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Original Article

Evaluation of the effects of *Streptococcus salivarius* M18 and K12 probiotic bacteria on the *Streptococcus mutans* in saliva: A randomized clinical trial

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ABSTRACT

Background: Various methods, including the use of probiotics, have been suggested to prevent caries. Caries, which is mainly caused by *Streptococcus mutans*, is one of the bacterial diseases that imposes a heavy cost on society. The present study was conducted to investigate the probiotic products available in Iranian pharmacies that are used for caries prevention.

Materials and Methods: In this double-blind randomized clinical trial, 40 students of medicine and pharmacy were randomly allocated to two equal groups of intervention and control using random allocation software. The intervention group used a probiotic pill containing *Streptococcus salivarius* M18 and K12 bacteria every night before going to bed. The control group used a mouth freshener tablet with the same flavor as the probiotic tablet every night before going to bed. The data were analyzed by SPSS (version 24) software using descriptive statistics (central tendency and dispersion) and inferential statistics (paired *t*-test and independent *t*-test). Data were collected using Excel software, and statistical analyses were performed by SPSS software (version 24).

Results: The mean number of *S. mutans* in the intervention group was 754.5 cfu/mm before the intervention and 1701.5 cfu/mm after the intervention, which showed a statistically significant difference ($P < 0.05$). In the control group, the mean *S. mutans* was 683 cfu/mm at the beginning of the intervention and 659 cfu/mm at the end of the intervention, which did not indicate a statistically significant difference ($P > 0.05$). Moreover, the normality of data was checked by the Kolmogorov–Smirnov test.

Conclusion: The mean number of *S. mutans* bacteria in the group using probiotic tablets was significantly increased compared to those of the control group. However, further studies are suggested to evaluate these products.

Key Words: Clinical trial, dental caries, probiotics, *Streptococcus mutans, Streptococcus salivarius*

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INTRODUCTION

Dental caries is defined as a bacterial disease in the calcified tissues of the tooth and is characterized by the depletion of minerals and destruction of the organic material of the tooth. When sucrose

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is consumed repeatedly, an organism known as *Streptococcus mutans* emerges as the dominant organism, which is uniquely associated with dental caries.[1] Plaque bacteria that ferment sucrose produce acids that lower the pH level to below 5.0 *in vitro* and cause enamel demineralization. However, only *S. mutans* of all these species significantly cause caries in germ-free animals with a high-sucrose diet. This shows that microbial acid production is not the sole determinant of caries, and *S. mutans* must have other characteristics that are responsible for its severity and make it the main cause of caries.[1] *S. mutans* form several complex glucans such as fructans, dextrans, and mutans. *In vitro* experiments have shown that these glucans enable *S. mutans* to adhere firmly to surfaces and cause tooth decay.[1] Adding fluoride to drinking water, producing different mouthwashes, and using pit and fissure sealants, topical products such as fluoride gels and varnishes, xylitol emulsifiers, and probiotics are among the procedures to prevent caries.

According to the definition of the World Health Organization (WHO), probiotics are living microorganisms that are prescribed in sufficient amounts and provide health benefits to the host.[2] *Streptococcus salivarius* M18 and K12 (S.S. K12, S.S. M18) are probiotic bacteria used in various food products. It has been shown that these species of *S. salivarius* are capable of producing bacteriocin and have a narrow range of effects on preventing the growth of some other bacteria.[3]

S.S. M18 is able to reduce the number of *S. mutans,* but has no specific effect on the health of the gingiva and periodontal tissues.[4] This caries prevention mechanism is attributed to the ability of S.S. M18 to produce urease and dextranase enzymes. These enzymes are able to deal with the formation of dental plaque and prevent caries.[5,6] It has also been shown that S.S. K12 is effective in preventing and treating diseases such as halitosis, acute otitis media, pharyngotonsillitis, and oral candidiasis by producing bacteriocin.[7-9] A study on the effect of S.S. M18 on the risk of caries and oral health showed a reduction in the amount of *S. mutans* and dental plaque.[10] In another study, the comparison of saliva sample cultures at the beginning and end of the intervention showed a slight difference in the number of *S. mutans* between the two groups and between each group compared to the baseline. However, nine children who had higher M18 salivary bacteria in their mouths showed a significant decrease in the number of *S. mutans*. Moreover, 87.5% of M18 salivary bacteria users who had a large amount of plaque at the beginning of the intervention showed a significant decrease in plaque formation, while those with a large plaque in the placebo group showed a 44% decrease in plaque formation.[4] Most of the studies conducted on probiotics have shown that their oral and dental effects are highly dependent on the type of probiotic bacteria used, and few studies have been done in this field, especially on S.S. K12 and S.S. M18 probiotic bacteria. Furthermore, very few academic studies have been done on Iranian probiotic products as well as their effects on the prevention of caries, which requires further studies of this type.

MATERIALS AND METHODS

Subjects

This double-blind randomized clinical trial was conducted on 40 students of medicine and pharmacy, with the age range of 19–35 years, at Isfahan University of Medical Sciences. A checklist of subjects was prepared at the beginning and end of this study. The exclusion criteria were students with orthodontic appliances or 6-month orthodontic treatments, students who had taken antibiotics in the last month, students undergoing incomplete dental treatments, and students with immune system diseases. Further, students who needed dental treatment during the intervention, used <90% of the consumables, needed to take antibiotics during the intervention, got bacterial and viral diseases during the intervention, and changed the number of times they brushed their teeth and the type of toothpaste and mouthwash or used a new oral hygiene method were excluded after the study. As shown in Figure 1, 47 volunteers enrolled for this study and 7 of them were excluded due to the study exclusion criteria. Four were undergone dental treatment and 3 had orthodontic appliances. The participants' flow diagram is represented in Figure 1.

Randomization and blinding

The subjects were randomly divided into two groups of 20 people each. To ensure the participants in both groups were equal in number, the block randomization method was used. The size of all the blocks was equal, and there were 40 blocks, including 20 participants in the intervention group (probiotic consumers) and 20 participants in the control group (mouth freshener tablets) in this two-group trial.

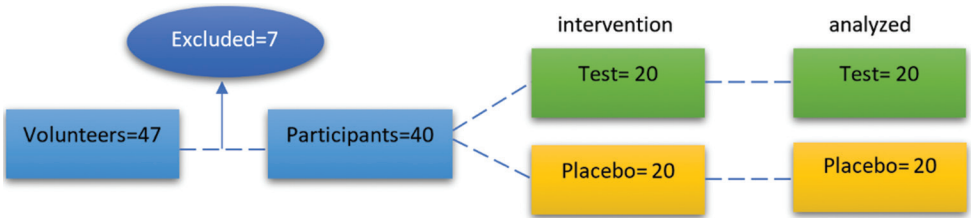

Figure 1: The participants flow diagram.

The randomization tool used was the random allocation software (version 2.0), which is able to perform block randomization in addition to simple randomization. Allocation concealment, which is used to implement a random sequence on the participants, was also used so that the assigned group was not known before the allocation of the individual. Sequentially, numbered, sealed, opaque envelopes were used, in which each of the random sequences created is recorded on a card, and the cards are placed inside the envelopes, respectively. To maintain a random sequence, the outer surface of the envelopes is numbered in the same order. Finally, the envelopes are glued and placed in a box. At the time of the participants' registration, based on the order, in which the eligible participants were included in the study, one of the envelopes was opened and the allocated group of that participant was revealed. In this study, the double-blind method was used. For this purpose, the participants did not know whether the product they were using contained salivary probiotics or xylitol fresheners and the products were not delivered to them in the company's original packaging. Furthermore, the statistician who analyzed the data was blind to the participants' information. The randomization and blinding process were done by the main author.

Products

During the equalization of oral hygiene before and during the study, signal toothpaste with a fluoride dose of 1450 ppm, manufactured in Iran and licensed by the Food and Drug Organization under license number 1515/Z/38, was used. The control group received xylitol freshener tablet (Iceberg tablet, a product of Shiva company, manufactured in Iran under health license number 7009/Z/56), and the intervention group received a probiotic tablet containing *S. salivarius* M18 and K12 (Lactogam tablet, Zist Takhmir Company, Company). It should be noted that the amount of xylitol in the mouth freshener tablets is much lower than the therapeutic and effective dose of xylitol and has no effect on the number of *S. mutans*. Both products have a mint flavor.

Ethical considerations

This study has been registered in the Ethics Committee of Isfahan University of Medical Sciences, with the ethics code IR.MUI.RESEARCH.REC.1400.297. It has also received the scientific code IRCT20210811052147N1 from the Center for Clinical Trials of Iran. All the information of the participants in this study was kept confidential, and only the main authors were aware of it. Moreover, the participants were fully aware of the research conditions. The results of the study were provided to the participants after the intervention.

Intervention

According to the instructions of the probiotic product, the intervention group sucked one probiotic tablet every night before going to bed and after brushing their teeth for 20 days. The control group sucked a mouth freshener every night after brushing their teeth before going to bed for 20 days. Further, both groups were required not to make any new changes in their oral hygiene habits and to continue the training process before the intervention. The preintervention process means that they should not change the number of times they brush their teeth, or if they did not use mouthwash or dental floss, they would not do this during this 20-day period and should follow the instructions given. Participants were informed of all these conditions, and these health habits were determined through a checklist before and after the intervention. The oral health level before and after the study was determined by the plaque index using the common Silness–Loe method based on the WHO Oral Health Surveys, 5th Ed., 2013. The students were contacted by phone once every 5 days and their cooperation was ensured. Furthermore, a text message was sent to the participants every night to remind them of taking probiotic pills and freshener tablets.

Collection and culturing of salivary samples

At the beginning and end of the intervention, unstimulated saliva samples of both groups were

collected in sterile containers and cultured. To collect unstimulated saliva, the participants were asked to sit and rest for at least 2 h after consuming food and water between 9 and 11 am and pour their saliva samples into sterile and dry polyethylene vials through spitting and without chewing. The samples were collected at the Dentistry Facility of Isfahan University of Medical Sciences.

The TYCSB medium, which is a selective medium for *S. mutans*, was used to culture the bacteria. Then, 0.1 mL of each sample's saliva was placed on the culture medium and cultured by a sterile loop in a linear manner. The plates were placed in an incubator at 35°C–37°C for 48–72 h under 5% carbon dioxide and 95% nitrogen. After incubation, the suspicious colonies were removed, heat-stained, and evaluated in terms of the presence of Gram-positive cocci. Then, catalase test was performed to confirm *Streptococcus*, and if catalase was negative, the presence of *S. mutans* was confirmed by performing differential sugar tests, including sorbitol and mannitol. Since the TYCSB culture medium is a specific medium for the growth of *S. mutans*, only this bacterium was expected to grow on the medium. Then, colony counting was performed, and the number of bacterial colonies was calculated based on cfu/mL and compared in both groups before and after the intervention. The samples were collected by the main author.

Statistical analysis

The data were collected through Excel software and analyzed by SPSS software (version 24, IBM, USA) using descriptive statistics (central tendency and dispersion) and inferential statistics (paired *t*-test and independent *t*-test). Furthermore, the normality of the data was checked using the Kolmogorov–Smirnov test. Since 0.1 mL of each sample's saliva was cultured, the number of bacteria at the end of counting was multiplied by 10 to report it based on the CFU/mm unit.

RESULTS

According to Table 1, based on the results of the Kolmogorov–Smirnov test, the data were normally distributed ($P > 0.05$). For inferential analysis, parametric paired and independent *t*-tests were used.

comparison of mean scores of research variables was done using the paired *t*-test.

In the control group, the mean score of *S. mutans* was 683 ± 420.9 before the intervention and 659 ± 360.9 after the intervention ($P > 0.05$). Therefore, the number of *S. mutans* in the control group did not change before and after the intervention. In the intervention group, the mean score of *S. mutans* was 754.5 ± 537.7 before the intervention and 1701.5 ± 926.7 after the intervention ($P < 0.05$). Therefore, in the intervention group, the number of *S. mutans* increased significantly after the intervention [Table 2].

The mean *S. mutans* in the first period was compared between the control and intervention groups, which showed no statistically significant difference ($P > 0.05$). The mean *S. mutans* in the second period was compared between the control and intervention groups, which indicated a statistically significant difference ($P < 0.05$) Table 3.

DISCUSSION

The present study is one of the first studies on probiotic products made in Iran and their effectiveness in preventing dental caries. The results showed that the number of *S. mutans*, which is one of the known factors in causing dental caries, and the dental plaque index in the intervention group that received probiotic

Table 1: Results of Kolmogorov–Smirnov test to check the normality of the data

Group	Sm 1	Sm 2
Observational		
N	20	20
Normal parameters, mean±SD	683±420.977	659±360.991
Test statistic	0.168	0.160
P	0.141	0.192
Intervention		
N	20	20
Normal parameters, mean±SD	754.500±537.797	1701.50±926.773
Test statistic	0.177	0.131
P	0.101	0.200

SD: Standard deviation; Sm: *Streptococcus mutans*

Table 2: Comparison of mean and standard deviation of *Streptococcus mutans* in each group before and after the intervention and their test results

Group	Mean±SD	*P*
Control		
Sm 1	683±420.97	0.303
Sm 2	659±360.99	
Test		
Sm 1	754.5±537.79	0.000
Sm 2	1701.5±926.77	

SD: Standard deviation; Sm: *Streptococcus mutans*

Table 3: Comparison of the average of *Streptococcus mutans* in the test and control groups with each other before and after the intervention

Group	Mean±SD	*P*
Sm 1		
Observational	683±420.97	0.642
Interventional	754.5±537.79	
Sm 2		
Observational	659±360.99	0.000
Interventional	1701.5±926.77	
Interventional	0.7590±0.406	

SD: Standard deviation; Sm: *Streptococcus mutans*

tablets (*S. salivarius* bacteria M18 and K12) were significantly increased. Di Pierro *et al*. reported that the 90-day consumption of the probiotic *S. salivarius* M18 could prevent dental caries,[10] which is completely different from the results of the present study. It should be noted that the products used in the present study are made in Iran and the manufacturer of the products is different from that of the study mentioned above.

Moreover, Söderling (2011) *et al*. demonstrated that four types of probiotic products could effectively reduce the number of *Streptococcus* bacteria,[11] which is not in line with the results of the present study. Further, Söderling (2011) *et al*. reported that the *in vitro* antibacterial activity of probiotic products against *S. mutans* is highly dependent on the pH of the environment, and the increase in the number of *S. mutans* in the present study can be because this product may have changed the pH of the environment in favor of *S. mutans*.

Contrary to the results of the current study, Burton *et al*. found probiotic products to be effective in reducing caries.[4] In the present study, the probiotic bacteria used may make food more easily accessible to harmful bacteria in the mouth and teeth, thereby increasing their population. In a systematic review conducted by Poorni *et al*. in 2019, the quality of the articles on this subject was very poor, so they recommended further studies in this regard.[12]

Babina *et al*. indicated that the probiotic product had a positive effect on reducing microbial plaque contamination, which is again different from the results of the present study.[13] In the present study, the probiotic bacteria used may have changed the pH of the environment, thereby balancing the normal flora of the area (Echo logic Nich change) and ultimately increasing harmful oral bacteria.

CONCLUSION

The mean number of *S. mutans* in the group using these Iranian probiotic tablets was significantly increased compared to the control group. Accordingly, further research is suggested to evaluate these Iranian products.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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