Original Article

A BITTER SWEET! Effectiveness of Chocolates and Probiotic Chocolates in Reduction of *Streptococcus mutans* – An *In vitro* Study

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Abstract

Context: Dental caries is the most common type of dental problem known to mankind, and the bacterial species-*Streptococcus mutans (S.mutans)* is known to initiate it, thus playing a significant role in dental caries formation. Theobromine in dark chocolate and probiotics are known for anticariogenic and antibacterial properties. **Aims:** The aim of this study is to find the effectiveness of white chocolate, dark chocolate, and probiotic-infused dark chocolate against *S. mutans*. **Subjects and Methods:** Blood-infused Mueller–Hinton agar was prepared with three wells. Twenty grams of chocolate were dispersed in each well. Chlorhexidine and saline were used as positive and negative control. The agar plate was then streaked with *S. mutans*. These prepared culture media was then allowed to incubate at 37°C for a time period of about 24 h under sterile environment. Finally, the diameters of the zone of inhibition were measured using the Hi Antibiotic Zone Scale. ANOVA and Tukey's *post hoc* test were used for statistical. **Statistical Analysis Used:** ANOVA and Tukey's *post hoc* test were used for statistical. **Results:** On comparison, probiotic-infused dark chocolate shows the greatest zone of inhibition (16.83 ± 1.329) followed by chlorhexidine (15.18 ± 1.458) which is then followed by dark chocolate (11.33 ± 1.751) and white chocolate (7.17 ± 1.835), and finally normal saline which showed no zone of inhibition. On overall evaluation using ANOVA, the statistically significant difference was found out ($P < 0.001$). Intergroup comparison was performed using Tukey's *post hoc* test, whose $P < 0.001$ thus being statistically significant. **Conclusion:** Among all the different kinds of chocolates used in the study, probiotic-infused dark chocolate showed the greatest anti-microbial activity against *S. mutans*.

Keywords: Chocolates, dental caries, probiotics, *Streptococcus mutans*, theobromine

Introduction

Dental caries can be defined as the irreversible destruction of tooth structure by acids as a result of fermentation of the carbohydrates present in the diet by oral bacteria and microbial flora.[1] Dental caries affects the entire human population irrespective of the factors such as age, sex, nationality, and thus presents as the world's most prevalent chronic disease.[2] Furthermore, it is a dynamic process caused by acids which are in turn produced by bacteria, which results demineralization and remineralization of minerals in the teeth. The rate of demineralization is significantly greater than that of the rate of remineralization.[3]

There are more than 10^3 species of bacteria identified in the oral cavity, and dental plaque serves as the hub for the bacterial species to thrive. It lodges approximately 4×10^{10} organisms with more than 400 species to it.[4] Dental plaque serves as the precursor for dental caries. Among all the species, *S. mutans* is known to initiate dental plaque formation, and it can thrive at a low pH level.[5] *S. mutans* initiates the metabolism of simple carbohydrates to produce acids, which cascades and eventually leads to the formation of dental caries.[4] In addition, it can synthesize a water-insoluble and adherent glucan from sucrose

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Figure 1: White chocolate (Group – I)

Figure 3: Probiotic and dark chocolate (Group – III)

by the action of enzyme glucosyltransferase. This accumulated adherent glucan aids in the adhesion of other bacteria and acids thus forming the dental plaque.[6]

On the other hand, there is a common misconception that chocolates are the one responsible for dental caries formation. The carbohydrate present in the chocolate is the one which actually causes dental caries. Although dental caries formation is a multifactorial process, carbohydrate is undoubtedly an important factor.[7]

Moreover, chocolate has various chemicals such as xanthine, caffeine, theobromine, phenolic, and mainly fats and sugars which constitute 30% of the normal chocolates. However, 99% of dark chocolate (the one used in the study) has only 1% of fats and sugars.

Chocolate, whose primary ingredient is cocoa, has various health benefits.[8] Theobromine (3, 7–dimethyl xanthine) present in cocoa has anticariogenic properties.[9] Theobromine is an alkaloid compound which enhances enamel microhardness.[10] The presence of theobromine during the development of teeth aids in the formation of large hydroxyapatite crystals, which contribute to the primary structure of the teeth (mineral/inorganic component), thus decreasing the loss of calcium and phosphorous ions from the enamel surface.[9] Moreover, it also aids in the treatment

Figure 2: Dark chocolate (Group – II)

Figure 4: Chlorhexidine (Group – IV)

of dentin hypersensitivity.[11] The enamel hardness obtained as a result of theobromine is nearly as effective as fluoride.[12] The antimicrobial property of the cocoa is ascribed to the concentration of cocoa content within it; cocoa is rich in catechins which are polyphenols of the flavanol group which bestows the anti-bacterial property. Thus, dark chocolate shows the greatest anti-microbial activity against the *S. mutans* species.[9] It is undoubtedly clear that theobromine by itself has anti-bacterial property.[13]

Probiotics are living microbes of various proportions which, when on administration provides various health benefits on the host.[14] When ingested daily, it shows decrease in the level of *S. mutans* count in the oral cavity.[15]

Probiotic containing *Lactobacillus* and *Bifidobacterium* species infused chocolate show significant inhibitory action against the growth of *S. mutans*.[1] Thus, it is clear that cocoa and the probiotic has anti-cariogenic property to it,[1] and *S. mutans* serves as the initiating factor for the dental caries formation.[6] However, there is a lack of literature stating the comparison of chocolate infused probiotics as an antimicrobial agent. Thus, the aim of the study is to find

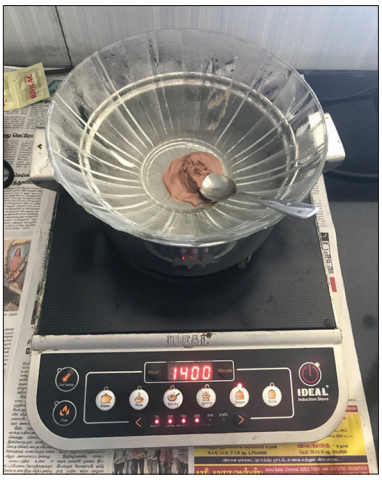

Figure 5: Preparation of probiotic-infused dark chocolate

the effectiveness of the probiotic-infused dark chocolate against *S. mutans*. The null hypothesis of the study is that the probiotic-infused dark chocolate does not have any effect on *S. mutans*.

Subjects and Methods

The current study was conducted in the Department of Public Health Dentistry, in a private institute at Chengalpattu, Tamil Nadu. Ethical approval taken from the institutional review board before the study was conducted. Three types of commercially available chocolates were used in the study.

- Group I: White chocolate [Figure 1]
- Group II: Dark chocolate [Figure 2]
- Group III: Probiotic-infused dark chocolate [Figure 3]
- Group IV: Chlorhexidine (control) [Figure 4].

Preparation of the probiotic-infused dark chocolate

The chocolates (10 gm) were melted in a glass bowl using the double boiling technique at 43°C–45°C, which was then tempered at 43°C for 10 min. The Bifilac sachet was then added and mixed homogenously to the dark chocolate, and thus, the probiotic-infused dark chocolate was prepared [Figure 5].

Preparation of the culture media

The procedure was done in the Department of Microbiology, in a private institute at Chengalpattu, Tamil Nadu. *S. mutans* was obtained from the patients undergoing orthodontic treatment, using the swab collection procedure. Blood-infused Mueller–Hinton agar was selected as the culture media for the study. The Petri dish containing blood-infused Mueller– Hinton agar was prepared with three wells (4 mm in diameter and 3 mm deep) using a sterile cone back. Using a sterile micropipette, 0.02 ml of white chocolate was dispersed into the first well, 0.02 ml of dark chocolate was dispersed into the second well, and 0.02 ml of probiotic-infused dark chocolate was dispersed into the third well, respectively. Chlorhexidine (0.2%) was used as positive control which was prepared and let for observation in a separate culture media.

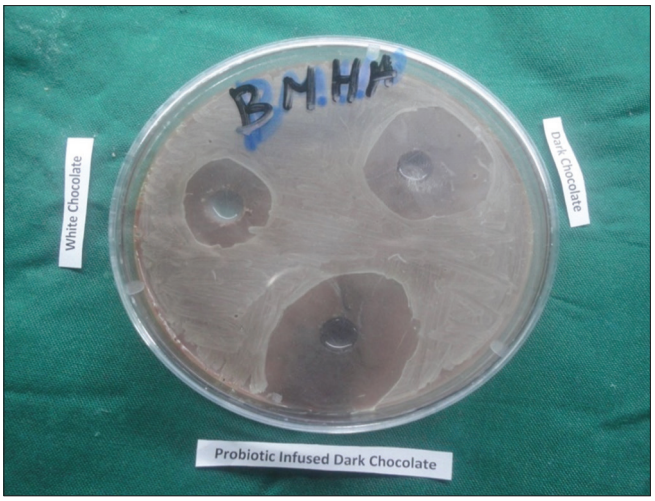

Figure 6: Observed zone of inhibition in white chocolate, dark chocolate, and probiotic-infused dark chocolate

These prepared culture media was then allowed to incubate at 37°C for 24 h under sterile environment to ensure uniform distribution of the respective chocolate groups around the wells into blood-infused Mueller–Hinton agar. This serves as quality control. The agar plates were then lawn cultured with *S. mutans*. The Petri dishes were then once again allowed to incubate at 37°C for 24 h. The petri dishes were then observed for zones of inhibition. Diameters of the zone of inhibition were measured using the Hi Antibiotic Zone Scale. Saline was used as a negative control in this study to ensure the contamination-free environment during the experiment.

Furthermore, the test was repeated six times to overcome any technical errors that might have occurred during a single attempt. The results were collected and tabulated in the excel sheet and statistical analysis was then performed using SPSS (version 25) software, manufactured by IBM, Chicago, USA. ANOVA and Tukey's *post hoc* test were performed to analyze and conclude the results.

Results

Table 1 shows the mean zone of inhibition values (mm) between different groups against *S. mutans*. Diameters of the zone of inhibition were measured using Hi Antibiotic Zone Scale and were recorded in an excel sheet and statistical analysis was then performed using SPSS (version 25) software. On overall evaluation using ANOVA, the statistically significant difference was found out ($P < 0.001$).

On comparison, probiotic-infused dark chocolate shows the greatest zone of inhibition (16.83 ± 1.329) followed by chlorhexidine (15.18 ± 1.458) which is then followed by dark chocolate (11.33 ± 1.751) and white chocolate (7.17 ± 1.835), and finally, normal saline which showed no zone of inhibition [Figure 6 and 7].

Table 2 shows the multiple comparisons between different chocolate groups against *S. mutans*. On intergroup

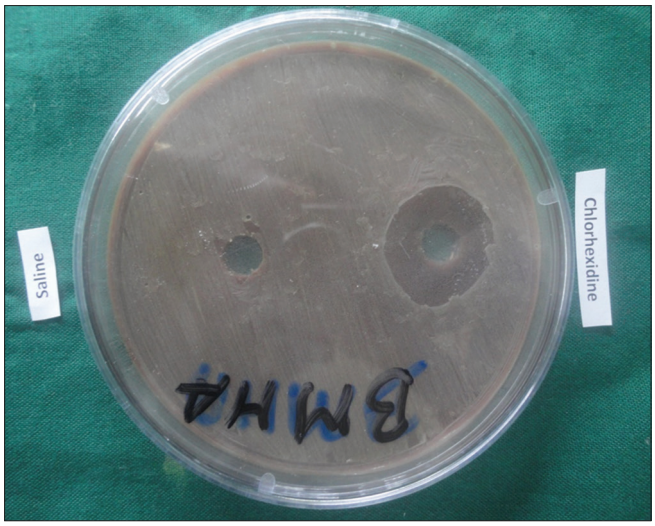

Figure 7: Observed zone of Inhibition in chlorhexidine and saline

Table 1: Mean zone of inhibition values (mm) between different groups against *streptococcus mutans*

Groups	*n*	Mean±SD	*F*	*P*
Group-I	6	7.1717±0.13167	1106.64	0.00*
Group-II	6	13.43±0.24924		
Group-III	6	16.83±0.43497		
Group-IV	6	15.1833±0.34361		
Total	24	13.1538±3.74693		

P is significant at 0.05 levels, ANOVA. SD: Standard deviation

Table 2: Multiple comparisons between different groups against *streptococcus mutans*

Comparison between groups	Mean difference	*P*
Group-I		
Group-II	−6.25833	0.00*
Group-III	−9.65833	0.00*
Group-IV	−8.01167	0.00*
Group-II		
Group-III	−3.4	0.00*
Group-IV	−1.75333	0.00*
Group-III		
Group-IV	1.64667	0.00*

P is significant at 0.05 level, Tukey's *post hoc* test

comparison of antimicrobial effect with probiotic-infused dark chocolate and white chocolate using Tukey's *post hoc* test *P* value was <0.05 and thus the result indicate the difference in antimicrobial efficacy of both the groups and the difference was statistically significant.

Discussion

Dental caries is considered to be one of the world's most well-known and prevailing bacterial diseases which is known to have caused by many bacterial species and mainly spearheaded by *S. mutans* which primarily resides on oral cavity, pharynx, and intestine.[16] Among several species, *S. mutans* is considered to be the most common initiator that produces acids from carbohydrates and causes demineralization of the enamel surface.[17,18] *S. mutans* plays a significant role in the etiology of dental caries formation.[19]

Dark chocolate is preferentially chosen over other chocolates for comparison, as it has the highest content of theobromine when compared to other chocolate groups. A single sachet of Bifilac (0.5 mg) contains *Lactobacillus sporogenes* (50 million), *S. fecalis* (30 million), *Clostridium butyricum* (2 million), and *Bacillus mesentericus* (1 million), respectively. Probiotics have proven to bestow many health benefits and have potential antimicrobial action against various bacterial species.[14]

In the study conducted by Malarvizhi Dakshinamoorthy *et al*.,[20] it was proven that *Bifidobacterium Longum*-infused probiotic dark chocolate showed the greatest zone of inhibition when compared to his other comparative study groups. This result obtained from the study coincides with that of the result shown in our study that is– probiotic-infused dark chocolate shows the greatest zone of inhibition when compared to other groups.

According to Khanafari *et al*.[21] probiotic chocolate containing *Lactobacillus rhamnosus* PTCC 1637 had a significant effect on *S. mutans* strains in the culture media with a 75 mm of the inhibitory zone, all the three chocolate groups used in the study successfully showed the zone of inhibition. However, probiotic-infused dark chocolate showed the greatest zone of inhibition, this is similar to the results of our study.

However, the limitations of the study include being an *in vitro* study it has to be proven effective on a larger scale and more clinical trials ought to be conducted to substantiate the results. Second, the chocolates used in the study still have sugar (carbohydrates) as sweetening agents which can be substituted by artificial sweetening agents.

Conclusion

Among all the different kinds of chocolates used in the study, probiotic infused dark chocolate showed the greatest anti-microbial activity against Streptococcus mutans.

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