ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 11, Issue, 12, pp.8973-8978, December, 2019

DOI: https://doi.org/10.24941/ijcr.37565.12.2019

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

COMPARATIVE ASSESSMENT OF THE EFFECT OF RAW BOVINE MILK, PACKAGED FULL CREAM MILK, PACKAGED SKIMMED MILK AND INFANT FORMULA ON STREPTOCOCCUS MUTANS BIOFILM FORMATION

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ARTICLE INFO

Article History:
Received 24th September, 2019
Received in revised form
28th October, 2019
Accepted 25th November, 2019
Published online 31st December, 2019

Key Words:
Cariogenicity,
Biofilm, *Streptococcus Mutans*,
Milk,
Infant Formula.

ABSTRACT

Background: Dental caries is one of the most common chronic diseases of early childhood and is considered as a multi-factorial infectious and transmissible disease. *Streptococcus mutans* is the major etiological agent of early childhood caries in children. The caries forming activity of *S. mutans* on teeth are carried out through the formation of dental biofilm containing *S. mutans* and subsequent production of lactic acid. Milk is a significant part of the human diet. For infants, it is the sole source of vital nutrients for the first six months of its life. An extensively used nutritional supplement for feeding babies and children are infant formulas. **Objectives:** This study investigated the effect of four different types of milk i.e., raw bovine milk, packaged full fat or whole milk, packaged skimmed milk and infant formula on biofilm formation in the presence of *Streptococcus mutans.* **Methods:** A 24-hour culture of *S. mutans* was treated with various concentrations of the milks and infant formula diluted in bacteriological media. The biofilms were washed, fixed, and stained with crystal violet. The absorbance was measured to evaluate biofilm growth and total absorbance. **Results:** Biofilm formation occurred in all the four experimental groups and was statistically significant when compared with the control group. Between the groups, the highest biofilm formation was observed in packaged skimmed milk and the least in bovine milk at all dilutions. **Conclusion:** Bovine milk is least cariogenic of all the experimental groups while packaged skimmed milk is the most cariogenic.

Citation: Dr. Sohini Sengupta, Dr. Babu, G.V. and Dr. Shilpy Dwivedi. **2019.** "Comparative assessment of the effect of raw bovine milk, packaged full cream milk, packaged skimmed milk and infant formula on streptococcus mutans biofilm formation", *International Journal of Current Research*, 11, (12), 8973-8978.

INTRODUCTION

Dental caries is one of the most common chronic diseases of early childhood and is considered as a multi-factorial infectious and transmissible disease (Mouradian, 2001; Gussy, 2006). Although it is not life threatening, dental caries seriously affects the quality of life and well-being of the child due to pain, limitations in mastication and food selection and reduced self-esteem. *Streptococcus mutans* is the major etiological agent of early childhood caries in children. (Fejerskov, 2003). The caries forming activity of *S. mutans* on teeth are carried out through the formation of dental biofilm containing *S. mutans* and subsequent production of lactic acid. Biofilm is a layer of slime that contains polymers, bacterial cells and debris from a person's diet (Seow, 1998).

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Biofilm is formed via a multi-step process. It starts out as an acquired salivary pellicle that contains no bacteria. Secondly, planktonic bacteria colonize the pellicle, via reversible binding to the salivary proteins. Finally, in what is known as the maturation stage, via polysaccharide or protein receptors, the first group of bacteria allows for binding of a second group of colonizers including *S. mutans*. This aggregation is crucial for the formation of a mature biofilm (Reisine, 1998). Biofilms offer specific advantages for bacteria, including resistance to antibiotics and protection from salivary immune factors due to the high density of cells. These advantages may be a contributing factor for the estimated 65% of human infections that come from biofilm entity (Milnes, 1996). The sucrose dependent virulence of an *S. mutans* biofilm is primarily due to the activity of Glucosyltransferases (GTF), glucans and glucan-binding proteins (GBP). Tooth enamel has cell-free and cell-associated GTFs attached to the salivary pellicle, which, when exposed to sucrose, synthesize glucans. These glucans, through the use of GBPs, aid in the attachment of *S. mutans*to tooth enamel.

They also allow for the ability of bacteria to attach to one another, thus creating a stronger and more virulent biofilm (American Academy of Pediatric Dentistry, 2008). Milk is a significant part of the human diet. For infants, it is the sole source of vital nutrients for the first six months of its life. When the child is slowly weaned off of breast milk, milk remains an essential source of nutrition and is consumed in different forms – both raw animal milk such as bovine milk and packaged milk, which is available in different concentrations and formulations (Aas *et al*., 2008). Milk could show cariogenic potential due to the presence of lactose, considering it is metabolized by the bacteria of the dental biofilm and organic acids that are released because of this process, favoring the demineralization (Forssten, 2010). This data has a great clinical relevance because the caries progression rate in deciduous enamel is higher than the permanent enamel (Huang, 2011). An extensively used nutritional supplement for feeding babies and children are infant formulas manufactured from cow's milk, the composition of which is altered to either add or remove a few components such as corn syrup, sucrose, lactose, etc. The cariogenic potential of liquids in baby bottles containing sucrose is well reported. Yet the potential cariogenicity of milk and infant formulas which are the most common bottle contents, remain uncertain (Krzyściak, 2014). Hence, this present study intended to determine the local effect that different types of milk and infant formula may exert on the oral cavity. This study investigated the effect of four different types of milk i.e., raw bovine milk, packaged full fat or whole milk, packaged skimmed milk and infant formula on biofilm formation in the presence of *Streptococcus mutans*.

MATERIALS AND METHODS

This study contained four main experimental groups

Group I: Bovine Milk
Group II: Packaged Full Cream Milk
Group III: Packaged Skimmed Milk
Group IV: Infant Formula

The contents of each of the groups are given in Table 1. *Streptococcus mutans* strain from the MTCC was used in the present study due to its completely sequenced genome and for being specific to the etiology of dental caries. The strain was stored at -80^{o}C in Tryptic Soy Broth with 20% glycerol before use. To determine biofilm formation, an overnight *S. mutans* culture (10^{6}CFU/ml) in TSB was treated for 24 hours with various concentrations of the four experimental groups diluted in TSB and incubated with 10 µl of an overnight culture of *S. mutans* for 24 hours in sterile 96-well microtiter plates. Each group was tested at the following dilutions – 1:10, 1:20 and 1:40.

The total absorbance of the wells indicating the relative amount of biofilm and planktonic cells were measured at 595 nm. Biofilm was washed twice with saline, fixed with 10% formaldehyde for 30 minutes, washed twice again with saline, and stained with 0.5% crystal violet for 30 minutes. After washing the biofilm three times with saline, crystal violet was extracted from the biofilm cells by 200 µl of 2-propanol for 1 hour. Then the absorbance of the biofilm was again read at 595 nm (Rugg-Gunn, 1993). Each experiment for the different types of formula was repeated six times. Summary statistics (mean, standard deviation, standard error) were calculated for each dilution for biofilm mass and total growth. Comparisons were made between each dilution and the control group, between each of the groups at each dilution. The tests used were Unpaired t-test and ANOVA. The level of significance was kept at less than 0.05. The softwares used were Graphpad and One-Way ANOVA Calculator.

RESULTS

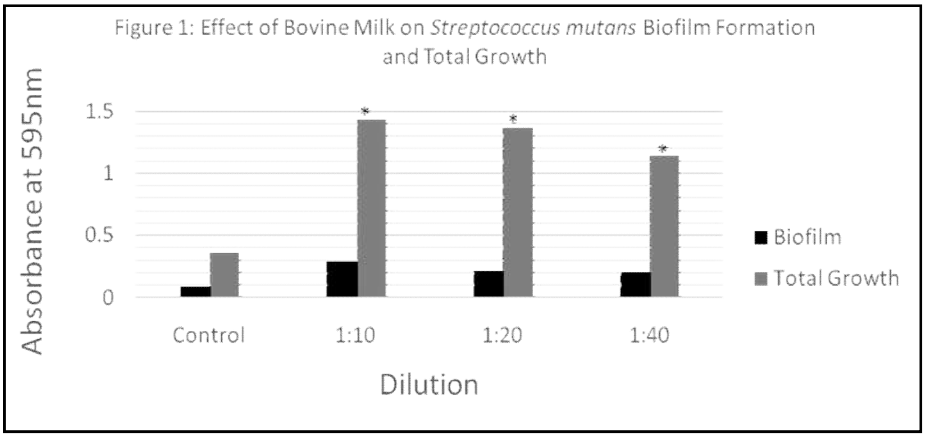
Figure 1 shows that when compared to the control group, the increase in biofilm formation in the presence of bovine milk has increased and the data is statistically significant, that is, the p-value is <0.05. Similarly, the total growth has also increased significantly as compared to the control group. The amount of biofilm formation and total growth decreases as the dilution increases. Figure 2 shows that when compared to the control group, there is significant increase in the biofilm formation in the presence of packaged full cream milk, which has decreased with the increase in dilution. Similarly, the total growth has also increased significantly as compared to the control group, with a decrease as dilution of the packaged full cream milk increases. Figure 3 shows that compared to the control group, there is significant increase in both total growth and biofilm formation in the presence of packaged skimmed milk. It can also be noticed that more the concentration of the skimmed milk higher is the total growth and biofilm formation. Figure 4 shows that when compared to the control group, the increase in biofilm formation in the presence of infant formula has increased and the data is statistically significant. Similarly, the total growth has also increased significantly as compared to the control group. However, the amount of biofilm formation and total growth decreases as the dilution increases. Figure 5 shows that at 1:10 dilution, the maximum biofilm formation has occurred in the presence of skimmed milk followed by infant formula, packaged full cream milk and bovine milk respectively. When statistically analysed the difference in biofilm formation between the four experimental groups was found to be statistically significant (p-value < 0.05). Figure 6 shows that packaged skimmed milk exhibits the highest amount of *Streptococcus mutans*biofilm formation followed by infant formula at 1:20 dilution. Packaged full cream milk and Bovine milk shows significantly less biofilm formation. Figure 7 similar to Figure 6 shows that at 1:40 dilution, the maximum biofilm formation takes place in the presence of packaged skimmed milk, followed by Infant formula, and significantly less in packaged full cream milk and bovine milk.

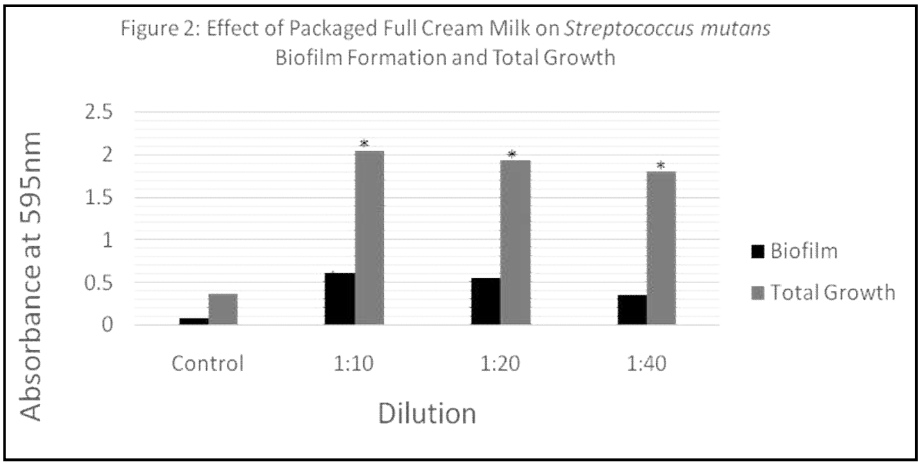
DISCUSSION

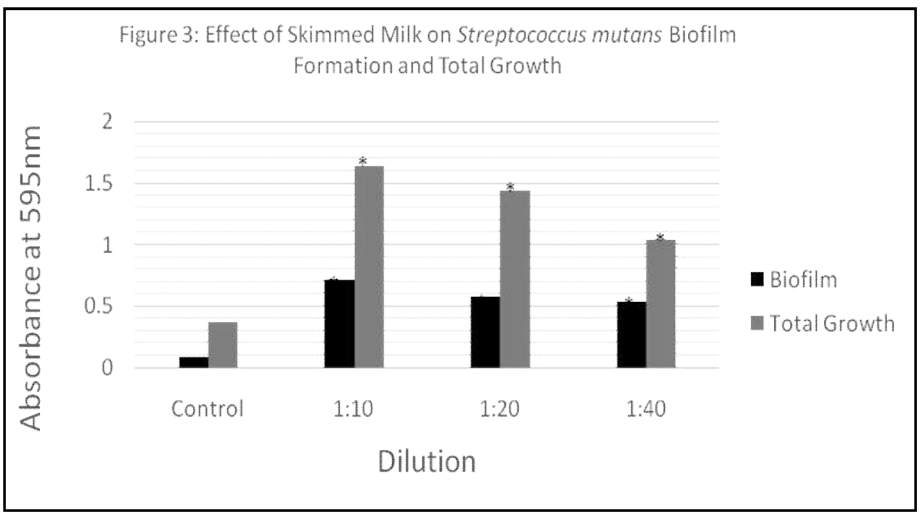
In the present study, there is an increase in the *Streptococcus mutans* total growth and biofilm formation in bovine milk. This is in accordance to the study conducted by Bowen *et al*. who concluded that lactose has the potential to promote caries. Lactose forms eighty percent of the carbohydrate present in milk, which accounts for 4.8g of lactose per 100ml of milk. Lactose is metabolised by *S. mutans* and reduces the biofilm pH favouring mineral loss from the teeth (Levine, 2001; Peres, 2009; Sheikh, 1996). Birkhed *et al*. in his study concluded that dental plaque microflora may adapt to lactose in milk leading to a greater ability to ferment lactose following frequent milk consumption (Erickson, 1998). When compared between packaged full fat milk and skimmed milk, full cream milk exhibits less biofilm formation in all three dilutions and this can be corroborated with study conducted by Giacaman *et al*. (1997)

Table 1. Contents of the Experimental Groups

Contents	Bovine milk	Packaged full fat milk	Packaged skimmed milk	Infant formula
CALORIES	61	76.8	35	474
PROTEIN (g/100ml)	3.29	3.6	3.5	14.3
FAT (g/100ml)	3.34	2.4	0.1	20
CARBOHYDRATE (g/100ml)	5.4	10.8	5	59.1
CHOLESTEROL (mg/100ml)	14	8	0	6
CALCIUM (mg/100ml)	119	26	150	420
PHOSPHORUS (mg/100ml)	93	0	0	270
SODIUM (mg/100ml)	49	44	0	150
POTASSIUM (mg/100ml)	152	0	0	480
VITAMIN C (mg/100ml)	0.94	1	0	48
VITAMIN A (mg/100ml)	37.8	9	0.075	0.35
VITAMIN D (mg/100ml)	0	0	0.0005	0.006
RIBOFLAVIN (mg/100ml)	0.162	0	0	1
Iron (mg/100ml)	0	7	0	6.80







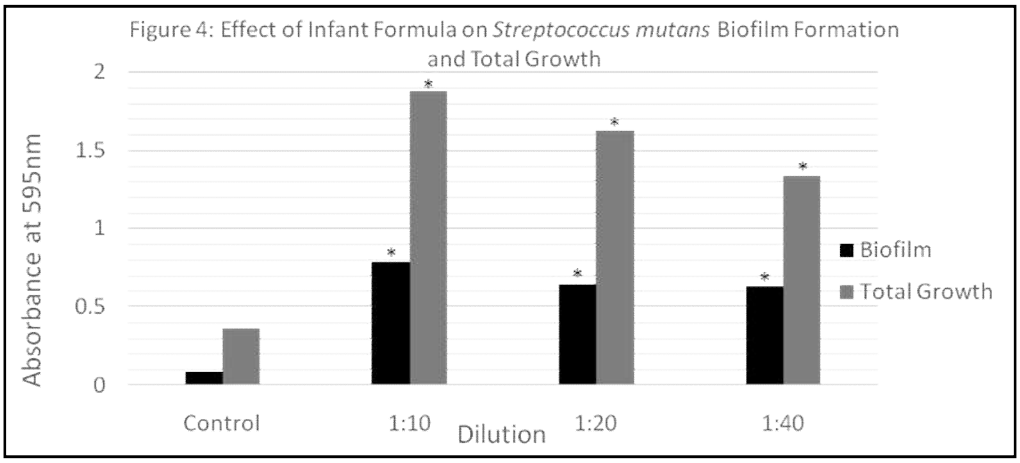


Figure 4: Effect of Infant Formula on Streptococcus mutans Biofilm Formation and Total Growth
Absorbance at 595nm
2
1.5
1
0.5
0
Control
1:10
1:20
1:40
Dilution
Biofilm
Total Growth

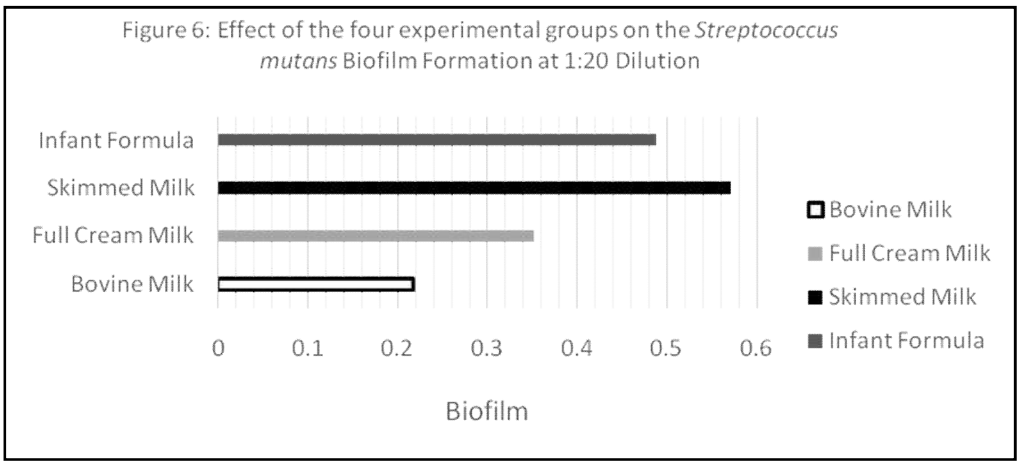


Figure 6: Effect of the four experimental groups on the Streptococcus mutans Biofilm Formation at 1:20 Dilution
Infant Formula
Skimmed Milk
Full Cream Milk
Bovine Milk
0
0.1
0.2
0.3
0.4
0.5
0.6
Biofilm
Bovine Milk
Full Cream Milk
Skimmed Milk
Infant Formula

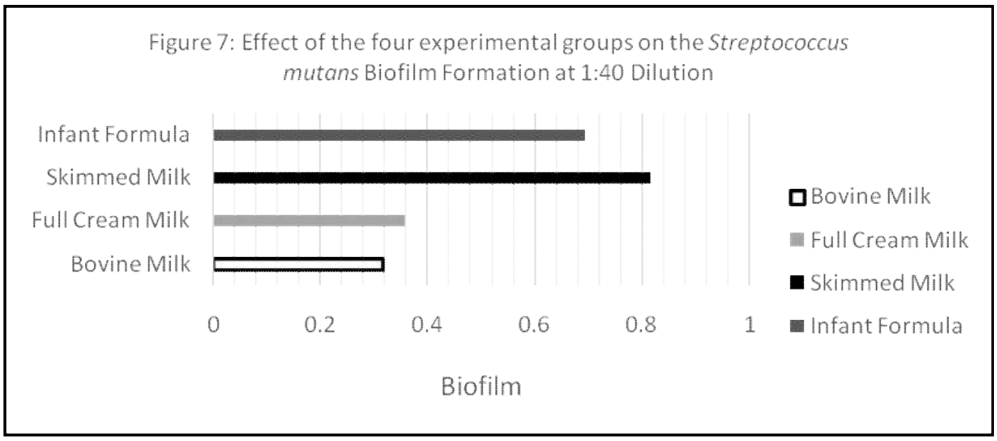


Figure 7: Effect of the four experimental groups on the Streptococcus mutans Biofilm Formation at 1:40 Dilution
Infant Formula
Skimmed Milk
Full Cream Milk
Bovine Milk
0
0.2
0.4
0.6
0.8
1
Biofilm
Bovine Milk
Full Cream Milk
Skimmed Milk
Infant Formula

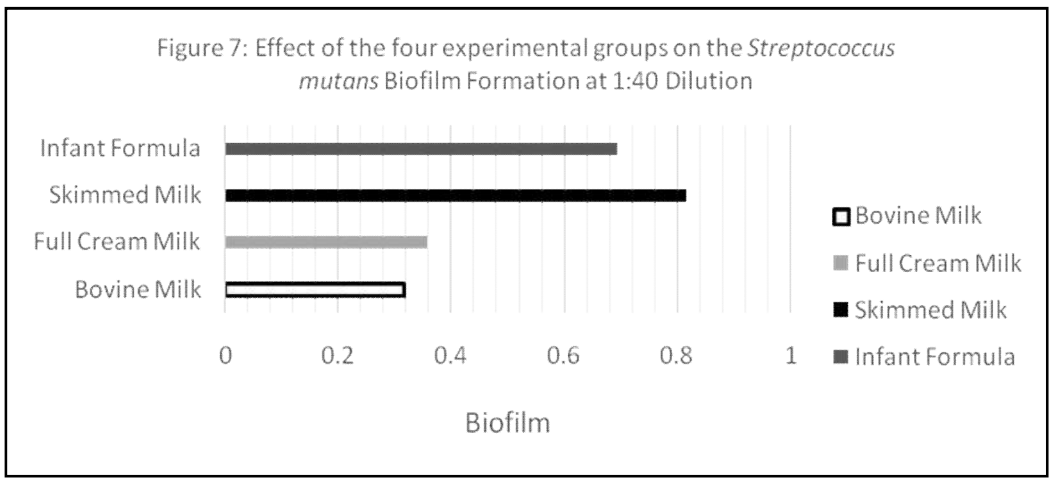


Figure 7: Effect of the four experimental groups on the Streptococcus mutans Biofilm Formation at 1:40 Dilution
Infant Formula
Skimmed Milk
Full Cream Milk
Bovine Milk
0
0.2
0.4
0.6
0.8
1
Biofilm
Bovine Milk
Full Cream Milk
Skimmed Milk
Infant Formula

In their study, it was found that skim and semi-skim milk showed similar biofilm mass as compared to the sucrose control group, while whole milk showed less biofilm formation, comparable to bovine milk. Infant formula has shown the highest biofilm formation at 1:10 dilution. Even at dilutions of 1:20 and 1:40, it exhibits biofilm formation comparable to skimmed milk. This finding is in accordance to the study conducted by Bowen *et al*. who concluded that infant formulas had greater potential to promote cariogenic properties when compared to plain bovine milk due to the higher carbohydrate variability which is ten times than that present in bovine milk (Hinds *et al*., 2016). When compared between the groups, bovine milk shows the least biofilm formation at all dilutions. This is in conjunction with several studies which claim that milk abolishes the adherence of *S. mutans* in solution and reduces the activity of glucosyltransferase, thus in turn reducing glucan formation (Levine, 2001; Bánóczy *et al*., 2009; Meurman, 2009; Stecksén-Blicks, 2009). Bovine milk has also been found to be less cariogenic because of its high content of calcium and phosphate. Hence bovine milk, if unsweetened with external agents, is not caries promoting. It contains various macro and micro-nutrients beneficial for dental structures (Dietrich *et al*., 2004). Moreover, lactose is metabolised slowly by oral micro-organisms and pH values below the critical pH of 5.5 are not commonly observed. Hence in accord with the study conducted by Bowen *et al*. is can be concluded that milk is only minimally caries promoting, if not completely non-cariogenic (Hinds *et al*., 2016).

Conclusion

The results of this study demonstrate that bovine milk is significantly less cariogenic than packaged whole milk, skimmed milk and infant formula. However, more detailed in-vivo studies are required for advanced knowledge the cariogenic potential of the different forms of milk available for infants.

Conflict of interest: The authors have no conflict of interest related to this study.

Funding: No funding sources.

GLOSSARY OF ABBREVIATIONS

CFU: Colony Forming Unit
g: Gram
GBP: Glucan Binding Protein
GTF: Glucosyltransferases
mg: milligram
ml: mililiter
nm: nanometer
S. mutans:Streptococcus mutans
TSB: Tryptic Soy Broth
µl:microliter

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