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ACID PRODUCTION RATE OF *STREPTOCOCCUS MUTANS* AFTER ADAPTATION IN HUMAN BREAST MILK

Miss Yart-ruetai Kosakul

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Pediatric Dentistry

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By	Miss Yart-ruetai Kosakul
Field of Study	Pediatric Dentistry
Thesis Advisor	Professor Chutima Trairatvorakul, D.D.S., M.S.
Thesis Co-Advisor	Assistant Professor Panida Thanyasrisung, D.D.S., Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Dentistry

(Assistant Professor Suchit Poolthong, D.D.S., Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Thipawan Tharapiwattananon, D.D.S., Ph.D.)

.....Thesis Advisor

(Professor Chutima Trairatvorakul, D.D.S., M.S.)

.....Thesis Co-Advisor

(Assistant Professor Panida Thanyasrisung, D.D.S., Ph.D.)

.....External Examiner

(Professor Rawee Teanpaisan, Ph.D.)

หยาดฤทัย โก้สกุล : อัตราการผลิตกรดของเชื้อสเตร็ปโตค็อกคัส มิวแทนส์ภายหลังจากการปรับตัวในนมแม่ (ACID PRODUCTION RATE OF *STREPTOCOCCUS MUTANS* AFTER ADAPTATION IN HUMAN BREAST MILK) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.(พิเศษ) ทพญ. ชุติมา ไตรรัตน์วรกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ทพญ. ดร. พนิดา ธัญญศรีสังข์, 91 หน้า.

วัตถุประสงค์ การศึกษานี้มีขึ้นเพื่อศึกษาการปรับตัวในการผลิตกรดของเชื้อสเตรปโตคอคคัส มิวแทนส์ ภายหลังจากการเพาะเลี้ยงในนมแม่เป็นระยะเป็นเวลานาน

วิธีวิจัย เชื้อสเตรปโตคอคคัส มิวแทนส์ ถูกเพาะเลี้ยงในนมแม่จากมารดาจำนวน 11 ราย เพื่อสร้างสภาวะในการปรับตัว เชื้อจะถูกเพาะเลี้ยงเป็นเวลา 15 รอบ (11 ชั่วโมงต่อ 1 รอบ) ในนมแม่ นมแม่ผสมอาหารเลี้ยงเชื้อบีเอชไอ และ อาหารเลี้ยงเชื้อบีเอชไอ(กลุ่มควบคุม) เก็บเชื้อรอบที่ 0 1 5 9 13 และ 15 จากนั้นนำเชื้อมาวัดการผลิตกรดและการเจริญเติบโตทันทีในนมแม่และในอาหารเลี้ยงเชื้อบีเอชไอผสมน้ำตาลซูโครสร้อยละ 7 ทำการวัดค่าความเป็นกรด-ด่าง และจำนวนเชื้อ ทุกๆ 1 ชั่วโมงเป็นระยะเวลา 6 ชั่วโมง และที่ 12 ชั่วโมง และคำนวณอัตราการผลิตกรดในช่วงที่มีการลดลงของค่าความเป็นกรด-ด่างมากที่สุด

ผลการทดลอง การผลิตกรดในนมแม่ และในอาหารเลี้ยงเชื้อบีเอชไอผสมน้ำตาลซูโครสร้อยละ 7 ของเชื้อสเตรปโตคอคคัส มิวแทนส์ มีการเปลี่ยนแปลงไปภายหลังเชื้อถูกเลี้ยงในอาหารทั้ง 3 ชนิด เป็นระยะเวลา 11 ชั่วโมง (1 รอบ) การผลิตกรดในนมแม่จากเชื้อที่ถูกเลี้ยงในนมแม่และในนมแม่ผสมอาหารเลี้ยงเชื้อบีเอชไอ สามารถทำให้ค่าความเป็นกรด-ด่างลดลงต่ำกว่าค่าความเป็นกรด-ด่างวิกฤตของเคลือบฟันภายใน 2-3 ชั่วโมง ซึ่งเร็วกว่าการผลิตกรดในนมแม่จากเชื้อรอบที่ 0 ที่ใช้เวลา 4 ชั่วโมง อัตราการผลิตกรดในนมแม่และในอาหารเลี้ยงเชื้อบีเอชไอผสมน้ำตาลซูโครสร้อยละ 7 ไม่มีความแตกต่างกันในทุกกลุ่มการทดลอง นอกจากนี้การเพาะเลี้ยงเป็นระยะเวลานานขึ้น ไม่ส่งผลต่อการเปลี่ยนแปลงการผลิตกรดในรอบที่ 1 ถึงรอบที่ 15

สรุปผลการศึกษา ภายหลังการเพาะเลี้ยงในนมแม่ เชื้อสเตรปโตคอคคัส มิวแทนส์ สามารถใช้นมแม่ได้ดีขึ้น นำไปสู่เวลาที่สั้นกว่าสภาวะปกติในการไปถึงค่ากรด-ด่างวิกฤต

ภาควิชา	ทันตกรรมสำหรับเด็ก	ลายมือชื่อนิสิต .....
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ปีการศึกษา	2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5775830032 : MAJOR PEDIATRIC DENTISTRY

KEYWORDS: HUMAN BREAST MILK / STREPTOCOCCUS MUTANS / ADAPTATION / ACID PRODUCTION RATE

YART-RUETAI KOSAKUL: ACID PRODUCTION RATE OF *STREPTOCOCCUS MUTANS* AFTER ADAPTATION IN HUMAN BREAST MILK. ADVISOR: PROF. CHUTIMA TRAIRATVORAKUL, D.D.S., M.S., CO-ADVISOR: ASST. PROF. PANIDA THANYASRISUNG, D.D.S., Ph.D., 91 pp.

Objective: This study aims to investigate an adaptation of acid production of *S. mutans* to long-term exposure to human breast milk (HBM).

Materials and Methods: *S. mutans* UA 159 were grown in pooled HBM from 11 mothers. To create the adaptation condition, *S. mutans* were sub-cultured up to the 15th passage (11 hours/passage) in HBM, BHI supplemented HBM (HBM+BHI) and BHI control. The bacterial cells were collected at the baseline (the 0 passage), the 1st, 5th, 9th, 13th and 15th passage to immediately determine the acid production and growth in HBM and 7% sucrose supplemented BHI (BHI+Sucrose). The pH and the number of bacteria were measured every hour for 6 hours and after 12 hours of incubation. The acid production rate was calculated at the fastest pH-dropping duration.

Results: The acid production in HBM and in BHI+Sucrose of *S. mutans* was changed after exposure to all three media for 11 hours (the 1 passage). The acid production in HBM of HBM and HBM+BHI grown cells reached the critical pH of enamel after 2-3 hours which was faster than the baseline HBM (4 hours). The acid production rate in HBM and in BHI+Sucrose of all tested group was not significant difference. Moreover, the longer period of exposure was performed; there was not obvious discrepancy of acid production among the 1st through the 15th passage.

Conclusion: After exposure to HBM, *S. mutans* increase its ability to utilize HBM leading to the shorter time to reach critical pH than its usual condition.

Department:	Pediatric Dentistry	Student's Signature .....
Field of Study:	Pediatric Dentistry	Advisor's Signature .....
Academic Year:	2016	Co-Advisor's Signature .....

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CHAPTER I

INTRODUCTION

Research background and rationale

Human breast milk (HBM) has several benefits for infants such as prevention of obesity, allergy and immunological diseases and providing economic and excellent nutrition for brain and body development (1, 2). 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 (3). Human breast milk is composed of macronutrients mainly fats, proteins and lactose. It also contains vitamins, minerals, growth factors and immunologic components. Previous studies show that lactoferrin, alpha-lactabumin and immunologic components found in HBM prevent, adhesion, growth and biofilm formation of *Streptococcus mutans*, a major pathogen of dental caries (4, 5). On the other hand, several clinical studies revealed that breastfeeding, especially night-time breastfeeding, is related to the prevalence of caries in preschool children (6-9).

Early childhood caries (ECC) is defined as the presence of one or more caries, missing (due to caries) or filled tooth surfaces in primary tooth in children from birth to 6 years old (10). ECC can cause dental pain, chewing problems, trouble sleeping and can be a source of infection. These adverse consequences have a great impact to the quality of life and development of children. According to a World Health Organization (WHO) report, 51 million worldwide aged 5 years old or younger have low weight-for-height, mostly as a consequence of poor feeding and repeated infections (3). A longitudinal study in the Songkhla province of Thailand revealed that caries can develop on recently erupted primary teeth and rapidly progress toward pulpal tissue in a few months. In addition, the prevalence of ECC rapidly increased from 2% of the population at 9 months old to 68.1%

at 18 months old (11). This finding concurs with the study in the Supan Buri province of Thailand, where researchers found a prevalence of ECC of 83% among children aged 6 to 19 months old (12). Moreover, according to the National Dental Survey of Thailand, the prevalence of the disease has been over 50% for preschool children for more than a decade (13). Due to its high prevalence among children; ECC is considered an oral health problem in Thailand.

An initial caries process occurs when the rate of tooth demineralization is greater than the rate of remineralization (14). Mutans streptococci (MS), mainly *Streptococcus mutans,* have been recognized as the key pathogens since they ferment sucrose and subsequently produce glucan and lactic acid (15). Using glucan, the bacteria adhere to tooth surfaces and even accumulate other bacteria to form a virulent dental plaque. In the dental plaque, acids are detained long enough to dissolve minerals in the tooth enamel (demineralization). Several studies demonstrate that children who acquired *S. mutans* at an early age tend to have more cavities (16-19).

Since a main sugar in HBM is lactose, *S. mutans* may not ferment this sugar as they ferment sucrose (20). Therefore, acid production and biofilm formation, which plays an important role in cariogenesis, may not be developed. However, there are several reports revealing a positive correlation between breastfeeding and ECC (8, 21-24). These findings suggest that there may be an adaptation of *S. mutans* to this unfavorable environment in order to survive and develop a cariogenic circumstance. Therefore, the aim of this study is to investigate an adaptation in acid production of *S. mutans* grown in lactose as a preliminary study for further investigation on HBM conditions.

Research question

Does *Streptococcus mutans* adapt its acid production rate in long-term exposure to HBM?

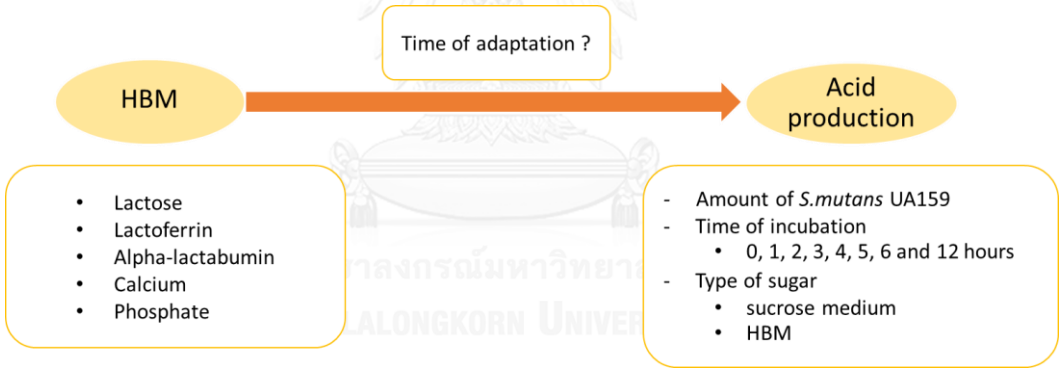
Research objectives

1. To investigate an adaptation in acid production rate of *Streptococcus mutans* after long-term exposure to HBM.
2. To compare the acid production rate of *Streptococcus mutans* in sucrose-culture condition and HBM after long-term exposure to HBM.

Hypothesis

1. The acid production rate of *Streptococcus mutans* increases after long-term exposure to HBM.
2. The acid production rate of *Streptococcus mutans* in HBM is similar to sucrose-culture condition after long-term exposure to HBM.

Conceptual framework



Research design

This is an *in vitro* study using *Streptococcus mutans* UA159 to determine its adaptation in acid production rate when culturing it in HBM.

Keywords

Streptococcus mutans, acid production rate, human breast milk, adaptation

Definition

An adaptation is a change in a characteristic that enhances the survival or reproduction of organisms that bear in a specific environment. It could be a change in genetic or physiologic condition.

Expected benefits of study

In this study, we investigate an adaptation in acid production of *Streptococcus mutans* cultured in HBM. If *S. mutans* increase ability of acid production in HBM, they will increase risk of caries development. Therefore, health care providers should pay more attention to motivate parents and caregivers to provide adequate oral hygiene for their children.

Limitation

This study will be performed in an *in vitro* condition, which is different from oral condition of children. Moreover, this study uses a single laboratory strain of *S. mutans* that may respond differently from clinical dental plaque, which contains complex species.

Ethical consideration

The study protocol was approved by the Human Research Ethic Committee of the Faculty of Dentistry, Chulalongkorn University (HREC_DUC 2016-050).

CHAPTER II

LITERATURE REVIEW

Human breast milk (HBM) and its composition

Human breast milk is the best nutrients for infants especially during the first six months. It protects infants from gastrointestinal infection and diarrhea, which is the second most common cause of mortality in young children and reduces the risk of pneumonia and respiratory tract infection (25, 26). Moreover, HBM has evidence in reducing risk of leukemia and chronic disease such as asthma, allergy, obesity, and diabetes (1, 2, 27, 28). Concurrently, children who were not exclusively breastfeeding at 6-8 weeks of age would have a greater hospitalization (29). Thus, WHO recommends that mothers should exclusively breastfeed their infant for 6 months and continue breastfeeding with proper complementary food up to the age of two or above (3). Exclusive breastfeeding is defined as giving infant only breast milk without any water, formula, and other liquid or solid foods. If mothers cannot breastfeed their infant, they can fed with appropriately-stored expressed breast milk (3).

Breast milk lactation is divided into 3 stages due to its difference in proportion of macronutrients and micronutrients. Macronutrients in milk are composed of carbohydrates, fats and proteins whereas micronutrients are composed of vitamins and minerals. In the first few days, mothers produce colostrum (also known as the first milk) that contains an enriched source of immunologic and antimicrobial components but is low in lactose and fat (30, 31). After one week, the proportion of HBM compositions gradually changes to transitional milk that provides a greater amount of lactose and fat, but decreases immunoglobulin and total proteins. The obvious change in the proportion of

human milk components is continued up to 4-6 weeks postpartum, after which it becomes stable, and the milk is referred to as mature milk (32) (Table 1).

Table 1 The average proportion of macronutrients and minerals of human milk in three stages (32)

Nutrients	Human milk, colostrums	Human milk, transitional	Human milk, mature
Protein, g/100ml	2	1.5	1.3
Fat, g/100ml	2.6	3.7	4.1
Lactose, g/100ml	6.6	6.9	7.2
Ca, mg/100ml	28	25	34
P, mg/100ml	14	16	15

In individuals, the proportion of HBM compositions after 4 months varies depending on the postpartum age, maternal body weight, period return and nursing frequency (33-35). Maternal dietary intakes have no influence on the amount of protein and lactose whereas fat in the maternal diet alters the proportion of fat in milk (32, 33). Among these factors, the postpartum age is the most influential factor (36). Mature milk at 6 months postpartum shows an obvious decrease of more than 25% in the proportion of protein and calcium, while lactose and fat increases more than 10% compared to mature milk at one month postpartum (35, 37). Lactoferrin and secretory immunoglobulin A (sIgA) levels also decrease 25% in the first month and remain constant until 26 months (38). Moreover, amenorrhea and high nursing frequency increases lactose production during the first 6-9 months postpartum (33). Besides the postpartum age, the weight of the mother was positively correlated with the proportion of fat and protein in mature milk after 6 months (33).

Human breast milk (HBM) and its controversies over the cariogenic potential

The association of HBM and dental caries has been widely investigated both in experimental and epidemiological studies and is still controversial. Several *in vitro* studies showed that HBM and its components have a caries-protective effect (4, 39, 40). A total of 33% of human milk samples had a potential to inhibit more than 40% of *S. mutans* cultures to colonize on salivary-coated hydroxyapatite discs (39). The components that play an important role in this property are casein, immunoglobulin, lactoferrin, and alpha-lactabumin. The most potent components are casein and immunoglobulin that can inhibit broader laboratory strains than lactoferrin and alpha-lactabumin (4). Milk fat also exhibits a role in caries prevention by reducing the quantity of stagnant food to the tooth surface, reducing hydroxyapatite dissolution having a bacteriostatic effect (41, 42). In addition, HBM was investigated for its acidogenicity in human dental plaque, with the minimum plaque pH after breastfeeding for 1 minute being 6.40 in 12-24 months old children and the pH after rinsing with HBM for 15 min being 6.26 in adult subjects. In both cases, the pH was not lower than the critical pH of enamel (pH = 5.5) (40, 43). Concurrently, an *in vitro* study revealed that incubating an *S. mutans* biofilm on premolar teeth together with HBM cannot develop dental caries as shown through radiography and clinical examination (40) . On the contrary, Prabhakar *et al* showed that *S. mutans* isolated from children with ECC could utilize HBM resulting in visible white spot lesions (44). Moreover, HBM samples increased 1.5-3.8 fold of *S. mutans* biofilm formation compared with tryptic soy broth (5). There are also evidences from animal experiments showed that on-demand feeding with HBM can develop carious lesion especially on smooth surfaces (45, 46).

Several epidemiological studies support that breastfeeding is not related to caries development (47-51). The analysis of the United State survey data during 1999-2002 in children 2 to 5 years old revealed that breastfeeding is not a single contributing factor for

dental caries as well as the duration of breastfeeding (51). In addition, longitudinal studies conducted in a United States population showed that breastfeeding at least 6 months can lower caries prevalence of second primary molars at 5 years old (50). A meta-analysis of 5 cross-sectional studies reported that breastfed children have a protective effect against caries development. However, studies that mention the influence of nocturnal feeding and prolonged feeding were excluded because the heterogeneity of the data among those studies (52).

In contrast, nocturnal, prolonged breastfeeding and high frequency of breastfeeding are significantly related with increased caries prevalence (6-8, 53). In Myanmar, 25-to-30-month-old children with history of nocturnal feeding more than twice or more than 15 minute per nocturnal feeding were associated with ECC (6). Prolonged breastfeeding for up to 2 years is associated with increasing caries prevalence (Odd ratio; OR 5.31) in a Cambodian population, in which nocturnal breastfeeding is common (53). In addition, a study about the relationship between breastfeeding behavior and ECC in Bangkok, Thailand revealed a prevalence of ECC of 42.5% in breastfed children. Subsequently, a multivariate analysis demonstrated that the factors related to a higher risk of caries were children with plaque covering over 1/3 of crown length (Odds ratio; OR 59.15), delaying of the first cleaning (OR 7.34), breastfeeding to sleep (OR 3.26) and breastfeeding on demand (OR 2.09) (54). A systematic review by Valaitis *et al* found that prolonged breast feeding for more than one year and nocturnal breastfeeding after tooth eruption are related with ECC, although the evidences is not consistent and shows a weak association (55).

Early childhood caries (ECC) and its impact on children

Early childhood caries is characterized by the presence of one or more decayed (non-cavitated or cavitated lesion), missing (due to caries), or filled tooth surfaces in primary teeth of children under the age of 6. Any signs of smooth surface caries acquired in children younger than 3 years also indicate severe early childhood caries (S-ECC). Additionally, from ages 3 through 5, one or more cavitated, missing (due to caries), or filled smooth surface in primary maxillary anterior teeth or a decayed, missing or filled score of ≥ 4 (age 3), ≥ 5 (age 4), ≥ 6 (age 5) surfaces are also diagnosed as S-ECC (56).

Early childhood caries is one of the most common diseases in children. The Seventh National Health Survey of Thailand in 2012 revealed that 51.7% of 3-year-old children and 78.5% of 5 year-old children have at least one decayed, filled or missing tooth (13). A longitudinal study in Songkhla province, Thailand revealed that caries develops on recently erupted primary teeth and rapidly progress toward the pulpal tissue in a few months. In addition, the prevalence of ECC rapidly increased from 2% of the population study at 9 months old to 68.1% at 18 months old (11). This finding concurs with the study in Supan Buri province, Thailand, where researchers found that the prevalence of ECC was 83% among children aged 6 to 19 months old (12).

Due to the high prevalence of ECC, its impact to the quality of life of children and its complicated treatment, ECC remains an important health concern in Thailand and most active caries lesions are left untreated. Pain and systemic infection are common consequences of untreated decay (57). Oral health problems also affect children's quality of life in many ways such as sleep disturbance, chewing problem, learning ability and school participation.

Dental caries and sugar metabolism of *Streptococcus mutans*

Dental caries is the disease process that destroys tooth structure by the acidic-end product from carbohydrate-fermentable bacteria (58). Prolonged exposure of acid attack is the initial process of mineral dissolution or demineralization. This process can be reversed by mineral precipitation or remineralization. These two processes occur dynamically and the mineral loss occurs when demineralization is greater than remineralization. If demineralization continues, it will lead to cavitated lesions. Therefore, the etiology of dental caries is linked to the acid production of oral microorganisms. However, it is not a single factor that contributes to the dental caries process. The high consumption of fermentable carbohydrate diet and abnormal tooth ultra-structure also accelerate the process (59), while the quantity and quality of saliva is essential for the caries defensive system (60).

Streptococcus mutans is considered to be an important pathogen causing dental caries since it can rapidly produce acid that lowers the environmental pH. Furthermore, even in a very low pH condition, *S. mutans* can adapt itself to maintain the glycolytic activity by many mechanisms (61). This high tolerance in very low pH called an aciduricity that is another essential virulence factor for cariogenic bacteria.

Besides cariogenic bacteria, dental caries is associated with the frequency of fermentable carbohydrate intakes especially that of sucrose, which has been claimed as the arch criminal of dental caries (62, 63). Sucrose can be extracellularly metabolized by 3 extracellular enzymes of *S. mutans*: glucosyltransferase, fructosyltransferase and alpha-glucosidase. Glucosyltransferase (GTF) converts sucrose into water-insoluble glucan. Bacteria use this sticky glucan to adhere to the tooth surface and also aggregate other bacteria to form a complex biofilm (64-66). Fructosyltransferase (FTF) is used for fructans

formation that serves as an extracellular storage source and degradation of fructans by fructosidase, which prolongs and increases the acid production (67). The other extracellular enzyme is alpha-glucosidase. It cleaves sucrose into glucose and fructose which are taken up directly and subsequently utilized to obtain energy. Sucrose can be transported directly into the cell and divided by intracellular alpha-glucosidase (68) (figure 1).

Sucrose is predominantly transported by sucrose specific phosphoenolpyruvate-mediated phosphotransferase transport system (PEP-PTS), while the multiple sugar metabolism system (Msm) is contributing less to sucrose metabolism (69, 70). PEP-PTS has a very high affinity to sucrose even in a limited sucrose condition and this pathway can reduce energy consumption by combining phosphorylation of the sugar with sugar uptake (68, 71) (figure 1).

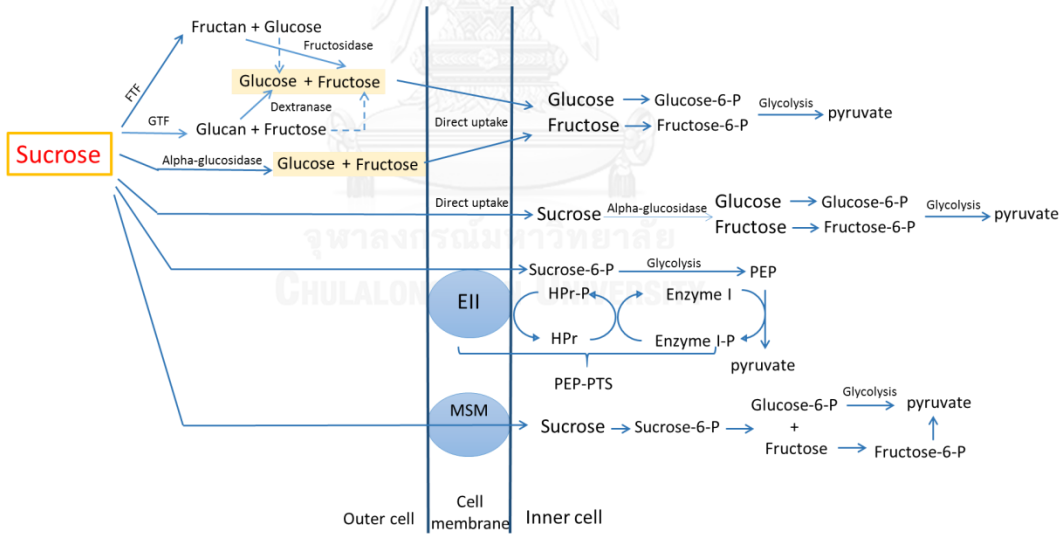


Figure 1 Pathway of sucrose metabolism by *Streptococcus mutans* (68).

Sucrose-specific PEP-PTS consists of Enzyme I (EI), Histidine rich protein (HPr) and sucrose-specific Enzyme II complex (EII). The phosphate group is transferred from phosphoenol pyruvate to EI, HPr and EII, and finally transferred to sucrose resulting in sucrose-6-phosphate formation (68). It is cleaved into glucrose-6-phosphate and fructose

by sucrose phosphate hydrolase. These metabolites are catabolized via the glycolytic pathway, resulting in pyruvate production (figure 1). The fate of pyruvate depends on the amount of sugar available outside the cell. When sugars are abundant, pyruvate is converted to lactate-by-lactate dehydrogenase, although formate, acetate and ethanol are obtained under a limited sugars condition (68, 72) (figure 2).

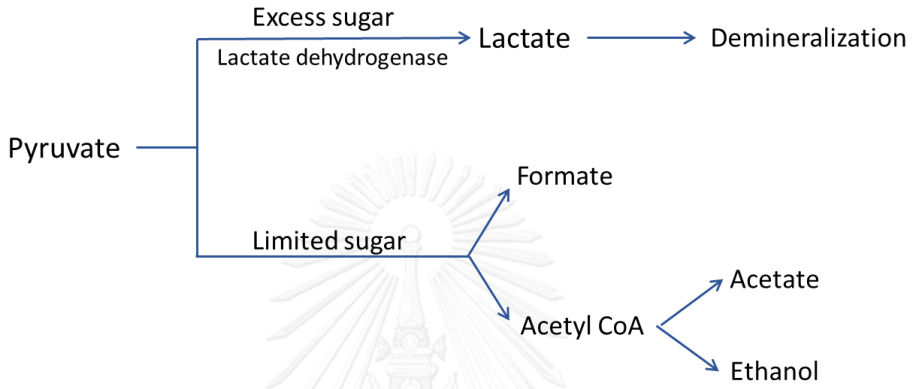


Figure 2 Pathway of pyruvate metabolism in excess and limited sugar conditions (68, 72).

***Streptococcus mutans* and lactose utilization**

Lactose, the main sugar in HBM, is less cariogenic than sucrose, glucose, and fructose (73, 74). It is not a primarily source of energy for *S. mutans* as it is utilized when sucrose and glucose are used up (75). Lactose is mainly internalized and phosphorylated into lactose-6-phosphate (Lac-6-P) via the lactose-specific PEP-PTS system and might be taken up by non-PEP-PTS in some strains (76). In contrast to sucrose-specific PEP-PTS that is constituent in *S. mutans*, lactose-specific PEP-PTS has to be induced during growth in a lactose medium (76-78). Subsequently, Lac-6-P is divided into glucose and galactose-6-phosphate (Gal-6-P) by phosphor-beta-galactosidase. Then Gal-6-P is metabolized via the tagatose pathway to obtain D-glyceraldehyde-3-phosphate, an intermediate metabolite of the glycolysis pathway (76, 79, 80) (figure 3).

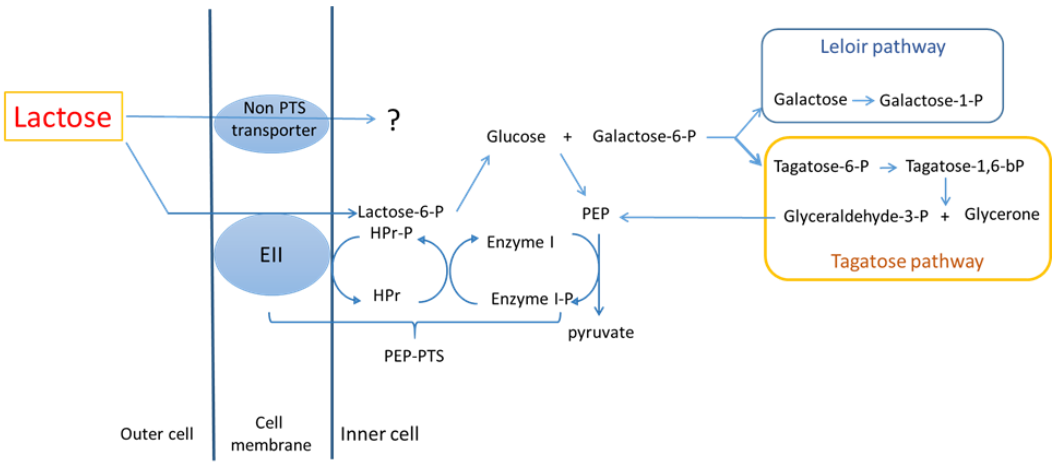


Figure 3 Pathway of lactose metabolism by *Streptococcus mutans* (76, 80).

One study showed that bacterial plaque can utilize lactose and produce a faster and more acidic condition after an adaptation period. In that study, healthy adult subjects were instructed to rinse 10% lactose for 2 min 6 times a day for 6 weeks. The authors found that dental plaque pH after 6-week adaptation significantly decreased comparing to that before adaptation ($P < 0.01$) (81). However, there are no studies directly demonstrating how long it takes for *S. mutans* to adapt itself for utilizing lactose to produce acid in order to reach lower pH levels than the enamel's critical pH (pH = 5.5). Therefore, the aim of our study is to determine the ability of *S. mutans* to adapt to produce acid after incubate in HBM.

CHAPTER III

MATERIALS AND METHODS

Bacterial strain and growth conditions

S. mutans UA159 was used since its genome was completely published (80). The strain had been stored at -80°C in Brain-Heart Infusion broth (BHI broth, HiMedia Laboratories, India) with 40% glycerol. The bacteria from a glycerol stock were recovered for 2 days in BHI agar at 37°C, 5% CO_2 incubator (Forma Steri-Cycle CO_2 Incubator, Thermo Scientific, USA). The isolated colony was grown overnight in BHI medium with continuous shaking at 240 rpm (IKA KS 130 basic Shaker, USA). Overnight cultures were diluted to optical density at 600 nm (OD_{600nm}) of 0.1 and incubated until the OD_{600nm} reach 0.4-0.6 (log phase) for the adaptation assay (figure 5).

Human breast milk collection and preparation

The study protocol was approved by the Human Research Ethic Committee of the Faculty of Dentistry, Chulalongkorn University (HREC_DUC 2016-050). Mature human breast milk samples were donated from 11 healthy lactating mothers who still breastfeed their child at least once a day and fulfilled with inclusion criteria shown below.

Table 2 Inclusion and exclusion criteria for HBM-donating mothers.

Inclusion criteria	Exclusion criteria
- Medically healthy (ASA I, II) - Age more than 18 years - 96-110% of Ideal body weight for height - Full-termed pregnancy - Still breastfeed their child aged between 6-18 months at least once a day - Willing to participate in this study and sign a consent form	- Medically compromised mothers - Taking alcohol, antibiotics or nonsteroidal anti-inflammatory drug within 3 months

The milk samples were expressed at one time by their preferred methods (breast pump or manually expressed) and transported in disposable breast milk-storage plastic bags (Marinda, My inspiration, Thailand) on ice to laboratory within 4 hours. This study used filtrated HBM prepared according to Allison L.M. *et al*. Briefly, the samples were centrifuged at 3000 x g for 10 minutes at 4°C. The supernatant was filter-sterilized through 2.5 pore-sized filter papers (Whatman Grade 5, UK) and followed by 0.45 μm pore-sized filter papers (Sartorius stedim, Germany) (5). The filtrated HBM were pooled and aliquot into sterile tubes and stored at -20°C until use (figure 4).

The major compositions of HBM such as lactose, fat and protein were analyzed by The Institute of Nutrition, Mahidol University, Bangkok, Thailand.

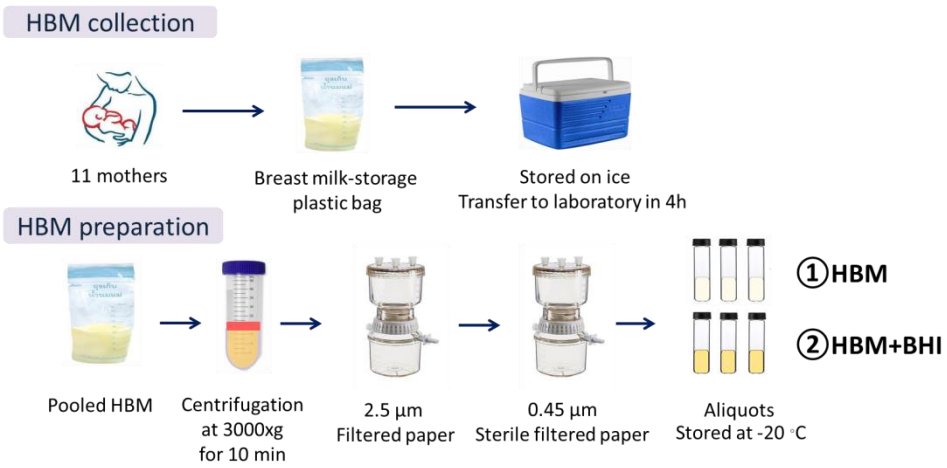


Figure 4 HBM collection and preparation.

Adaptation assay

The HBM stock was thawed at room temperature. The log-phase cells of *S. mutans* were washed 3 times with phosphate-buffered saline (PBS) and then separately re-suspended in 3 culture media: 1) HBM, 2) BHI-supplemented HBM (HBM+BHI) and 3) BHI control. To create an adaptation condition, all culture media were incubated at 37°C, 5%CO_2 without shaking for 11 hours (= 1 passage) and then were continuously sub-cultured to fresh media every 11 hours up to 15 passages. Since bacterial cells tend to form clumps in all media, the cultures were soaked in a sonicator bath (Elmasonic S 30H, Elma, Germany) for 10 minutes. To determine the adaptation, the certain amounts of bacterial cultures from each media (total 3 media) were collected at the baseline (the 0 passage), 1st, 5th, 9th, 13th and 15th passage. The collected samples of each passage were immediately examined the adaptation via the acid production assay. In this study, the level of pH represented the acid production and the number of bacteria during acid production assay represented the growth of bacteria.

Acid production assay

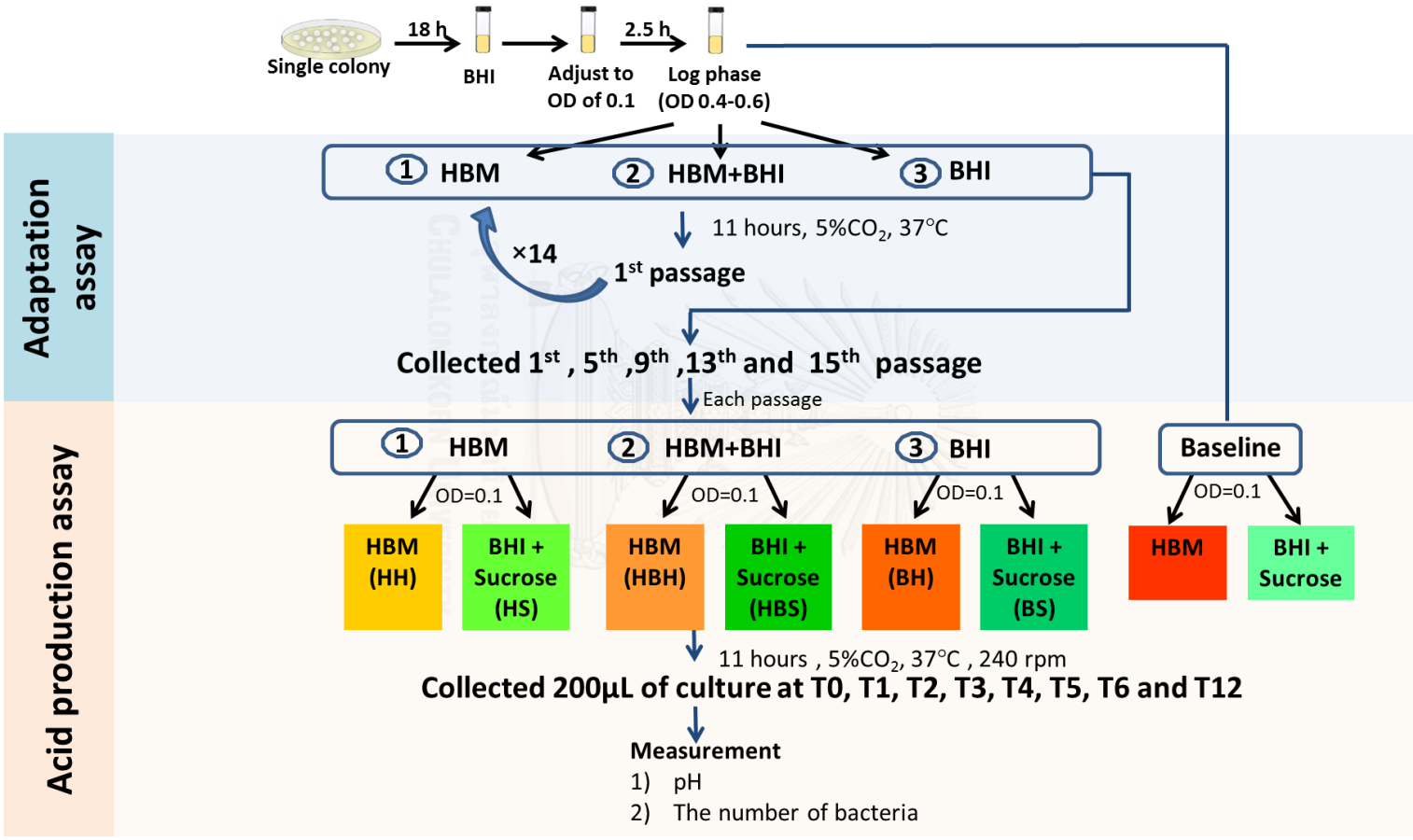
Three collected samples (HBM, HBM+BHI and BHI) of each passage from the adaptation assay were washed 3 times with PBS. After that they were adjusted OD_{600nm} of 0.1 (UV-Cuvette micro, Brand GMBH, Germany) (approximate 10^8 CFU/ml) separately in HBM and in 7% sucrose-supplemented BHI (BHI+Sucrose) in order to investigate their acid production in these two conditions. Finally, there were 6 experimental groups per one passage; 1) HBM-grown cells in HBM (HH) 2) HBM-grown cells in BHI+Sucrose (HS) 3) HBM+BHI-grown cell in HBM (HBH), 4) HBM+BHI-grown cell in BHI+Sucrose (HBS) 5) BHI-grown cell in HBM (BH), 6) BHI-grown cell in BHI+Sucrose (BS). The log phase cells of *S. mutans* were also collected and re-suspended in HBM and BHI+Sucrose in order to investigate their acid production as the baseline groups (the 0 passage) (figure 5). All 6 groups and the baseline groups 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 from 0, 1, 2, 3, 4, 5, 6, and 12 hour-cultures (T0-T12) in order to examine the adaptation by measuring the cultural pH and the number of bacteria (figure 5). The cultural pH was measured by pH meter (Compact pH Meter, Horiba, Japan) whereas the numbers of bacteria were determined by miniaturized plating method as CFU/ml (82). The experiments were performed 3 times.

The acid production rate was calculated at the fastest pH-dropping duration (Ti-Tj) following this equation modified from Piwat, S *et al* (83):

$$\text{Acid production rate}_{(\text{Ti}-\text{Tj})} = \frac{\Delta\text{pH}}{\text{average logCFU/ml}_{(\text{Ti}-\text{Tj})} \text{ x } (\Delta\text{T})}$$

ΔpH = The change in pH during Ti-Tj

ΔT = The change in time during Ti-Tj (hours)



Note HH= HBM-grown cells in HBM HBH = HBM+BHI-grown cell in HBM BH = BHI-grown cell in HBM

HS = HBM-grown cells in BHI+Sucrose HBS = pHBM+BHI-grown cell in BHI+Sucrose BS = BHI-grown cell in BHI+Sucrose

Figure 5 Workflow for measurement of the acid production.

Statistical analysis

The SPSS software package version 17.0 (SPSS 17.0, SPSS Inc., USA) was used in this study. Data were shown as mean ± standard deviation (SD). Statistical comparison of the numbers of bacteria (logCFU/ml) and the pH at the specific time point (T0-T12) among tested passages in HBM and in BHI+Sucrose and also the acid production rate of all tested groups was done by one-way ANOVA followed by post hoc Tukey. In case of the variances were not homogeneous, Welch and post hoc Tamhane tests were performed. The growth of bacteria during T0-T12 of all experiments was analyzed using one-way repeated ANOVA. A significant difference was determined when *P*-value was less than 0.05 ($P < 0.05$).

CHAPTER IV

RESULTS

Human milk composition

Human milk samples were donated from 11 mothers. The mean age of mothers and child were 31 ± 5 years and 11 ± 2 months, respectively. HBM was prepared and filtrated as mentioned in material and method in order to remove contaminated bacteria (5). The mean concentrations of major components in the HBM were 0.99 g/dL for protein, 0.03 g/dL for fat, 4.8 g/dL for lactose, 18.3 mg/dL for calcium and 14.52 mg/dL for phosphorus (table 3).

Table 3 The concentrations of major components in the HBM

Components	Concentrations
Protein, g/100ml	0.99
Fat, g/100ml	0.33
Lactose, g/100ml	4.80
Calcium, mg/100ml	18.30
Phosphorus, mg/100ml	14.52

Factors affecting the acid production

To assure that the decrease of pH causes from bacterial sugar metabolism, not from the effect of long-term incubation; we measured the pH of control HBM (no bacteria) and the pH of control BHI+Sucrose (no bacteria) from T0 to T12 for all passages. As seen in figure 6, the pH of both control HBM and control BHI+Sucrose were approximately 7.5-8.0 throughout 12 hours of incubation whereas those of the baseline groups

decreased from 7.0-7.5 to 4 after 12 hours of incubation. This result indicated that long-term incubation has no an effect on the pH of both media: HBM and BHI+Sucrose.

In addition, the initial pH and the initial number of bacteria (T0), which affect the acid production, were not significant difference among all passages including the baseline groups (figure 7 and 10).

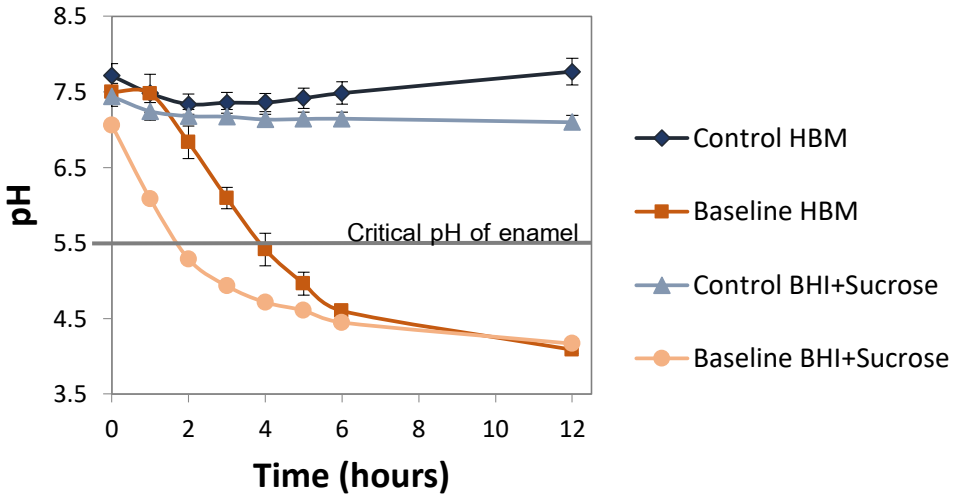


Figure 6 The pH of all tested- passages of control HBM (no bacteria) and control BHI+Sucrose (no bacteria) and also those of the baseline HBM (the 0 passage) and the baseline BHI+Sucrose (the 0 passage) from T0 to T12.

Acid production of *S. mutans* at the baseline (the 0 passage) in HBM and BHI+Sucrose

As presented in figure 6, the pH of the baseline HBM (a dark orange line) was lower than pH 5.5 after 4 hours of incubation whereas those of the baseline BHI+Sucrose (a light orange line) took approximately 2 hours to reach the same level.

Acid production in HBM of *S. mutans* after long-term exposure to designated conditions

Acid production in HBM of *S. mutans* after long-term exposure to HBM, HBM+BHI and BHI was presented in figure 7.

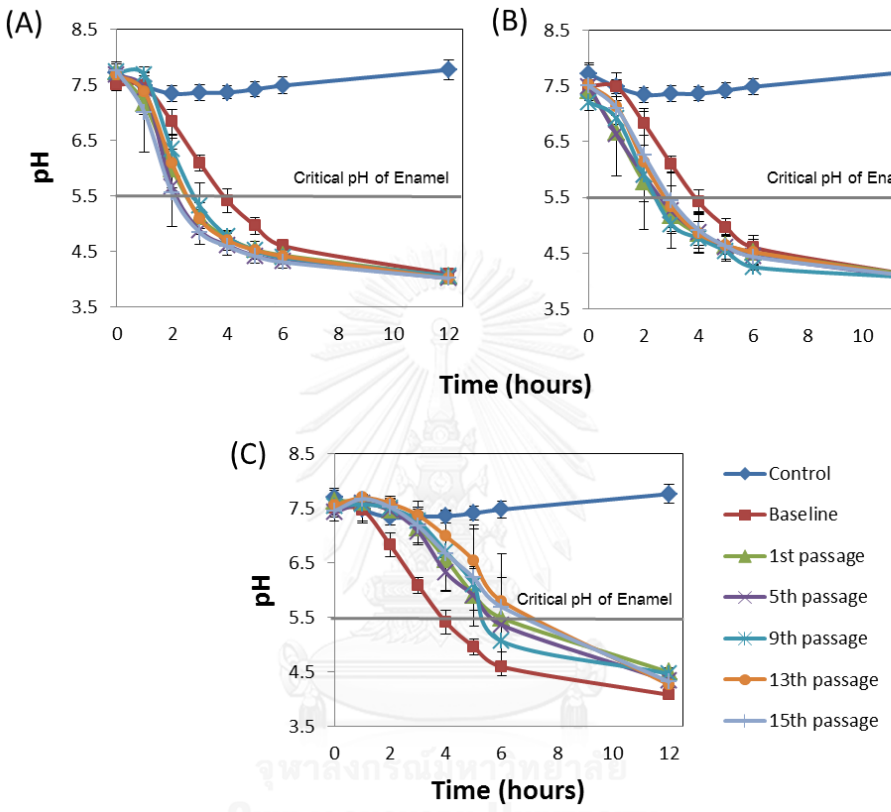


Figure 7 Acid production in HBM from T0 to T12 of *S. mutans* after exposure to HBM **(A)**, HBM+BHI **(B)** and BHI **(C)** including the control (no bacteria) and the baseline (the 0 passage).

The acid production in HBM of the 1st passage after exposure to HBM, HBM+BHI and BHI for 11 hours was different from the baseline HBM (the 0 passage) (figure7A, B and C, respectively). Moreover, in each group (figure 7A, B and C), there were no significant differences in pH at each time point (from T0 to T12) among passages (the 1st,5th ,9th ,13th, 15th passage) ($P > 0.05$). These two findings indicated that the adaptation

in acid production occurred after exposure to designated conditions for 11 hours (= the 1st passage). Therefore, the 1st passage of all groups (HBM, HBM+BHI and BHI) was used in further analysis.

The pH of the 1st passage of HBM and HBM+BHI groups reached the critical pH of enamel after 2-3 hours which were faster than the baseline HBM (4 hours) (figure 8). On the other hand, the pH of the 1st passage of BHI group took 6 hours to reach the same level (figure 8).

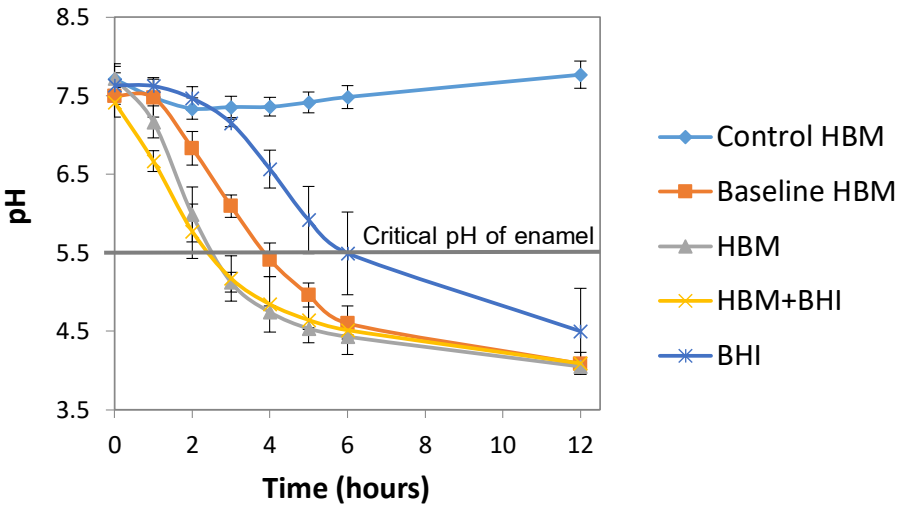


Figure 8 Acid production in HBM from T0 to T12 of *S. mutans* after exposure to HBM, HBM+BHI and BHI for 11 hours (the 1st passage) comparing to the control (no bacteria) and the baseline (the 0 passage).

The growth of *S. mutans* in HBM of all groups was showed in figure 9. There was no significant difference of the number of bacteria (logCFU/ml) throughout 12 hours of incubation except the 5th passage of HBM group ($P = 0.03$) (figure 9A), which the bacterial growth significantly increased after 4-12 hours of incubation comparing to T0.

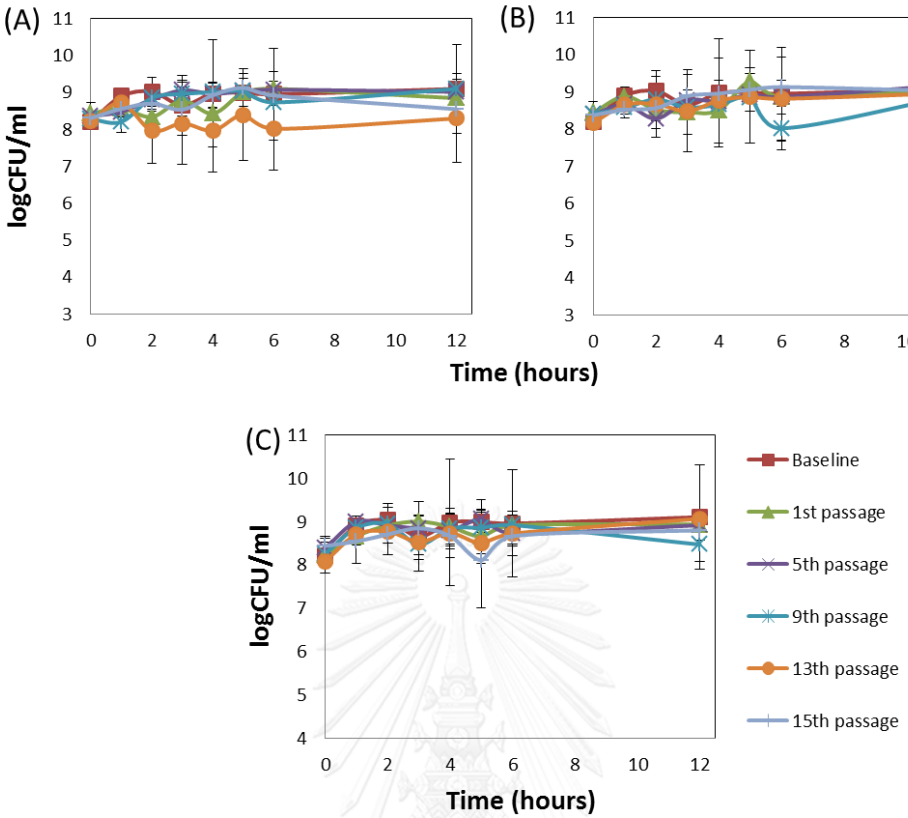


Figure 9 The growth of bacteria (logCFU/ml) in HBM from T0 to T12 of *S. mutans* after exposure to HBM **(A)**, HBM+BHI **(B)** and BHI **(C)** including the baseline (the 0 passage).

Acid production in BHI+Sucrose of *S. mutans* after long-term exposure to designated conditions

Acid production in BHI+Sucrose of *S. mutans* after long-term exposure to HBM, HBM+BHI and BHI was presented in figure 10.

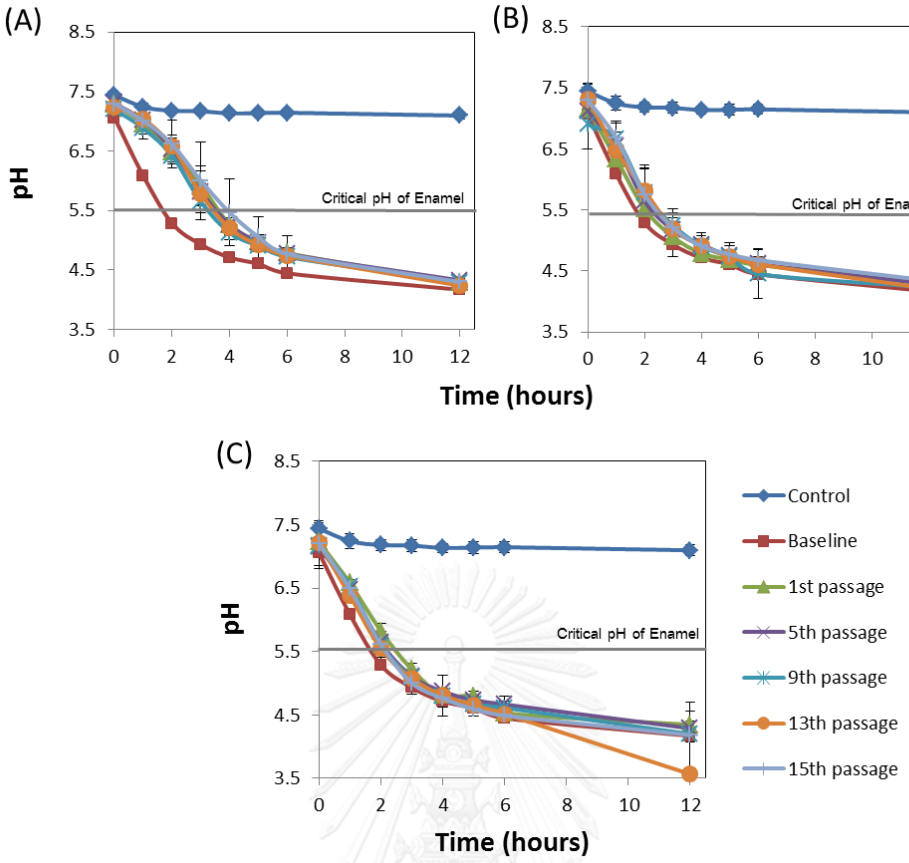


Figure 10 Acid production in BHI+Sucrose from T0 to T12 of *S. mutans* after exposure to HBM **(A)**, HBM+BHI **(B)** and BHI **(C)** including the control (no bacteria) and the baseline (the 0 passage).

The acid production in BHI+Sucrose of the 1st passage after exposure to HBM, HBM+BHI and BHI for 11 hours was different from the baseline BHI+Sucrose (the 0 passage) (figure10A, B and C, respectively). Moreover, in each group (figure 10A, B and C), there were no significant differences in pH at each time point (from T0 to T12) among passages (the 1st,5th ,9th ,13th, 15th passage) ($P > 0.05$). These two findings indicated that the adaptation in acid production occurred after exposure to designated conditions for 11 hours (= the 1st passage). Therefore, the 1st passage of all groups (HBM, HBM+BHI and BHI) was used in further analysis.

The pH of the 1st passage of HBM+BHI and BHI groups reached the critical pH of enamel approximately 2 hours which were slightly different from the baseline BHI+Sucrose (less than 2 hours) whereas the pH of the 1st passage of HBM group took approximately 4 hours to reach the same level (figure 11).

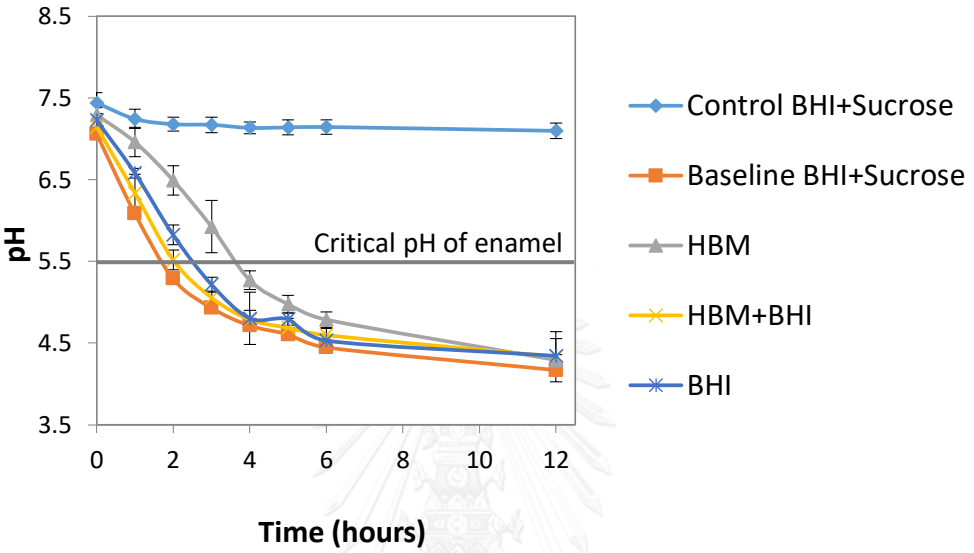


Figure 11 Acid production in BHI+Sucrose from T0 to T12 of *S. mutans* after exposure to HBM, HBM+BHI and BHI for 11 hours (the 1st passage) comparing to the control (no bacteria) and the baseline (the 0 passage).

The growth of *S. mutans* in BHI+Sucrose of all groups was showed in figure 12. There was no significant difference of the number of bacteria (logCFU/ml) throughout 12 hours of incubation ($P > 0.05$).

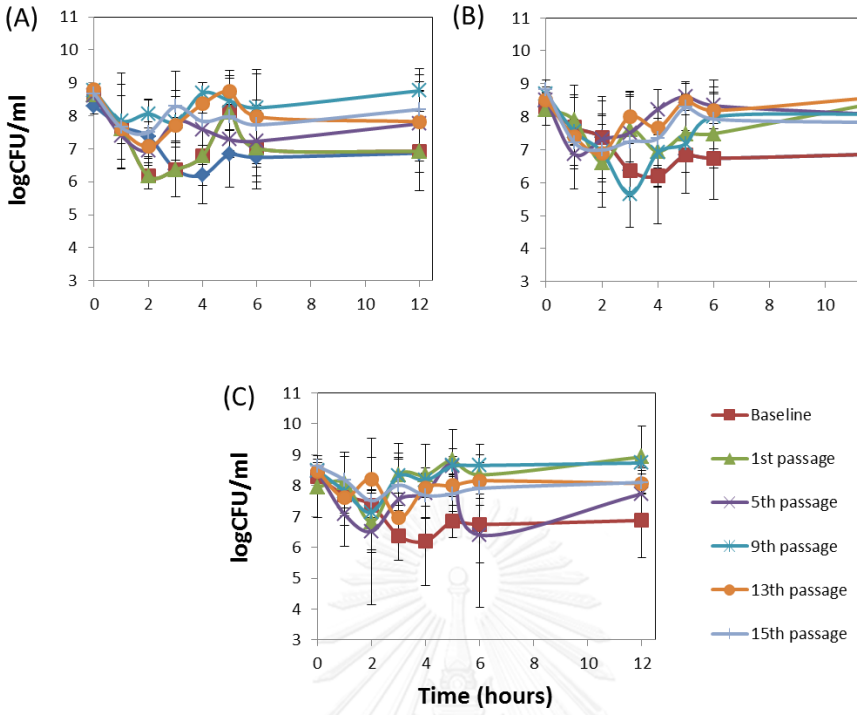


Figure 12 The growth of bacteria (logCFU/ml) in BHI+Sucrose from T0 to T12 of *S. mutans* after exposure to HBM (A), HBM+BHI (B) and BHI (C) including the baseline (the 0 passage).

Acid production rate

The acid production rates were calculated from the fastest pH-dropping duration of the pH of each group (acid production in HBM: the baseline HBM (T2-T4), HBM (T1-T3), HBM+BHI (T0-T2) and BHI (T3-T5) (figure 8) and acid production in BHI-Sucrose: the baseline BHI-Sucrose (T0-T2), HBM (T2-T4), HBM+BHI and BHI (T0-T2) (figure 11)). Since the numbers of bacteria during these specific durations of each group were not significant difference, the mean number of bacteria was used for calculation following the equation in material and method. As seen in figure 13, the cells which prior exposure to HBM showed the fastest acid production rate when measure in HBM condition. However, it was not statistical difference.

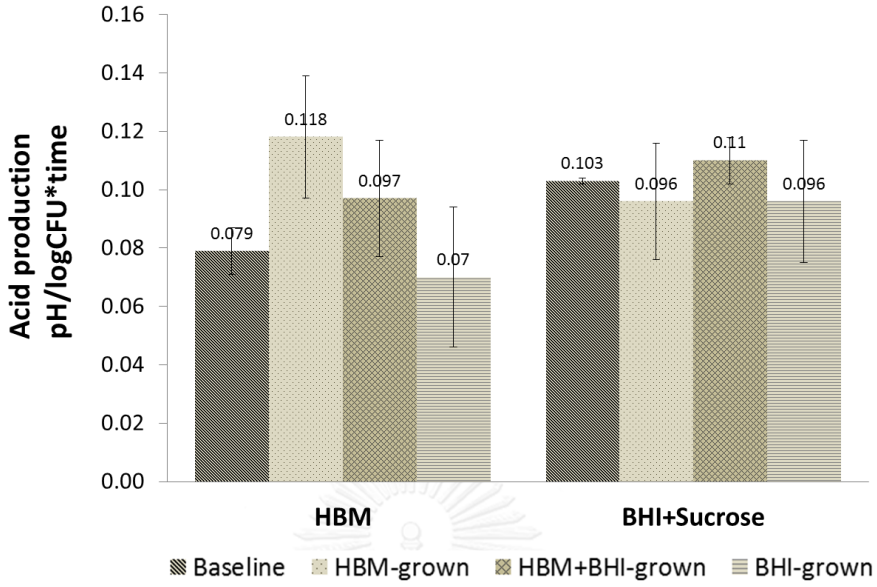


Figure 13 The acid production rate in HBM and in BHI+Sucrose of the 1st passage of each tested group. The number on the top of each bar represents the acid production rate (pH/logCFU*hours).

CHAPTER V

DISCUSSION AND CONCLUSIONS

It has been known for a long time that *S. mutans*, a major pathogen of human dental caries, metabolizes sucrose and releases acid as by product resulting in dental caries (84). However, the main sugar in HBM is lactose, which has less cariogenicity (20, 73, 85, 86). This information together with the knowledge about the caries-protective components of HBM leads to the conclusion that HBM is non-cariogenic (4, 39, 40, 43). However, there were clinical evidences showing that children who consume only HBM have dental caries (54, 87, 88). Based on this clinical finding, we assumed that when *S. mutans* exposes to HBM for enough time, it may improve its ability to metabolize lactose causing the increase of acid production. Therefore, to determine this assumption, *S. mutans* was exposed to HBM with various durations and its acid production was examined as level of pH. To simulate the poor oral hygiene condition, the number of bacteria at the beginning of acid production assay (T0) was adjusted to approximately 10^8 CFU/ml (89).

The HBM samples in the present study (child 11 ± 2 months of age) contain 4.8% lactose, which is slightly less than the HBM obtained from 11 months-postpartum mothers (5.7%) (90). This slight difference in lactose may result from the duration of freezing storage and thawing process of the samples (91). The previous study showed that lactose has less cariogenicity and may not play an important role in caries development unlike sucrose glucose or fructose (20, 73, 85). In contrast, the present study demonstrated that *S. mutans* could metabolize HBM resulting in pH reduction less than 5.5 after 4 hours even though the lactose content in the samples would less than the average. However,

the acid production of the baseline (the 0 passage) HBM was quite slower than those of the baseline BHI+Sucrose (figure 6).

After exposure to HBM and also to HBM+BHI for 11 hours (the 1st passage), *S. mutans* increase its ability to utilized HBM comparing to the baseline HBM group (the 0 passage) (figure 8). Moreover, the longer period of exposure was performed; there was not obvious discrepancy of acid production among the 1st through the 15th passage (figure 7A). Our findings indicated that *S. mutans* possibly adapts itself to metabolize lactose in HBM after a particular time of exposure to HBM condition (HBM alone or HBM+BHI). It is interesting that the acid production in HBM of HBM-grown *S. mutans* (HH) reached the critical pH of enamel earlier than the acid production in BHI+Sucrose (HS) (figure 8 and 11); this finding implies that long-term exposure to HBM causes *S. mutans* prefers HBM than sucrose.

As seen in figure 6, *S. mutans* in the baseline HBM (the 0 passage) could not ferment HBM immediately unlike BHI+Sucrose but it took approximately 1 hour to start decreasing the cultural pH. This finding was concurrent with the previous study that pre-exposure to lactose for 1 hour is required for acid production of *S. mutans* in lactose condition (74). The previous studies also reported that *S. mutans* could not readily ferment lactose since its enzymes for lactose catabolic pathway were not constituent (76-78). In addition, S. *mutans* has a regulatory system for uptake preferential sugar called Carbon Catabolite Repression (92-94). This system inhibits uptake of un-preferable sugar (for example lactose) when preferable sugar (for example glucose, fructose and sucrose) is presented. When the preferable sugar is used up or not presented, this system is prohibited and allows to activate other alternative pathway to uptake un-preferable sugar (95). The evidence revealed that enzymes for lactose metabolism of *S. mutans* could be induced after exposure to lactose for a while (76-78).

As BHI medium contains 0.2% glucose, the long-term expose to HBM+BHI is similar to long-term expose to both lactose and glucose. It was remarkable that HBM+BHI grown cells could metabolize both lactose in HBM and sucrose and glucose in BHI+Sucrose (figure 8 and 11). Therefore, long-term exposure to various sugars might result in an extended capacity of *S. mutans* to utilize various type of sugar.

The previous study reported the association between nocturnal breastfeeding and on-demand breastfeeding with ECC (6, 9). It is presumed that this association causes from the retention of HBM in the oral cavity, which promote *S. mutans* to increase its ability to produce acid from lactose. Our findings supported this assumption. We showed that when *S. mutans* exposes to HBM for enough time, it produces acid faster than usual condition. Base on this reason, the recommendation of WHO that breastfeeding should be continued to the age of 2 years or upon may not jeopardize the oral health of children, if there is no nocturnal feeding or sufficient daily oral hygiene is introduced.

It has to be considered that the present investigation was done in a single species condition whereas oral cavity compose of several species of microflora associated with dental caries such as *Lactobacillus* spp., *Actinomyces* spp., yeast and Bifidobacteria (96). Combination of *S. mutans* with some species of Bifidobacteria showed significant increase in acid production of lactose compared with *S. mutans* alone. (86) Therefore, it may increase risk of caries development due to prolonged retention of HBM, if the children harbor a variety of cariogenic bacteria.

Although the increment of acids found in this study, it may not develop tooth demineralization since dental caries is a multifactorial disease and the components of HBM also have protective properties. But additional caries risk as presented in this experiment is undeniable especially when overall protective factors in children mouth are

inadequate such as low saliva flow during sleep, poor oral hygiene etc. Since HBM is the most important source of nutrients and other beneficial factors for children, we still strongly agree with WHO recommendation to keep exclusive breastfeeding for 6 months and can continue with a proper supplementary food until the age of 2 or older (3). Therefore, the easiest way to prevent dental caries is just to remove bacteria (dental plaque) by brushing with additional fluoride toothpaste.

Conclusions

This study demonstrated that after exposure to HBM at least 11 hours (1 passage), *S. mutans* increase its ability to utilize HBM by reducing times to reach the critical pH of enamel comparing to the baseline group, which never been exposed to HBM. The findings emphasize an important of daily and adequate dental plaque control along with breastfeeding to decrease the risk of demineralization due to adaptive capacity of *S. mutans* in HBM utilization.

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APPENDIX

APPENDIX A

Study Protocol and Consent Form Approval

No. 070/2016

Study Protocol and Consent Form Approval

The Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol and patient/participant information sheet dated and/or amended as follows in compliance with the **ICH/GCP**

Study Title	: Acid production rate of *Streptococcus mutans* after adaptation in human breast milk
Study Code	: HREC-DCU **2016-050**
Study Center	: Chulalongkorn University
Principle Investigator	: Dr. Yart-ruetai Kosakul
Protocol Date	: May 19, 2016
Date of Approval	: July 5, 2016
Date of Expiration	: July 4, 2018

V. Lertchirakarn.

(Associate Professor Dr. Veera Lertchirakarn)
Chairman of Ethics Committee

K. Bhalang

(Assistant Professor Dr. Kanokporn Bhalang)
Associate Dean for Research

*A list of the Ethics Committee members (names and positions) present at the Ethics Committee meeting on the date of approval of this study has been attached (upon requested). This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Approval is granted subject to the following conditions: (see back of the approval)

APPENDIX B

Patient/Participant Information sheet

เอกสารข้อมูลคำอธิบายสำหรับอาสาสมัครที่เข้าร่วมในการวิจัย

(Patient/Participant Information Sheet)

1. โครงการเรื่อง อัตราการผลิตกรดของเชื้อสเตร็ปโตค็อกคัส มิวแทนส์ภายหลังจากการปรับตัวในนมแม่

2. ชื่อผู้วิจัยหลัก

1. ทพญ.หยาดฤทัย โก้สกุล

2. ศ.(พิเศษ)ทพญ.ชุติมา ไตรรัตน์วรกุล (อาจารย์ที่ปรึกษาวิทยานิพนธ์)

3. อ.ทพญ.ดร. พนิดา ธัญญศรีสังข์ (อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม)

สถาบันที่สังกัด คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

แหล่งทุนวิจัย กำลังดำเนินการขอทุนจากจุฬาลงกรณ์มหาวิทยาลัย

3. วัตถุประสงค์ของโครงการ เพื่อศึกษาอัตราการเกิดกรดของเชื้อสเตร็ปโตคอกคัส มิวแทนส์ภายหลังจากการปรับตัวในนมแม่

4. สถานที่ดำเนินการวิจัย คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

5. วิธีการที่เกี่ยวข้องกับการวิจัย

งานวิจัยในห้องปฏิบัติการเพื่อทดสอบการผลิตกรดของเชื้อก่อโรคฟันผุเมื่อเลี้ยงในนมแม่เป็นระยะเวลานาน

6. เหตุผลที่เชิญเข้าร่วมเป็นอาสาสมัครในโครงการ

เนื่องจากท่านเป็นผู้ที่กำลังให้นมบุตรอย่างน้อยวันละ 1 ครั้ง มีสุขภาพแข็งแรง ไม่มีโรคประจำตัวที่ไม่สามารถควบคุมได้ อายุมากกว่า 18 ปีขึ้นไป มีน้ำหนักอยู่ในเกณฑ์ 96-110% ของน้ำหนักในอุดมคติ (ideal body weight) (คำนวณจาก 45.5+2.3×(ส่วนสูงเป็นนิ้ว – 60) ไม่เคยได้รับยาปฏิชีวนะ ยาต้านอักเสบชนิดไม่ใช่-สเตียรอยด์ และ แอลกอฮอล์เป็นระยะเวลาอย่างน้อย 3 เดือนก่อนบริจาคนม และเต็มใจที่จะบริจาคน้ำนมของท่านเพื่อใช้ในการศึกษานอกจากนี้บุตรของท่านควรมีอายุระหว่าง 6-18 เดือน และคลอดครบกำหนด

7. ความรับผิดชอบของและระยะเวลาที่อาสาสมัครจะอยู่ในโครงการ

ขอให้ท่านปฏิบัติตามที่ผู้วิจัยแนะนำ โดยสามารถอยู่ในโครงการได้ตลอดระยะเวลา 6 เดือน และสามารถเดินทางมาที่สถานที่เก็บนมแม่ได้แก่ คณะทันตแพทยศาสตร์ จุฬาฯ หรือโรงพยาบาล

จุฬาฯ โดยอาสาสมัครดำเนินการปั๊มน้ำนมตามที่เคยปฏิบัติเป็นประจำ เมื่อปั๊มเสร็จแล้วผู้วิจัยจะขอแบ่งน้ำนมออกมาเก็บไว้อย่างน้อย 25 มิลลิลิตรต่อครั้ง โดยจะขอเก็บอย่างน้อย 3 ครั้ง แต่ละครั้งห่างกันอย่างน้อย 3 อาทิตย์

8. ประโยชน์ของการวิจัยที่อาสาสมัครและ/หรือผู้อื่นที่อาจได้รับ

ท่านจะไม่ได้รับประโยชน์โดยตรงในการร่วมการวิจัยครั้งนี้ แต่ผลการวิจัยที่ได้จะบ่งบอกถึงความสามารถของเชื้อที่สำคัญต่อการเกิดฟันผุในการผลิตกรดจากนมแม่ ซึ่งเป็นประโยชน์ในการสนับสนุนการดูแลอนามัยช่องปากอย่างสม่ำเสมอ ในเด็กปฐมวัยที่รับประทานนมแม่

9. ความเสี่ยงหรือความไม่สะดวกที่อาจจะเกิดขึ้นแก่อาสาสมัคร และในบางกรณีแก่ทารกในครรภ์หรือทารกที่ดื่ม

นมมารดา

อาสาสมัครอาจมีความไม่สะดวกในการเดินทางมาสถานที่เก็บน้ำนม รวมถึงเสียเวลาในการเก็บน้ำนม ซึ่งทางผู้วิจัยได้มีการตั้งงบประมาณในส่วนค่าเดินทาง และค่าเสียเวลาให้แก่อาสาสมัคร ในส่วนของการขอแบ่งน้ำนมนั้นจะเป็นไปตามความสมัครใจของอาสาสมัคร โดยน้ำนมที่ขอแบ่งมีปริมาณโดยประมาณ 25 มิลลิลิตรของการปั๊มในแต่ละครั้ง และผู้วิจัยจะขอแบ่งน้ำนมเพียงแค่ 3 ครั้ง โดยในแต่ละครั้งจะห่างกันประมาณ 3 อาทิตย์ ซึ่งตามปกติในระยะเวลา 1 วัน มารดาจะมีการปั๊มน้ำนมเกิน 1 ครั้งอยู่แล้ว ดังนั้นการขอแบ่งมาเพียงแค่ 1 ครั้งใน 1 วันจึงไม่น่าจะกระทบต่อปริมาณน้ำนมที่จะให้บุตร รวมถึงช่วงอายุของบุตรที่อยู่ในเกณฑ์ของการศึกษานี้ ก็เป็นช่วงวัยที่มีการรับประทานอาหารเสริมอื่น ๆ อยู่แล้ว

10. ค่าใช้จ่ายที่อาสาสมัครจะต้องจ่าย หรืออาจจะต้องจ่าย

อาสาสมัครไม่ต้องออกค่าใช้จ่ายใดๆ

11. การชดเชยใดๆ และการรักษาที่จะจัดให้แก่อาสาสมัครในกรณีที่ได้รับอันตรายซึ่งเกี่ยวข้องกับการวิจัย

เนื่องจากการศึกษานี้ไม่ได้บังคับให้อาสาสมัครเก็บน้ำนมผิดไปจากที่ทำตามปกติ แต่เป็นการขอแบ่งน้ำนมจากการปั๊มที่ทำอยู่แล้ว นอกจากนี้การศึกษานี้ทำในน้ำนมที่เก็บออกมาแล้ว ไม่ได้ทำการทดลองใดๆที่เกี่ยวข้องกับตัวอาสาสมัครโดยตรง จึงไม่มีความเสี่ยงในการได้รับอันตรายใดๆ

12. การจ่ายค่าเดินทาง ค่าเสียเวลาแก่อาสาสมัครที่เข้าร่วมในการวิจัย

ค่าเดินทางและค่าเสียเวลาของอาสาสมัครจะได้รับการตอบแทนเหมาจ่ายเป็นจำนวนเงิน 300 บาทต่อคน โดยไม่มีข้อแม้หรือเงื่อนไขใดๆทั้งสิ้นในการจ่ายเงิน

13. เหตุการณ์ที่อาจจะเกิดขึ้น หรือเหตุผลซึ่งผู้วิจัยจะต้องยกเลิกการเข้าร่วมในโครงการวิจัยของอาสาสมัคร

หากอาสาสมัครมีอาการเจ็บป่วย หรือรับประทานยาปฏิชีวนะ ยาต้านอักเสบชนิดไม่ใช่สเตียรอยด์ และ แอลกอฮอล์ในช่วงเวลาที่เก็บน้ำนม

14. มีการเก็บชิ้นตัวอย่างที่ได้มาจากอาสาสมัครเอาไว้ใช้ในโครงการวิจัยในอนาคตหรือไม่ เก็บจำนวนเท่าไหร่

อย่างไร และที่ไหน

นมแม่ที่ได้รับบริจาคมาจะถูกนำมาใช้ในการทดลองทั้งหมด โดยไม่มีการเก็บไว้สำหรับโครงการวิจัยในอนาคต

15. การกำกับดูแลและควบคุมการดำเนินโครงการ

ผู้กำกับดูแลการวิจัย ผู้ตรวจสอบ คณะกรรมการพิจารณาจริยธรรม และคณะกรรมการที่เกี่ยวข้อง สามารถเข้าไปตรวจสอบการดำเนินโครงการ รวมทั้ง ตรวจสอบบันทึกข้อมูลของอาสาสมัคร เพื่อเป็นการยืนยันถึงขั้นตอนในการวิจัยทางคลินิกและข้อมูลอื่นๆ โดยไม่ล่วงละเมิดเอกสิทธิ์ในการปิดบังข้อมูลของอาสาสมัคร ตามกรอบที่กฎหมายและกฎระเบียบได้อนุญาตไว้ นอกจากนี้ โดยการลงนามให้ความยินยอม อาสาสมัครหรือ ผู้แทนตามกฎหมายจะมีสิทธิตรวจสอบและมีสิทธิที่จะได้รับข้อมูลด้วยเช่นกัน

16. จริยธรรมการวิจัย

การดำเนินการโครงการวิจัยนี้ ผู้วิจัยคำนึงถึงหลักจริยธรรมการวิจัย ดังนี้

1. หลักความเคารพในบุคคล (Respect for person) โดยการให้ข้อมูลจนอาสาสมัครเข้าใจเป็นอย่างดี

และตัดสินใจอย่างอิสระในการให้ความยินยอมเข้าร่วมในการวิจัย รวมทั้งการเก็บรักษาความลับของ

อาสาสมัคร

2. หลักการให้ประโยชน์ไม่ก่อให้เกิดอันตราย (Beneficence/Non-Maleficence) ซึ่งได้ระบุในข้อ 8 และ 9

ว่าจะมีประโยชน์หรือความเสี่ยงกับอาสาสมัครหรือไม่

3. หลักความยุติธรรม (Justice) คือมีเกณฑ์คัดเข้าและคัดออกชัดเจน มีการกระจายความเสี่ยงและ

ผลประโยชน์อย่างเท่าเทียมกัน โดยวิธีสุ่มเข้ากลุ่มศึกษา

17. ข้อมูลที่อาจนำไปสู่การเปิดเผยตัวของอาสาสมัครจะได้รับการปกปิด ยกเว้นว่าได้รับคำยินยอมไว้โดยกฎระเบียบและกฎหมายที่เกี่ยวข้องเท่านั้น จึงจะเปิดเผยข้อมูลแก่สาธารณชนได้ ในกรณีที่ผลการวิจัยได้รับการตีพิมพ์ ชื่อและที่อยู่ของอาสาสมัครจะต้องได้รับการปกปิดอยู่เสมอ และอาสาสมัครหรือผู้แทนตามกฎหมายจะได้รับแจ้งโดยทันท่วงที ในกรณีที่มีข้อมูลใหม่ซึ่งอาจใช้ประกอบการตัดสินใจของอาสาสมัครว่าจะยังคงเข้าร่วมในโครงการวิจัยต่อไปได้หรือไม่

18. หากท่านมีข้อสงสัยต้องการสอบถามเกี่ยวกับสิทธิของท่านหรือผู้วิจัยไม่ปฏิบัติตามที่เขียนไว้ในเอกสารข้อมูล

คำอธิบายสำหรับผู้เข้าร่วมในการวิจัย ท่านสามารถติดต่อหรือร้องเรียนได้ที่ ฝ่ายวิจัย คณะทันตแพทยศาสตร์

จุฬาลงกรณ์มหาวิทยาลัย ตึกสมเด็จย่า 93 ชั้น 10 หรือที่หมายเลขโทรศัพท์ 02-218-8866 ในเวลาทำการ

19. หากท่านต้องการยกเลิกการเข้าร่วมเป็นอาสาสมัครในโครงการนี้ ให้ท่านกรอกและส่งเอกสารขอยกเลิกมาที่

ทพญ.หยาดฤทัย โก้สกุล ภาควิชาทันตกรรมสำหรับเด็ก คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330

20. อาสาสมัครสามารถติดต่อผู้วิจัยได้**<u>ตลอด 24 ชั่วโมง</u>** ที่:

ทพญ.หยาดฤทัย โก้สกุล โทรศัพท์ 086-352-3456, อ.ทพญ. ดร.พนิดา ธัญญศรีสังข์ โทร.081-655-8240,

ศ.(พิเศษ) ทพญ.ชุติมา ไตรรัตน์วรกุล 081-648-5756

.....

(ทพญ.หยาดฤทัย โก้สกุล)

ผู้วิจัยหลัก

วันที่ 16 / พฤษภาคม / 2559

APPENDIX D

Consent Form

เอกสารยินยอมเข้าร่วมการวิจัย (Consent Form)

การวิจัยเรื่อง อัตราการผลิตกรดของเชื้อสเตร็ปโตคอกคัส มิวแทนส์ ภายหลังจากการปรับตัวในนมแม่

ข้าพเจ้า (นาย/ นาง/ นางสาว/ เด็กชาย/เด็กหญิง).....

อยู่บ้านเลขที่.....ถนน.....ตำบล/แขวง.....

อำเภอ/เขต.....จังหวัด.....รหัสไปรษณีย์.....

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้

1. ข้าพเจ้ายินยอมที่จะบริจาคน้ำนมอย่างน้อย 3 ครั้ง ด้วยปริมาณครั้งละประมาณ 25 มิลลิลิตร และข้าพเจ้าได้รับทราบรายละเอียดข้อมูลคำอธิบายสำหรับอาสาสมัครที่เข้าร่วมในการวิจัย รวมทั้งได้รับการ

อธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการทำวิจัย อันตรายหรืออาการที่อาจเกิดขึ้นจากการทำวิจัย

หรือจากยาที่ใช้รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียดและมีความเข้าใจดีแล้ว

2. ผู้วิจัยรับรองว่าจะตอบคำถามต่างๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจไม่ปิดบังซ่อนเร้นจนข้าพเจ้าพอใจ

3. ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับและจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุป

ผลการวิจัย การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้องกระทำได้เฉพาะกรณีจำเป็น

ด้วยเหตุผลทางวิชาการเท่านั้น และผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆ จากการวิจัยดังกล่าว ข้าพเจ้าจะได้รับ

การรักษาพยาบาลโดยไม่คิดมูลค่า

4. ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมในโครงการวิจัยนี้เมื่อใดก็ได้และการบอกเลิกการเข้าร่วมการวิจัยนี้จะไม่มีผลต่อการรักษาโรคที่ข้าพเจ้าจะพึงได้รับต่อไป

ข้าพเจ้าจึงสมัครใจเข้าร่วมโครงการวิจัยนี้ตามที่ระบุในเอกสารข้อมูลคำอธิบายสำหรับอาสาสมัครและได้ลง นามในใบยินยอมนี้ด้วยความเต็มใจ และได้รับสำเนาเอกสารใบยินยอมที่ข้าพเจ้าลง

นามและลงวันที่ และเอกสารยกเลิกการเข้าร่วมวิจัย อย่างละ 1 ฉบับ เป็นที่เรียบร้อยแล้ว ในกรณีที่อาสาสมัครยังไม่บรรลุนิติภาวะจะต้องได้รับการยินยอมจากผู้ปกครองด้วย

ลงนาม..... (อาสาสมัคร) (.....) วันที่...../...../.....	ลงนาม..... (ผู้ปกครอง) (.....) วันที่...../...../.....
ลงนาม..... (ผู้วิจัยหลัก) (ทันตแพทย์หญิงหยาดฤทัย โก้สกุล) วันที่...../...../.....	ลงนาม..... (พยาน) (.....) วันที่...../...../.....

ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนาม หรือประทับลายนิ้วหัวแม่มือขวาของข้าพเจ้าในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม..... (อาสาสมัคร) (.....) วันที่...../...../.....	ลงนาม..... (ผู้ปกครอง) (.....) วันที่...../...../.....
ลงนาม..... (ผู้วิจัยหลัก) (ทันตแพทย์หญิงหยาดฤทัย โก้สกุล) วันที่...../...../.....	ลงนาม..... (พยาน) .....) วันที่...../...../.....

APPENDIX E

Withdrawal Form

เอกสารยกเลิกการเข้าร่วมวิจัย (Withdrawal Form)

การวิจัยเรื่อง อัตราการผลิตกรดของเชื้อสเตร็ปโตคอกคัส มิวแทนส์ ภายหลังจากการปรับตัวในนมแม่

ข้าพเจ้า (นาย/ นาง/ นางสาว/ เด็กชาย/ เด็กหญิง).....

อยู่บ้านเลขที่.....ถนน.....ตำบล/แขวง.....

อำเภอ/เขต.....จังหวัด.....รหัสไปรษณีย์.....

ขอยกเลิกการเข้าร่วมโครงการวิจัยนี้ โดยมีเหตุผลในการยกเลิกการเข้าร่วมวิจัยคือ

- ❑ ย้ายภูมิลำเนา
- ❑ ไม่สะดวกในการเดินทาง
- ❑ เหตุผลอื่น.....

ลงนาม.....ผู้ยกเลิก

(.....)

วันที่.....เดือน.....พ.ศ. .....

ลงนาม.....พยาน

(.....)

วันที่.....เดือน.....พ.ศ. .....

ลงนาม.....ผู้วิจัยหลัก

(ทันตแพทย์หญิงหยาดฤทัย โก้สกุล)

วันที่.....เดือน.....พ.ศ. .....

APPENDIX F

Statistical Analysis

The pH and the numbers of bacteria (logCFU/ml) at the beginning of acid production in HBM of all passages

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
pH_T0	48	6.62	7.98	7.5498	.23376
logCFU_T0	48	7.79	8.84	8.3150	.21486
Valid N (listwise)	48				

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
pH_T0	4.292	15	32	.000
logCFU_T0	1.898	15	32	.063

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
pH_T0	Between Groups	.926	15	.062	1.203	.319
	Within Groups	1.642	32	.051		
	Total	2.568	47			
logCFU_T0	Between Groups	.511	15	.034	.657	.805
	Within Groups	1.659	32	.052		
	Total	2.170	47			

Robust Tests of Equality of Means

		Statistic[a]	df1	df2	Sig.
pH_T0	Welch	2.336	15	11.851	.074
logCFU_T0	Welch	.517	15	11.350	.884

a. Asymptotically F distributed.

The culture pH and the numbers of bacteria (logCFU/ml) at the beginning of acid production in BHI+Sucrose of all passages

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
pH_T0	48	6.47	7.60	7.1962	.17205
logCFU_T0	48	7.94	9.07	8.5505	.27650
Valid N (listwise)	48				

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
pH_T0	2.850	15	32	.006
logCFU_T0	2.314	15	32	.023

Robust Tests of Equality of Means

		Statistic[a]	df1	df2	Sig.
pH_T0	Welch	2.106	15	11.745	.102
logCFU_T0	Welch	2.351	15	12.043	.071

a. Asymptotically F distributed.

The culture pH in HBM of HBM-grown cell (HH) 1st to 15th passage

Time 0

Test of Homogeneity of Variances[a]

HH_pH

Levene Statistic	df1	df2	Sig.
6.726	4	10	.007

a. Time = 0

Robust Tests of Equality of Means[a]

HH_pH

	Statistic[b]	df1	df2	Sig.
Welch	2.276	4	4.672	.203

a. Time = 0

b. Asymptotically F distributed.

Time 1

Test of Homogeneity of Variances[a]

HH_pH

Levene Statistic	df1	df2	Sig.
4.413	4	10	.026

a. Time = 1

Robust Tests of Equality of Means[a]

HH_pH

	Statistic[b]	df1	df2	Sig.
Welch	2.535	4	4.834	.171

a. Time = 1

b. Asymptotically F distributed.

Time 2

Test of Homogeneity of Variances[a]

HH_pH

Levene Statistic	df1	df2	Sig.
1.953	4	10	.178

a. Time = 2

ANOVA[a]

HH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.222	4	.305	1.983	.173
Within Groups	1.540	10	.154		
Total	2.761	14			

a. Time = 2

Time 3

Test of Homogeneity of Variances[a]

HH_pH

Levene Statistic	df1	df2	Sig.
2.960	4	10	.075

a. Time = 3

ANOVA[a]

HH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.450	4	.112	1.757	.214
Within Groups	.640	10	.064		
Total	1.090	14			

a. Time = 3

Time 4

Test of Homogeneity of Variances[a]

HH_pH

Levene Statistic	df1	df2	Sig.
1.583	4	10	.253

a. Time = 4

ANOVA[a]

HH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.080	4	.020	1.274	.343
Within Groups	.157	10	.016		
Total	.237	14			

a. Time = 4

Time 5

Test of Homogeneity of Variances[a]

HH_pH

Levene Statistic	df1	df2	Sig.
5.186	4	10	.016

a. Time = 5

Robust Tests of Equality of Means[a]

HH_pH

	Statistic[b]	df1	df2	Sig.
Welch	1.829	4	4.142	.282

a. Time = 5

b. Asymptotically F distributed.

Time 6

Test of Homogeneity of Variances[a]

HH_pH

Levene Statistic	df1	df2	Sig.
2.081	4	10	.158

a. Time = 6

ANOVA[a]

HH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.036	4	.009	1.053	.428
Within Groups	.085	10	.009		
Total	.121	14			

a. Time = 6

Time 12

Test of Homogeneity of Variances[a]

HH_pH

Levene Statistic	df1	df2	Sig.
1.975	4	10	.174

a. Time = 12

ANOVA[a]

HH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.006	4	.001	.359	.832
Within Groups	.040	10	.004		
Total	.046	14			

a. Time = 12

The culture pH in HBM of HBM+BHI-grown cell (HBH) 1st to 15th passage

Time 0

Test of Homogeneity of Variances[a]

HBH_pH

Levene Statistic	df1	df2	Sig.
5.536	5	12	.007

a. Time = 0

Robust Tests of Equality of Means[a]

HBH_pH

	Statistic[b]	df1	df2	Sig.
Welch	.205	5	5.460	.948

a. Time = 0

b. Asymptotically F distributed.

Time 1

Test of Homogeneity of Variances[a]

HBH_pH

Levene Statistic	df1	df2	Sig.
3.016	5	12	.054

a. Time = 1

ANOVA[a]

HBH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.542	5	.308	1.699	.209
Within Groups	2.177	12	.181		
Total	3.719	17			

a. Time = 1

Time 2

Test of Homogeneity of Variances[a]

HBH_pH

Levene Statistic	df1	df2	Sig.
3.124	5	12	.049

a. Time = 2

Robust Tests of Equality of Means[a]

HBH_pH

	Statistic[b]	df1	df2	Sig.
Welch	3.500	5	5.395	.090

a. Time = 2

b. Asymptotically F distributed.

Time 3

Test of Homogeneity of Variances[a]

HBH_pH

Levene Statistic	df1	df2	Sig.
2.712	5	12	.073

a. Time = 3

ANOVA[a]

HBH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.105	5	.421	2.237	.117
Within Groups	2.258	12	.188		
Total	4.364	17			

a. Time = 3

Time 4

Test of Homogeneity of Variances[a]

HBH_pH

Levene Statistic	df1	df2	Sig.
.410	5	12	.833

a. Time = 4

ANOVA[a]

HBH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.836	5	.167	1.873	.173
Within Groups	1.072	12	.089		
Total	1.908	17			

a. Time = 4

Time 5

Test of Homogeneity of Variances[a]

HBH_pH

Levene Statistic	df1	df2	Sig.
.538	5	12	.744

a. Time = 5

ANOVA[a]

HBH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.337	5	.067	1.454	.275
Within Groups	.556	12	.046		
Total	.893	17			

a. Time = 5

Time 6

Test of Homogeneity of Variances[a]

HBH_pH

Levene Statistic	df1	df2	Sig.
.960	5	12	.479

a. Time = 6

ANOVA[a]

HBH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.213	5	.043	.997	.460
Within Groups	.514	12	.043		
Total	.727	17			

a. Time = 6

Time 12

Test of Homogeneity of Variances[a]

HBH_pH

Levene Statistic	df1	df2	Sig.
2.572	5	12	.084

a. Time = 12

ANOVA[a]

HBH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	5	.001	.112	.987
Within Groups	.092	12	.008		
Total	.096	17			

a. Time = 12

The culture pH in of HBM of BHI-grown cell (BH) 1st to 15th passage

Time 0

Test of Homogeneity of Variances[a]

BH_pH

Levene Statistic	df1	df2	Sig.
.795	4	10	.555

a. Time = 0

ANOVA[a]

BH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.078	4	.020	.376	.821
Within Groups	.521	10	.052		
Total	.600	14			

a. Time = 0

Time 1

Test of Homogeneity of Variances[a]

BH_pH

Levene Statistic	df1	df2	Sig.
1.691	4	10	.228

a. Time = 1

ANOVA[a]

BH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.026	4	.007	.267	.892
Within Groups	.245	10	.025		
Total	.272	14			

a. Time = 1

Time 2

Test of Homogeneity of Variances[a]

BH_pH

Levene Statistic	df1	df2	Sig.
.590	4	10	.677

a. Time = 2

ANOVA[a]

BH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.034	4	.008	.308	.866
Within Groups	.275	10	.027		
Total	.309	14			

a. Time = 2

Time 3

Test of Homogeneity of Variances[a]

BH_pH

Levene Statistic	df1	df2	Sig.
2.196	4	10	.143

a. Time = 3

ANOVA[a]

BH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.156	4	.039	.525	.720
Within Groups	.743	10	.074		
Total	.899	14			

a. Time = 3

Time 4

Test of Homogeneity of Variances[a]

BH_pH

Levene Statistic	df1	df2	Sig.
2.543	4	10	.105

a. Time = 4

ANOVA[a]

BH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.692	4	.173	.986	.458
Within Groups	1.756	10	.176		
Total	2.448	14			

a. Time = 4

Time 5

Test of Homogeneity of Variances[a]

BH_pH

Levene Statistic	df1	df2	Sig.
1.633	4	10	.241

Time = 5

ANOVA[a]

BH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.818	4	.204	.574	.688
Within Groups	3.560	10	.356		
Total	4.378	14			

a. Time = 5

Time 6

Test of Homogeneity of Variances[a]

BH_pH

Levene Statistic	df1	df2	Sig.
2.303	4	10	.130

a. Time = 6

ANOVA[a]

BH_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.006	4	.251	.646	.642
Within Groups	3.892	10	.389		
Total	4.898	14			

a. Time = 6

Time 12

Test of Homogeneity of Variances[a]

BH_pH

Levene Statistic	df1	df2	Sig.
9.389	4	10	.002

a. Time = 12

Robust Tests of Equality of Means[a]

BH_pH

	Statistic[b]	df1	df2	Sig.
Welch	1.705	4	4.635	.292

a. Time = 12

b. Asymptotically F distributed.

The culture pH in BHI+Sucrose of HBM-grown cell (HS) 1st to 15th passage

Time 0

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
.441	4	10	.776

a. Time = 0

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.023	4	.006	1.063	.424
Within Groups	.053	10	.005		
Total	.075	14			

a. Time = 0

Time 1

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
1.596	4	10	.250

a. Time = 1

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.046	4	.012	.599	.672
Within Groups	.193	10	.019		
Total	.239	14			

a. Time = 1

Time 2

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
2.207	4	10	.141

a. Time = 2

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.067	4	.017	.301	.871
Within Groups	.558	10	.056		
Total	.625	14			

a. Time = 2

Time 3

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
2.839	4	10	.082

a. Time = 3

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.220	4	.055	.395	.808
Within Groups	1.392	10	.139		
Total	1.612	14			

a. Time = 3

Time 4

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
5.263	4	10	.015

a. Time = 4

Robust Tests of Equality of Means[a]

HS_pH

	Statistic[b]	df1	df2	Sig.
Welch	1.013	4	4.429	.488

a. Time = 4

b. Asymptotically F distributed.

Time 5

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
3.018	4	10	.071

a. Time = 5

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.041	4	.010	.314	.862
Within Groups	.327	10	.033		
Total	.368	14			

a. Time = 5

Time 6

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
2.503	4	10	.109

a. Time = 6

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.008	4	.002	.086	.985
Within Groups	.227	10	.023		
Total	.235	14			

a. Time = 6

Time 12

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
2.186	4	10	.144

a. Time = 12

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	4	.003	1.752	.215
Within Groups	.017	10	.002		
Total	.029	14			

a. Time = 12

The culture pH in the BHI+Sucrose of HBM+BHI-grown cell (HBS) 1st to 15th passage

Time 0

Test of Homogeneity of Variances[a]

HBS_pH

Levene Statistic	df1	df2	Sig.
2.847	4	10	.082

a. Time = 0

ANOVA[a]

HBS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.336	4	.084	1.402	.302
Within Groups	.599	10	.060		
Total	.936	14			

a. Time = 0

Time 1

Test of Homogeneity of Variances[a]

HBS_pH

Levene Statistic	df1	df2	Sig.
2.168	4	10	.146

a. Time = 1

ANOVA[a]

HBS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.236	4	.059	1.189	.373
Within Groups	.497	10	.050		
Total	.734	14			

a. Time = 1

Time = 2

Test of Homogeneity of Variances[a]

HBS_pH

Levene Statistic	df1	df2	Sig.
.998	4	10	.452

a. Time = 2

ANOVA[a]

HBS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.170	4	.043	.360	.831
Within Groups	1.181	10	.118		
Total	1.351	14			

a. Time = 2

Time = 3

Test of Homogeneity of Variances[a]

HBS_pH

Levene Statistic	df1	df2	Sig.
1.486	4	10	.278

a. Time = 3

ANOVA[a]

HBS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.063	4	.016	.419	.791
Within Groups	.373	10	.037		
Total	.436	14			

a. Time = 3

Time 4

Test of Homogeneity of Variances[a]

HBS_pH

Levene Statistic	df1	df2	Sig.
.847	4	10	.526

a. Time = 4

ANOVA[a]

HBS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.038	4	.010	.448	.772
Within Groups	.215	10	.021		
Total	.253	14			

a. Time = 4

Time 5

Test of Homogeneity of Variances[a]

HBS_pH

Levene Statistic	df1	df2	Sig.
1.393	4	10	.304

a. Time = 5

ANOVA[a]

HBS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	4	.003	.154	.957
Within Groups	.196	10	.020		
Total	.208	14			

a. Time = 5

Time 6

Test of Homogeneity of Variances[a]

HBS_pH

Levene Statistic	df1	df2	Sig.
4.204	4	10	.030

a. Time = 6

Robust Tests of Equality of Means[a]

HBS_pH

	Statistic[b]	df1	df2	Sig.
Welch	.153	4	4.555	.953

a. Time = 6

b. Asymptotically F distributed.

Time 12

Test of Homogeneity of Variances[a]

HBS_pH

Levene Statistic	df1	df2	Sig.
4.869	4	10	.019

a. Time = 12

Robust Tests of Equality of Means[a]

HBS_pH

	Statistic[b]	df1	df2	Sig.
Welch	.608	4	4.626	.676

a. Time = 12

b. Asymptotically F distributed.

The culture pH in BHI+Sucrose of BHI-grown cell (BS) 1st to 15th passage

Time = 0

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
.441	4	10	.776

a. Time = 0

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.023	4	.006	1.063	.424
Within Groups	.053	10	.005		
Total	.075	14			

a. Time = 0

Time 1

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
1.596	4	10	.250

a. Time = 1

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.046	4	.012	.599	.672
Within Groups	.193	10	.019		
Total	.239	14			

a. Time = 1

Time 2

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
2.207	4	10	.141

a. Time = 2

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.067	4	.017	.301	.871
Within Groups	.558	10	.056		
Total	.625	14			

a. Time = 2

Time = 3

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
2.839	4	10	.082

a. Time = 3

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.220	4	.055	.395	.808
Within Groups	1.392	10	.139		
Total	1.612	14			

a. Time = 3

Time 4

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
5.263	4	10	.015

a. Time = 4

Robust Tests of Equality of Means[a]

HS_pH

	Statistic[b]	df1	df2	Sig.
Welch	1.013	4	4.429	.488

a. Time = 4

b. Asymptotically F distributed.

Time 5

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
3.018	4	10	.071

a. Time = 5

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.041	4	.010	.314	.862
Within Groups	.327	10	.033		
Total	.368	14			

a. Time = 5

Time 6

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
2.503	4	10	.109

a. Time = 6

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.008	4	.002	.086	.985
Within Groups	.227	10	.023		
Total	.235	14			

a. Time = 6

Time = 12

Test of Homogeneity of Variances[a]

HS_pH

Levene Statistic	df1	df2	Sig.
2.186	4	10	.144

a. Time = 12

ANOVA[a]

HS_pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	4	.003	1.752	.215
Within Groups	.017	10	.002		
Total	.029	14			

a. Time = 12

Growth of HBM-grown cells in HBM (HH) at the 5th passage

Tests of Within-Subjects Effects[a]

Measure: CFU

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
time	Sphericity Assumed	1.660	7	.237	11.348	.000	.850
	Greenhouse-Geisser	1.660	1.763	.941	11.348	.030	.850
	Huynh-Feldt	1.660	7.000	.237	11.348	.000	.850
	Lower-bound	1.660	1.000	1.660	11.348	.078	.850
Error (time)	Sphericity Assumed	.292	14	.021			
	Greenhouse-Geisser	.292	3.526	.083			
	Huynh-Feldt	.292	14.000	.021			
	Lower-bound	.292	2.000	.146			

a. passage = HH5

Tests of Between-Subjects Effects[a]

Measure: CFU

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	1880.758	1	1880.758	29831.760	.000	1.000
Error	.126	2	.063			

a. passage = HH5

Estimates[a]

Measure: CFU

time	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
1	8.361	.075	8.040	8.682
2	8.480	.041	8.303	8.656
3	8.813	.155	8.144	9.482
4	9.061	.155	8.393	9.729
5	8.965	.066	8.682	9.248
6	9.042	.071	8.737	9.346
7	9.077	.062	8.809	9.346
8	9.020	.031	8.886	9.154

a. passage = HH5

Pairwise Comparisons[a]

Measure: CFU

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.[c]	95% Confidence Interval for Difference[c]	
					Lower Bound	Upper Bound
1	2	-.119	.040	.099	-.292	.055
	3	-.452	.164	.110	-1.158	.254
	4	-.700	.189	.066	-1.514	.113
	5	-.604*	.101	.027	-1.038	-.170
	6	-.681*	.145	.042	-1.304	-.057
	7	-.717*	.055	.006	-.951	-.482
	8	-.659*	.106	.025	-1.115	-.204
2	1	.119	.040	.099	-.055	.292
	3	-.333	.172	.191	-1.072	.405
	4	-.582	.184	.087	-1.372	.209
	5	-.486*	.091	.033	-.878	-.094
	6	-.562*	.111	.037	-1.041	-.083
	7	-.598*	.061	.010	-.862	-.334
	8	-.541*	.070	.016	-.843	-.239
3	1	.452	.164	.110	-.254	1.158
	2	.333	.172	.191	-.405	1.072
	4	-.248	.060	.053	-.504	.008
	5	-.152	.091	.236	-.543	.239
	6	-.229	.167	.304	-.947	.489
	7	-.264	.112	.143	-.747	.218
	8	-.207	.165	.335	-.916	.501
4	1	.700	.189	.066	-.113	1.514
	2	.582	.184	.087	-.209	1.372
	3	.248	.060	.053	-.008	.504
	5	.096	.093	.408	-.302	.494
	6	.019	.140	.903	-.584	.623
	7	-.016	.135	.915	-.595	.563
	8	.041	.153	.815	-.617	.698
5	1	.604*	.101	.027	.170	1.038
	2	.486*	.091	.033	.094	.878
	3	.152	.091	.236	-.239	.543
	4	-.096	.093	.408	-.494	.302
	6	-.077	.086	.467	-.447	.294
	7	-.112	.050	.154	-.327	.103
	8	-.055	.074	.534	-.375	.265
6	1	.681*	.145	.042	.057	1.304
	2	.562*	.111	.037	.083	1.041
	3	.229	.167	.304	-.489	.947
	4	-.019	.140	.903	-.623	.584

	5	.077	.086	.467	-.294	.447
	7	-.036	.118	.790	-.542	.470
	8	.021	.042	.658	-.157	.200
7	1	.717*	.055	.006	.482	.951
	2	.598*	.061	.010	.334	.862
	3	.264	.112	.143	-.218	.747
	4	.016	.135	.915	-.563	.595
	5	.112	.050	.154	-.103	.327
	6	.036	.118	.790	-.470	.542
	8	.057	.088	.584	-.323	.437
8	1	.659*	.106	.025	.204	1.115
	2	.541*	.070	.016	.239	.843
	3	.207	.165	.335	-.501	.916
	4	-.041	.153	.815	-.698	.617
	5	.055	.074	.534	-.265	.375
	6	-.021	.042	.658	-.200	.157
	7	-.057	.088	.584	-.437	.323

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. passage = HH5

c. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no djustments).

Acid production rate

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Baseline HBM	3	.07889	.007840	.004526	.05941	.09836
HBM_HBM	3	.11794	.020583	.011884	.06681	.16907
HBM+BHI_HBM	3	.09733	.019549	.011287	.04877	.14590
BHI_HBM	3	.07010	.023943	.013823	.01062	.12957
Baseline BHI+Sucrose	3	.10293	.000950	.000549	.10057	.10529
HBM_BHI+Sucrose	3	.09587	.020235	.011683	.04561	.14614
HBM+BHI_BHI+sucrsoe	3	.10976	.008227	.004750	.08932	.13020
BHI_BHI+Sucrose	3	.09631	.021062	.012160	.04399	.14863
Total	24	.09614	.020619	.004209	.08744	.10485

Test of Homogeneity of Variances

rate

Levene Statistic	df1	df2	Sig.
1.855	7	16	.145

ANOVA

rate

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.005	7	.001	2.445	.066
Within Groups	.005	16	.000		
Total	.010	23			

The number of bacteria at T1-T3 of acid production in HBM of HBM-grown cell (HH)

Time 1

Test of Homogeneity of Variances[a]

HH_logCFU

Levene Statistic	df1	df2	Sig.
1.499	4	10	.274

a. Time = 1

ANOVA[a]

HH_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.422	4	.106	3.072	.068
Within Groups	.344	10	.034		
Total	.766	14			

a. Time = 1

Time 2

Test of Homogeneity of Variances[a]

HH_logCFU

Levene Statistic	df1	df2	Sig.
.972	4	10	.464

a. Time = 2

ANOVA[a]

HH_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.499	4	.125	1.630	.242
Within Groups	.766	10	.077		
Total	1.265	14			

a. Time = 2

Time 3

Test of Homogeneity of Variances[a]

HH_logCFU

Levene Statistic	df1	df2	Sig.
.414	4	10	.795

a. Time = 3

ANOVA[a]

HH_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.324	4	.081	2.435	.116
Within Groups	.333	10	.033		
Total	.657	14			

a. Time = 3

The number of bacteria at T0-T2 of acid production in HBM of HBM+BHI-grown cell (HBH)

Time 0

Test of Homogeneity of Variances[a]

HBH_logCFU

Levene Statistic	df1	df2	Sig.
2.490	4	10	.110

a. Time = 0

ANOVA[a]

HBH_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.135	4	.034	.423	.789
Within Groups	.796	10	.080		
Total	.930	14			

a. Time = 0

Time 1

Test of Homogeneity of Variances[a]

HBH_logCFU

Levene Statistic	df1	df2	Sig.
1.017	4	10	.444

a. Time = 1

ANOVA[a]

HBH_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.222	4	.056	.874	.512
Within Groups	.636	10	.064		
Total	.858	14			

a. Time = 1

Time 2

Test of Homogeneity of Variances[a]

HBH_logCFU

Levene Statistic	df1	df2	Sig.
6.095	4	10	.009

a. Time = 2

Robust Tests of Equality of Means[a]

HBH_logCFU

	Statistic[b]	df1	df2	Sig.
Welch	3.398	4	4.043	.130

a. Time = 2

b. Asymptotically F distributed.

The number of bacteria at T4-T6 of acid production in HBM of BHI-grown cell (BH)

Time 3

Test of Homogeneity of Variances[a]

BH_logCFU

Levene Statistic	df1	df2	Sig.
1.509	4	10	.272

a. Time = 3

ANOVA[a]

BH_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.605	4	.151	2.430	.116
Within Groups	.622	10	.062		
Total	1.227	14			

a. Time = 3

Time 4

Test of Homogeneity of Variances[a]

BH_logCFU

Levene Statistic	df1	df2	Sig.
.638	4	10	.647

a. Time = 4

ANOVA[a]

BH_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.086	4	.021	.142	.962
Within Groups	1.508	10	.151		
Total	1.593	14			

a. Time = 4

Time 5

Test of Homogeneity of Variances[a]

BH_logCFU

Levene Statistic	df1	df2	Sig.
6.775	4	10	.007

a. Time = 5

Robust Tests of Equality of Means[a]

BH_logCFU

	Statistic[b]	df1	df2	Sig.
Welch	1.813	4	4.105	.286

a. Time = 5

b. Asymptotically F distributed.

The number of bacteria at T2-T4 of acid production in BHI+SUCROSE of HBM-grown cell (HS)

Time 2

Test of Homogeneity of Variances[a]

HS_logCFU

Levene Statistic	df1	df2	Sig.
3.242	4	10	.060

a. Time = 2

ANOVA[a]

HS_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.879	4	1.470	3.333	.056
Within Groups	4.409	10	.441		
Total	10.288	14			

a. Time = 2

Time 3

Test of Homogeneity of Variances[a]

HS_logCFU

Levene Statistic	df1	df2	Sig.
2.029	4	10	.166

a. Time = 3

ANOVA[a]

HS_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.307	4	1.577	2.259	.135
Within Groups	6.980	10	.698		
Total	13.287	14			

a. Time = 3

Time 4

Test of Homogeneity of Variances[a]

HS_logCFU

Levene Statistic	df1	df2	Sig.
3.347	4	10	.055

a. Time = 4

ANOVA[a]

HS_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.502	4	1.626	2.709	.092
Within Groups	6.001	10	.600		
Total	12.503	14			

a. Time = 4

The number of bacteria at T0-T2 of acid production in BHI+SUCROSE of HBM+BHI-grown cell (HBS)

Time 0

Test of Homogeneity of Variances[a]

HBS_logCFU

Levene Statistic	df1	df2	Sig.
3.180	4	10	.063

a. Time = 0

ANOVA[a]

HBS_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.606	4	.152	1.382	.308
Within Groups	1.096	10	.110		
Total	1.702	14			

a. Time = 0

Time 1

Test of Homogeneity of Variances[a]

HBS_logCFU

Levene Statistic	df1	df2	Sig.
2.795	4	10	.085

a. Time = 1

ANOVA[a]

HBS_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.786	4	.446	.737	.588
Within Groups	6.061	10	.606		
Total	7.847	14			

a. Time = 1

Time 2

Test of Homogeneity of Variances[a]

HBS_logCFU

Levene Statistic	df1	df2	Sig.
2.753	4	10	.088

a. Time = 2

ANOVA[a]

HBS_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.747	4	.187	.157	.955
Within Groups	11.870	10	1.187		
Total	12.617	14			

a. Time = 2

The number of bacteria at T0-T2 of acid production in BHI+SUCROSE of BHI-grown cell (BS)

Time 0

Test of Homogeneity of Variances[a]

BS_logCFU

Levene Statistic	df1	df2	Sig.
2.125	4	12	.140

a. Time = 0

ANOVA[a]

BS_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.253	4	.063	1.524	.257
Within Groups	.498	12	.042		
Total	.751	16			

a. Time = 0

Time 1

Test of Homogeneity of Variances[a]

BS_logCFU

Levene Statistic	df1	df2	Sig.
.410	4	13	.799

a. Time = 1

ANOVA[a]

BS_logCFU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.949	4	.737	.871	.507
Within Groups	11.000	13	.846		
Total	13.949	17			

a. Time = 1

Time 2

Test of Homogeneity of Variances[a]

BS_logCFU

Levene Statistic	df1	df2	Sig.
5.898	4	13	.006

a. Time = 2

Robust Tests of Equality of Means[a]

BS_logCFU

	Statistic[b]	df1	df2	Sig.
Welch	2.538	4	5.596	.155

a. Time = 2

b. Asymptotically F distributed.

VITA

Miss Yart-ruetai Kosakul was born on 7th September 1988 in Bangkok. She graduated with D.D.S (Doctor of Dental Surgery from the Faculty of Dentistry, Chulalongkorn University in 2012, and had worked at Sawee hospital and Lamae hospital in Choomphon province for 2 years. She started her Master degree program in Pediatric Dentistry at Graduate School, Chulalongkorn University in 2012.