Effect of Human Milk and its Components on *Streptococcus Mutans* Biofilm Formation

Allison LM* / Walker LA**/ Sanders BJ*** / Yang Z**** / Eckert G***** / Gregory RL******

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Objective: *This study investigated the effects of human breast milk and its components on the nutritional aspect of the caries process due to Streptococcus mutans UA159 biofilm formation.* ***Study design***: *Human breast milk was collected from 11 mothers during 3-9 months postpartum. To test for the effect on biofilm formation, a 16-hour culture of S. mutans was treated with dilutions of human breast milk and several major components of human breast milk, lactose, lactoferrin, IgA, and bovine casein in sterile 96-well flat bottom microtiter plates for 24 hours. The biofilms were fixed, washed, stained with crystal violet, and extracted. Absorbance was measured to evaluate biofilm growth mass.* ***Results***: *Dilutions 1:10-1:2,560 of the human breast milk samples increased biofilm formation by 1.5-3.8 fold compared to the control. Lactoferrin decreased biofilm formation significantly in all dilutions (average milk concentration of 3 mg/ml). Lactose had no effect at average breast milk concentrations (60 mg/ml) except at its lowest concentration (15 mg/ml) where it was increased. IgA significantly decreased biofilm formation at its highest concentration of 2,400 µg/ml (average milk concentration 600 µg/ml). Casein caused significantly increased biofilm formation at all concentrations tested above the average milk content (2.3 mg/ml).* ***Conclusions***: *The results of this study demonstrate an increase in S. mutans biofilm formation by human breast milk 3-9 months post partum. Among its major components, only casein significantly increased biofilm formation among the concentrations analyzed. Lactose had no effect except at 15 mg/ml. Lactoferrin and IgA significantly decreased S. mutans biofilm formation at their highest concentrations. This information expands the current knowledge regarding the nutritional influence of breastfeeding and validates the necessity to begin an oral hygiene regimen once the first tooth erupts.*

Key Words: human milk, breast milk, breastfeeding, lactose, lactoferrin, casein, secretory IgA, early childhood caries (ECC), S. mutans

From Indiana University School of Dentistry.

*Lesa M. Allison, DDS; Associate Pediatric Dentist; Former Pediatric Dental Resident; Indiana University School of Dentistry; Riley Hospital for Children at IU Health.

** LaQuia A. Walker, DDS, MPH; Department of Pediatric Dentistry, Indiana University School of Dentistry, Riley Hospital for Children at IU Health

***Brian J. Sanders, DDS, MS; Department of Pediatric Dentistry, Indiana University School of Dentistry, Riley Hospital for Children at IU Health

****Ziyi Yang, MS; Department of Biostatistics, Indiana University School of Medicine

*****George Eckert, MAS; Department of Biostatistics, Indiana University School of Medicine

****** Richard L. Gregory, PhD; Department of Oral Biology, Indiana University School of Dentistry

Send all correspondence to:

Lesa Allison and Richard Gregory
Indiana University School of Dentistry
1121 West Michigan Street
Indianapolis, Indiana 46202
Phone: 317-627-1755
lealliso@iupui.edu
lesa.allison@gmail.com
rgregory@iu.edu

INTRODUCTION

Human breast milk is the sole nutrition for many infants in the US and the world. The American Academy of Pediatrics (AAP) 2012 policy statement stresses the importance of breastfeeding stating it is the standard for infant nutrition.[1] The policy describes the many advantages of breastfeeding including several short and long term developmental advantages.[1] The AAP strongly considers breastfeeding a necessity for infant nutrition and that the benefits far outweigh not attempting or discontinuing breastfeeding due to lifestyle demands.[1] Their recommendation includes "exclusive breastfeeding for about 6 months, followed by continued breastfeeding as complementary foods are introduced, with continuation of breastfeeding for one year or longer as mutually desired by mother and infant".[1] The World Health Organization (WHO) also describes the importance of exclusive breastfeeding.[2] With this increased support of breastfeeding for infants and children, it has become more important for dental professionals to explain the effects of breastfeeding on the oral cavity. A recent article from the Journal of the American Dental Association stresses the responsibility of the dental profession to provide current, accurate information about the many health advantages of breastfeeding and even more so the obligation to encourage it.[3] The American Academy of Pediatric Dentistry revised the Guideline on Infant Oral Health Care in 2012

to include: "human breast milk is uniquely superior in providing the best possible nutrition to infants and has not been epidemiologically associated with caries" but that "breastfeeding greater than 7 times daily after 12 months of age is associated with increased risk for early childhood caries (ECC)".[4] Therefore, the AAPD recommends beginning oral hygiene measures after eruption of the first primary tooth.[4] Several studies are in agreement with this recommendation of early oral hygiene measures since their findings describe human breast milk as having high potential for causing dental caries, and demonstrate increased cariogenicity over cow's milk.[5-8]

With this focus on the infant's diet, the concern for dental health care professionals remains on the effect of human breast milk with oral health. Unfortunately, dental caries is still a common infectious disease among children. In particular, early childhood caries (ECC) is of concern since it may lead to immediate problems such as pain, infection, fever, malnutrition, and long-term problems including malocclusion, phonetic issues, low self-esteem, and possible systemic interactions.[9] Dental caries are associated with a bacterial biofilm which contains elevated levels of mutans streptococci, such as *Streptococcus mutans*. Caries development is characterized by early colonization of these organisms followed by biofilm formation and they are recognized as determinants for the caries process.[10] Dental biofilms mature to become complex biochemical and physiological structures mainly affected by dietary fermentable carbohydrates.[11] The principal sugar in human milk is the disaccharide lactose which demonstrates average concentrations in breast milk of approximately 6.4-7.6 g/dL.[12] Similar to sucrose, but not to the same degree, lactose is fermentable by *S. mutans*. In an experimental animal study comparing the cariogenicity of dietary sugars, lactose caused dental destruction but with a slower onset and less acute progression of the disease when compared to sucrose.[13] Many genetic, environmental, and behavioral risk factors interact but mutans streptococci are known as the leading etiological agents of caries.[14] Environmental factors include the availability or lack of nutrients, acidic pH, and exposure to organic acids.[14] These factors greatly affect the existing bacteria's ability to survive. Several strains of *Streptococcus mutans* have been isolated and identified from human carious lesions. Since all strains are phenotypically similar, identification of specific strains have been classified by serotypes and biotypes.[15] One important universal characteristic of all *S. mutans* strains is its acid tolerance with ability to grow at a pH of 4.0. Many other bacterial species are unable to survive in the same acidic environment produced by the byproducts of metabolism in carious lesions.[15] *S. mutans* preferred form of growth is in a biofilm which provides many advantages to reproduction, metabolism, and defense.[16] "Biofilms are defined as orientated aggregations of microorganisms attached to each other or to a surface and enclosed in extracellular polymeric substance (EPS) produced by themselves."[16] Lactic acid, then, is produced from *S. mutans* acting on a fermentable sugar in a biofilm which causes dissolution of minerals, such as calcium and phosphate, from teeth.[16,17]

In relation to early childhood caries and human breast milk, it is important to examine the composition of human milk and its effect on biofilm formation. Human milk composition contains nutritional and nonnutritive bioactive factors such as anti-infectious and anti-inflammatory agents, growth factors, and prebiotics.[12] Nutritional components include whey and casein proteins, palmitic and oleic acids providing the fat, and the principal sugar being lactose. Bioactive factors of human milk include several immunologic components such as secretory immunoglobulin (Ig)A, lactoferrin, leukocytes, and developmental factors such as epidermal growth factor.[12] Due to the wide array of components in human milk, cariogenicity and acidogenicity of human milk have been studied in comparison to other common infant nutrients such as cow milk, cola, and infant formula. Using an in vitro caries model in the absence of saliva, human breast milk and plain bovine milk were found to be relatively cariogenic.[18] In addition, bovine milk demonstrated greater bacterial growth, fermentation, and buffer capacity than human milk.[18] However, the authors speculate that if this study was conducted in an in vivo setting, for example in the presence of saliva, milk may have no cariogenic effects.[18] Peres et al. reported formula milk to be the most cariogenic, formula milk > human milk > cow's milk. [5] Bowen et al. described the relative cariogenicity of human and cow's milk, sucrose, cola, and honey.[8] The data from this study further confirm previous data of the low cariogenicity of cow's milk. Comparisons were made by giving a number to each milk or additive in terms of their cariogenicity in relation to each other. Cow's milk which displayed the lowest cariogenicity of the group was 0.01 with cola being the highest at 1.16 and human milk at 0.29.[8] Therefore, human milk was shown to be more cariogenic than cow's milk.[8] A related study using a desalivated rat model found similar results, that human milk was more cariogenic than cow's milk but not as much as infant formula.[5] In addition, Hegde et al. studied the cariogenic potential of stored human milk reporting an increase in *S. mutans* colony counts in human milk stored at various temperatures[19], demonstrating the potential for the ability of human milk to increase *S. mutans* growth and possibly biofilm formation.

Essential to investigating human milk's properties is to appreciate its complex interactions. It does, in fact, provide caries protective properties as well. The protective potential of human milk and whole bovine milk are comparable, but the individual milk fractions including casein, whey protein, lactose, and milk fat, were more protective in bovine milk than human breast milk.[20] Shetty et al. went further to order the fractions of human milk in the order of potential caries protective action, "casein>whey protein>lactose>milk fat".[20] Two studies have tested the effects of human milk and the casein components of milk on the adhesion of *S. mutans*.[10,21] Human breast milk was discovered to inhibit *S. mutans* binding to synthetic hydroxyapatite in 71% of 21 mothers' samples tested; although, there was never complete inhibition in any sample.[10] Caseinoglycomacropeptide and caseinophosphopeptide, both components of milk, reduced the adherence of *S. mutans* significantly.[21] According to Lönnerdal et al., the average concentration of casein in mature human milk is 2.33 mg/ml.[22] Human milk contains predominantly β-casein which differs qualitatively and quantitatively from bovine milk. Casein content of human milk is much lower than cow's milk amounting to about 20-40% of total protein content and the remainder being whey protein. Cow's milk also contains β –casein but the quality of human milk casein differs with smaller micelle size and lower phosphorus contents[23], however these differences are not thought to significantly affect *S. mutans*.

Additional components to consider are lactoferrin and the sIgA antibodies present in human milk. Lactoferrin is an important bioactive factor which chelates iron, and serves as an antibacterial factor

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and as an antioxidant.[12] The lactoferrin concentration in mature milk has been reported to be 2.6 g/L.[24] Experiments strongly suggest that sIgA is the crucial protective component of breast milk in providing protection in infants against epithelial infections.[25] Kawano et al. measured sIgA levels in postpartum mothers during the first 12 weeks after delivery. SIgA concentration in human milk peaked on day 3 and declined rapidly over the first 4 weeks of lactation.[26] The concentration then gradually declined from week 4 to week 12 of lactation.[26] Breast milk sIgA averaged 2,178.5 µg/ml on day 3, then decreased on week 8 to a concentration of 1,140.5 µg/ml.[26] sIgA concentrations measured approximately 939.5 µg/ml by 12 weeks postpartum.[26] Levels of IgA then generally decrease to an average of 518-853µg/ml dependent on the individual.[27] SIgA levels have been compared in studies of caries resistant and caries susceptible individuals. Gregory et al. discovered that caries resistant individuals had higher levels of salivary sIgA antibodies to *S. mutans* antigens and the saliva from these individuals inhibited growth, adherence, and metabolic activity of *S. mutans*.[28] Consequently, it is important to recognize the complex relationship that human milk provides with possible cariogenic and caries protective effects.

In order to help discern this association between breastfeeding and ECC, the purpose of the study was to explore the ability of human milk and its various components to affect *S. mutans* biofilm formation.

MATERIALS AND METHOD

The study was comprised of 11 female participants. Institutional Review Board approval was obtained. Inclusion criteria included the following: 3-9 months post partum, over 18 years old, willing and able to sign the informed consent and authorization form, generally healthy (ASA I and II; no systemic health issues or well controlled mild systemic conditions), minimum of once per day breast feeding, no known IgA deficiency, no illegal drug use, no tobacco use, and no antibiotic or anti-inflammatory drug use within the past 3 months.

Subjects were recruited at random by flyers around the Indiana University Purdue University Indianapolis campus, Indiana University Health Clinics and Hospitals (including Riley Dental Clinic), and emailing the aforementioned flyer to colleagues and friends. The subjects were de-identified and given subject numbers for labeling of sample collection tubes and coolers. Informed consent and authorization was obtained from the subjects.

Once the mother was recruited, she was consented by the investigators and given a subject number. After consent was obtained, each subject was given a sterile capped collection tube and cooler to keep the breast milk cold during transportation to the lab. The participant was then either given a specific timeline of when to collect (in accordance with 3-9 months postpatrum guideline) or breast milk was collected immediately. Each participant obtained a one time human breast milk sample of 30 ml at their desired place in the normal manner of collecting breast milk (i.e., breast pump, manual expression, etc). Samples were stored on ice until processed at the laboratory. Participant samples were delivered to or picked up by the principal investigator and taken to the laboratory within 4 hours of collection by the subject. After arrival at the lab, the samples were centrifuged for 10 minutes at 3,000 x g at 4°C upon receipt and the supernatant filter sterilized (0.45 um) and stored at -80°C until used.

S. mutans strain UA159 was used in the study due to its completely sequenced genome.[29] The strain was stored at -80˚C in tryptic soy broth (TSB, Acumedia, Baltimore, MA) with 20% glycerol before use. Mitis Salivarius Sucrose Bacitracin (MSSB, Anaerobe Systems, Morgan Hill, CA) agar plates were used to initially grow the strains. TSB was the sole media used and the growth conditions were incubation in 5% CO_2 at 37˚C.[17] Lactose (α-lactose monohydrate), lactoferrin (from human milk), casein (from bovine milk), and purified human colostral IgA were all obtained from Sigma Chemical Co., St. Louis, MO.

To determine biofilm formation, an overnight *S. mutans* culture (10^6 CFU/ml) in TSB was treated with various concentrations of human breast milk samples and macronutrients including lactose, casein, lactoferrin, and IgA. A preliminary test was conducted to determine the best concentrations to use. Breastmilk samples were tested at dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1,280, 1:2,560, and 1:5,120. Lactose, lactoferrin, casein, and IgA, were 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 lactose (60 mg/ml), lactoferrin (3 mg/ml), casein (2.3 mg/ml), and IgA (600 µg/ml) were established from current literature.[12,22,24,26] Controls included TSB with and without bacteria. The samples were combined with TSB (190 µl total volume) along with 10 µl of an overnight culture of *S. mutans* and incubated for 24 hours in sterile 96-well microtiter plates. Biofilms were fixed with 10% formaldehyde (Fisher Scientific, Co., Fair Lawn, NJ) for 1 hour, washed twice with distilled water, and stained with 0.5% crystal violet (Fisher Scientific) for 1 hour.[17] After washing the biofilm two times with distilled water, crystal violet was extracted from the biofilm cells by 200 µl of 2-propanol (Fisher Scientific, Co., Fair Lawn, NJ) for 1 hour. The extract was read at 490 nm with 2-propanol used as a blank control.

Statistical Analyses

Each experiment for the individual milks and milk components was repeated three times. Summary statistics (mean, standard deviation, standard error of the mean, range) were calculated for each milk and milk component at each dilution for biofilm mass. Comparisons against the controls were made using ANOVA, allowing different variances for each group, with a Sidak multiple comparisons adjustment used to control the overall significance level at 5%. A 5% significance level was used for all tests. The ratio of the data relative to the TSB control followed a log-normal distribution with a coefficient of variation of 1.0.

RESULTS

Human breast milk samples were tested separately by subject and representative graphs prepared for each individual (Fig. 1). Human breast milk from subjects 1 and 10 demonstrated an increase in biofilm formation above the control at the first 3 dilutions (1:10, 1:20, and 1:40). The remaining dilutions approached the control level or revealed decreased biofilm formation. Subjects 2 and 3 demonstrated the same trend but displayed increased biofilm in the first 3 dilutions. Subjects 4, 5, and 7 demonstrated higher biofilm formation similar to Subject 2 and 3 within the first several dilutions, but did not reach levels comparable to the control until later dilutions of the milk sample (1:320). Subjects 6, 8, 9, and 11 breast milk samples increased biofilm formation at lower dilutions but reached

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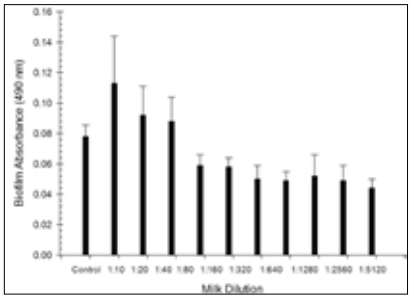
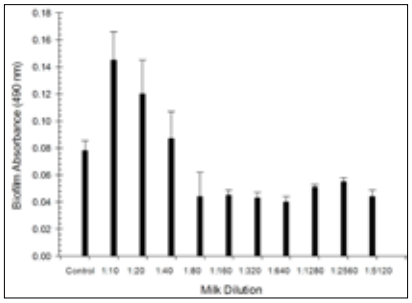
Figure 1. Effect of dilutions of eleven individual human breast milk samples on S. mutans biofilm formation. Each graph identifies the mean biofilm absorbance for each breast milk dilution.

Subject 1



Subject 2



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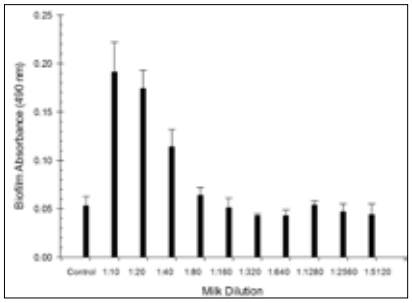

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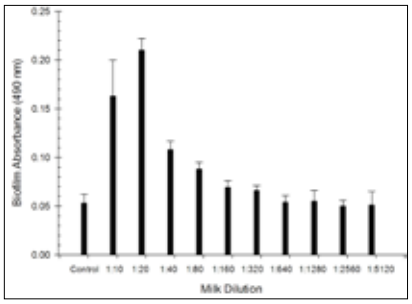

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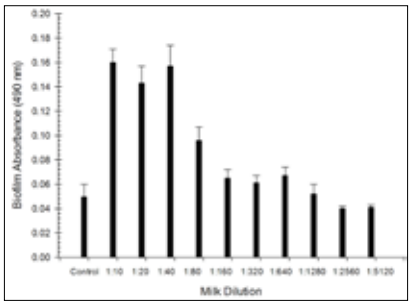

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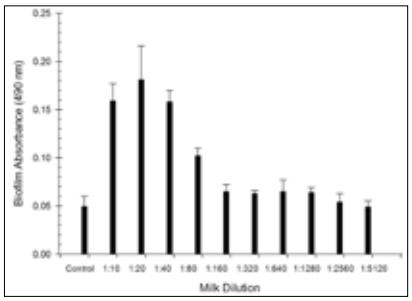

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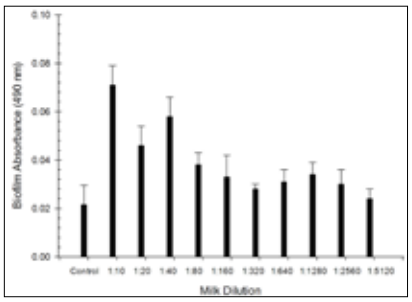

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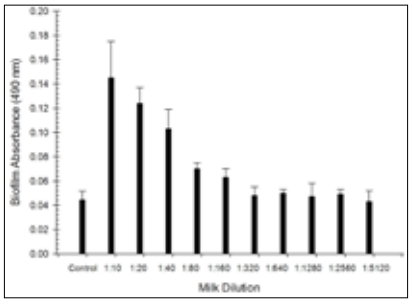

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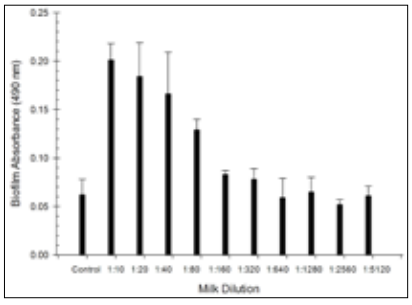

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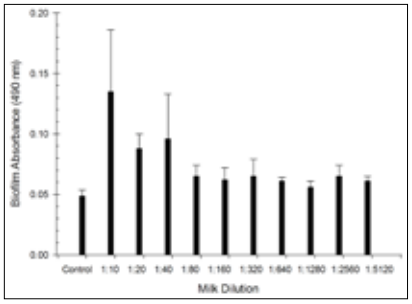
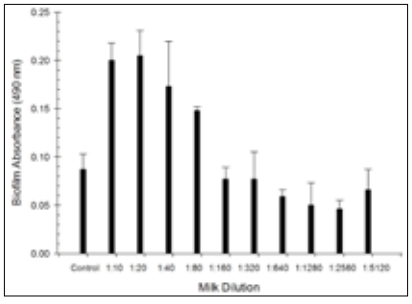


Subject 11



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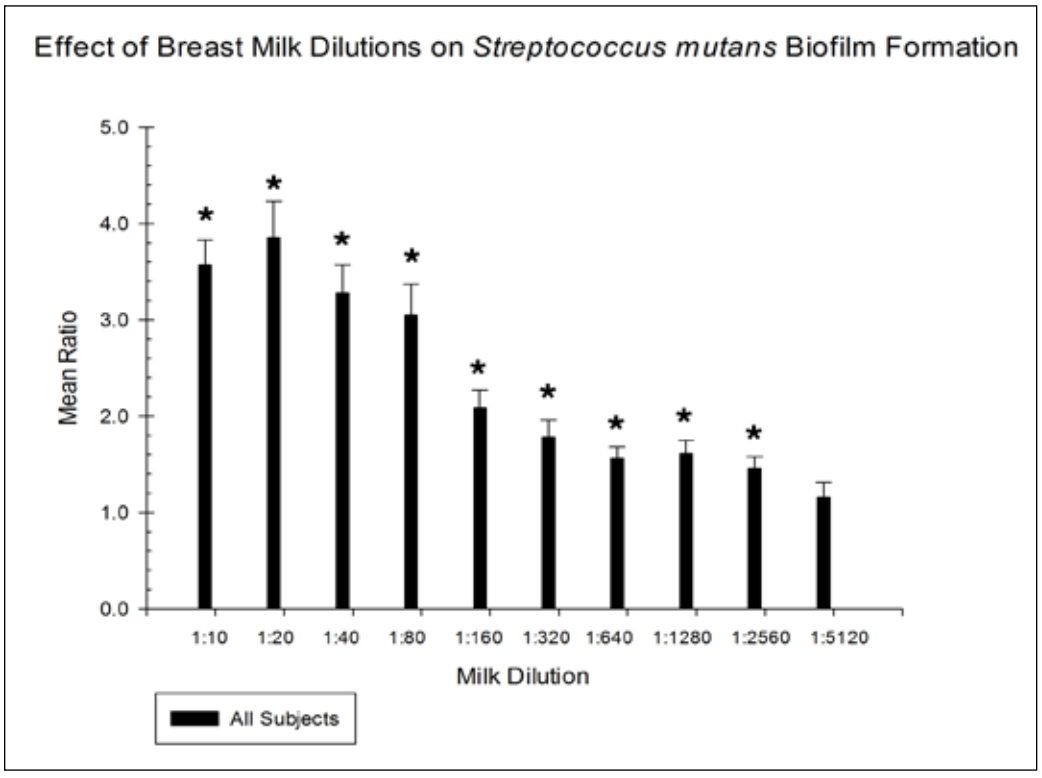
levels comparable to the control and lower at the 1:160 dilution and subsequent dilutions. All subjects' breast milk sample data were grouped by dilution and a mean ratio calculated for each dilution factor in comparison to the TSB control. This is depicted in a single graph to visualize how much biofilm formation was increased by the human breast milk samples (Fig. 2). Dilutions 1:10-1:2,560 of the human breast milk samples increased biofilm formation by 1.5-3.8 fold compared to the control.

Lactoferrin decreased biofilm formation significantly in all dilutions (0.075-12 mg/ml; except 1.5 mg/ml) analyzed (Fig. 3). Lactose had no effect at average breast milk concentrations, but significantly increased biofilm formation at its lowest concentration (15 mg/ml). IgA significantly decreased biofilm formation at its highest concentration (2,400 μg/ml) and remained comparable to the control at its lowest concentration (150 μg/ml). Lastly, 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.

DISCUSSION

It appears that human breast milk increases *S. mutans* biofilm formation. Samples from all 11 subjects resulted in an increase in biofilm, analogous to dental plaque. Results of this study validate the AAPD recommendation to begin an oral hygiene regimen once the first tooth erupts. However, due to many other factors and complex interactions, it is difficult to discern if human breast milk, in fact, causes early childhood caries directly. First, the mechanism of breastfeeding is different than drinking from a bottle. A breastfeeding child draws milk by compressing the nipple against the palate with soft peristaltic motions.[31] In contrast, a bottle fed child uses a much more powerful sucking motion of lips and cheeks in a "piston-like" motion to compress the bottle nipple.[31] Baby bottles have been identified as an associated risk factor to ECC in some countries. "Baby bottles predispose children to ECC because their nipple blocks the access of saliva to the upper incisors, whereas lower incisors are close to the main salivary glands and are protected from the liquid contents by the bottle, nipple and the tongue".[32] In bottle fed infants, the mechanics of sucking were fewer and of longer duration than breastfed infants.[33] Secondly, humans, in particular infants, have greatly varying amounts of saliva individually and also significant variations throughout the day. "Salivary flow is [also] decreased during sleep, and clearance of the liquid from the oral cavity is slowed".[34] Because of these high variations of salivary flow, it is extremely difficult to know an exact concentration at which breast milk would be present in the mouth at any given time for a given infant. For this reason, a range of concentrations was tested but appreciably, the amount of biofilm formation established across dilutions varied greatly. Thirdly, unless the child is sleeping with the mother throughout the night or breast milk is contained in a bottle, the child does not have a frequent source of breast milk. Next, the introduction of fermentable carbohydrates is closely associated with 6 months of age.[1] Van Palenstein Helderman and colleagues identified this difficulty in determining whether ECC can be contributed

Figure 2. Effect of all 11 human breast milk samples at each dilution on *S. mutans* biofilm formation represented as a mean ratio. The ratio is defined as the crystal violet absorbance of each milk dilution compared to the TSB control (set at 1.0). For example, the 1:10 dilution of milk increased biofilm formation 3.6 fold compared to the TSB control. Asterisks indicate statistically significant differences compared to control values ($p<0.05$).



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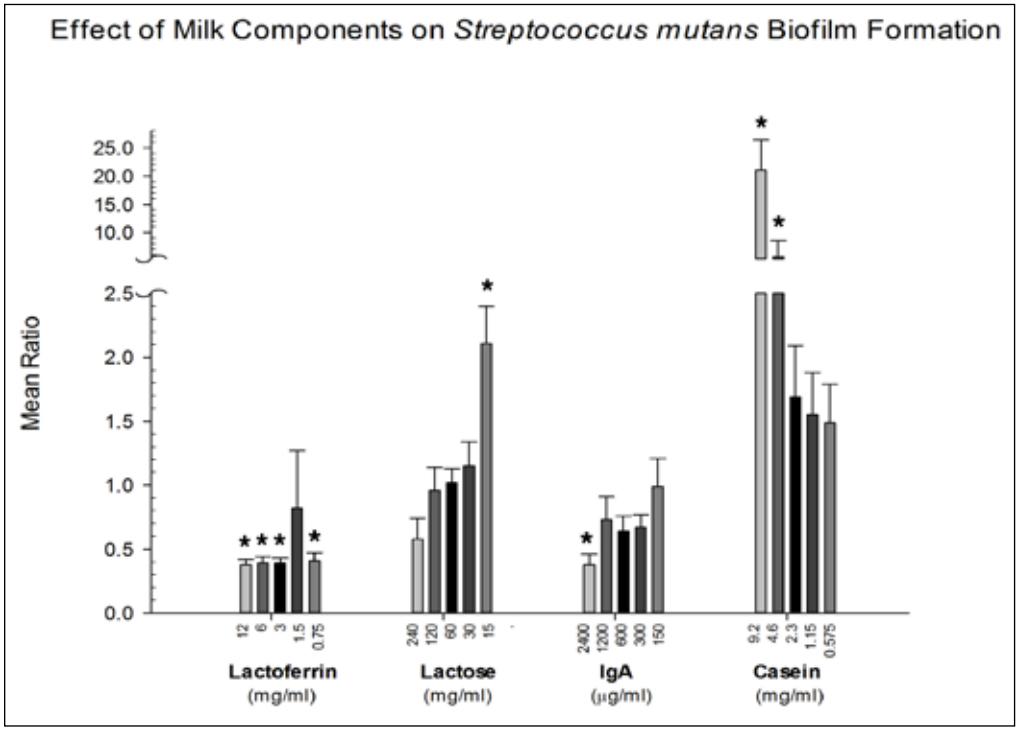
solely to breastfeeding since complementary foods, possibly highly cariogenic, are also being given. "Exclusive breast-feeding is only performed during the first 6 months of life, and subsequent introduction of new foods, especially those rich in sucrose, into the diet is a confounding factor when analyzing the association between breast-feeding and caries".[35] On the other hand, Hallonsten and colleagues reported "that children who experience prolonged breast-feeding tend to develop unsuitable dietary habits that put them at risk for caries at an early age".[36] Lastly, the fidelity of *S. mutans* transfer from the mother, siblings, or close playmates determines the virulence of *S. mutans* colonized in the infant's mouth.[14] Specific areas of further research would be beneficial in testing the *S. mutans* strains from the mother and infant, in conjunction with the human breast milk samples gathered from each mother-infant pair.

CONCLUSIONS

The results of this study demonstrate an increase in *S. mutans* biofilm formation by human breast milk. Among its major components, only casein significantly increased biofilm formation above average milk concentrations. Lactose at average breast milk concentrations and higher had no effect on biofilm formation, however, the lowest concentration (15 mg/ml) caused increased biofilm formation. Lactoferrin and sIgA significantly decreased *S. mutans* biofilm formation at the highest concentrations analyzed. This information expands the current knowledge regarding the nutritional influence of breastfeeding and validates the necessity to begin an oral hygiene regimen once the first tooth erupts.

Figure 3. Effect of major milk components (lactoferrin, lactose, IgA, and casein) on *S. mutans* biofilm formation. Average milk concentrations of each component were lactoferrin-3 mg/ml, lactose-60 mg/ml, IgA 600 µg/ml and casein-2.3 mg/ml. Each bar represents the ratio of biofilm formation for a specific dilution of the indicated component compared to the TSB control (set at 1.0). For example, lactoferrin at its first concentration of 12 mg/ml significantly decreased biofilm formation to 0.4 fold of the control. Asterisks indicate statistically significant differences compared to control values ($p<0.05$).



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