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ORIGINAL RESEARCH

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The role of sucrose incorporated into milk on biofilm formation, pH change, and enamel demineralization

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Abstract

Backgroud: While breastfeeding is endorsed for the overall health of infants and reduces the hazard of developing various illnesses, spontaneous breastfeeding should be considered a contributing element in the growth of early childhood caries (ECC). This investigation evaluated the effects of sucrose combined with milk on biofilm formation, pH change, and enamel demineralization. **Methods**: Biofilm formation and pH change of *in vitro* medium with human milk (HM), bovine milk (BM), and infant formula (IF) were measured with/without the presence of 10% sucrose and/or *Streptococcus mutans* (*S. mutans*). Enamel areas were made on extracted permanent molars and incubated using milk specimens. Demineralization of enamel and progression of caries were evaluated histologically after two weeks. **Results**: HM had less biofilm formation than BM and IF. However, adding 10% sucrose and *S. mutans* augmented biofilm formation in all three milk types. Sweetened HM exhibited the most significant change in pH and the most severe progression of carious lesions into the enamel. Enamel lesion depths were increased and pH was more acidic under a high load of sucrose and *S. mutans*. **Conclusions**: In conclusion, HM is recommended for health and reducing the threat of disease, but spontaneous breastfeeding after introducing additional nutritional carbohydrates is a risk factor for EEC.

Keywords

Milk; Sucrose; *Streptococcus mutans*; Biofilm; Enamel demineralization

1. Introduction

Breastfeeding and human milk (HM) are considered the gold standards for infant nourishment. However, the impact of breastfeeding on infant oral health, both in the short and long term, remains a subject of ongoing research. The American Academy of Pediatrics (AAP) recommends exclusive breastfeeding for six months after birth. It then advises that breastfeeding continues alongside the introduction of complementary foods for at least one year, or as long as both mother and child desire. The AAP also emphasizes the numerous health benefits of breastfeeding for both infants and mothers including nutritional, immunological, developmental, and psychological advantages [1–3]. Contrarily, a separate policy statement by the American Academy of Pediatric Dentistry (AAPD), updated in 2021, presents views that appear to diverge from those of the AAP [4]. While the AAP emphasizes prolonged breastfeeding for its health benefits, the AAPD warns against frequent and prolonged breastfeeding due to the risk of dental caries. The AAP's focus is on the overall health and developmental benefits of breastfeeding, whereas the AAPD focuses on the oral health and the prevention of early childhood caries (ECC) [3–5].

The breastfeeding guidelines of the AAP and the AAPD, specifically in the context of breastfeeding after the addition of other sources of carbohydrates to the infant's diet, may appear, especially to the general public, to directly conflict with each other, leaving parents unsure of whether they should continue to breastfeed their child after they begin to introduce other sources of carbohydrates to the child's diet.

The initiation and progression of cariogenic biofilm heavily depend on fermentable carbohydrates, with sucrose being the primary culprit. This is because oral bacteria ferment sucrose, leading to the generation of both intracellular and extracellular polysaccharides. These processes result in a lower pH, favoring the growth of plaque-causing microorganisms and altering the biofilm's structural makeup. Additionally, sucrose-based biofilms have been found to contain lower levels of vital minerals and fluoride, which play key roles in tooth health [5, 6].

The aim of this investigation was to address the current lack of proof needed to determine whether relations exist between breastfeeding and the development of ECC. Specifically, this study is intended to examine the role of introducing highly refined sugar to a breastfeeding child's diet in the development of ECC by comparing the cariogenic capabilities of refined sugar solutions prepared from HM, bovine milk (BM) and infant formula (IF). The null hypothesis was no difference in

the cariogenic capabilities of refined sugar solutions prepared from HM, BM and IF.

2. Methods

2.1 Experimental design

Institutional Review Board approval was obtained for this investigation. HM was collected from three healthy, non-smoking females ranging from 24–35 years of age in sterile plastic bottles (Medela, IL, USA) once gentle hand expression of the breast was used. The collected HM was either stored in a freezer (−80 °C) or used immediately as a sample [7]. Pasteurized whole BM was bought from a local grocery store (Prairie Farms). The cow's milk-based IF brand used was Similac Pro-Advance® (64682NT, Abbott Nutrition, Columbus, OH, USA), as it is the most commonly used formula in the US [7]. Of all Similac formulas, Pro-Advance® is the formula that most closely resembles breast milk, and it contains no added sugars.

S. mutans strain Clarke (ATCC 25175) was acquired from the American Type Culture Collection (Manassas, VA) and cultured at 37 °C in brain heart infusion media (BHI). To prepare for this experiment, control groups were established using various combinations of sterile distilled water, BHI medium, and 10% sucrose, along with either HM, BM, or IF. For the experimental groups, solutions were prepared using the same formulas as the control groups, but 1×10^7 colony-forming units (CFUs) mL^{-1} of *S. mutans* were included in the BHI medium added to each group. Table 1 shows the control and experimental groups using various combinations of sucrose, milk and *S. mutants*.

2.2 Biofilm growth

Once the solutions were prepared, the baseline capacity of *S. mutans* to form biofilm was assessed. The formation of biofilm in 96-well polystyrene tissue culture plates was recorded in the presence of bovine plasma (35010162, Sigma-Aldrich Canada Co., Oakville, ON, Canada; 100%, 50%, 25% and 12.5% in 10 mM phosphate-buffered saline (PBS), pH 7.2). A baseline overnight culture of *S. mutans* was diluted in fresh BHI to achieve an optical density (OD) of 0.2 at OD600, and similar overnight dilutions involving each of the experimental groups were cultured.

Approximately 200 μL samples of each dilution above were deposited in separate, labeled 96-well polystyrene tissue culture plates and left to incubate for 48 hours at 37 °C. After 48 hours, the culture medium and free-floating bacteria were eliminated via aspiration, and the wells were washed twice with phosphate-buffered saline.

The resulting biofilms were stained with a 0.05% crystal violet dye (100 μL) for 10 minutes, and the wells were washed two times with PBS to eliminate unbound remnants of the dye. The wells were then dried for 2 hours at 37 °C. Once the wells were dry, 100 μL of 95% (V/V) ethanol was added to each well, and the tissue culture plate was shaken for 10 minutes to release the stain from the biofilms. Upon conclusion of the 10 minutes, the absorbance of each sample at 490 nm was measured using a Spectrophotometer (UV-Vis, Beckman Coulter Inc., Brea, CA, USA) to quantify the formation of biofilm.

2.3 pH measurements

The pH was measured daily for up to 28 days following incubation at 37 °C to determine the degree of pH change in each solution throughout the incubation period. A digital pH meter (Accumet AB15 Plus) reads the pH to three decimal places.

TABLE 1. Control and experimental groups using various combinations of sucrose, milk and S. mutants.

Group	Compositions in common
Control	
Group 1	HM, sterile distilled water, and BHI medium
Group 2	BM, sterile distilled water, and BHI medium
Group 3	IF, sterile distilled water, and BHI medium
Group 4	HM, 10% sucrose, and BHI medium
Group 5	BM, 10% sucrose, and BHI medium
Group 6	IF, 10% sucrose, and BHI medium
Experimental	
Group 7	HM, sterile distilled water, and 1×10^7 CFU mL^{-1} *S. mutans* in BHI medium
Group 8	BM, sterile distilled water, and 1×10^7 CFU mL^{-1} *S. mutans* in BHI medium
Group 9	IF, sterile distilled water, and 1×10^7 CFU mL^{-1} *S. mutans* in BHI medium
Group 10	HM, 10% sucrose, and 1×10^7 CFU mL^{-1} *S. mutans* in BHI medium
Group 11	BM, 10% sucrose, and 1×10^7 CFU mL^{-1} *S. mutans* in BHI medium
Group 12	IF, 10% sucrose, and 1×10^7 CFU mL^{-1} *S. mutans* in BHI medium

HM: human milk; BM: bovine milk; IF: infant formula; BHI: brain heart infusion; CFU: colony-forming units.

2.4 Enamel demineralization

Permanent, fully-formed human third molars were (n = 6) obtained from the ATSU dental hard tissue specimen core according to the approved protocol. The teeth were stored in water at 4 °C following extraction and used within one month. The roots of each tooth were eliminated, and the crowns were divided mesiodistally using a low-speed diamond saw (Isomet; Buehler, Lake Bluff, IL, USA). The unground enamel surfaces were examined under an optical microscope (Olympus CX33, Tokyo, Japan) at 10× magnification for defects/decalcified areas. For demineralization, the mid-coronal buccal enamel was polished with flour pumice, cleaned with 70% ethanol before inoculation for biofilm growth, and rinsed with sterile deionized water. Each sample surface was sealed with nail varnish (Revlon, New York, NY, USA) to avoid demineralization, except for a 3 × 3-mm window. The enamel window of each tooth was then bathed and incubated in 10.0 mL of HM, BM or IF (with and without 10% added sucrose and with or without 1×10^7 CFUs mL^{-1} of *S. mutans*) for 14 days. Subsequently, each tooth was replenished with its respective 10.0 mL of the solution of HM, BM or IF (with and without 10% added sucrose) daily. After 14-day incubation, the tooth samples were embedded in epoxy (Epoxycure, Buehler, Bluff, IL, USA) at room temperature. Then, each sample was cross-sectioned and prepared for examination using an optical microscope (Olympus CX33) and ImageJ software (1.54j, NIH, Bethesda, MD, USA) to measure the depth of each lesion.

2.5 Statistical analysis

The biofilm formation, pH changes, and enamel demineralization data were statistically analyzed using a two-way analysis of variance (ANOVA) followed by *post-hoc* tests. The statistical significance level was set at $\alpha = 0.05$.

3. Results

Significant variance existed between the biofilm growth of *S. mutans* in HM, BM and IF. IF had significantly greater biofilm formation than BM, while HM resulted in substantially less biofilm formation. Adding 10% sucrose to each solution significantly increased biofilm growth (Fig. 1).

Conversely, unlike the biofilm formation mentioned above, our data showed that the starting range of pH of HM was the highest (least acidic) of the three milk sources, while the formula showed no difference compared to BM after 6 days in culture (Fig. 2). In the presence of sucrose, HM exhibited the greatest variability in pH, becoming the most acidic following incubation. Changes and reduction in pH persisted after 28 days (4 weeks) in a culture with high sucrose and *S. mutans* load. In the presence of sucrose, HM exhibited the greatest variability in pH, becoming the most acidic following incubation.

Histological data (Fig. 3A,B) indicate that, following a 2-week baseline period, sectioned permanent teeth exhibited not only a deeper lesion depth in enamel but also increased damage with high sucrose and *S. mutans* load.

4. Discussion

The null hypothesis was rejected as there was a significant difference between the biofilm growth of *S. mutans* in HM, BM and IF. IF had significantly greater biofilm formation than BM, while HM resulted in substantially less biofilm formation. Adding 10% sucrose to each solution significantly increased its biofilm growth. This study sought to gain insights into whether parents should continue to breastfeed their child after they begin to introduce other sources of carbohydrates to the child's diet. Previous studies [8–12] concluded that HM alone is not a cariogenic food source; therefore, breastfeeding should continue, providing significant benefits to both the child and the mother [12, 13]. However, the AAPD's policy statement identifies ad libitum breastfeeding as a causative factor in the development of ECC [4]. It can be hypothesized in this study that the greater cariogenic effect of HM is due to higher concentrations of lactose and decreased buffer capacity in comparison to BM [14]. In another study, when evaluated by pH titration, unsweetened almond milk showed the lowest buffering ability, while BM displayed the greatest buffering ability [15]. This study evaluated the effect of HM or BM exposure on biofilm composition and enamel demineralization using different methodologies, including bovine enamel slabs for surface hardness. In their study, enamel demineralization was assessed by the percentage of surface hardness loss, which differs from our method [15]. BM has a great buffering capacity and allows the development of *S. mutans* biofilms, revealing caries potential, which is enhanced by sugar supplementation [16, 17]. The anti-cariogenic or cariogenic potential of BM varies with its type and composition, indicating that BM may not always be anti-cariogenic and could become cariogenic under specific circumstances [17]. BM is rich in casein, phosphate, and calcium, which are useful for healthy enamel, and exhibits anti-cariogenic action [18, 19]. BM also comprises antimicrobial peptides such as peroxide, lysozyme, and lactoferrin, which may add to anti-cariogenicity and cause caries if consumed at high frequencies [20].

To bridge the conflicting evidence, we evaluated the simulated cariogenicity of HM, BM and IF, both with and without the presence of 10% sucrose and/or *S. mutans*. Our study found that, while all evaluated milk sources were associated with biofilm formation, HM had less biofilm formation than BM and IF. However, adding 10% sucrose and *S. mutans* augmented biofilm formation in all three milk types. The use of different types of milk resulted in a reduction in pH when given sucrose in combination with *S. mutans*, demonstrating that HM, BM and IF were each fermented differently by the bacterial biofilm. Sweetened HM exhibited the most significant drop in pH (4.3 ± 0.75; Fig. 2) and the most severe progression of lesions into the enamel (Fig. 3). The change in the concentration of H^+ in the solution could cause such pH changes. Another study attributed this buffering capacity of natural milk to its protein content, predominantly casein [18]. Casein is a main component of significantly increased biofilm formation between the concentrations evaluated [21]. It was reported that the maximum buffering in HM occurs at an approximate pH of 5.5 [18]. In comparison, the concentration of organic acids is two to three times greater in HM than in

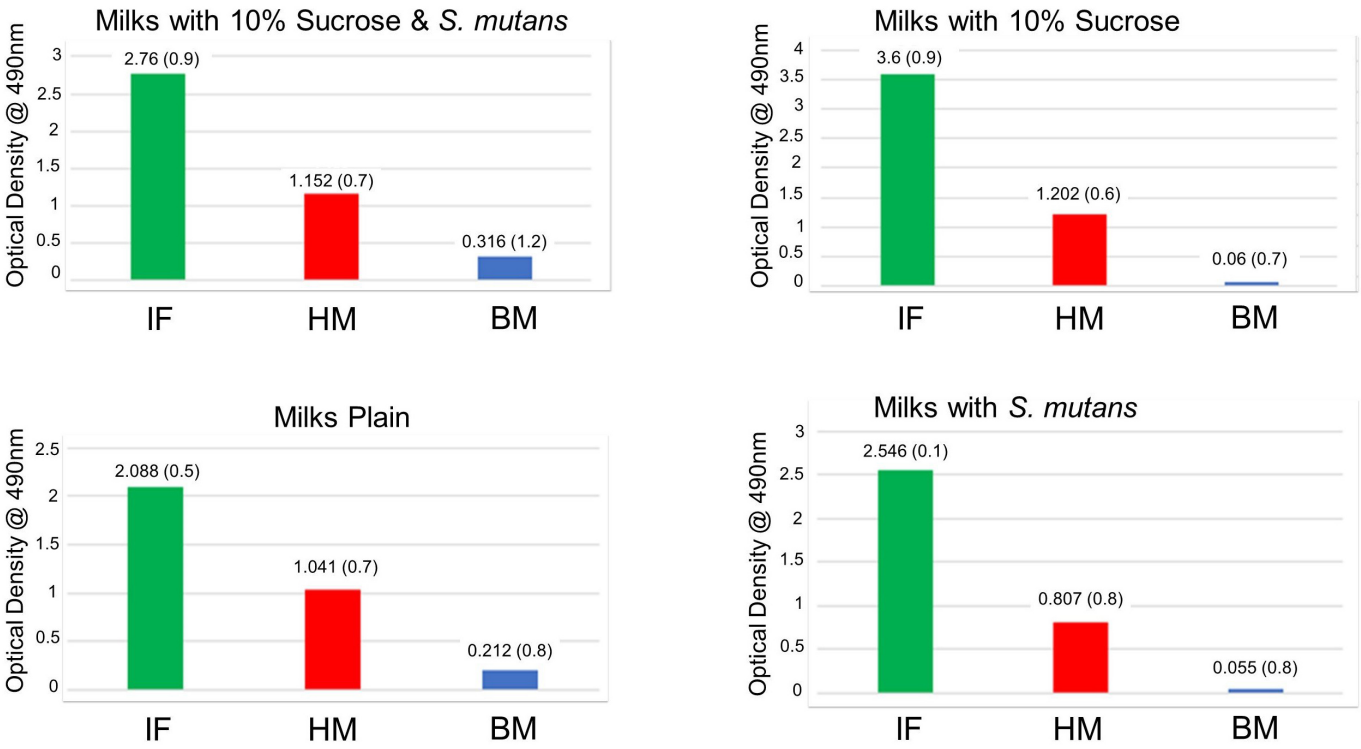

FIGURE 1. Spectrophotometer results. Absorbance results at 490 nm show the optical density (OD) of the biofilm after 48 hours of growth. BM: bovine milk; IF: infant formula; HM: human milk.

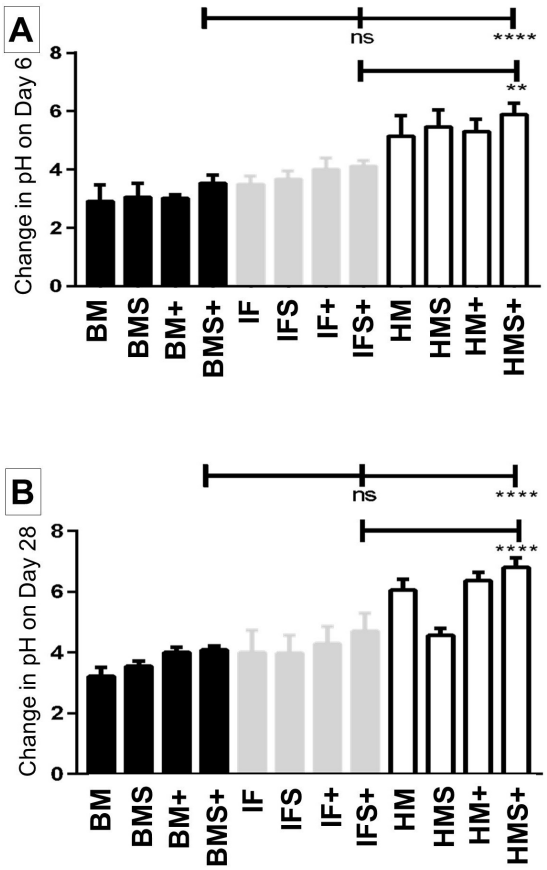

FIGURE 2. pH results. (A) Milk cultured with/without 10% sucrose and *S. mutans* pH was measured as fold change over 6 days of measurement of culture medium. (B) 28-day culture pH Changes. n = 3 per group, two-way ANOVA. $^{**}p < 0.01$, $^{****}p < 0.0001$. BM: bovine milk; IF: infant formula; HM: human milk; S: sucrose; +: *S. mutans*; ns: not significant.

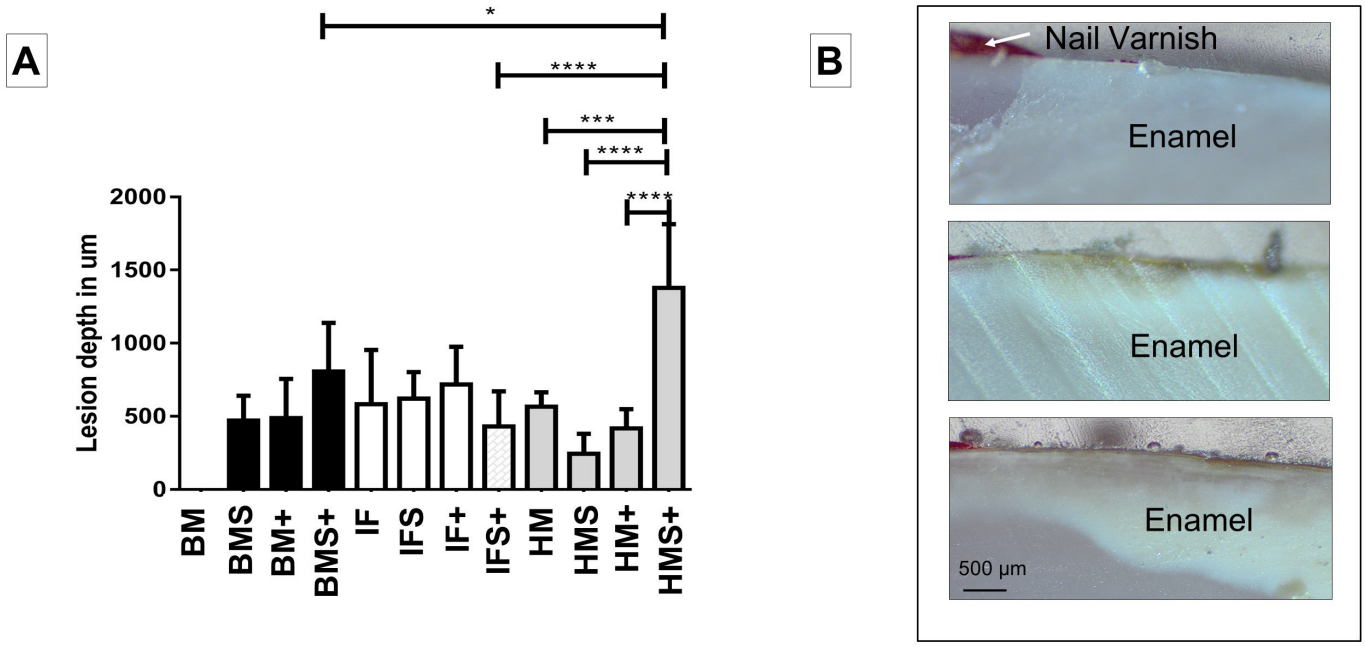

FIGURE 3. Lesion size results. (A) Milk cultured with/without 10% sucrose and *S. mutans* after 2 weeks in sectioned permanent teeth. (B) Representative sectioned teeth specimens are shown for BM, IF and HM (top to bottom, respectively) with sucrose added. Scale bar = 500 μm; n = 2; two-way ANOVA; *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. BM: bovine milk; IF: infant formula; HM: human milk; S: sucrose; +: *S. mutans*.

BM [9]. It is likely that the phosphates and proteins present within HM are capable of buffering the free hydrogen ions associated with these organic acids and thereby maintaining the pH near neutral when unchallenged by other acid sources. However, when additional acid is present, the buffering capacity is exceeded [9]. A study reported that HM is relatively less cariogenic in an *in vitro* caries model, but adding sugar increases its cariogenic potential [18]. This also could be due to the addition of sucrose. Sucrose was absent in the plain HM, BM and IF groups. Sucrose, a fermentable carbohydrate, feeds microorganisms, producing caries, increasing their progression and colonization on the newly erupted teeth of the infants, causing them to be more prone to acid attack and enamel demineralization. Some assumptions have been proposed to describe how sucrose modifies the inorganic concentrations in biofilms, such as continuous low-pH in the biofilm matrix owing to continuous sucrose fermentation, which melts mineral reservoirs or inhibits their storage. An investigation reported the influence of HM and its constituents on the dietary parts of the caries process due to *S. mutans* biofilm formation, resulting in amplification in *S. mutans* biofilm formation by breast milk 3–9 months post-delivery [22].

HM has significantly less phosphate and protein, of which the concentration of histidine with the highly buffering imidazole ring is essential, leading to higher dental demineralization. This also could be due to the addition of sucrose. Lactose does not decrease pH values as severely as sucrose. Sucrose was absent in the plain HM, BM, and IF groups. Sucrose, a fermentable carbohydrate, feeds microorganisms, producing caries, therefore increasing their progression and colonization on the newly erupted teeth of the infants, causing them to be more prone to acid attack and enamel demineralization. Some assumptions have been proposed to describe how sucrose modifies the inorganic concentrations in biofilms, such as continuous low-pH in the biofilm matrix owing to continuous sucrose fermentation, which melts mineral reservoirs or inhibits their storage, enamel could take these ions from the biofilm fluid, low bacterial density due to the high insoluble EPS could cause fewer binding sites for these ions, and low concentrations of specific ion-binding proteins could result in fewer mineral reservoirs in biofilms formed in the presence of sucrose [5]. Previous study demonstrated increased *S. mutans* biofilm formation by HM 3–9 months postpartum, which differs from our study [22]. Methodological differences could account for the discrepancies. Their study reported that only casein significantly increased biofilm formation above average milk concentrations. In addition, in their study, 11 mothers were participants, while only three individuals participated in our study. In their study breastmilk samples were tested at dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 and 1:5120. Casein was tested at concentrations representative of the average breast milk concentration along with two-fold dilutions above and below the average. The average breastmilk concentrations of casein was (2.3 mg/mL) and casein significantly increased biofilm formation in the two highest concentrations (4.6 and 9.2 mg/mL) more so than all the other components tested. A systematic review of the cariogenicity of infant formulas reported that different infant formulas still need to be studied in the future. Thus, no concrete recommendations can be made [22]. Furthermore, a study evaluated the change in salivary pH and plaque after drinking different IF and found changes in salivary pH and plaque after feeding with IF [23]. Another investigation assessed the change in plaque pH after fermenting four IF

reported that they were able to decrease the pH considerably below the pH before the rinse [24]. Furthermore, a study evaluated the change in salivary pH and plaque after drinking different IF and found changes in salivary pH (lower pH and more acidic) and plaque after feeding with IF [25].

The results of this investigation differ from other studies [25, 26], which suggested that HM is cariogenic. Studies have reported HM is considerably more cariogenic than cow milk, possibly because of its greater level of lactose and lesser mineral content [27, 28]. This *in vitro* experiment acts as a laboratory model for caries by trying to simulate many of the physical characteristics of the mouth. These models include all structures needed for caries formation/progression, *i.e.*, bacteria (*S. mutans*), the host (tooth), substrate, and time (2 weeks). It has been reported that mean pH of HM was slightly acidic at 6.60 $\pm$ 0.28 [29]. The elements that contribute to the pH buffering capacity of HM can be broadly divided into two types: Proteins and weak acids, bases, and their complexes with metal cations. Proteins and free amino acids are main contributors to pH buffering [30]. Of particular importance is the concentration of histidine, which has a highly buffering imidazole ring, which is essential, leading to higher dental demineralization [30]. A study evaluated the acidogenicity of HM by the dental biofilms of children with and without ECC [31]. This study was clinical in which biofilms of 16 children with ECC and caries-free were exposed to HM or 10% sucrose solution in the crossover design, and the biofilm pH was determined. The authors concluded that breastfeeding does not provoke a significant drop in pH levels in the biofilm of children who are caries-free or who present with ECC, but sucrose provoked a significant pH drop after 5 min for both groups. In contrast, our study was *in vitro* and pH levels dropped in the biofilm.

To our knowledge, this proof-of-concept study is the first of its kind. Our findings can serve as an additional resource for clinicians or parents who need clarification on the existing guidelines for infant feeding. Our investigation confirmed the necessity to instruct and educate parents about the damaging effects of adding an outside source of carbohydrates to all forms of milk. Parents must consider the benefits and risks before sweetening milk to make it more pleasant for children.

Though this study lays the groundwork for elucidating whether associations exist between breastfeeding and the development of ECC, it has limitations: (1) this investigation was *in vitro*. While an *in vivo* study of this nature would be unethical, this study needs to completely account for the complex environment of the oral cavity, such as the role of saliva; (2) continuous exposure to sucrose, as done in the study, may not reflect real-life conditions. In a feeding child, exposure to sucrose is mostly intermittent, allowing saliva to neutralize acids and maintain oral health. To accurately assess adverse reactions, a future study should mimic these natural feeding patterns. However, the term intermittent is relative, and in situations where infants continually consume milk (such as during sleep) without their teeth being brushed, leading to the formation of a thick biofilm with higher carbohydrate content [32]. In such cases, this constant exposure nearly replicates those conditions [33]; (3) permanent instead of primary teeth were used in this study to measure enamel demineralization. Thus, it may seem that the results may not be applied to primary teeth; (4) limitations of the present study also include only three HM donors. This limitation is reflected in breast milk, which differs in composition at different stages of development to meet a child's needs. Also, the mother's features, such as diet, health status, and genetics, shape variation in milk composition across lactating mothers. Future studies with multiple donors with their caries risk status, dietary intake data, and sugar consumption could help explain the relationship between cariogenic and HM.

5. Conclusions

This investigation concluded that:

1. Biofilm data suggests that HM may be protective against plaque formation *in vitro*.
2. There was an *in vitro* increased reduction in pH and increased enamel lesions under a high load of sucrose and *S. mutans*.
3. Nursing mothers should be educated on the potential increased hazard of caries in children due to the increased acidity of HM after introducing additional carbohydrates. They should consider the conditions contributing to the cariogenic environment for caries development.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

AUTHOR CONTRIBUTIONS

VS, HN and FS—designed the research study. LB, JB, RZ and JP—performed the research. VS and HN—provided help and advice on biofilm formation and caries lesion experiment. LB and RZ—analyzed the data. LB, JB, RZ, JP, HN, VS and FS—wrote the manuscript. HN and FS—revised the manuscript and checked for plagiarism; completed the necessary documents for submission and submitted the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of Missouri School of Dentistry and Oral Health, IRB: 45CFR46.104 (d)(4)(ii). All the procedures complied with the principles of the Helsinki Declaration. Written informed consent has been obtained for all donors prior to the start of the study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. Fouad Salama is serving as one of the Editorial Board members of this journal. We declare that Fouad Salama had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to SG.

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