RESEARCH ARTICLE

Comparative Evaluation of Efficacy of Kefir Milk Probiotic Curd and Probiotic Drink on *Streptococcus mutans* in 8–12-year-old Children: An *In Vivo* Study

Saida Reddy[1], Vasepalli Madhu[2], Rachuri Punithavathy[3], Martha Satyam[4], Uday Kumar Chowdary[5], Raparla Mythraiye[6]

Abstracts

Aim and objective: To compare the effect of probiotic products in reducing the levels of salivary *Streptococcus mutans* before and after their consumption.

Materials and methods: Eighty school children with initial carious lesions in the age group of 8–12 years were selected and divided into four groups. Children in group I (control) were undergone with restorations without supplementation of probiotics. Children in group II (kefir milk), group III (probiotic curd), and group IV (probiotic drink) supplemented 100 mL of their respective probiotics for 1 month. Assessment of saliva sample was done at baseline, 1 hour after administration of probiotics followed by weekly intervals till 1 month.

Results: The study showed a marked reduction in colony-forming units (CFUs) at a 1-hour time interval in all four groups when compared to baseline. On the 30th-day, children in group II and group III have shown an equal reduction of CFUs when compared to group IV and group I.

Conclusion: Probiotic products like kefir milk and probiotic curd have shown an equal and marked decrease in CFUs when compared to the probiotic drink group.

Clinical significance: The administration of probiotics along with dairy products can be used as an adjuvant to routine preventive treatment procedures in the prevention of dental caries along with the remineralization of the demineralized tooth structure.

Keywords: ANOVA, Kefir milk, *Post hoc*, Probiotic curd, Probiotic drink, Streptococcus *mutans*.

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Introduction

The oral cavity is a complex ecosystem that provides shelter to different species of microflora. The disturbance in the homeostatic condition between the host and microflora results in the progression of oral diseases like dental caries and periodontitis.[1]

Dental caries is a multifactorial disease that occurs predominantly in childhood that interferes with speech, emotional conditions, and nutritional intake, which influences the development of the overall health status of the child.[2] The microflora and the chief pathogenic bacteria present in oral biofilm which causes dental caries is *Streptococcus mutans*.[1] Bacteriotherapy, by probiotics, is an alternative to routine preventive therapies of dental caries that helps to fight against pathogenic bacteria.[3]

The term "probiotics" was coined by Lilley and Stillwell in 1965 means "For life". Probiotics can be defined as "live microorganisms which, when administered in adequate amounts, confers a health benefit on the host"—Food and Agriculture Organization (FAO) and World Health Organization (WHO).[4] *Bifidobacterium* was the first isolated probiotic bacteria.[3] Consumption of probiotics that are available in various forms helps in the prevention of dental caries by competing with the pathogenic bacteria and also helps to modulate the defense mechanism.[4]

Materials and Methods

In this study, the levels of salivary *S. mutans* were compared before and after consumption of probiotic products.

The kefir drink used in this study was homemade. After boiling milk and cooling it at room temperature, 5% of kefir grains (containing *Lactobacillus casei* subsp. *Pseudoplantarum* and *Saccharomyces cerevisiae* and pH = 4) were added. The fermentation was done at 25°C for 12 hours, and then the kefir grains were separated by filtering.

Eighty school-going children of 8–12 years were selected. Permissions were taken from the college and the respective schools to evaluate the children. Selected children were divided into group I (control group), group II (kefir milk group), group III (probiotic curd group), and group IV (probiotic drink group) (Figs 1 to 3). All the initial carious lesions in the included children were restored before the supplementation of probiotics. Children in each group were supplemented with 100 mL of their respective probiotics once daily for 1 month along with the restorations except in group I. All the children were instructed to maintain normal oral hygiene measures and were instructed not to consume any food till 1 hour after taking of probiotic products.

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Conflict of interest: None

Fig. 1: Anaerobic gas packs fermented Kefir milk

Fig. 2: Probiotic curd

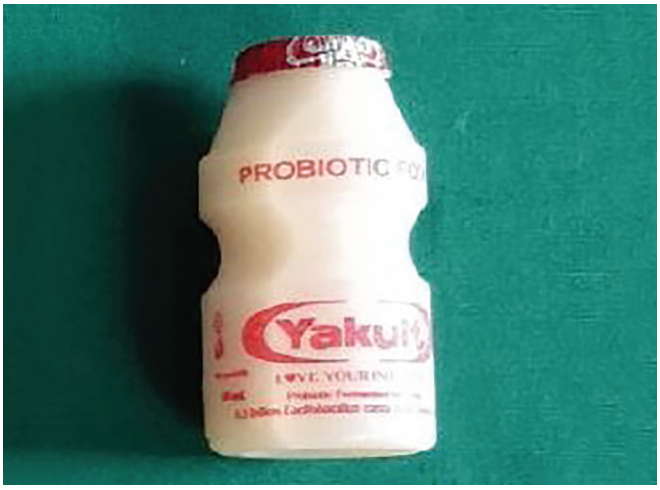

Fig. 3: Probiotic drink

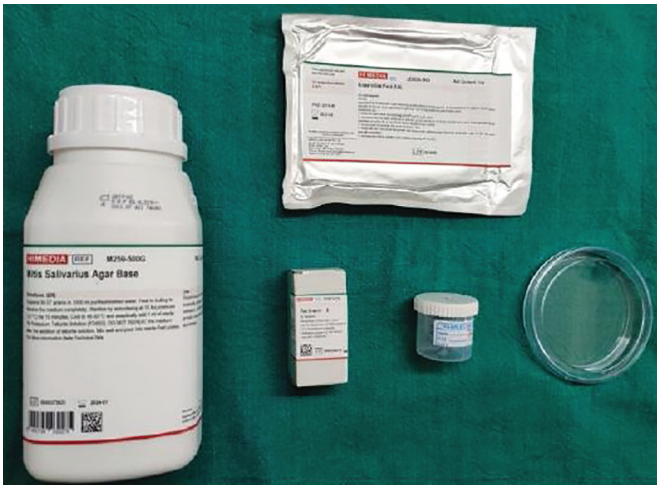

Fig. 4: *Mitis salivarius* agar, Bacitracin disks, sample collecting container, Pertri dish

Inclusion Criteria

- Eighty healthy children of age group 8–12 years.
- Initial carious lesions.
- Permanent maxillary central incisors, lateral incisors, along with permanent first molar, should have erupted.
- No signs of periodontal diseases.
- No history of any preventive dental treatment.

Exclusion Criteria

- Lactose intolerance subjects.
- Children with extensive carious lesions.
- Children under antibiotic treatment during the study.
- Medically compromised children.
- Children undergoing orthodontic treatment.

Ethical clearance was obtained before the start of the study from the ethical committee of Lenora Institute of Dental Sciences, Rajahmundry.

It was instructed to the children as well as their parent/guardian that the children given probiotics were to intake it once daily for 1 month along with treatment with restorations.

Collection of saliva was done before and after performing restorations and consumption of probiotics. Before the collection of saliva, each child in the respective group was coded, and salivary sample containers were allocated based on their codes. The children were instructed to swallow the preexisting saliva to clear the mouth of any residual saliva. A sterile plastic saliva container (HI-MEDIA) was given to each student and asked to spit saliva into it. The collected samples were sent to the laboratory on the same day. The transferred samples were stored at a room temperature of (17–25°C) before the analysis.

Subjects in group I were taken as control. The subjects were treated with restorations only; without supplementation of probiotics. Subjects in group II were given kefir milk; group III was given probiotic curd, group IV only probiotic drink. The second salivary sample from subjects was collected at 1 hour after the consumption of probiotics, followed by weekly intervals for a month. *Mitis salivarius* agar media, along with bacitracin disks, is used as culture media for identifying the *mutans streptococci* in the salivary samples (Figs 4 to 6).

The collected salivary samples were transferred to the respective culture medium containing Petri dish plates, and the

Fig. 5: Anaerobic gas packs

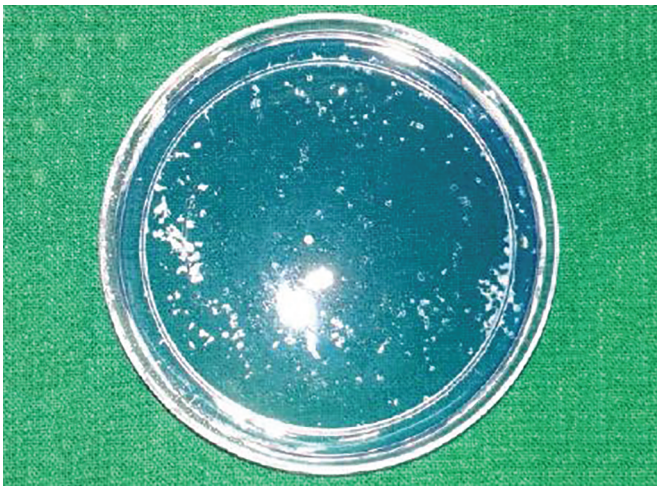

Fig. 6: Petri dish containing cultural medium

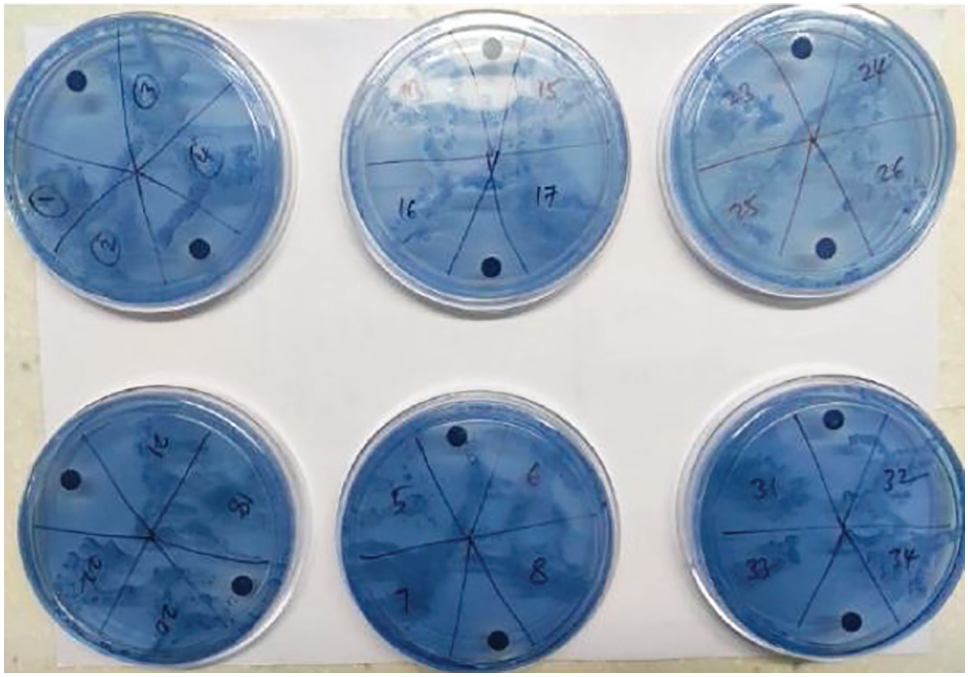

Fig. 7: Cultured salivary samples before the consumption of probiotics

plates were incubated anaerobically at 37°C for 72 hours. After 72 hours, colonies of *S. mutans* formed characteristics of *S. mutans* were studied and determined by using a colony counter, and the number of colony-forming units (CFUs) was counted. The levels of salivary mutans streptococci were compared before and after consumption of probiotic products (kefir milk, probiotic curd, probiotic drink) along with the restorations (Figs 7 to 9).

Results

In this study, the comparison of mean CFU of salivary mutans streptococci between four different groups from baseline till 1 month at weekly interval periods was depicted in Table 1.

At baseline, the mean CFUs of *S. mutans* in a salivary sample of the control group was higher (170.5 ± 22.11) than the kefir milk group, probiotic curd group, and probiotic drink group (121.75 ± 27.54, 123.75 ± 28.78, and 116.50 ± 20.71). On the 30th day of the follow-up period, the mean CFUs of *S. mutans* in the kefir milk group and probiotic group (88.50 ± 6.9, 89.50 ± 8.56) have shown an equal and significant reduction when compared to the probiotic group and control group (93.50 ± 6.90, 176.75 ± 19.81). There is no significant difference between the kefir milk group and the probiotic curd group.

Table 2 depicts ANOVA test value "*F*" has shown a statistically significant difference between the means of the control group and the probiotic group (kefir milk group, probiotic curd group, and probiotic drink group) from baseline to 30th-day time-interval.

Post hoc test in Table 3 has shown the mean difference between the control group and the kefir milk group, probiotic curd group and probiotic drink group (26.60, 26.10, 31.10) are statistically significant, but the mean difference between the probiotic group with

other probiotic groups does not show any statistical significance (intragroup comparison).

Table 3 infers that Kefir milk group and probiotic curd group shows better results than remaining two groups (Figs 10 to 13).

Discussion

The prevalence of dental caries in schoolchildren is about 60–90%. It is one of the most common oral diseases that affect childhood, which does not only intervene with speech and daily routine activities. It also affects the nutrition intake because of pain caused due to dental caries which results in the development of children with underwent abnormal cognitive condition. In developing countries like India and Thailand, the prevalence of dental caries is increasing when compared to western states. It could be due to the increased availability and usage of processed sugars and the decrease in the utilization of preventive services in developing countries.[2]

The oral cavity is a complex ecosystem in which the evolutions of various microbial species have taken place. The existing microorganisms vary from one species to the other species based on the nutritional, atmospheric, and physic-chemical factors. Alteration in the ecology of these microorganisms due to diet or medications affects the homeostasis of the oral cavity. It causes certain endogenous infections like dental caries and periodontal diseases in the oral cavity of the host.[3] The formation of biofilm was directly associated with the development of dental caries; *S. mutans* was the most causative organism present in the biofilm that causes dental caries. It produces exopolysaccharides (glucans) from glucose and synthesizes glucans through glycosyltransferases such as Gtf B, Gtf C, Gtf C, and Gtf D that helps in oral biofilm formation. The synthesized glucans decrease the salivary pH below 4 in the oral cavity after the establishment of oral biofilm.[5]

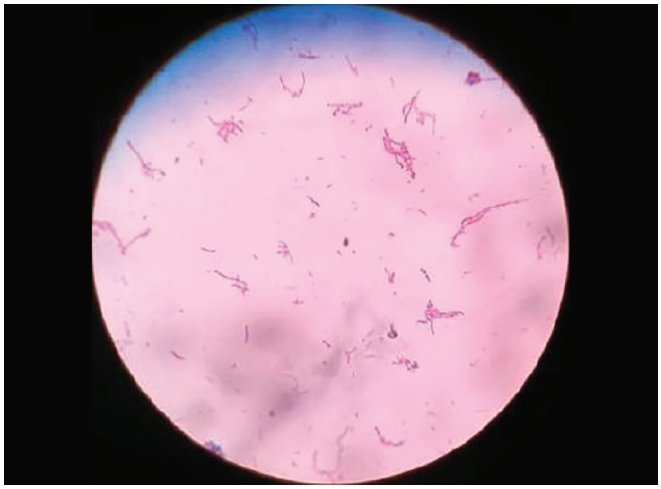

Fig. 8: Histological appearance of *Streptococcus mutans*

A new alternative method in caries management apart from the routine conventional physical and chemotherapeutic methods was by replacement therapy by genetically modified "effector strains" of the cariogenic bacterial species. They might act as a probiotic and helps in preventing the colonization of the cariogenic bacteria by producing ammonia and also helps in maintaining internal pH homeostasis.[6] The most commonly used probiotic bacteria are belonging to the species *Bifidobacterium* and *Lactobacillus* species. A study conducted by Yousuf et al. in 2016 has stated that administration of *Bifidobacterium* and *Lactobacillus* species as probiotics by orally has shown a short-term effect in reducing the *S. mutans* count that helps in the prevention of dental caries.[7] They are commercially available in various forms like a probiotic drink, probiotic milk, probiotic curd, probiotic yogurt, kefir, probiotic ice cream, probiotic fruit drink, probiotic cheese, recovery drink, probiotic lozenges, and probiotic tablets. Elie Metchnikoff, in the 20th century, had discovered that fermented milk contains lactic acid bacteria that help in inhibiting the growth of proteolytic bacteria because of its low pH, which is caused by the fermentation of lactose.[3] An *in vitro* study conducted by Xiolong et al. in 2017 proved that probiotic *Lactobacillus* species had shown an inhibitory effect on the growth of *S. mutans* by changing the composition of salivary biofilm which plays an essential role in causing dental caries.[8] Kim and Lee in 2016 conducted a study on the inhibitory effect of *Lactococcus lactis HY 449* on cariogenic biofilm, and they

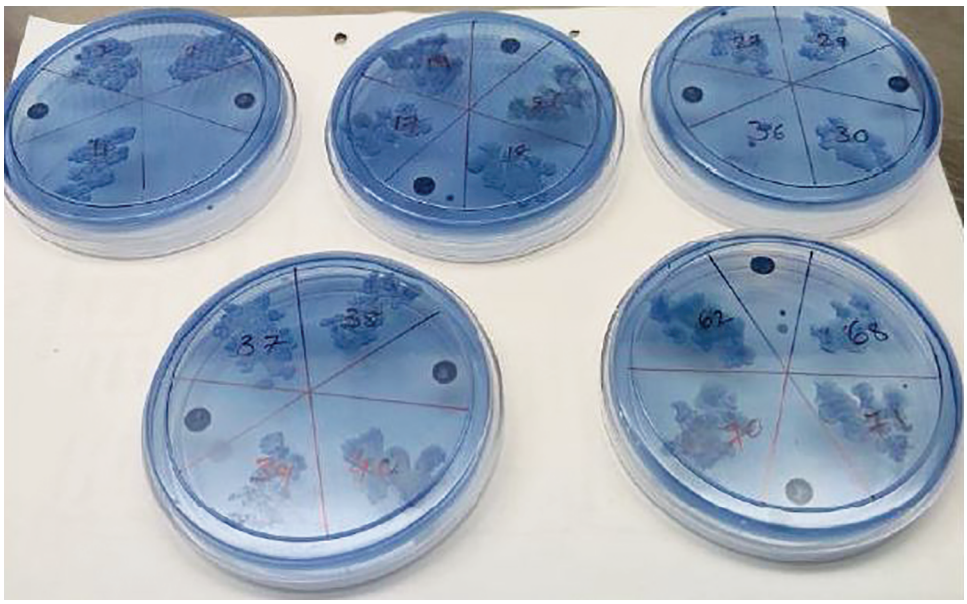

Fig. 9: Cultured salivary samples after the consumption of probiotics

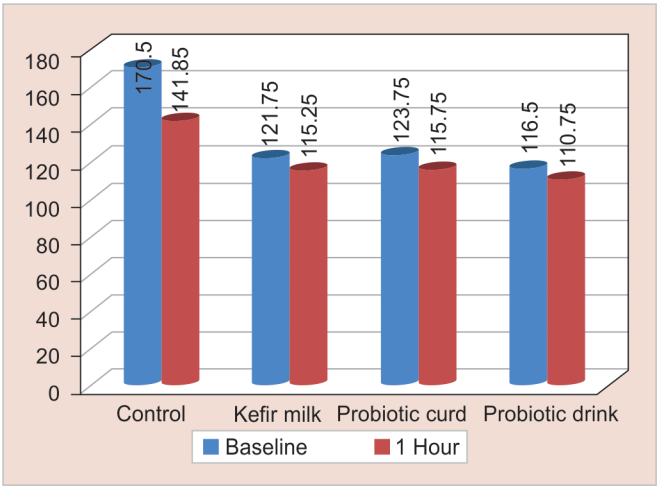

Fig. 10: Colony-forming units of *Streptococcus mutans* at baseline and after 1 hour

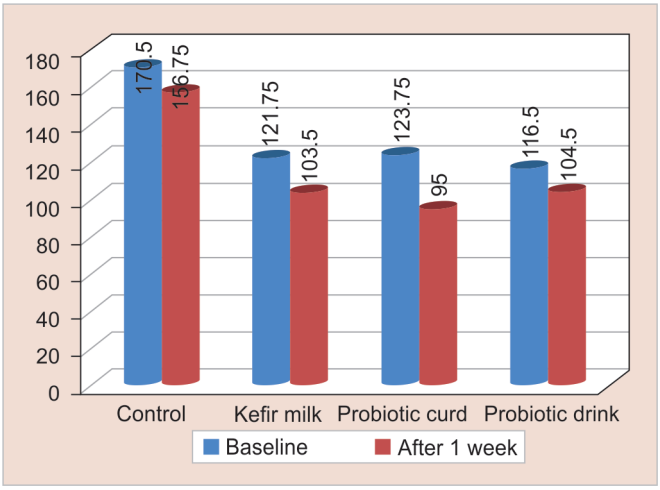

Fig. 11: Colony-forming units of *Streptococcus mutans* at baseline and after 1 week

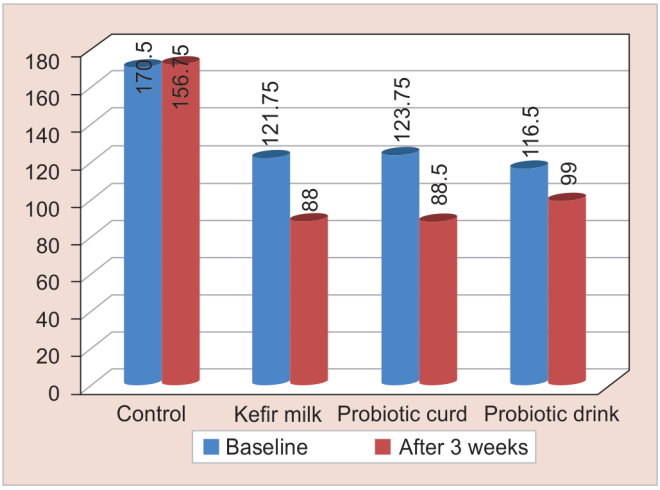

Fig. 12: Colony-forming units of *Streptococcus mutans* at baseline and after 3 weeks

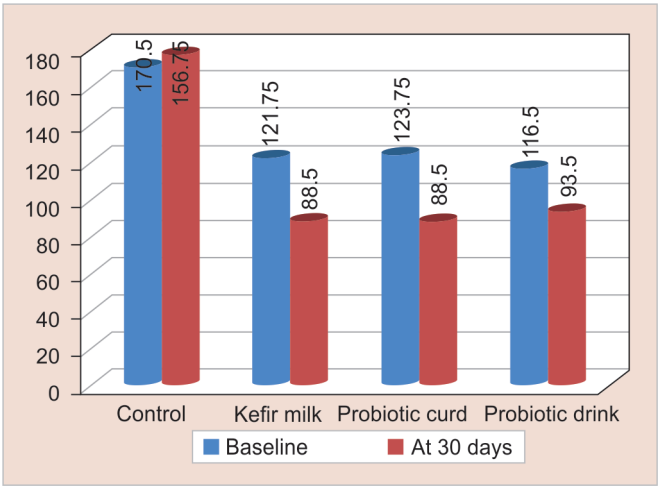

Fig. 13: Colony-forming units of *Streptococcus mutans* at baseline and after 30th day

Table 1: Comparison in mean colony-forming units (CFU) of mutans *Streptococcus* in four different groups from baseline to 1 month follow-up period at a weekly interval period

Group	*Baseline (mean ± SD)*	*1 hour (mean ± SD)*	*After 1 week (mean ± SD)*	*After 2 weeks (mean ± SD)*	*After 3 weeks (mean ± SD)*	*At 30 days (mean ± SD)*
Control	170.5 ± 22.11	141.85 ± 24.2	156.75 ± 21.04	162.25 ± 21.48	171.00 ± 20.23	176.75 ± 19.81
Kefir milk	121.75 ± 27.54	115.25 ± 22.79	103.50 ± 16.23	91.25 ± 12.44	88.00 ± 8.33	88.50 ± 6.9
Probiotic curd	123.75 ± 28.78	115.75 ± 23.91	95.00 ± 13.17	86.50 ± 12.25	88.50 ± 10.64	89.50 ± 8.56
Probiotic drink	116.50 ± 20.71	110.75 ± 16.87	104.50 ± 12.34	98.00 ± 10.05	99.00 ± 8.97	93.50 ± 6.90
Total	133.12 ± 32.88	120.90 ± 24.97	114.937 ± 29.19	109.50 ± 34.13	111.625 ± 37.03	112.0625 ± 39.38

stated that probiotic strain of *L. lactis HY 449* helps in the prevention of cariogenic biofilm formation. It was due to the inhibitory effect of glycosyltransferase (gtf) gene expression *by L. lactis*, which plays an essential role in metabolizing the sucrose by *S. mutans*.[9]

Dairy products were selected as probiotics administration because they act as an ideal delivery system of food for probiotics to the human gut, and these probiotic products offer a suitable environment and nutrients to promote the growth and viability of probiotic cultures. Probiotics, when incorporated along with dairy products, neutralize the acidic conditions in the mouth and inhibit the growth of cariogenic bacteria. Ferrazzano et al. in 2008 recommended that dairy products are the ideal vehicles for the administration of probiotics because these products contain casein phosphopeptides (CPPs) that inhibit the demineralization process, and helps in promoting the remineralization of the enamel of the tooth.[4]

Twelve years age group children were included in this study because according to World Health Organization (WHO 2013), it is the age group that children leave from primary school; and in many countries, it is the last and important age group at which

Table 2: ANOVA

		Sum of squares	*DF*	*Mean square*	*F*	*Sig.*
@1_hour_	Between groups	12,007.400	3	4,002.467	8.159	0.000
	Within groups	37,281.800	76	490.550		
	Total	49,289.200	79			
After_1_week	Between groups	47,710.938	3	15,903.646	61.624	0.000
	Within groups	19,613.750	76	258.076		
	Total	67,324.688	79			
After_2nd_week	Between groups	75,537.500	3	25,179.167	116.030	0.000
	Within groups	16,492.500	76	217.007		
	Total	92,030.000	79			
After_3rd_week	Between groups	95,553.750	3	31,851.250	189.339	0.000
	Within groups	12,785.000	76	168.224		
	Total	108,338.750	79			
At_30th_day_	Between groups	111,865.938	3	37,288.646	265.630	0.000
	Within groups	10,668.750	76	140.378		
	Total	122,534.688	79			
Gender	Between groups	1.100	3	0.367	1.474	0.228
	Within groups	18.900	76	0.249		
	Total	20.000	79			
At_base_line_cfumL	Between groups	37,811.250	3	12,603.750	20.120	0.000
	Within groups	47,607.500	76	626.414		
	Total	85,418.750	79			

Table 3: Multiple comparisons of the groups by *post hoc* test

Dependent variable	*(I) group_*	*(J) group_*	*Mean difference (I–J)*	*Sig.*
At_base_line_cfumL	Control group	Kefir milk group	48.75000*	0.000
		Probiotic curd group	46.75000*	0.000
		Probiotic drink group	54.00000*	0.000
	Kefir milk group	Control group	−48.75000	0.000
		Probiotic curd group	−2.00000*	0.996
		Probiotic drink group	5.25000	0.932
	Probiotic curd group	Control group	−46.75000*	0.000
		Kefir milk group	2.00000	0.996
		Probiotic drink group	7.25000	0.840
	Probiotic drink group	Control group	−54.00000*	0.000
		Kefir milk group	−5.25000	0.932
		Probiotic curd group	−7.25000	0.840
At_30th_day	Control group	Kefir milk group	88.25000*	0.000
		probiotic curd group	87.25000*	0.000
		probiotic drink group	83.25000*	0.000
	Kefir milk group	Control group	−88.25000*	0.000
		Probiotic curd group	−1.00000	0.995
		Probiotic drink group	−5.00000	0.621
	Probiotic curd group	Control group	−87.25000*	0.000
		Kefir milk group	1.00000	0.995
		Probiotic drink group	−4.00000	0.768
	Probiotic drink group	Control group	−83.25000	0.000
		Kefir milk group	5.00000	0.621
		Probiotic curd group	4.00000	0.768

*These groups performs better

data can be easily obtained through a reliable sample of the school system. Therefore, the age group 12 changed into the age of global monitoring of caries for international comparisons and monitoring of disease trends.[10]

Kefir is a fermented drink homogenous to the yogurt inconsistency that made from Kefir grains, which is a specific type of mesophilic symbiotic culture.[11] Different types of probiotic bacterial strains that are present in the kefir are—*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Streptococcus thermophilus*, *Lactobacillus lactis*, and *Lactobacillus kefiranofaciens*. Among all the bacterial species, the predominant bacterial species are *Lactobacillus* species that responsible for the synthesis of polysaccharide kefiran.[6,12] The synthesized kefiran has an antimicrobial property that helps in the inhibition of various types of pathogenic bacteria and yeast.[13] Cogulu et al. in 2010 conducted a study on the potential effect of a multistrain probiotic-kefir on salivary *S. mutans* and *Lactobacillus* species. They stated that short-term consumption of probiotic kefir inhibits the growth of *Streptococcus* and *Lactobacilli*.[14]

A dairy product like curd is a natural and useful vehicle for the administration of probiotics.[4] In this study, probiotic curd (Epigamia Artisanal Probiotic curd) was chosen because of its lactose-free content and presence of probiotic bacterial strains such as *L. acidophilus* and *Bifidobacteria* which maintains a gut-friendly environment that is important for a healthy digestive system. A study conducted by Srivastava et al. in 2016 on the effect of probiotic curd on salivary pH and *S. mutans* has shown the short-term consumption of probiotic curd reduces dental caries and enamel demineralization by increasing the salivary pH level.[4]

The commercially available fermented probiotic drink Yakult was used in the study, which contains live cultures of *L. casei Shirota* as probiotic bacteria. Other ingredients present in Yakult apart from probiotic bacteria are water, skimmed milk, glucose-fructose syrup, sucrose, and essence. Yadav et al. in 2014 stated that the presence of sucrose in Yakult had decreased the salivary *S. mutans* in children whose levels at baseline are $>10^5$ CFUs.[15] The type of culture medium that was selected for the growth of *S. mutans* was Mitis salivarius bacitracin agar. It has the composition of sucrose and bacitracin as main components.[16] Bacitracin is a mixture of related cyclic peptides that disrupt gram-positive bacteria by interfering with the cell wall and peptidoglycan synthesis[17] Shiklair, had stated that bacitracin proved to be the best antibiotic in differentiating the five variants of *S. mutans* strains.[17]

The result of this study has shown that the mean CFUs of mutans streptococci were higher initially at the baseline sample in all four groups, and it decreased gradually during their follow-up time interval periods (Table 1). In the control group, the mean CFUs of mutans streptococci were greater before undergoing restoration procedure and decreased at the 1-hour time interval after performing the restorations, and the mean colony-forming again increased at the subsequent follow-up periods. An *in vivo* study conducted by Patidar et al. in 2018 on salivary levels of *S. mutans* and *Streptococcus sanguinis* in early childhood caries before and after performing restorations. The result of the study has shown that reduction of the CFUs of *S. mutans* due to the antagonism between the *S. mutans* and *S. sanguinis* that competes for the early colonization on the tooth surface of the oral cavity. They concluded that the complete dental treatment has shown a significant effect in the reduction of *S. mutans* in the salivary sample.[1]

The mean CFUs of *S. mutans* in experimental groups (kefir milk group, probiotic curd group, and probiotic drink group) were significantly reduced in their follow-up periods when compared to the control group. However, comparing the mean values within the experimental group has shown that the mean CFUs of *S. mutans* in the probiotic curd group were less in number when compared to kefir milk and probiotic drink groups at the 1-hour and 1-week follow-up periods but at the 30th day of the follow-up period, both the kefir milk group and probiotic curd have shown an equal inhibition on *S. mutans* CFUs.

Probiotic drink shows less markedly reduction in CFUs of *S. mutans* at weekly time interval periods. It was due to the probiotic bacteria (*L. casei Shirota*) present in the probiotic drink (Yakult). A study conducted by Lima et al. in 2005 had shown that the adhesion potential of *L. casei Shirota* to the tooth surface is significantly less than the other probiotic bacteria *L. acidophilus*.[18] The obtained mean values of four different groups at different time interval periods were compared by using ANOVA and a statistically significant difference was noticed in the experimental group when compared to the control group in all-time interval periods.

According to the results of the present study, the mean CFUs of *S. mutans* were reduced in all four groups during their follow-up period in all time intervals when compared to baseline. In the control group, the mean CFUs in the salivary sample obtained 1 hour after the restoration procedure shown a reduction of CFUs of *S. mutans* than baseline values, but there is a marked increase in the mean CFUs in the remaining time interval periods till the 30th day in the control group. The increase in mean CFUs was due to the re-colonization of the *S. mutans* in the salivary sample of control group children during their remaining follow-up periods, and it might be due to the poor oral hygiene maintenance by the children which facilitates the colonization capability of *S. mutans*.

Conclusion

From this study, it was concluded that probiotic products along with restorations have shown an efficient role in the reduction of mean CFUs of *S. mutans* compared to the control group. Further studies with a longer follow-up period are necessary to achieve more beneficial effects by using probiotics.

Clinical Significance

The administration of probiotics along with dairy products can be used as an adjuvant to routine preventive treatment procedures in the prevention of dental caries along with the remineralization of the demineralized tooth structure. From this study, it was concluded that probiotic products along with restorations have shown an efficient role in the reduction of mean CFUs of *S. mutans* compared to the control group.

Manufacturer Name

- Yakult Probiotic Drink, Manufactured at 402–405 and 410–413, Food park, HSIDC, Ph-V, Rai, Sonepat, Haryana-131029, India.
- Epigamia Artisanal Curd, Manufactured by Fidelo Farms Pvt. Ltd, Khasra no. 482-490-492, Thikariya village, Jaipur-303106, Rajasthan, India.
- Milk Kefir Granules.

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