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# Effects of a *Lactobacillus salivarius* probiotic short-term intervention on *S. mutans*, *Lactobacillus* spp. and *C. albicans* – a randomized pilot study with pre-school children

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## Abstract

**Introduction and Objective.** The aim of the study is to evaluate the effect of short-term, oral supplementation of non-viable *Lactobacillus salivarius* (HM6 Paradens) on the number of caries-related microorganisms in the supragingival plaque biofilm of pre-school children.

**Materials and Method**. The study is prospective, randomized and observational in design performed on 2 parallel groups (test and control) of 72 childrenaged between 3 – 6 years, with or without early childhood caries (ECC). The primary outcomes measured are changes in *S. mutans* counts (number of colony-forming units – CFU) and the percent of the total viable counts) in dental plaque biofilm within the groups between baseline and 2-weeks follow-up, while secondary outcomes are changes in *Lactobacilli* spp. and *C. albicans* counts.

**Results.** For analysis performed only on the children with detectable bacterial counts at baseline, the reduction in the level of *S. mutans* increased in the control group, and decreased in the probiotic group, *Lactobacillus* spp. increased in the probiotic group and decreased in the control group, and for *C. albicans,* the level decreased in both the control and probiotic groups after 2 weeks.

**Conclusions.** The presence of *L.salivarius* (HM6 Paradens) may modify the interactions between *S. mutans* and *Lactobacillus* spp., which could support microbiological homeostasis and have a beneficial impact on ECC prevention.

## Key words

*Lactobacillus salivarius*, *S.mutans*, *C. albicans*, ECC prevention, paediatric dentistry

## INTRODUCTION AND OBJECTIVE

Early childhood caries (ECC) is one of the most common diseases in children under 6 years of age world, currently being the 10th most common health condition [1].

The etiopathogenesis of caries depends mainly on host-related factors, such as oral hygiene and diet [2, 3]. Frequent consumption of the most cariogenic non-milk sugars (NMES), metabolized by oral microorganisms which take part in the creation of cariogenic biofilms on the tooth surface [4, 5]. The initiation and progression of dental caries has been attributed to only a few gram-positive bacteria in the biofilm, i.e. *S. mutans*, along with some *Lactobacillus spp.* Next to *S. mutans*, *Streptococci* and *Lactobacilli spp, Candida spp.* are considered as a strong opportunistic secondary cariogenic agents [6, 7] *C. albicans* and *S. mutans* have been shown to not only co-exist but also interact in early childhood caries [8].

In view of current epidemiological data, it seems reasonable to use supplementary tools in addition to the established caries prevention procedures, in order to maintain symbiotic homeostasis of the oral microbiome (primary prevention), or restore it (secondary prevention) in the youngest groups of children [9]. The possibility of using probiotics in the prevention of dental caries has only recently been investigated, and no type has yet been described in the literature as being most effective against the imbalance in favour of caries micro-organisms. Probiotic strains mainly used in the prevention of oral diseases are the genera *Lactobacillus* and *Bifidobacterium* [10].

The mechanisms behind the beneficial impact of probiotics on caries are similar to that found in the gastrointestinal tract and are unique to each strain, being the result of a combination of competing actions which are not entirely clear. The principal inhibitory mechanisms include the synthesis of active metabolites, inhibition of cariogenic microbial biofilm,

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competitive adhesion and colonization, co-aggregation with pathogens, and regulation of the immune system [11].

Probiotic bacteria, such as *L. salivarius*, that colonize the oral cavity of naturally born children may play protective role based on the stimulation of the host's immune system to produce antibodies and immunoglobulins. All predicted mechanisms of action lead to a reduction in pathogenicity and caries potential of oral microorganisms, as well as a reduction in the potential pathogen load of the biofilms formed [11]. This may indicate their potential usefulness in modulating oral ecology of micro-organisms in terms of caries prevention. However, their clinical effectiveness in this aspect seems to remain limited and controversial. Although *in vitro* research brought promising results, clinical trials have not shown clear effects.

Recent studies in children aged 0–6 years show that probiotic supplementation can slightly reduce the risk of early childhood caries by reducing *S. mutans* levels and the incidence of new lesions, currently do not outperform established preventive methods [10].

Traditional strategies-diligent oral hygiene with fluoride toothpaste, fluoride prophylaxis (systemic and topical), and dietary sugar restriction, have a more pronounced effect on reducing caries in young children (often by 25–35% or more) [9].

In practice, probiotics should be considered a complementary measure, as part of a comprehensive caries prevention plan, especially for children at increased risk of early childhood caries or when traditional methods are insufficient. Probiotics are not recommended for use in only a few serious medical conditions, e.g. in individuals who are severely immunosuppressed (those with AIDS, lymphoma, or on long-term corticosteroids) due to the risk of serious infection. Other contraindications include acute pancreatitis, patients in Intensive Care Units, those with a central venous catheter, and infants with short bowel syndrome. Probiotics are also not recommended for those with melaena indicating gastrointestinal bleeding, or for patients with open wounds following major surgery [10].

The deficiency of published clinical trials assessing the effectiveness of probiotics in the prevention and control of ECC, i.e. in children up to 6-years-old, is remarkable [12]. There have only been *in vivo* studies on this subject in relation to the *L. salivarius* strain that showed a significant reduction in the level of salivary *S. mutans* immediately after short term supplementation of the probiotic containing this strain, but they concerned adults [13, 14].

A preliminary *in vitro* study of the *L. salivarius (*HM6 Paradens) strain demonstrated that this probiotic inhibited the ability of *S.mutans* and *C.albicans* strains derived from the dental plaque of children with early childhood caries (ECC), to form a common structure. The evaluation of the biofilm formed under the influence of the test preparation showed a decrease in both the number of colonies grown and the biomass of the biofilm, as well as reduced cross-linking of its structure [15].

The aim of the study was to evaluate the effect of short-term, oral supplementation of non-viable *L. salivarius* (HM6 Paradens), in the form of lozenges, on the number of *S. mutans, Lactobacillus spp.* and *C. albicans* in the supra-gingival plaque biofilm.

## MATERIALS AND METHOD

**Study design and permissions.** The study is of a prospective, randomized observational design [16]. It was carried out at the Department of Paediatric Dentistry, Institute of Dentistry, Jagiellonian University Medical College in Kraków, Poland, in accordance with the guidelines outlined in the Helsinki Declaration of 2013, and the Consolidated Standards of Reporting Trials (CONSORT) [17].

Permission for the study and the protocol of the clinical trial was approved by the Bioethics Committee of the Jagiellonian University in Kraków, Poland (Consent No. 1072.6120.31.2018).

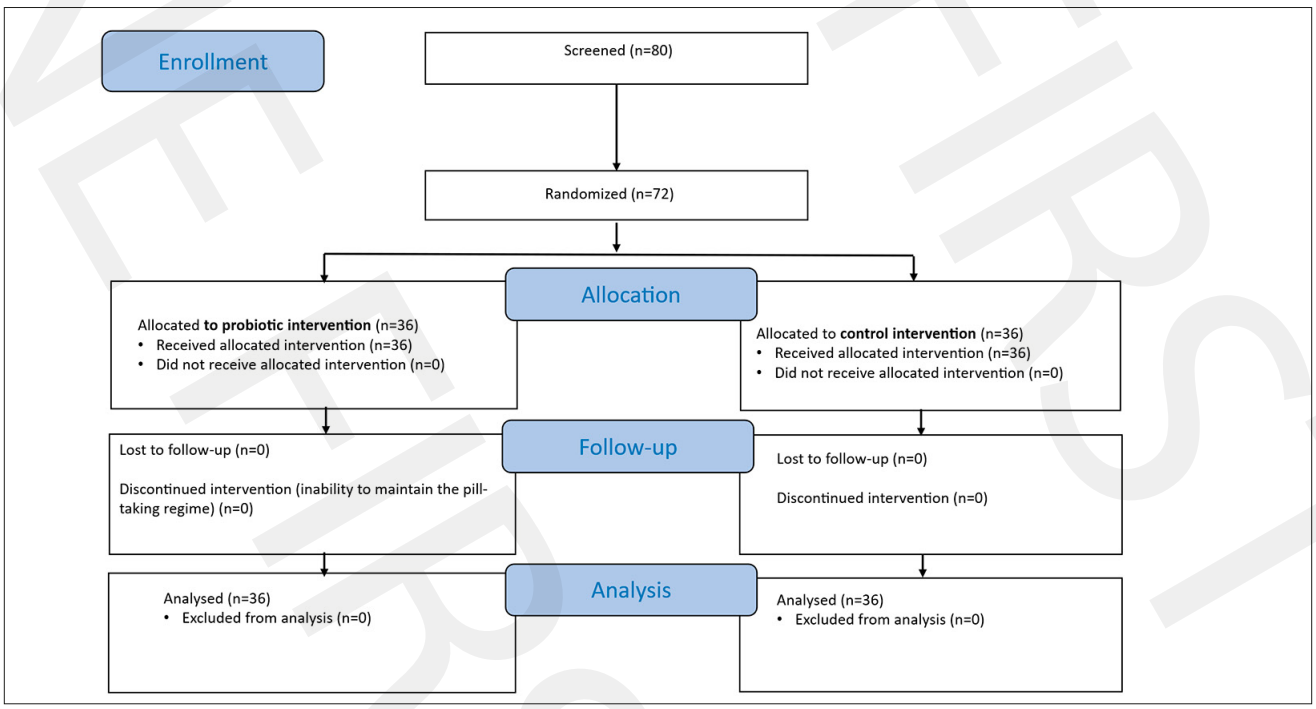


**Figure 1.** Research flow diagram

**Group characteristic and randomization.** The study group consisted of 80 healthy 3 – 6-year-old children with ECC were covered with screening, from which 8 were excluded due to not meeting the inclusion criteria. The remaining 72 children were included into the study and allocated to a study group (n=36) or the control group (n=36) after a randomization process (Fig. 1).

Inclusion criteria: not taking antibiotics or probiotics in any form for 3 months prior to the study, willing to chew the tablets and participate in the study, and written informed consent provided by parents or guardians. Exclusion criteria: children with severe infections, systemic diseases, weakened immune systems, congenital abnormalities, food allergies, inflammatory oral diseases, other oral diseases and periodontal pathology, and those who had had an orthodontic correction device installed. Other criteria for exclusion included the use of endogenic fluoride therapy, antibiotics, anti-inflammatory drugs or corticosteroids, probiotic products and supplements (vitamins, probiotics) within 3 months prior to the study, and partial or complete rejection of the dental examination by the child or legal guardian.

One of the researchers excluded from following stages of the study generated a random 1:1 allocation sequence using the IBM SPSS Statistics (version 27.0) programme, with the aid of computer-generated numbers (Excel randomization tool). Using random numbers, the participants were assigned to 1 of 2 parallel groups – the study (probiotic) group or to the control group. The participants were allocated 0 or 1 code. The code was kept by the same author in sealed envelopes to ensure allocation concealment, and was not disclosed until all data had been analyzed. The envelopes assigned to individual groups were delivered to the clinical centre. Other sealed bubble envelopes containing packets with tablets or empty packets, but with similar weight and marked with number codes, were used to keep the study blinded to the researchers caring for the participants, collecting plaque samples, and to the healthcare personnel of the clinic.

Before the start of the study, all parents and legal guardians of the recruited children received instructions according to the methods of brushing the children's teeth, and the appropriate amount of fluoridated toothpaste (1,000 ppm) to be used. They were asked to brush their child' teeth twice a day (morning and evening) during the entire study period, as the basis of home caries prophylaxis and an element of 'usual' care. The child's caregiver also recorded the hygiene procedures in a brushing booklet, and the child received a sticker on the board.

The children from the study group received commercially available chewable tablets containing 10 mg of heat-inactivated *L. salivarius* HM-6 Paradens. The tablets also included isomalt, sucralose, natural strawberry flavour, magnesium stearate and xylitol (200mg/1 tablet; Acidolac Dentifix Kids; Polpharma, Poland). The parents or guardians were instructed to give their child 2 tablets per day for 14 days – 1-after breakfast and 1 before bedtime, always after brushing their teeth according to the design of the clinical trial (ClinicalTrials.gov identifier, NCT number: 02752594). After intake of the tablets, ingestion of water or food was not allowed for 2 hours. The parents ort guardians were asked to return all non-used tablets to the clinical team. If ≤ 2 tablets per week were forgotten, compliance was rated as 'acceptable', and if this happened more frequently as 'questionable'. They were also asked to report to the clinical staff any possible harmful effects, sickness, or other adverse circumstances.

The control group was not administered any placebo tablets, but other hygienic and dietary recommendations (including compliance with appropriate breaks between meals) were the same in both groups.

## MATERIALS AND METHOD

Dental plaque bacteria were sampled at 2 identical time points – immediately before starting probiotic supplementation (baseline) and 2 weeks later, after its completion at the Department of Paediatric Dentistry, University Dental Clinic in Kraków. Material was collected from the fasted participants in the morning, 2 h after eating breakfast and toothbrushing, and after rinsing their mouths for 30 s with distilled water, but before any dental examination according to the Manual of Procedures for Human Microbiome Project (http://hmpdacc.org/resources/tools_protocols.php). Supragingival plaque samples from all tooth surfaces were collected using a sterile toothbrush mounted on the contra-angle of the dental unit's micromotor, which were then placed in 1 mL sterile saline pH 7.0 (PBS) at room temperature and transported within 2 h to the microbiology laboratory.

Plaque samples were then initially prepared for analysis by gentle vortexing and sonication for 30 s at room temperature. Serial dilutions of the stock solution were performed in sterile physiological saline. The 100 μL samples from the obtained dilutions were cultivated on 5% blood agar plates (Columbia medium) and the total viable count was estimated. Afterwards, the following selective media were used to allow the growth of certain groups of microorganisms while inhibiting the growth of others for further identification: CHROMagar™,chromogenic agar supporting the growth of Candida and yeast; BD MacConkey II selective agar for the isolation and differentiation of Enterobacteriaceae; BD™ LBS agar for the isolation and differentiation of Lactobacillus; chocolate agar plates for challenging Gram-negative bacteria; MacConkey agar; OPA agar plates.

Based on the colour and morphology of the yeast colonies on CHROMagarTM Candida, isolates were identified as Candida albicans. Inoculated media were incubated in microaerophilic conditions in the presence of 5% $CO_2$ at 37°C for 24–48 h. Bacterial and fungal species obtained during the cultivation were identified by means of MS MALDI TOF mass spectrometry (Bruker Daltonik, Germany), in accordance with methods described previously [18]. Isolated microorganism species were analyzed by mass spectrometry (MS) using a MALDI-TOF MS Biotyper 3.0 Microflex system (Bruker Biotyper; Bruker Daltonics, Bremen, Germany) connected to a database of microorganism profiles. Cultures in combination with MALDI-TOF are well established methods used in the identification of microorganisms from clinical specimens [19]. Identification of microbial species was possible by comparing obtained protein profiles with molecular mass, charge, and time-of-flight distribution spectra with reference spectra. The raw spectra were then analyzed automatically using the MALDI software package Biotyper 3.1 (Bruker Daltonics GmbH, Bremen, Germany, Biotyper® database version renewed 2020). Logarithmic results ranging from 0- 3.00 were obtained, which were interpreted according to the manufacturer's recommended

criteria of ≥1.7 as a reliable identification for the species, with <1.7 as a reliable identification at the genus level. The quantity (number of colony-forming units – CFU) of individual species of microorganisms were assessed at baseline and 2 weeks later, and the values obtained at both time points compared.

The primary measures of outcomes of the study were changes in *S. mutans* counts (the number of colony-forming units (CFU) and the percent of the total viable counts) in dental plaque biofilm within the groups of pre-school children between baseline and 2-weeks follow-up. Secondary outcomes were changes in *Lactobacilli spp.* and *C. albicans* counts – the number of colony-forming units (CFU) and the percent of the total viable counts in the dental plaque biofilm within the groups of pre-school children between baseline and 2-weeks follow-up.

**Statistical analysis.** All statistical analyses were performed using Statgraphics Centurion XIX. To account for variability in microbiological data, bacterial counts were log-transformed before statistical processing, a transformation that ensured a more normal distribution of the data, facilitating the application of parametric tests. For between-group comparisons, independent sample t-tests were employed to assess differences in log-transformed colony-forming unit (CFU) counts of *S. mutans, Lactobacillus spp.,* and *C. albicans* at baseline and after the 2-week intervention period. To evaluate within-group changes over time, paired t-tests were applied, determining the significance of microbial load variations in response to supplementation.

The prevalence of detectable microbial growth was analysed using the proportion test, which allowed comparisons of the frequency of *S. mutans, Lactobacillus spp.,* and *C. albicans* presence between the intervention and control groups. Additionally, regression analysis was conducted to explore relationships between bacterial species reduction rates. All statistical tests were 2-tailed, with a significance level set at $p < 0.05$. Data analyses were conducted following the principles of reproducibility and methodological rigour, aligning with current standards for clinical microbiological research.

## RESULTS

The baseline general, demographic and clinical characteristics of the children in the intervention group and the control group were compared to confirm the homogeneity of the 2 groups (Tab. 1). There were no statistically significant differences between the groups in age and gender distribution at the baseline (p=0.13 and p=0.43; p=0,50, respectively). The number of children with a detectable growth of *S. mutans* and *Lactobacillus spp.* was the same in both groups (61% and 53%, respectively), but not with any detectable growth of *C. albicans* (47% in probiotic and 89% in the control group). There were also no significant differences between the groups in the viable counts of *S. mutans* and *Lactobacillus spp.* at the start of the study, but there were significant differences between the groups in the viable counts of *C. albicans.* At baseline, the groups were also balanced according to the mean percentage of total cultivable microflora for *S. mutans* and *Lactobacillus spp.* (p=0.98 and p=0.80, respectively), but not for *C. albicans* (p=0.000) (Tab.1)

Microbial data at baseline and at the end of the study are shown in Tables 2–6. At baseline, the groups were not balanced according to the number of viable *C. albicans*, being significantly higher in the control group (p=0.000) (Tab. 2).

The number of viable *Streptococcus mutans* in the control and the probiotic group as well as *Lactobacillus spp.* in the control group were balanced at each time point (p=0.51, p=0.15 and p=0.39, respectively), but the number of viable *Lactobacillus spp.* in the probiotic, *C. albicans* in the control and the probiotic group differed significantly baseline and 2 weeks later (p=0.04; p=0.000 and p=0.001, respectively) (Tab. 3). Similarly, there was *C. albicans* prevalence of detectable growth in the probiotic group at both study points, but not in the control group (89% baseline vs. 47%, 2 weeks later) (Tab. 4). The proportion of *C. albicans* in relation to the total cultivable microflora (TVC) was lower in the intervention units compared with the control units baseline, and the difference reached statistical significance (p=0.0000) (Tab. 5). Similarly the difference between time points for the mean proportion of *Lactobacillus spp.* in relation to TVC in both the control and probiotic groups, and for the level of *C. albicans* in the probiotic group, reached statistical significance (Tab. 6).

**Table 1.** Baseline characteristics of the children in the probiotic and control group.

| Characteristic | | Control group | Probiotic group | control vs probiotic |
|---|---|---|---|---|
| **Participants (n)** | | **36** | **36** | |
| Male, n (%) | | 17 (43.59%) | 22 (56.41%) | p=0.43[1] |
| Female, n (%) | | 19 (57.58%) | 14 (42.42%) | p=0.50[1] |
| Age, mean±sd | | 4.39±0.69 | 3.97±1.08 | p=0.13[2] |
| $\log_{10}$ CFU/ml, mean±sd | *S. mutans* | 3.62±2.13 | 3.63±2.14 | p=0.98[2] |
| | *Lactobacillus spp.* | 3.27±2.18 | 3.25±2.16 | p=0.98[2] |
| | *C. albicans* | 3.66±0.96 | 2.36±1.46 | **p=0.000[2]** |
| Prevalence of detectable growth | *S. mutans* | 61% | 61% | |
| | *Lactobacillus spp.* | 53% | 53% | |
| | *C. albicans* | 89% | 47% | |
| Mean percentage (%) of total cultivable microflora (TVC) | *S. mutans* | 16.71±15.6 | 16.82±15.47 | p=0.98[2] |
| | *Lactobacillus spp.* | 13.01±13.05 | 12.24±12.74 | p=0.80[2] |
| | *C. albicans* | 1.14±0.57 | 0.44±0.51 | **p=0.000[2]** |

[1] proportion analysis test; [2] t-test independent sample; SD – standard deviation. **Boldface** indicates statistical significance (p<0.05) *Streptococcus mutans* = ***S. mutans;*** *Lactobacillus species*= ***Lactobacillus spp.*** *Candida albicans*= ***C. albicans***

**Table 2.** Number of viable microorganisms: *S. mutans, Lactobacillus spp., C. albicans* at baseline and at end of the study

| | Group | N | Mean | Median | SD | Min | Max | t-test for independent samples |
|---|---|---|---|---|---|---|---|---|
| *S. mutans* $\log_{10}$ CFU/ml, baseline, mean±sd | control | 36 | 3.62 | 5.2 | 2.13 | 1 | 5.82 | p=0.98 |
| | probiotic | 36 | 3.63 | 5.22 | 2.14 | 1 | 5.82 | |
| *S. mutans* $\log_{10}$ CFU/ml, 2 wks, mean±sd | control | 36 | 3.62 | 5.2 | 2.13 | 1 | 5.52 | p=0.99 |
| | probiotic | 36 | 3.62 | 5.24 | 2.13 | 1 | 5.51 | |
| *actobacillus spp.* $\log_{10}$ CFU/ml, baseline, mean±sd | control | 36 | 3.27 | 5.08 | 2.18 | 1 | 5.45 | p=0.98 |
| | probiotic | 36 | 3.25 | 5 | 2.16 | 1 | 5.45 | |
| Lactobacillus spp. $\log_{10}$ CFU/ml, 2 wks, mean±sd | control | 36 | 3.26 | 5.04 | 2.17 | 1 | 5.45 | p=0.99 |
| | probiotic | 36 | 3.26 | 5.08 | 2.17 | 1 | 5.46 | |
| *C.albicans* $\log_{10}$ CFU/ml, baseline, mean±sd | control | 36 | 3.66 | 4.02 | 0.96 | 1 | 4.02 | p=0.000 |
| | probiotic | 36 | 2.36 | 1 | 1.46 | 1 | 4.2 | |
| *C.albicans* $\log_{10}$ CFU/ml, 2 wks mean±sd | control | 36 | 2.4 | 1 | 1.52 | 1 | 4.92 | p=0.87 |
| | probiotic | 36 | 2.34 | 1 | 1.44 | 1 | 4.15 | |

T test independent sample; SD – standard deviation. **Boldface indicates statistical significance (p<0)**

**Table 3.** Number of viable microorganisms in each group at each time point

| | Group | N | Mean | Median | SD | Min | Max | paired t-test |
|---|---|---|---|---|---|---|---|---|
| *S. mutans* $\log_{10}$ CFU/ml, baseline, mean±sd | control | 36 | 3.62 | 5.2 | 2.13 | 1 | 5.82 | p=0.51 |
| *S. mutans* $\log_{10}$ CFU/ml, 2 wks, mean±sd | control | 36 | 3.62 | 5.2 | 2.13 | 1 | 5.52 | |
| *S. mutans* $\log_{10}$ CFU/ml, baseline, mean±sd | probiotic | 36 | 3.63 | 5.22 | 2.14 | 1 | 5.82 | p=0.15 |
| *S. mutans* $\log_{10}$ CFU/ml, 2 wks, mean±sd | probiotic | 36 | 3.62 | 5.24 | 2.13 | 1 | 5.51 | |
| *Lactobacillus spp.* $\log_{10}$ CFU/ml, baseline, mean±sd | control | 36 | 3.27 | 5.08 | 2.18 | 1 | 5.45 | p=0.39 |
| *Lactobacillus spp.* $\log_{10}$ CFU/ml, 2 wks, mean±sd | control | 36 | 3.26 | 5.04 | 2.17 | 1 | 5.45 | |
| *Lactobacillus spp.* $\log_{10}$ CFU/ml, baseline, mean±sd | probiotic | 36 | 3.25 | 5 | 2.16 | 1 | 5.45 | p=0.04 |
| *Lactobacillus spp.* $\log_{10}$ CFU/ml, 2 wks, mean±sd | probiotic | 36 | 3.26 | 5.08 | 2.17 | 1 | 5.46 | |
| *C. albicans* $\log_{10}$ CFU/ml, baseline, mean±sd | control | 36 | 3.66 | 4.02 | 0.96 | 1 | 4.02 | p= 0.000 |
| *C. albicans* $\log_{10}$ CFU/ml, 2 wks, mean±sd | control | 36 | 2.4 | 1 | 1.52 | 1 | 4.92 | |
| *C. albicans* $\log_{10}$ CFU/ml, baseline, mean±sd | probiotic | 36 | 2.36 | 1 | 1.46 | 1 | 4.2 | p=0.001 |
| *C. albicans* $\log_{10}$ CFU/ml, 2 wks, mean±sd | probiotic | 36 | 2.34 | 1 | 1.44 | 1 | 4.15 | |

Paired t test; SD – standard deviation. Boldface indicates statistical significance (p<0.0)

**Table 4.** Prevelance of detectable growth of *S. mutans, Lactobacillus spp.* and *C. albicans* at baseline and at end of the study

| | Group | N | n | % |
|---|---|---|---|---|
| *S. mutans* prevelance of detectable growth, baseline, % | control | 36 | 22 | 61 |
| | probiotic | 36 | 22 | 61 |
| *S. mutans* prevelance of detectable growth, 2 wks, % | control | 36 | 22 | 61 |
| | probiotic | 36 | 22 | 61 |
| *Lactobacillus spp.* prevelance of detectable growth, baseline, % | control | 36 | 19 | 53 |
| | probiotic | 36 | 19 | 53 |
| *Lactobacillus spp.* prevelance of detectable growth, 2 wks, % | control | 36 | 19 | 53 |
| | probiotic | 36 | 19 | 53 |
| *C. albicans* prevelance of detectable growth, baseline, % | control | 36 | 32 | 89 |
| | probiotic | 36 | 17 | 47 |
| *C. albicans* prevelance of detectable growth, 2 wks, % | control | 36 | 17 | 47 |
| | probiotic | 36 | 17 | 47 |

N – total number, n-number of children with detectable levels of bacteria

**Table 5.** Mean percentage (%) of total cultivable microflora (TVC) between the control and probiotic groups.

| | Group | N | Mean | Median | SD | Min | Max | t-test independent samples |
|---|---|---|---|---|---|---|---|---|
| *S. mutans* mean percentage (%) of total cultivable microflora (TVC), baseline, mean±sd | control | 36 | 16.71 | 20.35 | 15.6 | 0 | 50.21 | p=0.98 |
| | probiotic | 36 | 16.82 | 21.65 | 15.47 | 0 | 46.32 | |
| *S. mutans* mean percentage (%) of total cultivable microflora (TVC), 2 wks, mean±sd | control | 36 | 16.84 | 21.74 | 15.68 | 0 | 51.1 | p=0.98 |
| | probiotic | 36 | 16.73 | 21.19 | 15.6 | 0 | 44.8 | |
| *Lactobacillus spp.* mean percentage (%) of total cultivable microflora (TVC), baseline, mean±sd | control | 36 | 13.01 | 15.34 | 13.05 | 0 | 35.29 | p=0.80 |
| | probiotic | 36 | 12.24 | 12.71 | 12.71 | 0 | 39.23 | |
| *Lactobacillus spp.* mean percentage (%) of total cultivable microflora (TVC), 2 wks, mean±sd | control | 36 | 12.66 | 14.2 | 12.67 | 0 | 33.17 | p=0.98 |
| | probiotic | 36 | 12.74 | 14.14 | 12.97 | 0 | 35.83 | |
| *C. albicans* mean percentage (%) of total cultivable microflora (TVC), baseline, mean±sd | control | 36 | 1.14 | 1.2 | 0.57 | 0 | 2.33 | p=0.0000 |
| | probiotic | 36 | 0.44 | 0 | 0.51 | 0 | 1.56 | |
| *C. albicans* mean percentage (%) of total cultivable microflora (TVC), 2 wks, mean±sd | control | 36 | 0.83 | 0 | 1.95 | 0 | 9.3 | p=0.2 |
| | probiotic | 36 | 0.4 | 0 | 0.46 | 0 | 1.43 | |

T test independent sample; SD – standard deviation. **Boldface** indicates statistical significance (p<0.05)

**Table 6.** Mean percentage (%) of total cultivable microflora (TVC) at each time point

| | Group | N | Mean | Median | SD | Min | Max | Test t-paired samples |
|---|---|---|---|---|---|---|---|---|
| *S. mutans* mean percentage (%) of total cultivable microflora (TVC). baseline. mean±sd | control | 36 | 16.71 | 20.35 | 15.6 | 0 | 50.21 | p=0.65 |
| *S. mutans* mean percentage (%) of total cultivable microflora (TVC). 2 wks. mean±sd | control | 36 | 16.84 | 21.74 | 15.68 | 0 | 51.1 | |
| *S. mutans* mean percentage (%) of total cultivable microflora (TVC). baseline. mean±sd | probiotic | 36 | 16.82 | 21.65 | 15.47 | 0 | 46.32 | p=0.75 |
| *S. mutans* mean percentage (%) of total cultivable microflora (TVC). 2 wks. mean±sd | probiotic | 36 | 16.73 | 21.19 | 15.62 | 0 | 44.8 | |
| *Lactobacillus spp.* mean percentage (%) of total cultivable microflora (TVC). baseline. mean±sd | control | 36 | 13.01 | 15.34 | 13.05 | 0 | 35.29 | p=0.07 |
| *Lactobacillus spp.* mean percentage (%) of total cultivable microflora (TVC). 2 wks. mean±sd | control | 36 | 12.66 | 14.2 | 12.67 | 0 | 33.17 | |
| *Lactobacillus spp.* mean percentage (%) of total cultivable microflora (TVC). baseline. mean±sd | probiotic | 36 | 12.24 | 12.71 | 12.71 | 0 | 39.23 | p=0.052 |
| *Lactobacillus spp.* mean percentage (%) of total cultivable microflora (TVC). 2 wks. mean±sd | probiotic | 36 | 12.74 | 14.14 | 12.97 | 0 | 35.83 | |
| *C. albicans* mean percentage (%) of total cultivable microflora (TVC). baseline. mean±sd | control | 36 | 1.14 | 1.2 | 0.57 | 0 | 2.33 | p=0.36 |
| *C. albicans* mean percentage (%) of total cultivable microflora (TVC). 2 wks. mean±sd | control | 36 | 0.83 | 0 | 1.95 | 0 | 9.3 | |
| *C. albicans* mean percentage (%) of total cultivable microflora (TVC). baseline. mean±sd | probiotic | 36 | 0.44 | 0 | 0.51 | 0 | 1.56 | p=0.005 |
| *C. albicans* mean percentage (%) of total cultivable microflora (TVC). 2 wks. mean±sd | probiotic | 36 | 0.4 | 0 | 0.46 | 0 | 1.43 | |

Paired t test; SD – standard deviation. **Boldface indicates statistical significance (p<0.05)**

For analysis performed only for children who had detectable bacterial counts at baseline, the change in mean bacterial count after 2 weeks is shown in Figure 2 a-c.

Analysis of the reduction in the level of microorganisms showed that for *S. mutans* the log10 CFU/ml level increased in the control group and decreased in the probiotic group, for *Lactobacillus spp.* the log10 CFU/ml level increased in the probiotic group and decreased in the control group, and for *C. albicans* the log10 CFU/ml level decreased in both the control and probiotic groups after 2 weeks. The same results can be presented as % reduction and similarly as above, if the percentage is positive it means reduction and negative it means 'no reduction', i.e. increase (Fig. 2 d).

Comparison of regression lines for the percentage reduction in *S. mutans* (SM) and *Lactobacillus spp.* levels between the control (0) and probiotic (1) groups reveals distinct trends. In the control group, there was a direct proportional relationship between the reduction of *S. mutans* and *Lactobacillus spp.* This indicates that a greater reduction in *S. mutans* was associated with a greater reduction in *Lactobacillus spp.* Conversely, in the probiotic group, the relationship was inversely proportional to a lower reduction in *Lactobacillus spp* corresponding to a higher reduction in *S. mutans.* The difference in the regression line slopes between the 2 groups was statistically significant (p = 0.0211). The regression equations for these groups are as follows: control group: *S. mutans* proc_red= −2.80048+0.528238× *Lactobacillus spp* proc_red. and probiotic group: *S. mutans* proc_red=1.09689−0.293596× *Lactobacillus spp* proc_red. (Fig. 2e).

## DISCUSSION

Probiotics are increasingly being recognized for their potential to modulate the oral microbiome and inhibit pathogenic microorganisms, such as *S. mutans* and *C.albicans,* which are associated with dental caries in children. The use of probiotics, which are designed to protect the native microflora, is in line with the concept of searching for potential new agents for

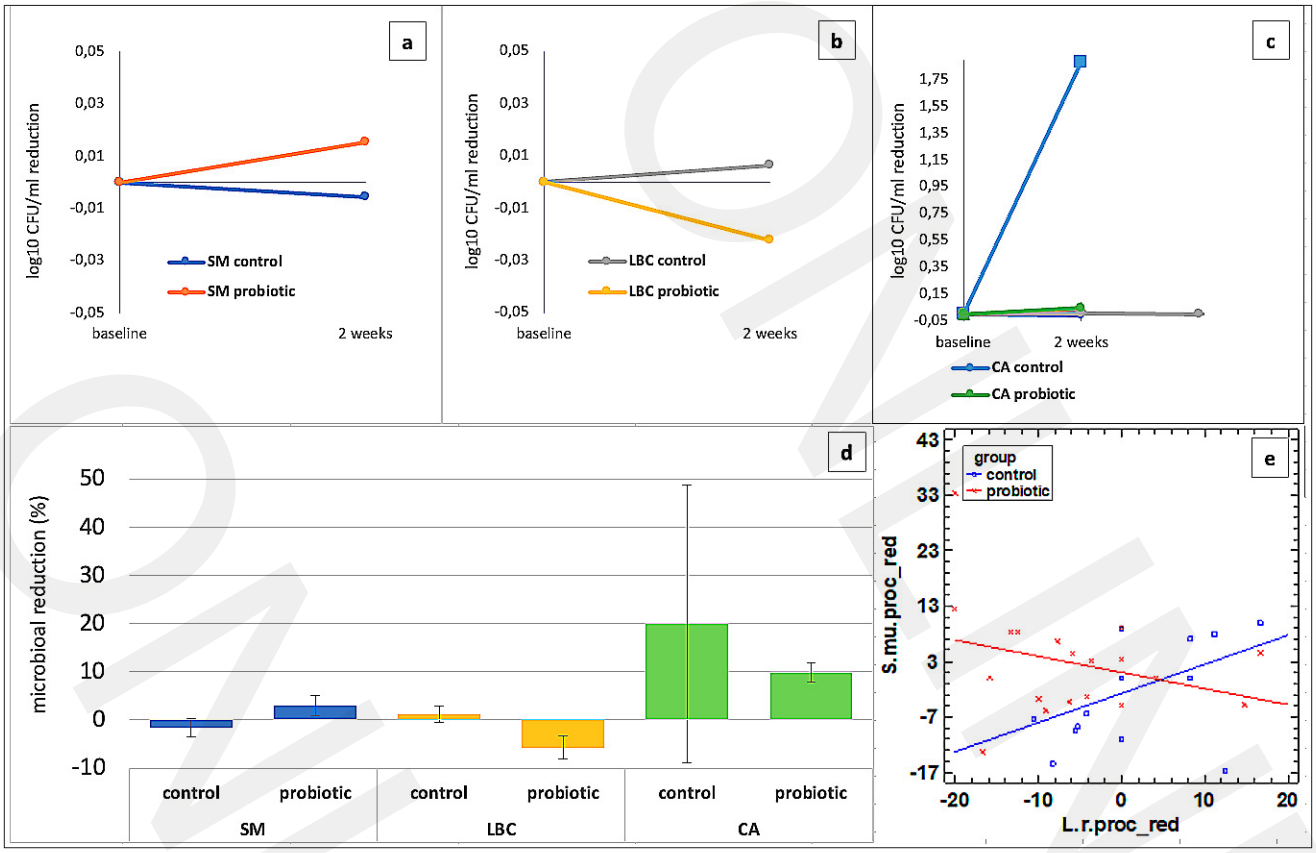


**Figure 2.** Results of reducing the level of *S. mutans* (a), *Lactobacillus spp.*(b), *Candida albicans* (c). Mean microbial reduction (%),whiskers indicate SE (standard error) (d). Comparison of regression lines for the percentage reduction in *Streptococcus mutans* (S. mu) and *Lactobacillus spp.* (L.r) levels between the control (0) and probiotic (1) groups reveals distinct trends (e). Analyses performed only for children who had detectable bacterial counts at baseline (a-e).

the prevention of early childhood caries (ECC) by restoring the correct ratio of physiological to cariogenic flora [20].

The research results concerning children under 6 years of age, showed a significant decrease in *S. mutans* after the use of different probiotic strains [21]. Similarly, a significant reduction in caries risk in children and adults by inhibiting caries bacteria and enriching commensal microorganisms in the oral cavity was shown by Sivamaruthi et al. [22].

There are considerable differences with some studies evaluating the short-term effect after 7–14 days of consumption, and others on the impact of long-term consumption between 6–9 months. The mostly short-term effect was due decrease of level *Streptococcus spp.*, and *Lactobacillus spp.* and long term-intake achieving no significant effects in adults. Between children, the results are more divergent, and depend not only on the duration of intake, but also on the form of consuming the probiotic. Some studies report a positive effect of probiotic consumption (probiotic intake as a mouthwash, milk or cereals) on supra-gingival plaque and salivary levels of *S. mutans* and/or *Lactobacillus spp.* [23, 24]. Notably, a common feature in studies reporting a positive effect was the evaluation of the effect immediately after short-term intake (7–14 days) [25]. In the presented study, the probiotic was administered for 2 weeks, also a short observation period, and analysis of the reduction in the level of microorganisms showed that for *S. mutans* the log10 CFU/ml level increased in the control group and decreased in the probiotic group, for *Lactobacillus spp.* the log10 CFU/ml level increased in the probiotic group and decreased in the control group.

The results of an *in vitro* study on the probiotic strain *L. salivarius* (HM6 Paradens), in which it was shown to reduce the formation of a 2-species biofilm of *S. mutans* and *C. albicans*, suggest that this bacterium competes with *S. mutans* for nutrient substrates, and also inhibits the aggregation of oral streptococci and yeast [21]. In addition, it may produce signalling molecules or indirectly inhibit caries-forming dual-species biofilm formation by inhibiting *S. mutans*. Attention was also drawn to the probiotic's mechanism of action related to the inhibition of proteolytic enzymes, i.e. aspartyl proteases (Saps) of *C. albicans*, which are considered to be a major factor in bacterial-fungal interactions. *L. salivarius* inhibited the morphological transformation of pathogenic fungi in a co-culture of *S. mutans* and *C.albicans*, strains isolated from children with early childhood caries, and therefore reduced the pathogenicity of *C. albicans*. The inhibitory effects of probiotics are attributed to both direct interactions with *C. albicans*, cells and the secretion of metabolites that impact its pathogenic attributes. From that data, it can be concluded that probiotics – particularly strains like *L. plantarum, S. salivarius, and S. thermophilus* – have shown significant potential in reducing *C. albicans* growth and biofilm formation in saliva.

In relation to the interpretation of the results of own pilot study for C. albicans, the prevalence of detectable growth differ in the control group (89% baseline vs. 47%, 2 two weeks later). The proportion of *C. albicans* in relation to the total cultivable microflora was lower in the intervention units compared with the control units baseline, and the

difference reached statistical significance. The difference in *C.albicans* levels between the study and control groups at the first measurement point may be attributed to individual variability in oral microbiota composition, which can be influenced by a range of factors, including diet, oral hygiene habits, previous antibiotic use, or environmental exposure – even in clinically healthy individuals. Although the groups were matched for general health and age, such microbiological variations can still occur naturally. *C.albicans* the log10 CFU/ml level decreased in both the control and probiotic groups after 2 weeks. Additionally, the study found significant association between the presence of *C. albicans* and ECC. In both groups (study and control), a decrease in the average % of *S. mutans* was observed over the 2 week period.

Based on the results of own research, it was concluded that the reduction in the levels of *S. mutans* in the control group was caused by improved hygiene, i.e. mechanical cleaning of dental plaque from teeth as a result of detailed hygiene instructions given to parents before the examination, and the motivation of the children to perform these procedures.

In summary, the findings of the study indicate that probiotics selectively reduce *S. mutans* counts and restore plaque microbiome balance, and are consistent with recent studies and meta-analyses. Concurrently, the significant reduction in *S. mutans* counts observed in the control group after parental/caregiver guidance on oral hygiene, supports randomized evidence that supervision improves oral hygiene behaviours in children of pre-school age. Compared with fluoride prophylaxis, the pillar of ECC prevention, the date in the presented study confirms that fluoride varnishes, and toothpastes provides the greatest and most consistent benefits when closely integrated with supervised care. In the Polish context of high ECC burden, comprehensive programmes combining caregiver-focused health care and fluoride with supplemental probiotics for selected high-risk children seem the most appropriate [24].

## CONCLUSIONS

Short-term oral supplementation of non-viable *Lactobacillus salivarius* selectively influences the interactions between *Lactobacillus spp.* and *S. mutans* in the supra-gingival plaque biofilm. The use of probiotics inhibits the reduction of beneficial *Lactobacillus spp.* while contributing to a greater reduction in *S. mutans*. This relationship may play a significant role in the prevention of caries by stabilizing the oral ecosystem.

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