Nutrafoods (2019) 1:49-57. DOI 10.17470/NF-019-0008

Original Research

Probiotics – a final countdown for caries

Abstract

The development of contemporary knowledge in the field of oral microbiology and probiotics was commenced by Elie Metchnikoff's report on *Lactobacillus bulgaricus* in 1907. Since then, multiple indications for the use of probiotics have been established, following the WHO definition: "probiotics are live microorganisms, which when applied in adequate amount may benefit the host health status".

In accordance with a general classification, several types of bacteria and fungi have been distinguished and, moreover, their mechanisms of action run parallel in both the gut and oral cavity.

The possible use of probiotics in dentistry is a relatively new idea.

Until now, three species prevailed in available research: *Bifidobacterium*, *Lactobacillus rhamnosus* GG and *Lactobacillus reuteri*. Current research focuses on the application of probiotics and the natural displacement of cariogenic bacteria within the oral cavity, and the subsequent alteration of health status in both adults and children.

Carioblis BLIS M18 probiotic, which contains freeze-dried cultures of *Streptococcus salivarius* M18, was introduced to the market as a supplement indicated for patients with a high caries rate.

The aim of this study was to investigate the influence of the BLIS M18 strain on oral cavity microflora and estimate possible health outcomes.

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Keywords: Probiotics, *Streptococcus salivarius*, caries, oral hygiene, Carioblis, oral diseases

Introduction

During the last 70 years, the number of products containing probiotics, that is, live bacteria with proven beneficial effects on the host organism, has significantly increased.

Traditionally, probiotics were associated with gut microbiome improvement, and therefore, the majority of clinical research was focused on this aspect. Notwithstanding this, the last decade has seen a significant increase in the number of reports on the pro-health effects of probiotic bacteria, including enhancement of the adaptive immune response in the treatment or prevention of genitourinary diseases and respiratory infections, and in the prevention or alleviation of allergies and atopic diseases in children. Probiotics are also recommended for patients using voice prostheses to increase their durability by inhibiting the adhesion of unwanted microorganisms. The most innovative possible application of probiotics is their use in the treatment of oral pathological conditions, with particular emphasis on caries.

The mechanisms underlying the functioning of probiotics in the mouth and intestine are analogous. They are primarily based on the production of bacteriocins, modulating the inflammatory response, stimulating local immunity, competing with pathogens for nutrients and binding sites, as well as modulating the ecological niche. Commonly used probiotic strains are bacteria classified as *Lactobacillus* and *Bifidobacterium*, considered to be permanent elements of normal human flora. In the oral cavity, lactic acid bacteria usually account for less than 1% of the total bacterial colonies, but species specific to the oral environment have not been found.

The presence of some species in saliva and faecal samples has been established [1]. Those species commonly isolated from saliva samples include: *L. paracasei*, *L. plantarum*, L. rhamnosus and *L. salivarius*. The available literature suggests that *Bifidobacteria* belong to the group of the first anaerobes inhabiting the oral cavity. Species isolated from saliva samples include *B. bifidum*, *B. dentium* and *B. longum* [2].

Research on the composition of human milk has shown the presence of probiotic bacteria in the diet of mothers who received supplementation with products containing probiotic bacterial strains during pregnancy. The microorganisms *Lactobacillus* and *Bifidobacterium* are present in human milk, indicating earlier oral exposure to these bacteria [3]. Another species with probiotic activity – *Streptococcus salivarius* – has innate ability to bind and persist on the dorsal part of the tongue. Some of these strains release large amounts of bacteriocins into saliva, which can be a targeted way of removing harmful bacteria [4, 5]. The production of bacteriocins in vivo is often mentioned as the main mechanism by which health benefits are achieved after the consumption of probiotic products [6].

The passage of these organisms through the mouth and intestine reduces the production of bacteriocins by the host microflora. In order to obtain sustained release of bacteriocins, it seems appropriate to maintain the stability of probiotic bacteria [7].

Materials and methods

The study included 65 patients, 45 women and 20 men, aged from 21 to 45 years.

Patients were divided into an experimental group, which contained 42 people, and a control group with 23 participants. The clinical trials were conducted in the Department of Conservative Dentistry and Endodontics and the microbiological analysis was performed at the Department of Medical Microbiology.

Both divisions belong to the Medical University of Lublin, Poland. The approval of the Bioethics Committee of the Medical University in Lublin

was obtained prior to conducting the research. All patients provided written informed consent in order to participate in the study. Due to the nature of the research, participants with a high level of awareness, mainly working in medical professions, participated in the experiment. Those individuals undergoing antibiotic therapy were excluded from the research, both at the time of commencement of the study and during treatment. In all participants enrolled in the study, early hard tissue demineralization was diagnosed using the DIAGNOdent Pen. The results with a final score of >25 were noted. The level of the oral hygiene of patients was determined using the Approximal Plaque Index (API). The API is determined after staining the plaque with an erythrosine tablet. The assessment covers interdental spaces examined from the palatal/lingual side in the first and third quarters and vestibular ones in the second and fourth quarters. After obtaining the final results, the number of interdental spaces in which plaque has been found should be divided by the total number of all examined spaces and multiplied by 100%. Interpretation of the results followed the standardized API scale. A biological material – saliva collected from patients – was evaluated using CRT bacteria tests. These tests allow selective multiplication and simultaneous identification of two species – *Streptococcus mutans* and *Lactobacillus*. The test procedure was conducted as follows: saliva was collected from patients about 2 hours after the last meal, then, with the use of an automatic pipette and sterile tip, this was applied to the medium. Samples were placed in sealed vials in which $NaHCO_3$ tablets were present. Vials were incubated in a vertical position at 37°C for 48 hours. The CRT bacteria tests enable simultaneous growth observations of both pathogenic species: *S. mutans* bacteria grow as blue colonies on the blue substrate and *Lactobacillus* spp. as white colonies on transparent media. Demonstration of microorganisms in an amount of 10^5 CFU in a millilitre of saliva indicates a high risk of carious disease. The CRT bacteria test was accompanied by pattern result imaging, according to which the number of *S. mutans* and *Lactobacillus* bacterial colonies were assessed. For the sake of simplicity and for uniformity of results, the image of the bacterial colonies obtained in each study was compared to one of the four levels of the pattern and characterized according to the scale. Evaluation of the saliva results was performed five times for each patient: before the initiation of BLIS M18 supplementation; in the 6^{th} and 12^{th} weeks of therapy; and also, in the follow-up period, that is, 6 and 12 weeks post-BLIS M18 supplementation.

Carioblis

This study tested the efficacy of Carioblis. This supplement contains BLIS M18 *Streptococcus salivarius* bacteria. The strain was isolated from the saliva of healthy adults and the probiotic is composed of freeze-dried cultures of Streptococcus salivarius M18 NLT (Not Less Than) 1×10^{11} CFU/g in a food coating of maltodextrins, trehalose and lactitol. Carioblis represents Level 1 (the safest) according to the American Type Culture Collection. It is a gluten-free product, and it is recommended that it should be stored at a temperature of 2–8°C; in addition, it should be protected from sunlight and freezing is contraindicated.

The supplement was administered to the patients from the experimental group who received instructions beforehand. Carioblis was consumed daily for 3 months. After evening brushing, the lozenge was inserted into the mouth and sucked until the preparation was completely dissolved. Patients were advised not to rinse the mouth afterwards and to also resist from eating and drinking after using the probiotic. Changing the position of the tablet in the mouth was recommended in order to avoid local irritation. During the examination, the bio-

logical material (saliva) was collected into sterile containers after stimulation of its secretion by chewing paraffin pellets included in the kit.

Data synthesis and analysis

The obtained results were subjected to statistical analysis. The obtained values are given as means ± standard deviation (SD). API, DIAGNOdent and CRT results were analyzed by ANOVA with replicates, repeated measures factorial ANOVA and Tukey's post hoc test (for different N) as a correction for multiple comparisons. Normal distribution of data was checked using the Shapiro–Wilk test, while the homogeneity of variance was assessed using Hartley's, Cochran's and Bartlett's tests. If data had no normal distribution and/or homogeneous variance, the Kruskal–Wallis ANOVA test and the median test with correction for replicates were used as well as post hoc comparisons for middle ranks with the Bonferroni correction for multiple comparisons. The analysis of survey data was based on multidisciplinary tables with the following tests: Pearson's chi-square, chi-square of the highest credibility and Spearman's rank correlation. Differences were accepted as being statistically significant for $p<0.05$. All statistical analyses were carried out using Statistica 12 (StatSoft Poland, Cracow, Poland).

Results

In the experimental group, a significant decrease in the S. mutans titre, as determined by the use of CRT SM tests, was observed at week 12 of supplementation, as well as at 6 and 12 weeks after the end of probiotic intake. A summary of the data regarding the count of Streptococcus mutans species is shown in **Table 1**.

The difference between the control group and the test group, based on probiotic supplementation, accounted for approximately 12% of the variability of the data – other factors influenced this change but these were not analyzed during the experiment. The amount of *S. mutans* was altered in patients who received the Cariobiotic BLIS M18 probiotic. Subsequent repeated measurement of the number of cariogenic bacteria colonies made in patients during the experiment accounted for 9% of the variability, while the interaction between successive trials and groups was responsible for an average of approximately 22% of the variability. Upon using the CRT test for lactobacilli, similar results were obtained. An interaction was observed between the control and test groups and subsequent repetitions. The number of lactobacilli

Table 1 CRT bacteria test results for *Streptococcus mutans*

		CRT SM										
Effect	Factor level	N	1 Mean (CFU)	1 SD	2 Mean (CFU)	2 SD	3 Mean (CFU)	3 SD	4 Mean (CFU)	4 SD	5 Mean (CFU)	5 SD
Total		65	2.4	1.1	2.1	0.8	1.9	0.7	1.9	0.8	1.9	0.8
Group	control	23	2.2	0.9	2.3	0.9	2.4	0.7	2.4	0.8	2.4	0.8
Group	study	42	2.5	1.1	2.0	0.8	1.6	0.5	1.6	0.5	1.6	0.5

1 = Beginning of the experiment (baseline); 2 = 6 weeks of supplementation; 3 = 12 weeks of supplementation; 4 = 6 weeks after the end of supplementation; 5 = 12 weeks after the end of supplementation. CRT SM = CRT bacteria test for *Streptococcus mutans*; SD = standard deviation; N = number of participants; CFU = colony forming unit in 1 ml of saliva

remained constant in the control group. In the test group, the amount of *Lactobacillus* was reduced over time after *S. salivarius* BLIS M18 supplementation. In the 3rd repetition, a significant reduction in *Lactobacillus* was noticed in the experimental group, that is, at the end of Carioblis treatment, and a reduction was also seen at the 4th and 5th measurements, that is, at 6 and 12 weeks after the end of *S. salivarius* BLIS M18 supplementation. **Table 2** presents data depicting the shift of the count of lactobacilli in both experimental and control groups before, during and after Carioblis BLIS M18 supplementation.

The difference between the control and test groups justified 10% of the observed variability. The interaction between groups and subsequent studies in the cycle explained 9% of dependencies. Based on statistical analysis of the data, it can be assumed that other factors such as oral hygiene, a carbohydrate-rich diet, addictions and so on, have also affected the results. Along with the decline in bacterial titres, a significant difference in the API before and after the beginning of the study was observed. The control and test groups were selected analogically to identify existing correlations. There was a significant interaction between the group and the repetition – in the experimental group, there was a significant decrease in the value of the API (p=0.022). The observed difference in the obtained results was – according to statistical data – explained by successive repeats of the API indicator measurement (38%), whereas the dependence of the probiotic supply and the Hygiene Index explained 25% of data variability. There were other factors affecting the API but they were not investigated. It seems probable that the patient's hygienic procedures directly affected the results of the experiment. In people using probiotics who had thoroughly cleansed their mouths, better results (lower DIAGNOdent Pen indications) were obtained in comparison to patients in the control group who neglected oral hygiene. A higher titre of *Lactobacillus* bacteria was found in men using the CRT bacteria diagnostic tests (p=0.022).
In addition, patients from the experimental group presented with significantly lower API values in the second (last) trial in comparison with the results of the first study (p=0.022). Statistical data were characterized by the occurrence of a significant interaction between the sex of patients and subsequent repetitions, which accounted for 10% of the variability of the obtained results. Other factors probably influencing the results include: hygiene, level of social awareness, profession and so on.

Table 2 *CRT bacteria test results for Lactobacillus*

CRT LB

Effect	Factor level	N	1 Mean (CFU)	1 SD	2 Mean (CFU)	2 SD	3 Mean (CFU)	3 SD	4 Mean (CFU)	4 SD	5 Mean (CFU)	5 SD
Total		65	2.4	1.0	2.1	0.8	2.0	0.8	1.9	0.7	2.0	0.8
Group	control	23	2.4	1.0	2.3	0.9	2.4	0.9	2.4	0.8	2.4	0.9
Group	study	42	2.4	1.0	2.0	0.8	1.7	0.7	1.7	0.6	1.7	0.6

1 = Beginning of the experiment (baseline); 2 = 6 weeks of supplementation; 3 = 12 weeks of supplementation; 4 = 6 weeks after the end of supplementation; 5 = 12 weeks after the end of supplementation. CRT LB = CRT bacteria test for *Lactobacillus*; SD = standard deviation; N = the number of participants; CFU = colony forming unit in 1 ml of saliva

The direct impact of each factor has not been studied. The results of the DIAGNOdent Pen measurement were lower for women, which testified to a higher degree of advancement of enamel remineralization changes. The interdependence of the repetitions and sex of the subjects allowed for the interpretation of 7% of the variability of the obtained statistical data (p=0.022).

Figure 1 presents the mean indications of the DIAGNOdent Pen assessment in both groups, the experimental and the control, over time. According to the graph, the mean results in both groups at the beginning of the experiment were comparable. With time, the level of enamel demineralization of the patients supplemented with Carioblis BLIS M18 (red line) decreased (lower numbers were achieved), whereas the level of the same parameter measured among the participants within the control group was elevated over the same period of time, that is 6 months.

R1*group; expected marginal average; current effect: F(4,252)=23.238, p=0.00000. Vertical bars represent 0.95 confidence intervals

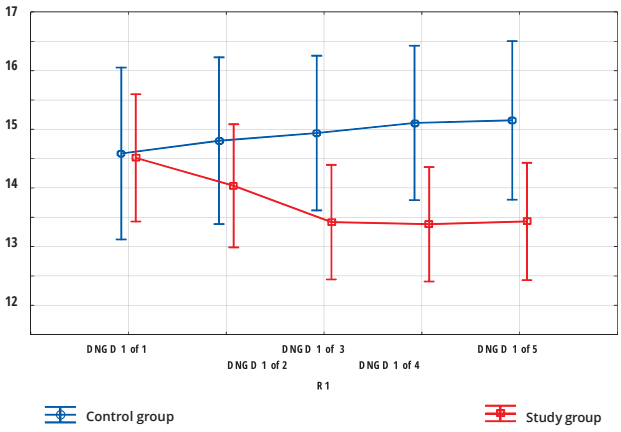

Figure 1 DIAGNOdent Pen indications over time in both groups. DNGD 1 of 1 = indication of DIAGNOdent at the beginning of the experiment (baseline); DNGD 1 of 2 = indication of DIAGNOdent after 6 weeks of supplementation; DNGD 1 of 3 = indication of DIAGNOdent after 12 weeks of supplementation; DNGD 1 of 4 = indication of DIAGNOdent at 6 weeks post-supplementation; DNGD 1 of 5 = indication of DIAGNOdent at 12 weeks post-supplementation

Discussion

To date, the ability of *S. salivarius* to produce bacteriocins has been mainly attributed to genetic determinants located on megaplasmids (size 160 to 220 kbp). Naturally occurring transmission of these megaplasmids has been demonstrated in both *in vitro* and *in vivo* studies. Their flexible ability to acquire, answer and demobilize many different loci can help explain the significance of *S. salivarius*. It has also been suggested that *S. salivarius* megaplasmids may be a reservoir of bacteriocin determinants obtained from various species found in the mouth by transposition of elements [8]. Preliminary molecular analysis indicated that these megaplasmids may also encode molecules that support cell adhesion without inducing antibiotic resistance [9]. Tooth decay is still one of the most widespread diseases in the world, despite the decline in its prevalence in Western societies [10]. This disease is caused by the interaction of microorganisms (mainly *S. mutans* and Lactobacillus), a diet rich in fermenting carbohydrates and factors such as reduced salivation rate, as well as its buffering capacity. Originally, *Streptococcus mutans* was considered the main pathogen responsible for caries onset. However, in recent years, it has been proven that the microflora remaining on hard tooth tissues changes depending on the severity of lesions, with an initial predominance of streptococci other than *S. mutans* and *Actinomyces* spp. This leads to the dominance of *S. mutans* and other bacteria including lactobacilli and *Bifidobacterium* spp. [11]. The issue of the influence of probiotics on the pathomechanism of caries is still new and little understood. Probiotics are administered primarily in order to maintain or restore the natural saprophytic microflora protecting against the invasion of pathogens, which are crucial for the development of serious diseases of the mouth (periodontal disease and caries).

The probiotic strains administered to improve oral hygiene are also used to gain benefits via the gastrointestinal tract. Due to the differences between the oral cavity and intestines, these strains may not specifically act against tooth and gum disease [12, 13]. The effect of probiotics on caries and the associated risk factors have been evaluated in several experimental studies using different strains: *Lactobacillus rhamnosus* GG, *L. reuteri*, *L. casei*, *L. brevis* CD2, *L. plantarum*, *Bifidobacterium* spp. and so on [14–16]. These strains were used to bring about reduction of *S. mutan*s and *Lactobacillus* titres, control of plaque pH changes and remineralization of carious lesions on the root surface. Different forms of probiotic supply were used in the research. Dairy products with the addition of probiotics were considered the simplest to introduce to the daily diet of children and adults [15, 17]. Based on the analysis of the available literature, it was found that in order to prevent oral diseases, and due to the necessity of slow release of probiotic bacteria, it seems essential to develop supplements with prolonged degradation. Clinical trials should be extended in order to induce the colonization of the oral cavity by these organisms and enable the stability of the new microbiome in which they will remain. The phenomenon of the short existence of such microorganisms in the gastrointestinal tract has been described by Ravn *et al* [18]. Researchers administered three bacterial strains contained in milk eight times within three days to patients. The presence of inorganic microorganisms was confirmed only in a small number in saliva samples and on the mucosal surface by fluorescence hybridization and using a confocal laser scanning microscope. Bacteria were not found on tooth surfaces. In another experiment, Caglar et al used a strain of *Lactobacillus reuteri* ATCC 55730. The formulation that was administered to patients in the form of tablets contained 10^8 CFU *L. reuteri* ATCC 55730 [16]. The therapy included three 14-day periods of purification, intervention and post-exposure follow-up. A total of 25 volunteers who took tablets at the appointed time participated in the clinical trial. In the saliva samples obtained, the amount of *L. reuteri* significantly decreased, and then, after a week, bacteria were detected in only 8% of the subjects. After five weeks, the presence of these microorganisms in the experimental group was not detected.

Therefore, it is advisable to administer probiotics in a form that enables extension of the secretion period and, preferably, remodeling of the structure of the oral environment. Experiments conducted in recent years prove that the potential utility of probiotic bacteria against cariogenic strains is still underestimated.

Research conducted by Lee and Kim using the RT-PCR technique found that a group of patients supplemented with probiotic L. rhamnosus had a reduced titre of *S. mutans* [19]. The authors demonstrated the inhibitory effect of *Lactobacillu*s probiotics on the growth of streptococci. Inhibition of glucosyltransferase expression was observed due to *Lactobacillus rhamnosus* activity. The integration of this strain into the biofilm did not occur, although *L. casei* and *L. acidophilus* were included in its composition. The obtained results led to the conclusion that *Lactobacillus rhamnosus* may induce inhibition of biofilm formation by reducing production of *Streptococcus mutans* glucans.

The ability to inhibit caries-forming *Streptococcus mutans* in vitro by naturally occurring *Lactobacillus* bacteria in the oral cavity of young adults has also been demonstrated by Simark-Mattsson *et al* [20]. The reduction of *S. mutans* after supplementation with another probiotic strain, *S. salivarius*, was observed by Burton *et al*. Their research involved a three-month observation of 100 students (in the age range of 5 to 10 years). In those patients qualified to take part in the experiment, at least three fillings had to be found, the last of which was not more than one year old. Patients received *S.*

salivarius probiotic over a three-month period. The authors showed a reduction in the number of *S. mutans* in patients in the experimental group with reference to the control group [21]. Probiotics are a new area of scientific research in the field of oral medicine. Preliminary data from pilot studies are encouraging, but randomized trials will require clear identification of the potential of probiotics for the prevention and treatment of oral infections. These tests will identify organisms with properties that are most suitable for oral administration, together with the most adequate carriers: food (cheese, milk, yogurt) or supplements (chewing gum, lozenges). There is still little known regarding the interactions between cariogenic pathogens and probiotics. Long-term observation and properly planned research will deliver answers to many questions related to the phenomenon of substitution of some strains by others.

Conclusions

The use of the probiotic strain *Streptococcus salivarius* BLIS M18 decreases the amount of *Lactobacillus* and *Streptococcus mutans*, and its effect lasts over time. Therefore, supplementation of the daily diet with a probiotic containing *S. salivarius* bacteria leads to inhibition of the process of demineralization of hard dental tissues.

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