivary pH and IgA

No significant differences in salivary pH were found between groups in any time point analysed (baseline and end of intervention). However, in the three groups there was a significant ($p<0.05$) increase of salivary pH at the end of intervention when compared to baseline (Figure5). With regard to sIgA at the end of intervention the levels were significantly higher ($p<0.05$) that at baseline for the three groups, without observing any significant differences among groups at any time point analysed (Figure 6).

Microbiological analysis

No significant differences neither at baseline nor at time point 4 between groups were found for any of the *Bifidobacterium* species and no differences were detected when time point 4 was compared with baseline. With regard to *L. rhamnosus* GG there were significant differences at time point 4, where the occurrence of *L. rhamnosus* GG positive samples was significantly higher in group C (61.1% of positive samples for LGG) than in groups A and B (15.79% and 26.67% of samples positive, respectively).

Subjective symptoms (questionnaires)

The evaluated symptoms were dry mouth sensation, oral burning sensation, chewing difficulty, swallowing difficulty, speaking difficulty, changes in voice, alteration of taste, halitosis, dry lips and dry extra-oral mucosa (nose, eyes). No significant differences among groups in any time point in any variable were found, but chewing gum, either probiotic or placebo, alleviated dry mouth sensation, this symptom occurring more often at baseline than in later sampling points. The difference was statistically significant ($p< 0.05$) already at time point 2 (1 month of intervention) and the sensation decreasing month by month, from 54.9% of the volunteers suffering it at baseline frequently or almost always, to 15.39% of the volunteers suffering it after 3

months of intervention (time point 4). Dry lips occurred significantly more often at the first three measurements (32.7% at baseline, 34.6% and 19.2% after 1 and 2 months of intervention, respectively) than at the last two (11.5% at both times; 3 months of intervention and end of follow-up). The other symptoms analysed were very rare even at baseline which prevented the use of statistical analyses.

Adverse effects

One volunteer complained about having more gastrointestinal gases accumulation during the chewing period, presumably due to aerophagia while chewing. No other adverse effects were reported.

Discussion

We report the impact of daily chewing during 12 weeks of probiotic gums compared with placebo, on saliva flow rate, saliva IgA levels and saliva pH in patients with hyposalivation. In general, salivary flow was increased during the ingestion of all chewing gum preparations.

Reduced salivary flow constitutes an important risk for oral health which is present more often in women than in men and it is further reduced with ageing.[36] Decline of salivary flow makes oral soft tissues more susceptible to drying, de-epithelization and insults from the environment, facilitates colonization by opportunistic microbes, and, therefore, it promotes inflammation of the mucous membranes (mucositis), the presence of painful ulcers, infection (candidiasis), hyperesthesia, angular cheilitis and burning mouth sensation. Consequently hyposalivation causes difficulties at the time of eating, talking, using prosthesis and sleep affecting the general quality of life.[38] When salivary

flow decreases, also the function of clearing that the saliva normally holds is compromised favouring the accumulation of dental plaque and food deposits, which is associated with an increase of caries and periodontal problems.[31] Thus, there is a keen interest on developing solutions which increase salivary flow in such subjects.

Our results support that gum-chewing, with and without probiotics, has a positive impact on salivary flow rate, saliva pH and IgA levels. Apart from the flavour of a chewing gum that may lead to a gustatory stimulation of saliva, mastication is generally agreed to be the main reason for saliva stimulation during gum-chewing.[39] A potential benefit of probiotic supplementation of chewing-gum has been previously reported.[23] A study carried out in 2007 suggested that unstimulated salivary flow rate increased with the administration of probiotic cheese, reducing the risk of hyposalivation in elderly subjects.[26] On the contrary a recent study reported a lack of effect in healthy adults' salivary flow of *Lactobacillus salivarius*-containing tablets.[14] Similarly we did not observe any statistically significant improvement in hyposalivating subjects following supplementation of the gum with probiotics, as the probiotic and placebo groups did not differ for any of the parameters analysed. Therefore, all our chewing gums improved hyposalivation and related symptoms, which is in agreement with previous reports on chewing gum,[40] without observing statistically significant differences among the experimental groups. The fact that all of the chewing gums contained xylitol could be responsible of not having observed statistically significant differences between groups, as the placebo was not completely inert, one more group of non-xylitol chewing gums could have given interesting data. In addition, we provided data on the oral colonization by the probiotic strains used. The presence of the probiotics was not observed prior to the study but during probiotic gum intake the presence was verified in saliva. Probiotic bacteria are known to inhibit the growth of many pathogenic microbes and therefore,

further studies are needed to assess the impact of probiotic chewing gum on oral pathogens and caries associated bacteria.

The results of the present work agree with other studies about xylitol-chewing gums, observing that chewing them (if they have no sugar added) have positive effects in oral health.[41] There are some studies concluding that gum-chewing per se is a potent stimulant of salivary flow rate and it could increase unstimulated saliva flow rate decreasing thereby the subjective symptom of dry mouth sensation.[42-45] These findings, however, are not in agreement with those of other reports that showed no effect of sorbitol and xylitol chewing gums on salivary flow rates.[46,47] The reason for such conflicting results is not known, but might be related to the duration of chewing or the portion size of the gums used. Sugar-free and sugar-containing chewing gums were shown to stimulate salivary flow rate equally well.[48] However, sugar is not advisable because of caries risk. It is generally accepted that saliva has a major effect in controlling plaque pH, and that stimulation of saliva by foods affects their acidogenic potential.[49] The chewing of sugar-free gums after meals and snacks can promote remineralization of enamel.[50]

Conclusions

In conclusion, while xylitol chewing gum seems to be beneficial by increasing salivation, IgA levels and pH and reducing symptoms associated with hyposalivation, the addition of the probiotic strains included in this study did not provide any additional benefit. However, the effect of probiotics on dental pathogens should be further associated.

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References

1 Food and Agriculture Organization of the United Nations/World Health Organization (FAO-WHO). Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO working group on drafting Guidelines for the evaluation of probiotics in food, 2002, Available at: ftp://ftp.fao.org/es/esn/food/wgreport2.pdf.

2 K. Brandt and T. Alatossava. Specific identification of certain probiotic *Lactobacillus rhamnosus* strains with PCR primers based on phage-related sequences. *International Journal of Food Microbiology,* 2003, **84**, 189-196.

3 A. C. Ouwehand, S. Salminen and E. Isolauri. Probiotics: an overview of beneficial effects, *Antonie Van Leeuwenhoek*, 2002, **82**, 279-289.

4 M. Saxelin, S. Tynkkynen, T. Mattila-Sandholm and W. M. De Vos. Probiotic and other functional microbes: from markets to mechanisms, *Current Opinion in Biotechnology*, 2005, **16**, 204-211.

5 K. L. Chhour, M. A. Nadkarni, R. Byun and F. E. Martin. Molecular analysis of microbial diversity in advanced caries, *Journal of Clinical Microbiology*, 2005, **43**, 843-849.

6 M. M. Velraeds, H. C. van der Mei, G. Reid and H. J. Busscher. Inhibition of initial adhesion of uro pathogenic *Enterococcus faecalis* by biosurfactans from *Lactobacillus* isolates, *Applied and Environmental Microbiology*, 1996, **62**, 1958-1963.

7 S. Hudault, V. Lievin, M. F. Bernet- Camard and A. L. Servin. Antagonistic activity exerted in vitro and in vivo by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium* C5 infection, *Applied and Environmental Microbiology*, 1997, **63**, 513-518.

8 S. Guandalini, L. Pensabene, M. A. Zikri, J. A. Dias, L. G. Casali, H. Hoekstra, S. Kolacek, K. Massar, D. Micetic-Turk, A. Papadopoulou, J. S. de Sousa, B. Sandhu, H. Szajewska and Z. Weizman. *Lactobacillus* GG administered in oral rehydration solution to children with acute diarrhea: a multicenter European trial, *Journal of Pediatric Gastroenterology and Nutrition*, 2000, **30**, 54-60.

9 V. Ojetti, G. Gigante, M. Gabrielli, M. E. Ainora, A. Mannocci, E. C. Lauritano, G. Gasbarrini and A. Gasbarrini. The effect of oral supplementation with *Lactobacillus reuteri* or tilactase in lactose intolerant patients: randomized trial, *European Review for Medical and Pharmacological Sciences*, 2010, **14**, 163-170.

10 J. H. Meurman, H. Antila and S. Salminen. Recovery of *Lactobacillus* strain GG (ATCC 53103) from saliva of healthy volunteers after consumption of yoghurt prepared with the bacterium, *Microbial Ecology on Health and Disease*, 1994, **7**, 295-298.

11 H. J. Busscher, A. F. Mulder and C. H. Van der Mei. *In vitro* adhesion to enamel and in vivo colonization of tooth surfaces by lactobacilli from a bio-yoghurt, *Caries Research*, 1999, **33**, 403-404.

12 A. J. Ahola, H. Yli-Knuuttila, T. Suomalainen, T Poussa, A. Ahlström, J. H. Meurman, and R. Korpela, R. Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors, *Archives of Oral Biology*, 2002, **47**, 799-804

13 W. Teughels, G. Loozen and M. Quirynen. Do probiotics offer opportunities to manipulate the periodontal oral microbiota? *Journal of Clinical Periodontology*, 2011, **38**, 159-177.

14 T. Nishihara, N. Suzuki, M. Yoneda and T. Hirofuji. Effects of *Lactobacillus salivarius*-containing tablets on caries risk factors: a randomized open-label clinical trial, *BMC Oral Health*, 2014, **14**, 110.

15 E. Caglar, N. Sandalli, S. Twetman, S. Kavaloglu, S. Ergeneli and S. Selvi, S. Effect of yoghurt with *Bifidobacterium* DN-173 010 on salivary mutans streptococci and lactobacilli in young adults, *Acta Odontologica Scandinavica*, 2005, **63**, 317-320.

16 E. Caglar, S. K. Cildir, S. Ergeneli, N. Sandalli and S. L. Twetman. Salivary mutans streptococci and lactobacilli level after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets, *Acta Odontologica Scandinavica*, 2006, **64**, 314-318.

17 E. Caglar, S. C. Kavaloglu, O. O. Kusku, N. Sandalli, P. L. Holgerson and S. Twetman, S. Effect of chewing gums containing xylitol or probiotic bacteria on salivary mutans streptococci and lactobacilli, *Clinical Oral Investigations*, 2007, **11**, 425-429.

18 M. K. Keller, P. Hasslöf, G. Dahlén, C. Stecksén-Blicks and S. Twetman. Probiotic supplements (*Lactobacillus reuteri* DSM 17938 and ATCC PTA 5289) do not affect regrowth of mutans streptococci after full-mouth disinfection with chlorhexidine: a randomized controlled multicenter trial, *Caries Research*, 2012, **46**, 140-146.

19 L. Näse, K. Hatakka, E. Savilahti, M. Saxelin, A. Pönkä, T. Poussa, R. Korpela and J. H. Meurman. Effect of long-term consumption of a probiotic bacterium *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children, *Caries Research*, 2001, **35**, 412-420.

20 H. Nikawa, S. Makihira, H. Fukushima, H. Nashimura, Y. Ozaki, K. Ishida, S. Darmawan, T. Hamada, K. Hara, A. Matsumoto, T. Takemoto and R. Aimi. *Lactobacillus reuteri* in bovine milk fermented decreases the oral carriage of mutans streptococci, *International Journal of Food Microbiology*, 2004, **95**, 219-223.

21 H. Ishikawa, Y. Aiba, M. Nakanishi, Y. Ohhashi and Y. Koga. Supression of periodontal pathogenic bacteria in the saliva of humans by the administration of *Lactobacillus salivarius* TI 2711, *Journal of the Japanese society of Periodontology*, 2003, **45**, 105-112.

22 H. Shimauchi, G. Mayanagi, M. Minamibuchi, Y. Ito, K. Yamaki and H. Hirata. Improvement of periodontal condition by probiotics with *Lactobacillus salivarius* WB21: a randomized, double-blind, placebo-controlled study, *Journal of Clinical Periodontology*, 2008, **35**, 897-905.

23 S. Twetman, B. Derawi, M. Keller, K. Ekstrand, T. Yucel-Lindberg and C. Stecksen-Blicks. Short-term effect of chewing gums containing probiotic *Lactobacillus reuteri* on the levels of inflammatory mediators in gingival crevicular fluid, *Acta Odontologica Scandinavica,* 2009, **67**, 19-24.

24 S. Elahi, G. Pang, A. Claney, and R. Claney. Enhanced clearance of Candida albicans from the oral cavities of mice following oral administration of *Lactobacillus acidophilus, Clinical and Experimental Immunology*, 2005, **141**, 29-36.

25 A. Haukioja, H. Yli-Knuuttila, V. Loimaranta, K. Kari, A. C. Ouwehand, J. H. Meurman and J. Tenovuo. Oral adhesion and survival of probiotic and other lactobacilli and bifidobacteria *in vitro*, *Oral Microbiology and Immunology*, 2006, **21**, 326-332.

26 K. Hatakka, A. J. Ahola, H. Yli-Knuuttila, M. Richardson, T. Poussa, J H. Meurman and R. Korpela. Probiotics reduce the prevalence of oral *Candida* in the elderly- a randomized controlled trial, *Journal of Dental Research*, 2007, **86**, 125-130.

27 A. Haukioja, V. Loimaranta and J. Tenovuo. Probiotic bacteria affect the composition of salivary pellicle and streptococcal adhesion *in vitro*, *Oral Microbiology and Immunology*, 2008, **23**, 336-343.

28 P. Brandtzaeg. Synthesis and secretion of secretory immunoglobulins: with special reference to dental diseases, *Journal of Dental Research*, 1976, **55**, C102–114.

29 Y. Kotani, S. Shinkai, H. Okamatsu, M. Toba, K. Ogawa, H. Yoshida, T. Fukaya, Y. Fujiwara, P. H. M. Chaves, K. Kakumoto and N. Kohda. Oral intake of *Lactobacillus pentosus* strain b240 accelerates salivary immunoglobulin A secretion in the elderly: a randomized, placebo-controlled, double-blind trial, *Immunity and Ageing*, 2010, **7**, 11.

30 M. S. Kang, B. G. Kimm, H. C. Lee and J. S. Oh. Inhibitory effect of *Weissella cibaria* isolates on the production of volatile sulphur compounds, *Journal of Clinical Periodontology*, 2006, **33**, 226-232.

31 I. Mandel. Impact of saliva on dental caries, *Compendium of Continuing Education in Dentistry*, 1989, **13**, 476-481.

32 S. L. Bahn. Drug-related dental destruction, *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 1972, **33**, 49–54.

33 C. Hoppe and C. Nexmann Karsen, in *Handbook of probiotics and prebiotics*, ed. YK Lee and S. Salminen, John Willey & Sons, Inc. New Jersey, USA, 2nd edition, 2009, Chapter 6.12 *Bifidobacterium animalis* ssp. *lactis* BB-12, pages 480-485.

34 M. Saxelin and K. Kajander, in *Handbook of probiotics and prebiotics*, ed. YK Lee and S. Salminen, John Willey & Sons, Inc. New Jersey, USA, 2nd edition, 2009, Chapter 6.8 *Lactobacillus rhamnosus GG*, pages 469-470.

35 S. Lahtinen and S. Salminen, in *Handbook of probiotics and prebiotics*, ed. YK Lee and S. Salminen, John Willey & Sons, Inc. New Jersey, USA, 2nd edition, 2009, Chapter 6.15 *Bifidobacterium longum* strains BL46 and BL2C- Probiotics for adults and ageing consumers, pages 492-494.

36 J. Aitken Saavedra, A. Maturana Ramírez, I. Morales Bozo, M. Hernández Rías and G. Rojas-Alcayaga. Study of reliability of the sialometry test for non-stimulated flow in clinically healthy adult individuals. *Revista Clínica de Periodoncia, Implantología y Rehabilitación Oral,* 2013, **6,** 25-28.

37 M. M. Rinne, M. Gueimonde, M. Kalliomäki, U. Hoppu, S. J. Salminen and E. Isolauri. Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding in infant gut microbiota, *FEMS Immunology and Medical Microbiology*, 2005, **43**, 59-65

38 I. Valdez and P. Fox. Diagnosis and managment of salivary dysfunction, *Oral Biology and Medicine*, 1993, **4**, 271-277.

39 F. Odusola. Chewing gum as aid in treatment of hyposalivation, *NY State Dentistry Journal*, 1991, **57**, 28-31.

40 H. Olsson, C. –J. Spak and T. Axell. The effect of a chewing gum on salivary secretion, oral mucosal friction, and the feeling of dry mouth in xerostomic patients, *Acta Odontologica Scandinavica*, 1991, **49**, 273-279.

41 European Food Safety Agency (EFSA). Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies on a request from Sunstar Suisse, S.A. on the scientific substantiation of a health claim related to Gum Periobalance™ tablets and

chewing gum and oral health pursuant to Article 13(5) of Regulation (EC) No 1924/2006, *The EFSA Journal*, 2009, **1178**, 1-8

42 G. N. Jenkins and W. M. Edgar. The effect of daily gum-chewing on salivary flow rates in man, *Journal of Dental Research*, 1989, **68**, 786-790.

43 M. W. J. Dodds, S. C. Hsieh and D. A. Johnson. The effect of increased mastication by daily gum-chewing on salivary gland output and dental plaque acidogenicity, *Journal of Dental Research*, 1991, **70**, 1474-1478.

44 C. Dawes. The unstimulated salivary flow rate after prolonged gum chewing, *Archives of Oral Biology*, 2005, **50**, 561-563.

45 X. P. Wang, B. Zhong, Z. K. Chen, M. E. Stewart, C. Zhang, K. Zhang, J. Ni, M. W. Dodds, A. B. Hanley and L. E. Miller. History of frequent gum chewing is associated with higher unstimulated salivary flow rate and lower caries severity in healthy Chinese adults, *Caries Research*, 2012, **46**, 513-518.

46 E. Söderling, K. K. Mäkinen, C. –Y. Chen, H. R. Jr. Pape and P. L. Mäkinen. Effects of sorbitol, xylitol, and xylitol/sorbitol chewing gums on dental plaque, *Caries Research*, 1989, **23**, 378-384.

47 O. Aguirre-Zero, D. T. Zero and H. M. Proskin. Effect of chewing xylitol chewing gum on salivary flow rate and acidogenic potential of dental plaque, *Caries Research*, 1993, **27**, 55-59.

48 C. Dawes and C. Dong. The flow rate and electrolyte composition of whole saliva elicited by the use of sucrose-containing and sugar-free chewing gums, *Archives of Oral Biology*, 1995, **40**, 699-705.

49 W. M. Edgar and S. M. Higham. In *Saliva and Oral health*, ed. W. M. Edgar and D. M. O´Mullane, British Dental Journal Publications, London (UK), Second edition, 1996, Saliva and the control of plaque pH, 81-94.

50 R. H. Manning, W. M. Edgar. Salivary stimulation by chewing gum and its role in the remineralization of caries-like lesions in human enamel in situ, *Journal of Clinical Dentistry*, 1992, **3**, 71-74.

Figure 1. Study design.

Figure 2. Number of bacteria in chewing gums during storage; filled triangle sample B (gum with *B. lactis* Bb-12) and open triangle sample C (gum with *L. rhamnosus* GG, *B. longum* 2C and *B. longum* 46).

Figure 3. Basal (A) and stimulated (B) salivary flow rates obtained for the different intervention groups. Black columns; Group A (placebo), grey columns; group B (gum with *B. lactis* Bb-12), white columns; group C (gum with *L. rhamnosus* GG, *B. longum* 2C and *B. longum* 46). * Indicate statistically significant differences between groups a and C.

Figure 4. Effect of chewing gum on salivary flow rate. * Indicates statistically significant differences when compared with baseline.

Figure 5. pH levels in the three experimental groups at baseline (the day before beginning the chewing-gum intervention) and at the end of intervention (the day after the end of the 3-months intervention phase). * Indicates statistically significant differences between end of intervention and baseline.

Figure 6. Saliva sIgA levels in the three experimental groups at baseline (the day before beginning the chewing-gum intervention) and at the end of intervention (the day after the end of the 3-months intervention phase). * Indicates statistically significant differences between end of intervention and baseline.

Figure 1. Flowchart of the subjects in the study

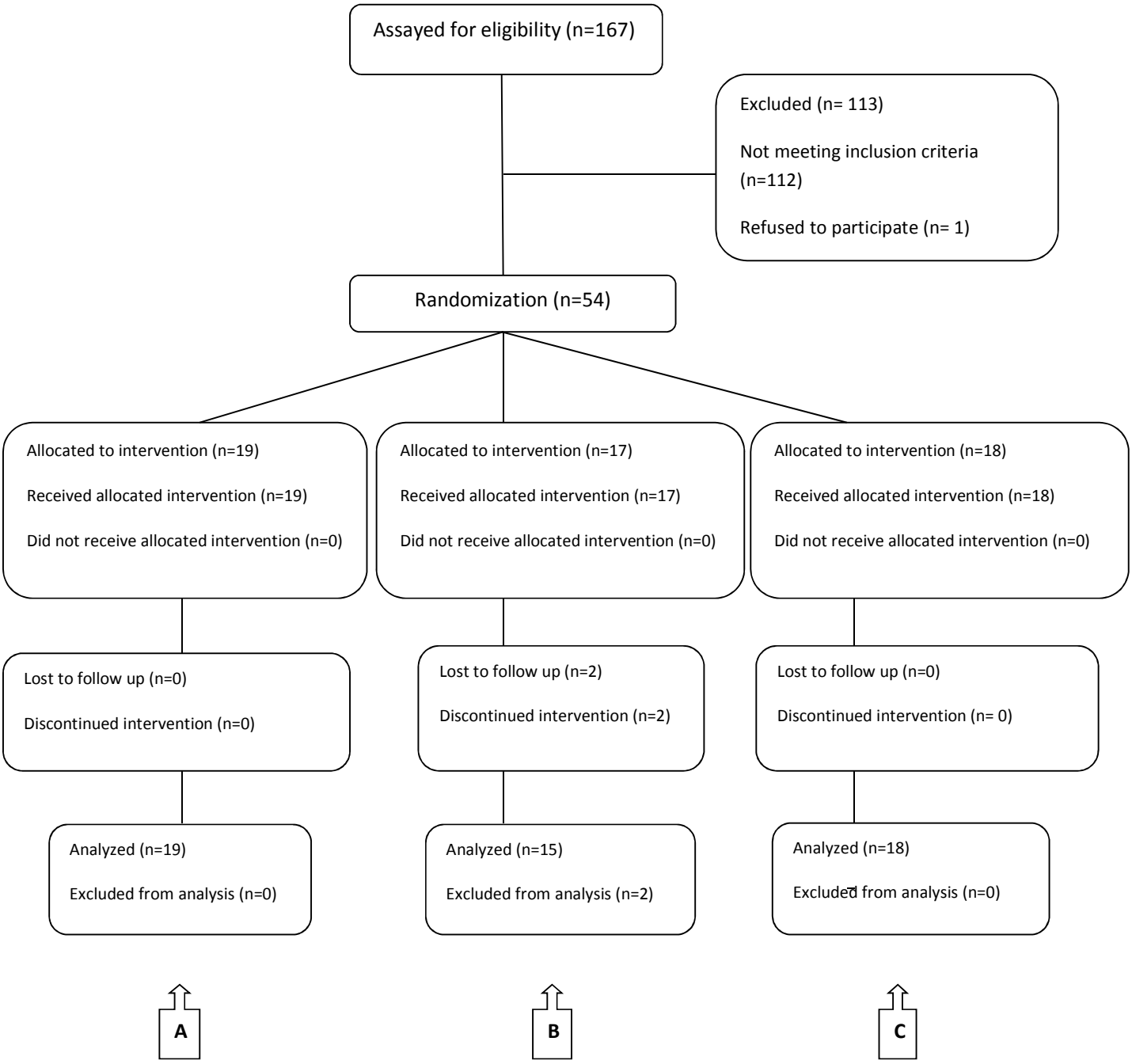

Figure 2

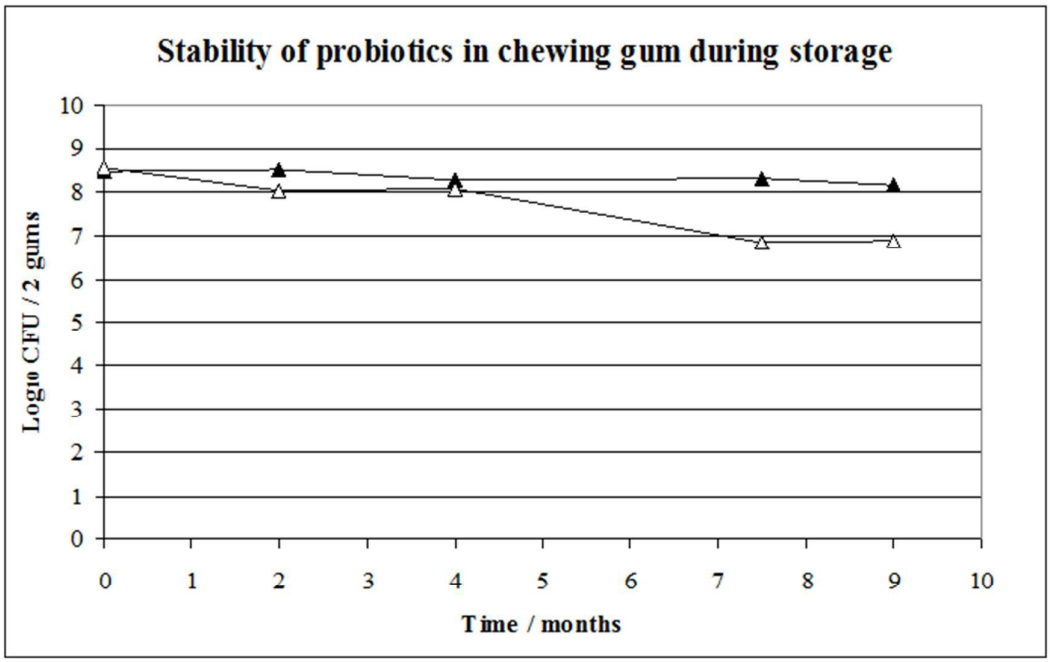

Figure 3

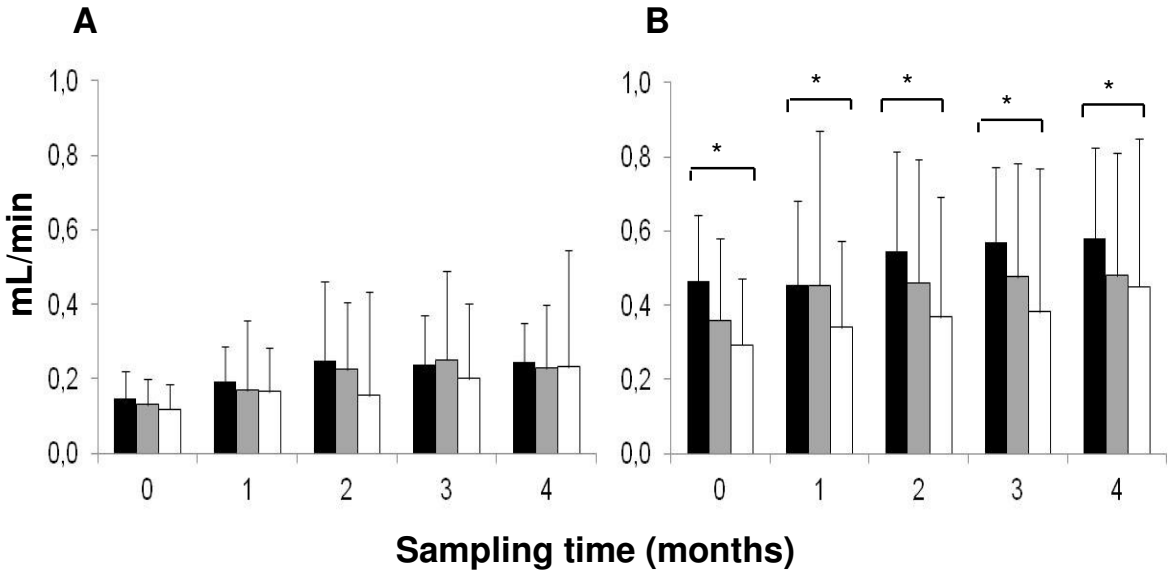

Figure 4

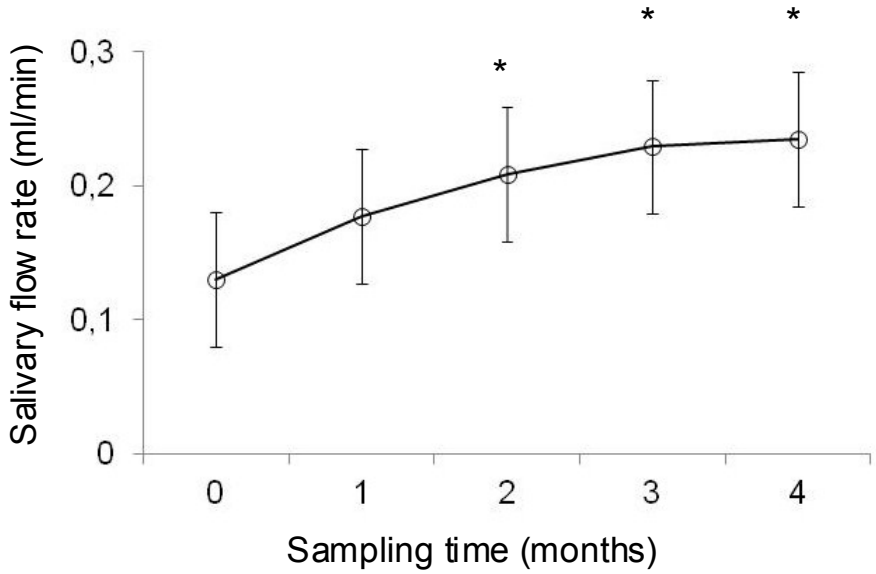

Figure 5

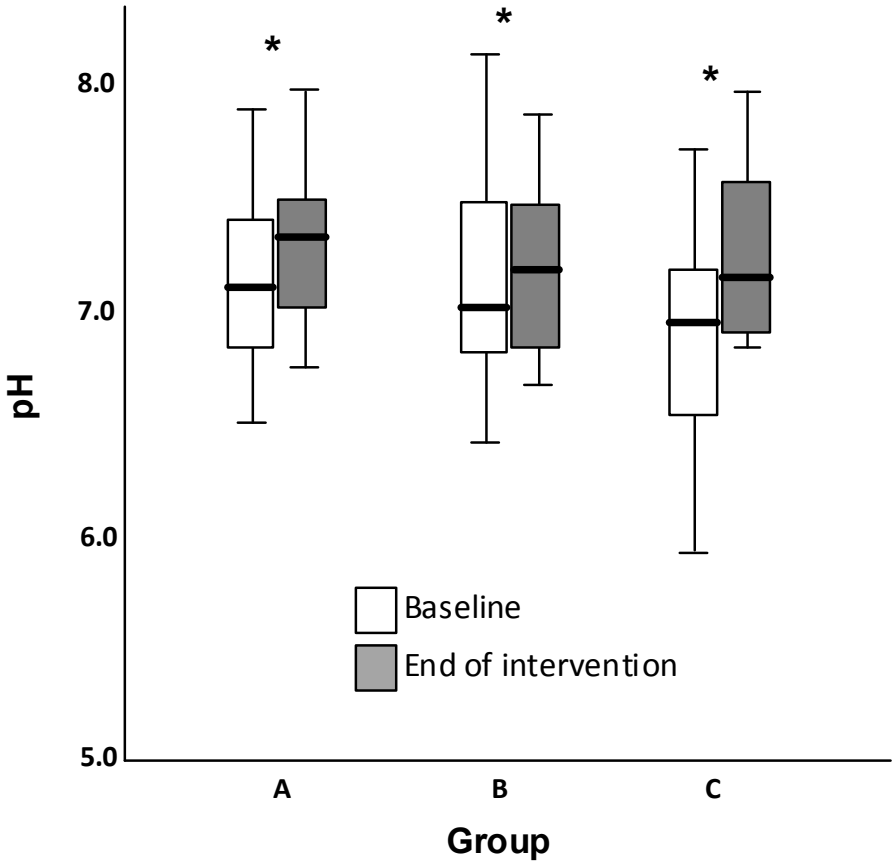

Figure 6