Clinical Oral Investigations (2021) 25:3823–3830
https://doi.org/10.1007/s00784-020-03712-8

ORIGINAL ARTICLE

The consumption of milk supplemented with probiotics decreases the occurrence of caries and the salivary concentration of hβD-3 in children

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Received: 2 September 2019 / Accepted: 25 November 2020 / Published online: 6 January 2021

Abstract

Objectives This study evaluated the effect of milk supplemented with *Lactobacillus rhamnosus* SP1 on the occurrence of caries and the salivary concentration of human β-defensin-3 (hβD-3) in preschool children with high caries risk.

Materials and methods A sample of 42 children was randomly assigned to two groups; children in the intervention group were given 150 mL of milk supplemented with 10^7 CFU/mL of *Lactobacillus rhamnosus* SP1, while children in the control group were given standard milk, for 10 months. The occurrence of dental caries was assessed using the International Caries Detection and Assessment System (ICDAS), and the concentration of hβD-3 was measured in unstimulated saliva using an ELISA test at baseline and after the intervention.

Results There was an increase in the number of teeth with carious lesions ($d_{ICDAS2\text{-}6}$ mft) in the control group, and this increase was statistically significant ($p = 0.0489$). The concentration of hβD-3 in saliva from the intervention group decreased from 597.91 to 126.29 pg/mL ($p = 0.0061$), unlike in the control group, where no change in hβD-3 salivary concentration was found.

Conclusions These findings showed that regular intake of probiotic-supplemented milk in preschool children with high caries risk decreased the occurrence of caries and the salivary levels of hβD-3.

Clinical relevance Our results suggest the need for developing and implementing probiotic supplementation, as adjuvants to the conventional treatments for caries and allow to considerate the salivary levels of hβD-3 as markers of oral tissue homeostasis.

Keywords *Lactobacillus rhamnosus* SP1 · Human beta-defensin-3 · Caries prevention · Supplemented milk · Children

Introduction

Dental caries is a chronic, multifactorial disease that generally progresses slowly and has a high prevalence worldwide [1, 2]. According to the "Ecological Plaque Hypothesis", dental caries is a result of changes in the environment due to acid production from the fermentation of dietary carbohydrates, which finally selects during biofilm formation, and acidogenic and acid-tolerating species such as *mutans streptococci* and *lactobacilli* [3, 4].

There are several preventive strategies for dental caries, including some mechanisms that modify the biofilm and reduce the cariogenic challenge, like the probiotic bacteria [5]. The World Health Organization (WHO) defines probiotics as live microorganisms, mainly bacteria, that are safe for human consumption and have beneficial effects on human health when ingested in sufficient quantities [6]. Now, probiotics also must have "defined contents, appropriate viable count at the end of shelf life, and suitable evidence for health benefits", and further stated that all probiotics must be "safe for their intended use" [7, 8]. Several clinical trials have reported on the use of different probiotic strains such as *Lactobacillus rhamnosus* GG, *L. casei*, *L. reuteri*, *L. plantarum*, *L. brevis* CD2, and *Bifidobacterium* spp. for the prevention of caries [9–14]. Among the genus *Lactobacillus*, *L. rhamnosus* SP1, also known as *L. rhamnosus* GG, has become one of the most

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studied probiotic bacteria in the world with decades of safe use that supports its efficacy and either health benefits [15–17]. Specifically, in the oral health field, *Lactobacillus rhamnosus* SP1 have been well studied, showing beneficial effects in dental caries or periodontal diseases [18, 19]. Although the multiple mechanisms of action of probiotics are not fully understood, it has been proposed that probiotics may work through systemic and local effects involving adherence, co-aggregation, competitive inhibition, organic acid production, bacteriocin-like compound production, and immune response modulation [20], or by stimulating non-specific and specific immune responses [21].

It is known that saliva and its components play an essential role in homeostasis and the prevention of caries. Salivary defense mechanisms are numerous and include local and systemic production of immunoglobulins, lysozymes, mucins, and antimicrobial peptides [22]. Antimicrobial peptides are natural antibiotics that deliver a first-line defense against a broad spectrum of pathogens, including Gram+ and Gram− bacteria, viruses, and fungi [23, 24]. There are 3 leading families of antimicrobial peptides, which are defined by their amino acid composition and three-dimensional structure: cathelicidins or LL-37, histatins, and defensins [25]. In humans, three β-defensins (BD) are predominantly expressed in the mucosal epithelium (hβD-1, 2, and 3) [26]. hβD-3 stands out among BD because it has the most powerful antibacterial action, and it is widely distributed in the oral epithelia in the gums, tongue, salivary glands, and oral mucosa [27, 28]. In epithelial cell line derived from colorectal adenocarcinoma (Caco-2), BD expression or secretion is induced by several *Lactobacillus* species [29]. Indeed, probiotic consumption modulates innate intestinal immunity, stimulating the synthesis of hβD-2 [29–32]. Recently, Kobayashi et al. [33] showed that gastric administration of LG2055 could control the oral inflammation and bone resorption caused by *P. gingivalis* infection by producing BD in the oral cavity. However, there is no evidence about whether the expression of hβD-3 is affected by the consumption of probiotic strains. Therefore, the present study examined whether the use of milk supplemented with *L. rhamnosus* SP1 for 10 months affects the salivary concentration of hβD-3 and caries scores.

Materials and methods

Study design

This experimental study enrolled forty-two children aged 2 to 3 years old, with an allocation ratio of 1:1. The participants were recruited from 16 nursery schools from the Integra Foundation located in the northwestern area of Metropolitan Region, Santiago, Chile. Informed consent was requested from parents and guardians. The inclusion criteria were healthy children without milk intolerance or food allergies. This project was guided by the principles of the Declaration of Helsinki, and was approved by the ethics committee of the Faculty of Dentistry at the University of Chile (certificate no. 2011/14).

Intervention

During their afternoon break, children were given 150 mL of 2% milk, which was prepared by the nursery school staff by adding 500gm of powdered milk (Macro Food, Santiago, Chile) to 5 L of previously boiled water at 40 °C. After stirring the preparation, a sachet of probiotics was added to the milk to attain a final concentration of 10^7-CFU/mL *L. rhamnosus* SP1 (Sacco, Cadorago, Italy) for the probiotic group. The placebo sachet for the control group only contained medium-fat milk. The probiotic-supplemented milk and the placebo milk were prepared and given to the children only on weekdays. During the intervention period, samples of milk were taken, and microbiological tests were performed to assess the presence of probiotic bacteria. The children were exposed to the intervention for a total of 10 months. The school staff filled in a logbook every day with information regarding the attendance of each child or his or her absence due to sickness or any other circumstances.

Clinical examination

Clinical dental examinations were performed at baseline and at the end of the intervention after 10 months. All children were examined by two qualified dentists (trained specifically for the study examination) at the nursery schools with an artificial light, a mouth mirror, and WHO periodontal probe. The examiners were trained in the clinical setting and using literature related to International Caries Detection and Assessment System (ICDAS). The assessment of intra- and inter-examiner reliability values resulted in a kappa value of 0.71, respectively. Visual and tactile detection of dental caries followed the ICDAS to describe lesion severity.

Salivary samples

The salivary samples were taken at baseline and the end of the intervention in the morning session at each nursery school. The procedure was performed by the same operator and always under the same conditions. Unstimulated saliva samples were taken as reported in Naše et al. [34]. Using a sterile pipette, a 1.5-mL sample of saliva was collected and deposited in a sterile, plastic microcentrifuge tube. The samples were taken and refrigerated (4 °C) in the Laboratory of Cellular and Molecular Biology, Faculty of Dentistry, University of Chile, and later stored at − 80 °C before biochemical analysis. At the time of analysis, the samples were thawed at room

temperature (RT) and cleared by centrifugation at 10,000 rpm at 4 °C for 10 min.

hβD-3 analysis

The levels of hβD-3 in the saliva samples were determined using an enzyme-linked immunosorbent assay (ELISA) (US Biological®, USA) according to the manufacturer's instructions. The concentration of each saliva sample was expressed in pg/mL. In brief, undiluted saliva samples were applied to a plate pre-coated with the specific primary antibody to hβD-3 in triplicate and incubated for 1 h at room temperature (RT). After washing four times with a wash solution, each well was incubated with biotinylated antibody for 1 h at RT. After another four washings with a wash solution, streptavidin-peroxidase (HRP) conjugate was added and incubated for 1 h. After five washings with a wash solution, 3,3′,5,5′-tetramethylbenzidine (TMB) was added for 15 min in the dark, and the reaction was then stopped by the addition of 1% H_2SO_4. The concentrations of hβD-3 in the saliva samples were calculated from a standard curve established by known concentrations of an hβD-3 standard.

Statistical analysis

Differences between groups were analyzed for statistical significance using the Pearson χ^2 test for categorized/dichotomized variables, and differences between groups at baseline and at the end of the study were analyzed for statistical significance using the Student t test. $p < 0.05$ was considered statically significant. The data were GraphPad software (GraphPad software Inc, La Jolla, CA USA).

Results

During the 10-month intervention, no adverse reactions to the daily consumption of milk supplemented with probiotic *L. rhamnosus* SP1 were detected. The number of children who participated is illustrated in the flowchart (Fig. 1). The baseline characteristics of the children in the intervention group and the control group are presented in Table 1. There were no statistically significant differences between the groups in terms of sex. At baseline condition, the mean salivary concentration of hβD-3 for ICDAS 2-6 individuals was 627.64 pg/mL (SD 41.92), and for caries-free individuals (ICDAS 2-6) is 353.23 pg/mL (SD 510.79). Individuals with cavitated caries lesions (ICDAS 5-6) have a salivary concentration of hβD-3 of 750.81 pg/mL (SD 966.61).

Table 1 Baseline characteristics of the children who entered the study

Characteristic	Probiotic group	Control group
Age, mean ± SD	2.95 ± 0.34	2.92 ± 0.28
Male sex, %	52.3	52.3

SD standard deviation

Effect on dental caries

The caries data at baseline and at the end of the study are shown in Table 2. The mean numbers of caries lesions in the children in the intervention group and the control group were equal at baseline in terms of both cavitated ($d_{ICDAS5\text{-}6}$ mft) ($p = 0.45$) and all carious lesions ($d_{ICDAS2\text{-}6}$ mft) ($p = 0.474$). The difference between the means of the number of caries lesions at the start and end of the intervention ($d_{ICDAS5\text{-}6}$ mft) was 0.76 ± 1.22 and 1.24 ± 1.92 for the probiotic group and the control group, respectively, and no statistically significant difference was found ($p = 0.12$) (Table 2). When examined at the all carious lesion level ($d_{ICDAS\ 2\text{-}6}$ mft) after the 10-month intervention period, the difference between the means of the number of caries lesions in the probiotic group was 1.29 ± 1.85, and in the control group was 2.38 ± 3.11. There was an increase in the number of teeth with carious lesions in the control group, and this increase was statistically significant ($p = 0.0489$) (Table 2).

Effect of probiotic consumption on the salivary concentration of hβD-3

At baseline, there were no statistically significant differences in salivary concentration between the intervention and control groups ($p = 0.27$) (Table 2). After the intervention, the salivary level of hβD-3 in the control group showed no statistically significant changes between baseline and after 10 months ($p = 0.9815$) (Fig. 2), which is different from the probiotic group, where the level decreased from 597.91 ± 743.28 to 126.29 ± 69.3 pg/mL ($p = 0.0061$) (Fig. 2). Similarly, a statistically significant difference ($p = 0.0140$) was found between both groups at 10 months (Table 2). In addition, the probiotic group had a wide range of hβD-3 saliva concentrations, with a large standard deviation before the initiation of the intervention ($m = 0$). After 10 months of probiotic consumption ($m = 10$), the salivary concentration of the peptide and the standard deviation in the group decreased. In the control group, the intragroup variability was maintained over time, as shown in the high standard deviation values.

Discussion

Probiotic use offers a current strategy to fight oral disease, including the development of caries [9, 10, 12–14, 35–37]. In our study, after 10 months of an intervention with a

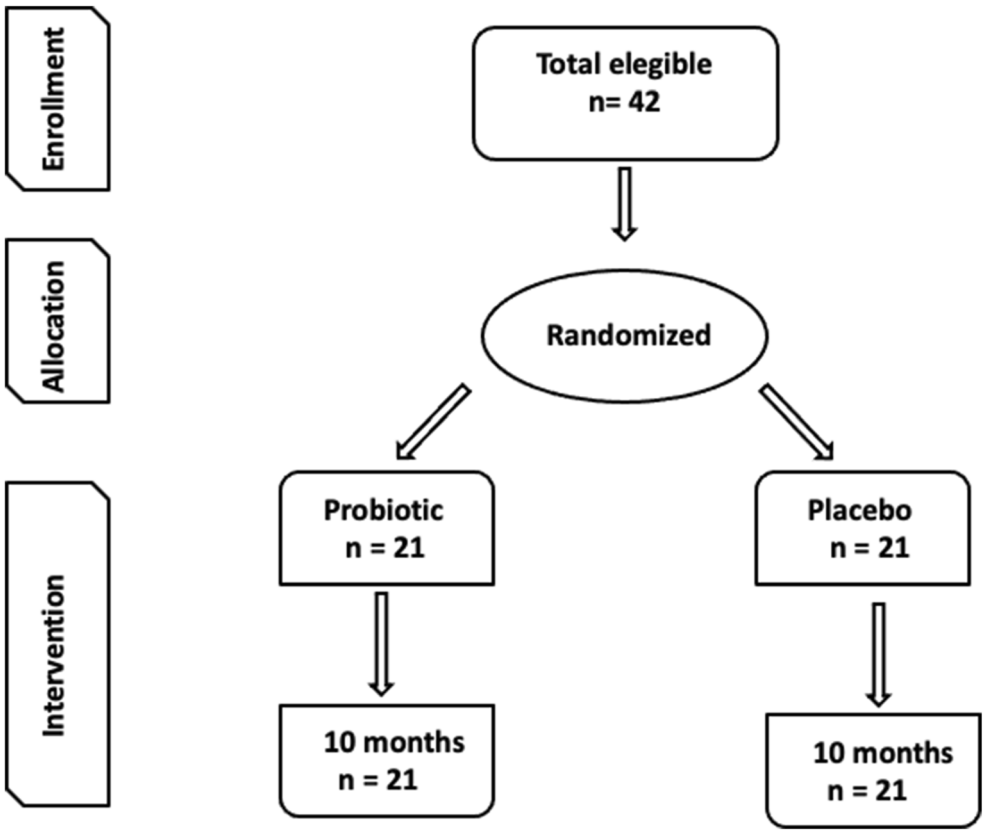

Fig. 1 Flowchart of the randomization of participants throughout the 10-month intervention period

probiotic, the number of teeth with carious lesions ($d_{ICDAS\ 2\text{-}6}$ mft) in the group treated with probiotic was lower than in the control group. This result confirmed that milk supplemented with probiotic *L. rhamnosus* SP1 will reduce the caries prevalence in this group of children with high caries risk, as shown in previous studies [11, 34, 38, 39]. In our study, the control and experimental groups were well balanced at baseline for dICDAS 2-6 mft caries lesions, 2.33 (SD 2.98), and 2.38 (SD 2.97). Although dICDAS 2-6 mft is an extensive range, we observed that dICDAS 5-6 mft caries lesions were well balanced too, 0.81 (SD 1.6) and 0.86 (SD 1.52).

Probiotics have multiple mechanisms of action that affect pathogenic bacteria in different aspects, such as metabolic activity [40], co-aggregation [37], microbial growth inhibition [35], and bacteriocins production [41]. In addition, probiotics can modulate the immune response [42]. On this subject, it has been reported that the use of *Lactobacilli* as a probiotic supplement increased salivary human neutrophil peptide 1–3 (HNP1-3) and was negatively related to the development of pit and fissure caries [43]. Since beta-defensin (BD) family members hβD-2 and hβD-3 are also associated with an oral immune response [44], we hypothesized that, similar to other

Table 2 Salivary concentration of hβD-3 and caries severity in the intervention group and control group at baseline and at the end of the study

Characteristic	Probiotic group Mean ± SD	Control group Mean ± SD	*p*
hβD-3 concentration (pg/mL), baseline	597.91 ± 743.28	373.49 ± 541.82	0.2700
hβD-3 concentration (pg/mL), 10 months	*126.29 ± 69.3*	*370 ± 468.97*	*0.0140*
$d_{ICDAS2\text{-}6}$ mft, baseline	2.33 ± 2.98	2.38 ± 2.97	0.4740
$d_{ICDAS2\text{-}6}$ mft, 10 months	3.61 ± 3.62	4.76 ± 3.90	0.1100
$\Delta d_{ICDAS2\text{-}6}$ mft 10 months—baseline	*1.29 ± 1.85*	*2.38 ± 3.11*	*0.0489*
$d_{ICDAS5\text{-}6}$ mft, baseline	0.81 ± 1.60	0.86 ± 1.52	0.4500
$d_{ICDAS5\text{-}6}$ mft, 10 months	1.57 ± 2.06	2.10 ± 2.90	0.2100
$\Delta d_{ICDAS5\text{-}6}$ mft 10 months—baseline	0.76 ± 1.22	1.24 ± 1.92	0.1200

ICDAS International Caries Detection and Assessment System, *SD* standard deviation

Italicized values indicate statistical significance. *T* test

antimicrobial peptides [43], probiotic supplementation might help prevent dental caries by augmenting basal production of hβD-3. Unexpectedly, we observed that regular consumption of milk enriched with *L. rhamnosus* SP1 caused a decrease in the salivary concentration of hβD-3 when compared with individuals who did not consume the probiotic.

This study compared groups with similar state of health related to dental caries. The hβD-3 levels indicated no statistical difference between the two groups at baseline and are in accordance with levels previously reported (0–6.21 μg/mL) [45].

Since microbial pathogens stimulate hβD-3 expression in oral epithelial cells [28, 46, 47] and hβD-3 function as a direct antibiotic agent to maintain homeostasis of microbes in the oral cavity, we think that the effect of probiotics on hβD-3 levels is probably associated with decreased caries prevalence and the re-establishment of a healthy oral microbiome. In periodontal disease, it has been observed that reducing the microbial burden decreases the inflammation of localized oral epithelial cells and supports disease resolution [48]. Although we [49–52] did not perform microbiota analyses, several clinical studies show that regular consumption of probiotics decreases the number of cariogenic *Streptococci* in bacterial plaques and in saliva [9–12, 34, 53, 54]. Besides, hβD-3 expression is also induced in epithelial cells by pro-inflammatory cytokines, such as (IL)-1β, tumor necrosis factor (TNF)-α, and IL-17 [55]; previous studies have shown high salivary levels of IL-6 and TNF-α in children with caries compared with healthy children [49–52]. Then, the suppression of inflammatory cytokines production in oral tissues by the consumption of *L. rhamnosus* SP1 might correlate with the hβD-3 levels observed at the end of the study. For instance, *L. rhamnosus* GG has proven to be immunostimulatory [56] showing several anti-inflammatory effects through the modulation of the gut microbiota and the downregulation of pro-inflammatory molecules in model of infection and cancer [57, 58]. Furthermore, probiotics have shown improving clinical symptoms in patients with immune dysregulation and autoimmune diseases, such as rheumatoid arthritis [59], systemic sclerosis [60], inflammatory bowel disease [61], and multiple sclerosis [62].

The ingested probiotics produce a change in the gut microbiota and promote health through various mechanisms, including the maintenance of the intestinal barrier function and the modulation of the host's immune system [63–65]. These studies related to the human intestine suggest that defensins are critical regulators of bacterial diversity and tissue homeostasis [66]. In our study, we observed hβD-3 in saliva, which is a remote place from the intestine; it may suggest that intestinal cells sensitized with *L. rhamnosus* SP1 could migrate and act on oral cavity tissues, and regulate hβD-3 production locally. In this context, there are subsets of migrating intestinal lymph dendritic cells (DCs) that play immunogenic or tolerogenic roles according to the microenvironment [67]. Kobayashi et al. [33] showed in a mice model of periodontal disease that gastric administration of *L. gasseri* STB2055 increased the DCs number among gingival mononuclear cells; however, the exact phenotype of DCs subset and its function was not studied. Further studies are required regarding to elucidate the exact mechanisms underlying the communication between intestine and mouth.

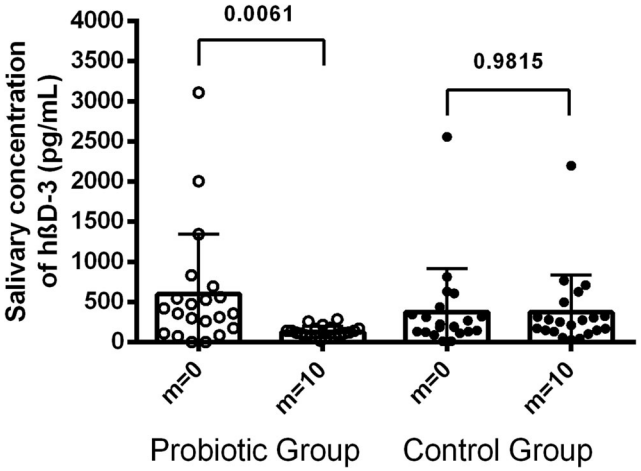

Fig. 2 Salivary concentration of hβD-3, expressed as pg/mL, of the groups at baseline ($m = 0$) and the end of the intervention ($m = 10$). The probiotic group showed a significant decrease in salivary concentration of hβD-3 ($p = 0.0061$) and a decrease in variability once the probiotic was consumed. No changes were observed in the control group

The findings of this study have to be seen in light of some limitations. We were focusing on hβD-3 because of the higher specificity and activity for streptococci, and salt-tolerant activity [68, 69]. However, there are other salivary proteins involved in host defense in the mouth with overlapping functions in addition to defensins such as histatins [70] and cathelicidins [71]. Although the role of cathelicidins in protection against caries is inconclusive [69, 72], naturally occurring histatins in AEP have a crucial role to acid injury [73] and derivatives of histatins appears to have improved activity against several potential pathogens, including *S. mutans* [74]. Up to now, there are no studies that relate probiotics defensins with other antimicrobial proteins which will be interesting to consider in future studies.

Finally, our results suggest the need for developing and implementing different oral health strategies, such as probiotic supplementation, as adjuvants to the conventional treatments for caries. However, additional studies are required to fully elucidate the influence of probiotic consumption on the oral immune response.

Funding The work was supported by the Grant FIOUCH 17/011 from Dirección de Investigación, Facultad de Odontología, Universidad de Chile.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the ethics committee of the Faculty of Dentistry at the University of Chile (certificate no. 2011/14) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained in writing from the children's parents or legal guardian.

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