The Acid Production of *Streptococcus mutans* after Prolonged Culturing in Human Breast Milk

Yart-ruetai Kosakul[1*] Chutima Trairatvorakul[2] and Panida Thanyasrisung[3]

Abstract

The study aims to investigate an adaptation of acid production of *Streptococcus mutans* (a cariogenic pathogen) after prolonged exposure to human breast milk (HBM). *S. mutans* UA159 were grown in pooled HBM (pHBM) from 9-11 mothers. The bacterial cultures were then sub-cultured in pHBM up to the 5th passage (1 passage = 11 hours). The bacterial cells were collected at the 0 passage as a control and at the 1st and 5th passages to determine the alteration of acid production. The bacterial cells from each passage were then inoculated in pHBM. The pH and the number of colony forming units (CFU) were determined every hour for 6 hours (T0-T6). There was a similar number of CFU at T0 for all tested-passages (approximate 10^8 CFU/ml). Moreover, in each tested-passage, there was no significant difference of bacterial growth during T0-T6 (approximate 10^8 CFU/ml). Therefore, the acid production was determined based on pH without disturbance from the amount of bacteria. The adaptation of acid production was defined as time duration for pH reduction to the pH less than a critical pH of enamel (pH=5.5). At the 0 passage, the pH was lower than 5.5 after 4 hours whereas the 1st and 5th passages took 3 hours to reach the lower critical pH. The average pH of the 5th passage after 3 hours was 5.03 lower than the 1st passage (pH=5.39) and the 0 passage (pH=6.09). The pH of pHBM control (no bacteria) was not significant different during T0-T6 (pH≈7.39). In conclusion, *S. mutans* can gradually lower the pH to the level which could demineralize teeth after 4 hours of incubation in pHBM. Furthermore, the prolonged exposure to pHBM enhances an ability of bacteria to reduce the pH faster than a usual condition. Therefore, health care providers should pay more attention to motivate parents and caregivers to provide adequate oral hygiene for their children.

Keywords: Human Breast Milk, *Streptococcus mutans*, Acid Production, Adaptation

[1] Graduated student, Department of Pediatric Dentistry, Faculty of Dentistry Chulalongkorn University, Bangkok 10330

[2] Prof., Department of Pediatric Dentistry, Faculty of Dentistry Chulalongkorn University, Bangkok 10330

[3] Assist. Prof., Department of Microbiology and Research Unit on Oral Microbiology and Immunology, Faculty of Dentistry Chulalongkorn University, Bangkok 10330

* Corresponding author: Tel.: 086-3523456 E-mail address: yartfon@gmail.com

Introduction

Human breast milk (HBM) can prevent infants from many common infectious diseases and also provides economic and excellent nutrition for brain and physical development [1-3]. Therefore, exclusive breastfeeding has been recommended worldwide at least through the age of six months and can continue with a proper supplementary food until the age of 2 or older [4]. There is still an argument on the cariogenic potential of HBM since most of its composition seems to have less cariogenic properties. For example, casein, immunoglobulin, lactoferrin and alpha-lactabumin prevent adhesion, growth and biofilm formation of *S. mutans* (a major pathogen of dental caries) [5, 6]. Although, lactose (the main sugar in HBM) has cariogenic potential, its acid by-product is less acidic than those of sucrose, glucose, and fructose [7]. Many epidemiological studies also supported that breastfeeding is not related to caries development [8-11]. In contrast, several clinical studies revealed the association between feeding habits such as nocturnal, prolonged and on-demand breastfeeding and early childhood caries (ECC) [12-15]. The animal study also showed that on-demand feeding with HBM could promote caries [16, 17]. These studies suggested that *S. mutans* may improve their acid production ability from lactose when they are prolonged exposed to HBM.

An adaption in acid production of *S. mutans* after exposure to lactose for a particular time has been reported [18, 19]. *In vitro* study showed that *S. mutans* which pre-cultured in lactose media for 1 hour can cause tooth demineralization within 1.5 hours whereas non-lactose-induced *S. mutans* has no effect on tooth [18]. Moreover, *S. mutans* grown in lactose media for 3 weeks increase their acid production ability from lactose in a similar level as acid production from glucose of *S. mutans* glucose-grown cells [19]. Concordantly, a clinical study found marked decrease of plaque pH after frequent exposures to lactose for 6 weeks comparing to non-lactose-induced dental plaque [19].

To survive in HBM which is an unfavorable condition, *S. mutans* adaptation time for utilizing HBM may be required. Therefore, this study aims to investigate the adaptation in acid production of *S. mutans* after prolonged exposure to HBM.

Materials and Methods

The study protocol was approved by the Human Research Ethic Committee of the Faculty of Dentistry, Chulalongkorn University (HREC_DUC 2016-050). HBM samples were donated by 9-11 healthy lactating mothers who still breastfeed their child at least once a day and fulfilled with inclusion criteria as following: 6-18 months postpartum, over 18 years old, full-termed pregnancy and willing to participate in this study and sign a consent form. Mothers who had taken alcohol, antibiotics or non-steroidal anti-inflammatory drug within 3 months were excluded. The milk samples were expressed at one time by their preferred methods (breast pump or hand expression) and transported in disposable breast milk-storage plastic bags (Marinda, My inspiration, Thailand) on ice to laboratory within 4 hours. The samples were centrifuged at 3,000 xg for 10 minutes at 4°C. The supernatant was filter-sterilized through 2.5 µm pore-sized filter papers (Whatman Grade 5, UK) and followed by 0.45 µm pore-sized filter papers (Sartorius stedim, Germany) [6]. The sterile samples were pooled and aliquot into sterile tubes and stored at -20°C until use.

S. mutans UA159 from a glycerol stock were inoculated on a Brain-Heart Infusion (BHI) agar plate (HiMedia Laboratories, India) and incubated at 37°C, 5%CO_2 for 2 days. The isolated colony then was grown overnight in BHI broth with continuous shaking at 240 rpm. The overnight cultures were diluted to optical density at 600 nm (OD_{600nm}) of 0.1 and continued to incubate until the log phase (OD_{600nm} = 0.4-0.6) was reached. The cells were then obtained for further assay.

In an adaptation assay, the pooled HBM (pHBM) stock was thawed at room temperature. The log-phase cells were washed 3 times with phosphate-buffered saline (PBS) and then suspended in pHBM to obtain an OD_{600nm} of 0.1 (approximate 10^8 cfu/ml). To promote an adaptation, the cultures were incubated at 37°C, 5%CO_2 for 11 hours (= 1 passage) and then sub-cultured into pHBM up to 5 passages.

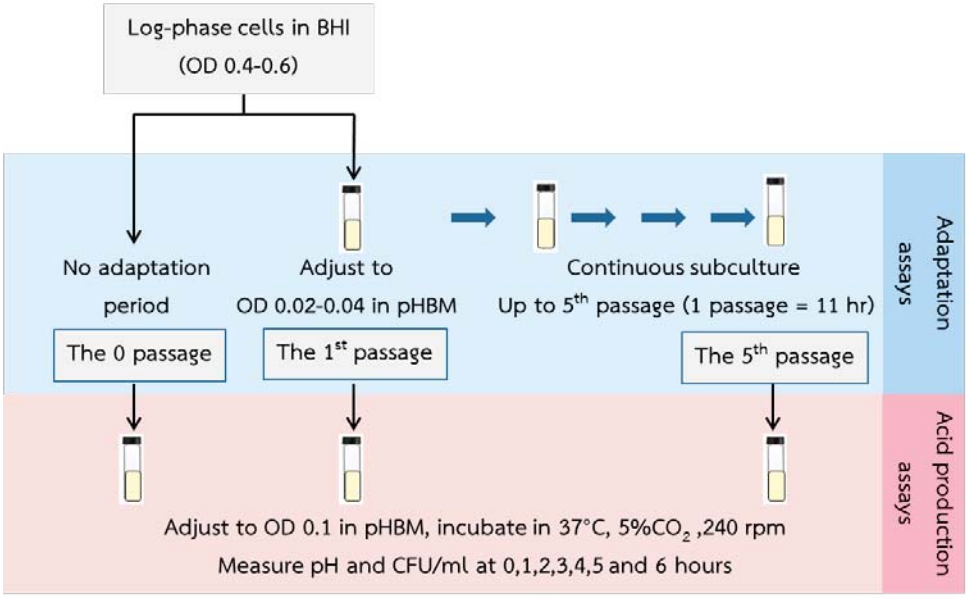

Figure 1 Workflow for measurement of acid production and the number of bacterial cells.

To investigate the adaptation, an acid production assay was performed. If bacteria increase ability to utilize lactose in HBM, they will produce more acid. In the assay, the log-phase cells obtained from BHI culture were incubated in pHBM for 6 hours as a control (the 0 passage). The cells were collected at the 1st and 5th passages and washed 3 times with PBS. After that, they were adjusted to OD_{600nm} of 0.1 in 7%-sucrose-supplemented BHI (BHI-S) and also in pHBM. Then both cultures were further grown at 37°C, 5% CO_2 with shaking at 240 rpm. Two hundred microliters of bacterial culture from each group were obtained at 0, 1, 2, 3, 4, 5 and 6 hours (T0-T6) in order to measure the number of bacterial colony and pH in each time point. (Figure 1)

The acid production of *S. mutans* will be determined from cultural pH value measured by pH meter (Compact pH Meter, Horiba, Japan). The CFU counts were measure by miniaturized plating method [20].

The experiments were performed 3 times. Data were shown as mean ± standard deviation (SD). Statistical comparison of the number of CFU at T0 was done by ANOVA. The bacterial growth during T0-T6 of each passage was compared using a General Linear Model for repeated measures. The adaptation of acid production was defined as time duration for pH reduction to the pH less than a critical pH of enamel (pH=5.5). The SPSS software package version 17.0 (SPSS 17.0, SPSS Inc., USA) was used in this study. A significant difference was determined when *P*-value was less than 0.05.

Results and discussion

The mean age of mothers and child were 31 ± 5 years and 11 ± 2 months, respectively. Since the amount of initial bacteria attributed to pH-dropping rate of the sample, the numbers of bacteria at the beginning of acid production measurement were analyzed for statistical difference. All tested-passages showed no significant difference of initial bacterial counts with approximate 10^8 CFU/ml ($P>0.05$) (Table 1). The initial pH was also no significant difference among each passage (Table 1). Previous studies reported that children who harbored *S. mutans* more than 1×10^6 CFU/ml had severe early childhood caries [21]. Therefore, the initial number of *S. mutans* in this study (approximate 10^8 CFU/ml, Table 1) simulated the poor oral hygiene condition.

Table 1 Mean comparison of initial *S. mutans* counts, initial pH and pH after 3 hours of acid production measurement using one-way ANOVA

S. mutans cells	Initial log CFU/ml	Initial pH	pH_{3hrs}
The 0 passage	8.21 ± 0.15	7.48 ± 0.02	6.09 ± 0.14
The 1st passage	8.08 ± 0.34	7.77 ± 0.10	5.39 ± 0.37 *
The 5th passage	8.34 ± 0.16	7.57 ± 0.40	5.03 ± 0.19 *

*$P < 0.05$ compared with the 0 passage.

Moreover, each tested-passage was no significant different of bacterial growth during T0-T6 (Figure 2). Hence, the acid production was determined based on pH without disturbance from the amount of growing bacteria. The other factor that influenced the pH value was spoilage process of HBM. Bacterial spoilage was prevented by filtration of pHBM. Although contaminated bacteria were removed, HBM spoilage could occur from chemical or thermal reaction. Thus decontaminated-pHBM pH values were measured as a control and it was not significant different during T0-T6 (pH≈7.39) (Figure 3). It could be implied that pH change in tested groups directly arose from HBM metabolism of *S.mutans*. The mean pH of HBM of each tested-passage during 6 hours was presented in figure 3.

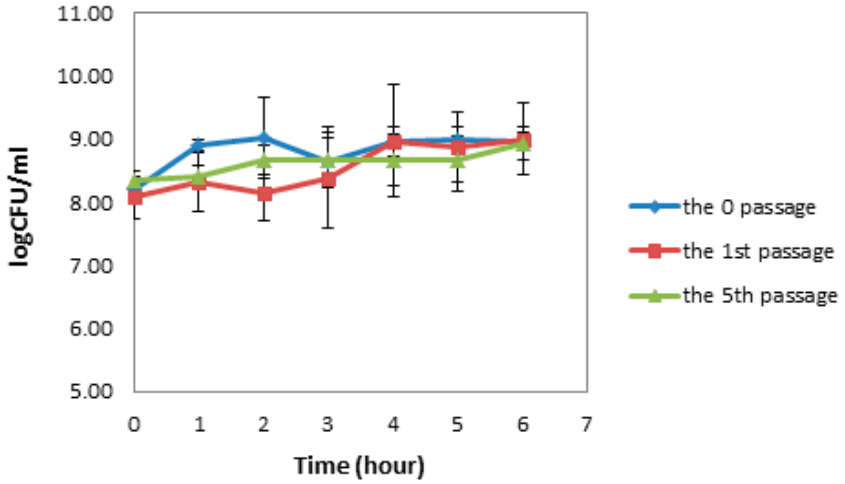

Figure 2 The numbers of bacteria (log CFU/ml) from the beginning to 6 hours (T0-T6).

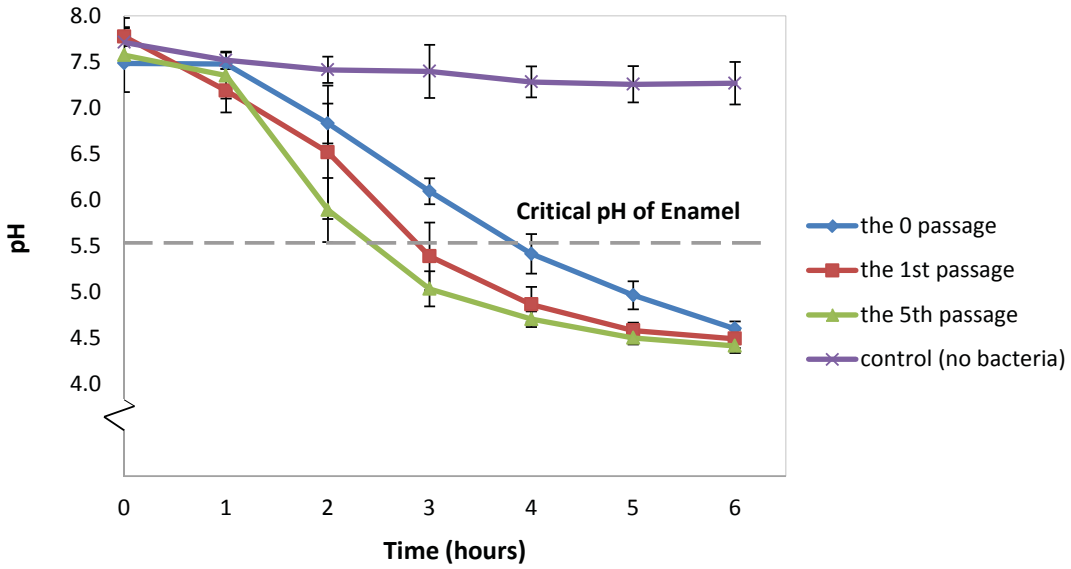

Figure 3 The mean pH of HBM of each tested-passage from the beginning to 6 hours (T0-T6).

At the 0 passage, the pH was lower than 5.5 (the critical pH of enamel) at 4 hours whereas the 1st and 5th passages took approximately 3 hours. The average pH of the 1st and 5th passages at 3 hours were 5.39± 0.37 and 5.03± 0.19, respectively which were statistically less than the 0 passage (P=0.042 and P=0.006, respectively; Table 1). The result of the 0 passage confirmed that *S. mutans* can metabolize sugars in HBM (mainly lactose) and produce acids but it takes time to be lower than the critical pH of enamel. Moreover, the increase of the period of exposure to pHBM (the 1st and 5th passages) tended to enhance the acid production ability of bacteria (Figure 3). This result indicated that the prolonged exposure to HBM may create an opportunity for *S. mutans* to adapt itself to utilize lactose in HBM. Concordantly, the previous evidence showed that *S. mutans* has ability to adapt itself in many stressful conditions [22]. For instance, prolonged consumption of xylitol, a sugar that could inhibit *S. mutans* metabolism, resulted in increased amount of xylitol-resistant *S. mutans*. Hence it had greater ability to utilize glucose and fructose more than xylitol-sensitive *S. mutans* [23]. HBM is a starving condition for *S. mutans* since it contains lactose which is a non-preferred carbohydrate source. The possible mechanism of adaptation in a limiting-nutritional circumstance such as HBM may involve in an increase of gene expression contributing to lactose catabolic pathways [24-26].

The components of HBM have both protective and cariogenic properties and dental caries is a multifactorial disease. Hence, the increment of acids found in this study may not initiate the dental caries but it increases the caries risk, especially when the overall protective factors in children mouth are inadequate such as low saliva flow rate during sleep, poor oral hygiene etc. Our study may also help to explain the previous clinical findings on the relationship between the nocturnal feeding situation and early childhood caries. Since HBM is the most important source of nutrients and other beneficial factors for children, we still strongly agree with WHO recommendation to keep breastfeeding for 6 months and can continue with a proper supplementary food until the age of 2 or older [4]. Therefore, the easiest way to prevent dental caries is just to remove bacteria (dental plaque) by brushing.

Conclusion

Our study exhibited that *S. mutans* after prolonged exposure to HBM can alter itself to utilize lactose in HBM which results in the decrease of pH to the level below critical pH of enamel faster than usual. The findings of this study will hopefully convince parents, caregivers, human breast milk advocates and pediatricians to pay more attention on children's plaque control along with breastfeeding, thus it leading to a decrease in the risk of developing ECC and consequently improving children's quality of life.

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