Effects of Non-*albicans Candida* species on biofilm formation and acidogenicity of dual-species biofilms and on cariogenic virulence gene expression of *Streptococcus mutans*

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ผลของ Non-*albicans Candida* species ต่อการสร้างไบโอฟิล์ม และการสร้างกรดในไบโอฟิล์มสองสายพันธุ์ และต่อการแสดงออกของยีนส์ที่เกี่ยวข้องกับปัจจัยก่อโรคฟันผุของ *Streptococcus mutans*

น.ส.วิรัลพัชร แสนเสนาะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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วิรัลพัชร แสนเสนาะ : ผลของ Non-*albicans Candida* species ต่อการสร้างไบโอฟิล์มและการสร้างกรดในไบโอฟิล์มสองสายพันธุ์ และต่อการแสดงออกของยีนส์ที่เกี่ยวข้องกับปัจจัยก่อโรคฟันผุของ *Streptococcus mutans*. (Effects of Non-*albicans Candida* species on biofilm formation and acidogenicity of dual-species biofilms and on cariogenic virulence gene expression of *Streptococcus mutans*) อ.ที่ปรึกษาหลัก : รศ. ทญ. ดร.พนิดา ธัญญศรีสังข์, อ.ที่ปรึกษาร่วม : รศ. ทญ. ดร.อรนาฎ มาตังคสมบัติ

โรคฟันผุในเด็กปฐมวัยเป็นหนึ่งในโรคช่องปากที่พบได้บ่อยในเด็กเล็กซึ่งส่งผลกระทบต่อสุขภาพร่างกายของเด็ก เชื้อ *Streptococcus mutans* เป็นเชื้อก่อโรคฟันผุที่สำคัญเพราะมีคุณสมบัติก่อโรคฟันผุซึ่งก็คือ การผลิตกรด การทนกรด และการสร้างไบโอฟิล์ม การศึกษาเมื่อไม่นานนี้แสดงให้เห็นว่า *Candida albicans* ซึ่งมักพบร่วมกับ *S. mutans* ในคราบจุลินทรีย์ของเด็กที่มีฟันผุอาจมีบทบาททำให้โรคฟันผุเพิ่มขึ้น นอกจากนั้น มีรายงานความชุกที่เพิ่มขึ้นของเชื้อ non-*albicans Candida* species (NACs) ในเด็ก และผู้ป่วยภูมิคุ้มกันบกพร่อง ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของเชื้อ NACs เมื่อเลี้ยงร่วมกับ *S. mutans* ต่อคุณสมบัติการก่อโรคฟันผุ และการแสดงออกของยีนก่อโรคของ *S. mutans* ผลการทดลองพบว่าการเลี้ยง *S. mutans* ร่วมกับ *C. albicans* หรือ NACs ทำให้มวลไบโอฟิล์มเพิ่มขึ้น โดย *Candida tropicalis* เพิ่มมวลไบโอฟิล์ม และจำนวนเชื้อที่มีชีวิต อย่างเห็นได้ชัดของไบโอฟิล์มสองสายพันธุ์ แต่ไม่ทำให้ความเป็นกรดเพิ่มขึ้น สำหรับการแสดงออกของยีนของ *S. mutans* พบว่า ยีนที่สัมพันธ์กับการสร้างเอ็กโซโพลีแซคคาไรด์ (*gtfBCD*) การขนส่งน้ำตาล (*scrAB*) และการใช้น้ำตาล (*pdhA* และ *pfl*) เพิ่มขึ้นในไบโอฟิล์มสองสายพันธุ์ เมื่อเลี้ยงร่วมกับ *C. albicans*, *C. tropicalis* และ *Candida krusei* ในทางตรงข้าม การแสดงออกของยีนที่เกี่ยวข้องกับการทนกรด (*atpD* และ *fabM*) ในไบโอฟิล์มสองสายพันธุ์ คล้ายกับในไบโอฟิล์มของ *S. mutans* เดี่ยว อย่างไรก็ตาม การแสดงออกของยีนที่เกี่ยวกับ ระบบการส่งสัญญาณ (*ciaRH*) เพิ่มขึ้นในไบโอฟิล์มสองสายพันธุ์ระหว่าง *S. mutans* และ *C. albicans* หรือ NACs, ยกเว้น *C. dubliniensis* โดยสรุปการศึกษานี้แสดงให้เห็นว่าเชื้อ NACs โดยเฉพาะ *C. tropicalis* และ *C. krusei* สามารถเพิ่มปัจจัยในการก่อโรคฟันผุของ *S. mutans* คล้ายกับ *C. albicans*

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Wirunphat Sansanoa : Effects of Non-*albicans Candida* species on biofilm formation and acidogenicity of dual-species biofilms and on cariogenic virulence gene expression of *Streptococcus mutans*. Advisor: Assoc. Prof. PANIDA THANYASRISUNG, DDS, PhD. Co-advisor: Assoc. Prof. ORANART MATANGKASOMBUT, DDS, PhD.

Early childhood caries is a common oral disease found in children which could affect their general health. *Streptococcus mutans* is an important cariogenic pathogen since it possesses cariogenic properties, which are acid production, acid tolerance and biofilm formation. Recent studies showed that *Candida albicans*, which is frequently found together with *S. mutans* in plaques of children with dental caries, enhances cariogenicity. At the same time, increasing prevalence of non-*albicans Candida* species (NACs) in children and immune-compromised patients has been reported. Therefore, this study aims to investigate the effects of NACs when co-cultured with *S. mutans* on cariogenic properties and virulence gene expression of *S. mutans*. The results showed that *C. albicans* and NACs, especially *Candida tropicalis,* increased biofilm mass and cell viability of dual species biofilm, but did not increase acid production. For *S. mutans* gene expression, the genes related to exopolysaccharides synthesis (*gtfBCD*), sugar transportation (*scrAB*) and sugar utilization (*pdhA* and *pfl*) were up-regulated in dual species biofilm when co-culture with *C. albicans*, *C. tropicalis* and *Candida krusei*. In contrast, the expression levels of genes related to acid stress tolerance (*atpD* and *fabM*) in all dual-species biofilm were similar to *S. mutans* in single-species biofilm. In addition, the expression of genes involved in the signal transduction system (*ciaRH*) were upregulated in dual-species biofilms of *S. mutans* and *C. albicans* or NACs, except *C. dubliniensis*. In conclusion, the presence of NACs, especially *C. tropicalis* and *C. krusei,* could enhance cariogenic virulence factors of *S. mutans* similarly to *C. albicans*.

Field of Study:	Medical Microbiology	Student's Signature .....
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Wirunphat Sansanoa

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LIST OF ABBREVIATIONS

ATCC	American Type Culture collection
BHI	Brain Heart Infusion
CFU	Colony-forming unit
CT	Cycle threshold
°C	Degree Celsius
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
ECC	Early Childhood caries
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharide
g	Gram
h	Hour
KCl	Potassium Chloride
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter
M	Molar
mg	Milligram

ml	Milliliter
mm	Millimeter
mM	Millimolar
MS	Mitis-Salivarius
MTT	((3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide)
NaCl	Sodium chloride
$Na_2HPO_42H_2O$	Disodium hydrogen phosphate dihydrate
nm	Nanometer
OD	Optical density
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulfate
µg	Microgram
µl	Microliter

CHAPTER I

INTRODUCTION

Dental caries is a multifactorial disease occurring when oral ecology shifts to acidic conditions that favor the change of microbial profile towards cariogenic pathogens (1). It is one of the most common diseases found in children, which is called early childhood caries (ECC). ECC affects children not only on their general health but also on their quality of life (2). One of the important factors causing ECC is dental biofilm, known as dental plaque. The biofilms consist of diverse microorganisms embedded in extracellular polymeric substances (EPS) (2-5). *Streptococcus mutans* (*S. mutans*) has been known for a long time as a major cariogenic pathogen (6) since it is commonly found in dental plaque of children with ECC (7, 8). Moreover, the bacteria showed their ability on acid production, acid tolerance and biofilm formation, which are the main virulence characters of cariogenic pathogens (8). *S. mutans* produce lactic acid as a by-product from sucrose metabolism. To survive in this acidic condition, they maintain pH in their cells via several mechanisms such as increased the levels of monounsaturated and longer chain fatty acids in membrane composition, increasing proton extrusion via the proton-extruding F_1F_0-ATPase pump and end-product efflux (8, 9). For biofilm formation via the sucrose dependent pathway, *S. mutans* produce glucosyltransferases (Gtfs) to convert sucrose to glucan and fructose. Water-insoluble

glucan (produced by GtfB) is one of the key molecules which play a role in adhering microbes to microbes and microbes to tooth surfaces (10).

Candida albicans (*C. albicans*) is the most prevalent species found in oral fungal infections (11). Recent clinical studies revealed that *C. albicans* is frequently co-isolated with *S. mutans* from plaque in children with dental caries (12-14). The study of interactions between these two microbes revealed that *S. mutans*–derived GtfB has high affinity and binding strength to mannoproteins of *C. albicans* cell wall. This interaction enhances the production of glucan matrix leading to biofilm development (15). Moreover, the presence of *C. albicans* in dual-species biofilms increases cariogenic virulence gene expressions of *S. mutans* especially genes related to the process of sugar utilization (15-18).

While most of studies focus on the mechanisms of interaction between *S. mutans* and *C. albicans*, increasing prevalence of non-*albicans Candida* species (NACs) found in children and immune-compromised patients is reported (12, 19-22). In case of ECC, the various NACs, including *Candida glabrata* (*C. glabrata*), *Candida tropicalis* (*C. tropicalis*) and *Candida dubliniensis* (*C. dubliniensis*), were also found in the dental plaque (12, 19, 22, 23). NACs possess different cell wall mannoproteins from *C. albicans* (24-26). These differences may impact pathogenic potential of dual-species biofilms and cariogenic virulence of *S. mutans*. Therefore, the aim of this study is to

determine the effect of NACs on biofilm formation and acidogenicity in dual-species biofilms and on cariogenic virulence gene expression of *S. mutans.*

Research hypothesis

Co-culture of non-*albicans Candida* species (NACs) and *S. mutans* modulates biofilm formation and acidogenicity in dual-species biofilms and cariogenic virulence gene expression of *S. mutans*.

Research objectives

1. To investigate the effects of non-*albicans Candida* species (NACs) on biofilm acidogenicity, biofilm mass and cell viability of dual-species biofilms.

2. To investigate the effects of non-*albicans Candida* species (NACs) on cariogenic virulence gene expression of *S. mutans* in dual-species biofilms.

CHAPTER II

LITERATURE REVIEW

2.1 Dental caries and early childhood caries (ECC)

Dental caries is a disease resulting from dysbiosis of microorganisms in oral biofilm induced by the changes of oral environment (1). In heathy condition, the tooth surfaces are covered by commensal microbial biofilm that mainly consist of non-mutans streptococci. The disease initially occurs when there are excessive fermentable carbohydrates, which are metabolized by microbes in biofilm producing acid as a by-product. As a result of acid accumulation in biofilm, microbial composition is shifted to cariogenic pathogens, which can grow in this acidic condition and can produce more acid. The pathogens, moreover, synthesize glucans, which help the cells to adhere to tooth surfaces or to other cells and finally creating a cariogenic biofilm (1, 8).

Early childhood caries (ECC) is dental caries found in primary teeth of children younger than 6 years old. Although there have been a lot of efforts to manage the disease, ECC is still one of the most prevalent diseases found in children worldwide (2, 27). The etiology of ECC is similar to dental caries in permanent teeth but the disease progression is faster. This faster caries progression is due to thinner and lesser organized ultrastructure of enamel in primary teeth than that of permanent teeth (27). There are many clinical and experimental studies showing the association between ECC and mutans streptococci (MS) especially, *Streptococcus mutans* (2, 7, 8, 28). Two-year

follow-up study found that children with salivary *S. mutans* over 10^5 CFU/ml have higher caries progression than children with low bacterial count (29). Furthermore, several recent studies showed that *Candida albicans* is frequently detected with *S. mutans* in dental plaque from children with ECC but not from caries free children (12, 18, 23). Moreover, an *in vivo* study revealed that this bacterial-fungal interaction occurs in the presence of sucrose (28). Based on current knowledge, ECC is a result of excessive consumption of sucrose together with the activities of complex microbes that lead to acidic environment in the biofilm.

2.2 *Streptococcus mutans* and its cariogenic virulence factors

Streptococcus mutans has been considered as one of the primary etiological pathogens associated with dental caries, particularly in ECC, since it is commonly found in carious lesions. Besides, it has cariogenic capabilities, i.e. acid production (acidogenicity), acid tolerance (aciduricity) and synthesis of exopolysaccharide (EPS) in the presence of sucrose (1, 5).

To gain energy for living, *S. mutans* transports sugars into cells via mainly two systems including phosphoenolpyruvate phosphotransferase (PEP-PTS) transport system and non-PTS system. Once sugars are inside the cells, they are metabolized via glycolysis pathway to pyruvate and energy. In case of sucrose, sucrose-6-phosphate hydrolase (encoded by scrB) cleaves sucrose-6 phosphate to glucose-6-phosphate in order to enter glycolysis (30). Under anaerobic condition and excess sugars (similar to

the conditions inside biofilms), *S. mutans* uses lactate dehydrogenase to convert pyruvate to lactate, which is pumped out of the cells together with protons in form as lactic acid (31) as shown in **Figure 1**. *S. mutans* possesses an extraordinary ability to transport sugars into the cells faster than other bacteria in biofilm, which resulting in faster acid production (32).

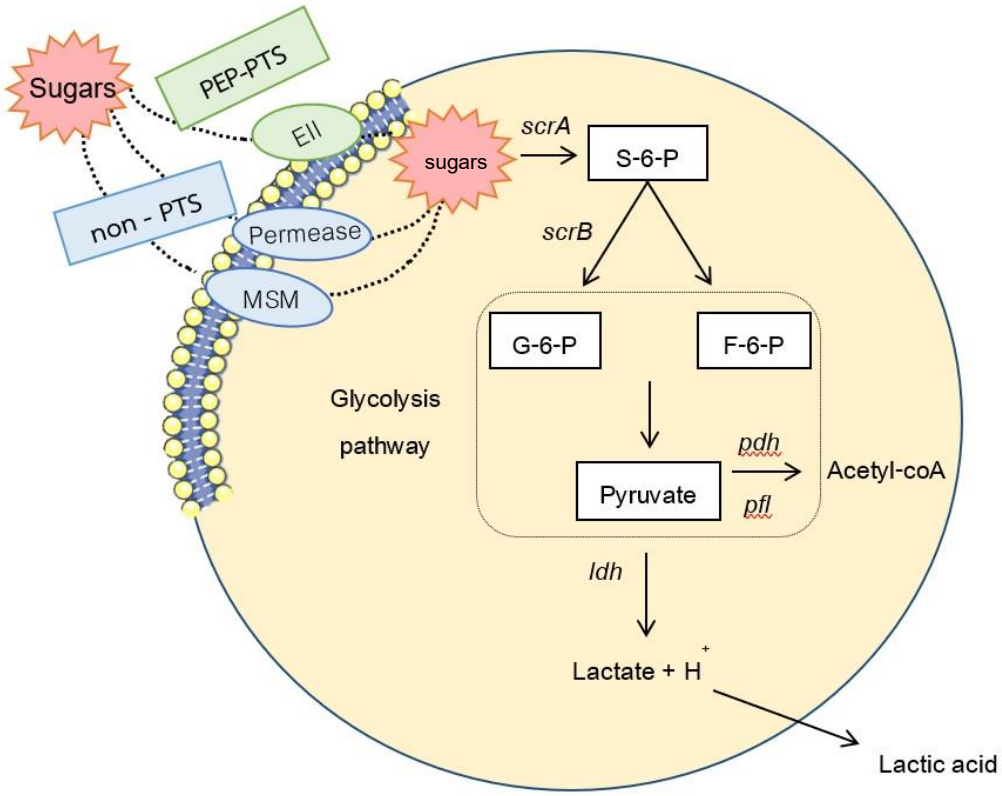


Figure 1. Sugar transportation of *Streptococcus mutans*.

Sugar transportation of Streptococcus mutans modified from Marsh PD et al. and He J et al. (31, 33). In S. mutans, two major incorporation systems, the phosphoenolpyruvate phosphotransferase (PEP-PTS) and non-PTS system, have been reported (31). After sugars are transport into cell, the sugar molecule is processed and

finally metabolized to enter the glycolysis pathway. ***EII:*** *Enzyme II;* ***MSM:*** *multiple sugar metabolism;* ***S-6-P:*** *sucrose-6-*phosphate*;* ***G-6-P:*** *glucose-6-phosphate;* ***F-6-P:*** *fructose-6-phosphate.*

Besides the ability to produce acid, *S. mutans* has ability to survive, grow and function in low pH condition. This acid tolerance is a consequence of mechanisms that maintain intracellular pH homeostasis, including prevention of proton influx by membrane composition alteration and increase proton extrusion by F_1F_0-ATPase activity and acid end product efflux (8, 9). *S. mutans* prevents proton influx by increasing the production of monounsaturated and longer chain fatty acids, which are regulated by *fabM* gene (34), to a level of four-time higher than that of saturated fatty acids. The key mechanism to maintain intracellular pH, not only found in *S. mutans* but also other acid tolerant species, is F_1F_0-ATPase proton pump. The F_1F_0-ATPase complex is encoded by the *atpHAGDC* operon (35) and *AtpD* plays an important role in the complex construction (36). The F_1F_0-ATPase complex is produced in larger amounts in *S. mutans* compared to other commensal streptococci in low pH condition and consequently pumps protons out of the cells. For acid end product efflux, as mentioned above, lactate is excreted together with protons out of the cells as lactic acid (8, 9).

The production of exopolysaccharide (EPS) is one of key pathogenic roles of *S. mutans* as mentioned above. *S. mutans* produces 3 types of exoenzyme glucosyltranferases (Gtfs): GtfB (insoluble glucans), GtfC (soluble and insoluble

glucans) and GtfD (soluble glucans), to turn sucrose into glucans and fructose. The secreted Gtfs can bind to the surfaces of other microbes and change them to glucan producers. Glucans, particularly insoluble glucans, are the main components of biofilm matrix which bind to tooth surfaces and to bacterial cell surface proteins (glucan-binding proteins; Gbps) (8, 10, 28, 37, 38). *S. mutans* produces at least 4 types of Gbps including GbpA, GbpB, GbpC and GbpD. Among these, GbpB is the most interesting, since it can induce protective immune responses to dental caries in animal models and has highest glucan binding property, which indicated that it may be one of cariogenic virulence factors (30, 39). Previous studies, including data from Human Oral Microbiome Database, revealed that *S. mutans* is the main among only a few oral species that can synthesize insoluble glucans (8, 28). The EPS matrix prevents saliva to neutralize acid inside the biofilm leading to accumulation of acids. The acidic environment enhances the growth of other aciduric and acidogenic pathogens (28). Thus, with this unique ability, *S. mutans* may be considered as a cariogenic biofilm initiator.

2.3 *S. mutans* and *C. albicans* dual-species biofilms and their influences on cariogenic virulence

Several clinical studies revealed that *C. albicans* can be frequently co-isolated with *S. mutans* from dental plaque in children with ECC (12, 18). Moreover, the presence of *C. albicans* together with *S. mutans* cause more severity of carious lesion than *S.*

mutans single species infection (17, 23). The mechanisms underlying this intriguing increase in cariogenicity of dual species biofilm are not yet clearly understood.

As mentioned above, the virulence factors of cariogenic pathogens consist of aciduricity, acidogenicity and biofilm formation (9). Previous evidences showed that the presence of sucrose mediates the interaction between *S. mutans* and *C. albicans* via glucosyltransferase B (GtfB) (40, 41). *S. mutans* produces GtfB, which binds strongly to *C. albicans* cell wall mannnans (15) and hyphae (41). At the same time, *C. albicans* induces *S. mutans gtfB* gene expression (17). When sucrose is available, large amounts of glucans are produced from *S. mutans*-secreted GtfB and also *C. albicans* surface-bound GtfB. This phenomenon promotes the adhesion between microbes to microbes and microbes to tooth surface leading to the increase in the mass of dual-species biofilm (15). Furthermore, not only *S. mutans,* but also *C. albicans,* are aciduric and acidogenic microorganisms. Thus, both microbes could contribute to the cariogenic ability of dual species biofilm (42).

Moreover, a recent transcriptome study showed that the presence of *C. albicans* up-regulated expression of *S. mutans* genes related to sugar utilization (Fig. 1) such as *scrA* (Enzyme II) which related to sugar transportation via PEP-PTS system, *ldh* (lactate dehydrogenase), *pdh* (pyruvate dehydrogenase) and *pfl* (pyruvate formate lyase) which related to acid production (33). The study also showed that the expression of *ciaRH*

genes which regulate acid tolerance, sucrose-dependent adhesion, and biofilm formation are increased (33).

2.4 *Candida* cell wall mannan

Cell wall is the dynamic structure which involves in cell division and cell expansion. Moreover, as the outermost structure, it protects the microbes from harmful environment and interacts with other microbes and host cells (43). The cell wall of *Candida* species is mainly composed of chitin and β1,3-linked glucan in the inner layer, and β1,6-glucan, mannan and proteins in the outer layer (Fig. 2, (15, 44)). Mannan locates at the outermost layer of cell wall which mediate adhesion activity (45). *Candida albicans* cell wall mannan composes of *O*-mannan and *N*-mannan linked to proteins as shown in Fig. 2 (46). *O*-mannan is unbranched mannan that consists of a few mannose units joined together through α-1,2 linkages and linked to proteins via the hydroxyl group of serine and threonine (43). *N*-mannan is branched mannose polymers that are joined through α-1,6 linkages and linked to asparagine through an N-acetyl-D-glucosamine (43). The characteristics and arrangement composition of mannoproteins are different among various *Candida* species (24, 47, 48).

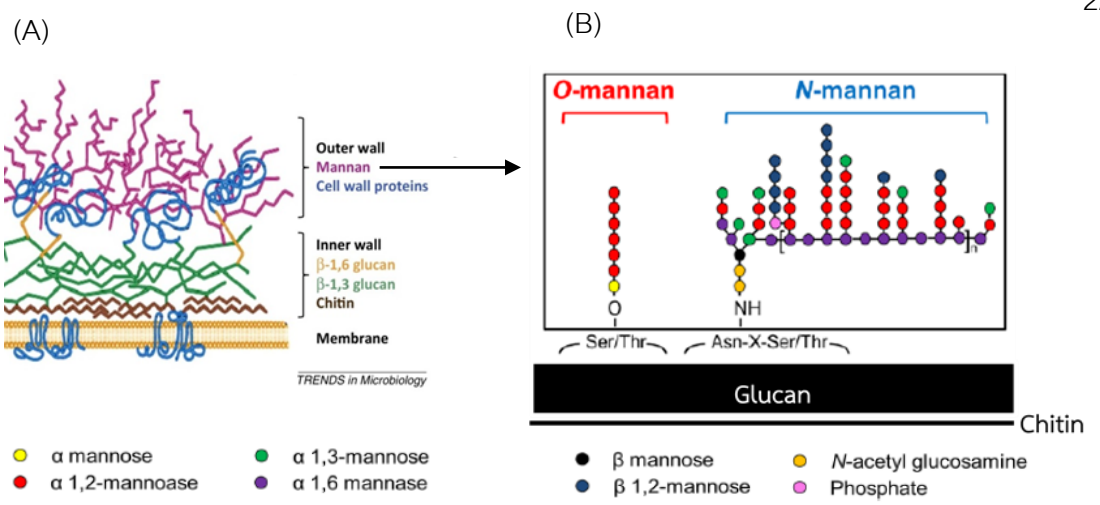


Figure 2. *Candida albicans* cell wall.

Candida albicans cell wall (A, reproduced from (44), CC-BY 3.0) and outer wall (B, reproduced from (49), CC-BY 3.0).

Previous study showed that *C. albicans* mutant strains lacking both *O*-mannan and *N*-mannan lost binding to GtfB of *S. mutans*. These observations point out that the mannan is important for interaction between these two microbes, which may affect the cariogenic potential of dual-species biofilm (15).

2.5 Non-*albicans Candida* species (NACs) in oral infection and their cell wall mannan structure

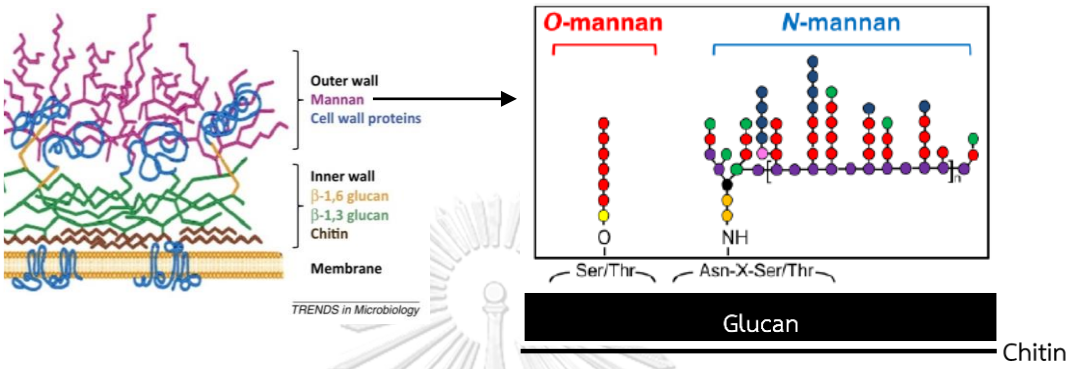
Candida species are commensal oral microbiota, but can also be the most common fungal pathogens which can cause oral candidiasis and dental caries (50). Although *C. albicans* is the most prevalent species, there have been increasing reports of clinical isolations of NACs from children, elderly and immunocompromised patients, who generally have higher caries risk (20, 22, 51, 52). Several ECC studies revealed the

presence of NACs including *C. tropicalis, C. krusei, C. parapsilosis, C. glabrata* and *C. dubliniensis* in children with dental caries (12, 22, 23). de Carvalho and colleagues reported a high frequency of *C. albicans,* followed by *C. tropicalis* and *C. krusei,* in dental plaque of children with dental caries (12). In another study, Raja and colleagues showed that the most prevalent NACs in ECC were *C. tropicalis* and *C. parapsilosis* (53). Xiao and colleagues showed that *C. albicans* is the most prevalent, followed by *C. krusei* and *C. glabrata,* in ECC. (23). In addition, although most studies focused on *C. albicans, C. dubliniensis* have phenotypic similarities to *C. albicans* (54) and may also be involved in dental caries. Al-Ahmad and colleagues reported that, while *C. albicans* was detected in both caries-free and caries-active dental plaque, *C. dubliniensis* was not detected in caries-free group but occurred in approximately 25% of caries active subjects (22).

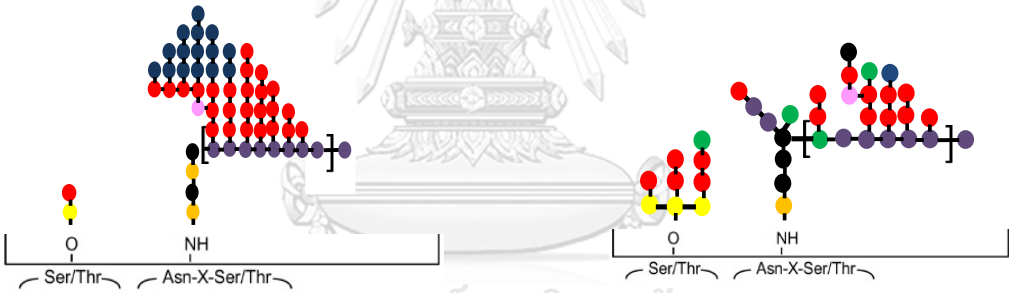
Interestingly, these NACs possess different mannan structures and composition from *C. albicans* (47, 48, 55). *C. albicans* mannan consists of short-chain *O*-linked mannan oligosaccharides and branching *N*-linked mannan polysaccharide moieties (15) as shown in **Figure 3**. *C. tropicalis* cell wall also possesses *O*- and *N*- mannan structures similarly to *C. albicans,* but *N*-mannans of *C. tropicalis* are more abundant of phosphomannan and β 1,2-mannose residues than those of *C. albicans* (Fig. 3B) (47). *C. glabrata* cell wall was shown to have diversity of mannan composition in *O*- and *N*-

mannan structures (Fig. 3C) (48), whereas cell wall mannan of *C. parapsilosis* contains short-chain branching in *N*- mannan structure (Fig. 3D) (55).

(A) *Candida albicans*



(B) *C. tropicalis* IFO1647 (C) *C. glabrata* NRBC0622



(D) *C. parapsilosis* IFO1396

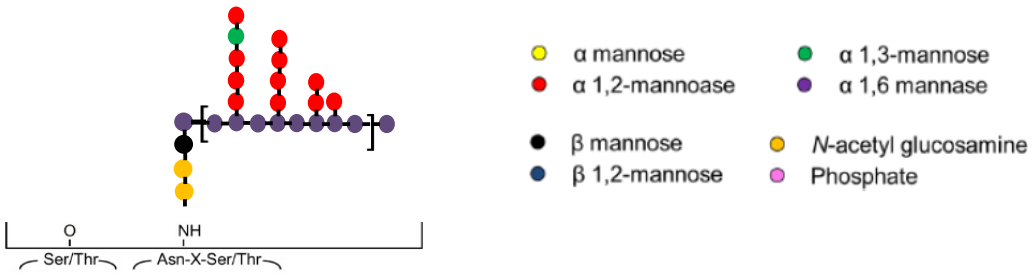


Figure 3 Structure of the cell wall mannan of *Candida albicans* and NACs.

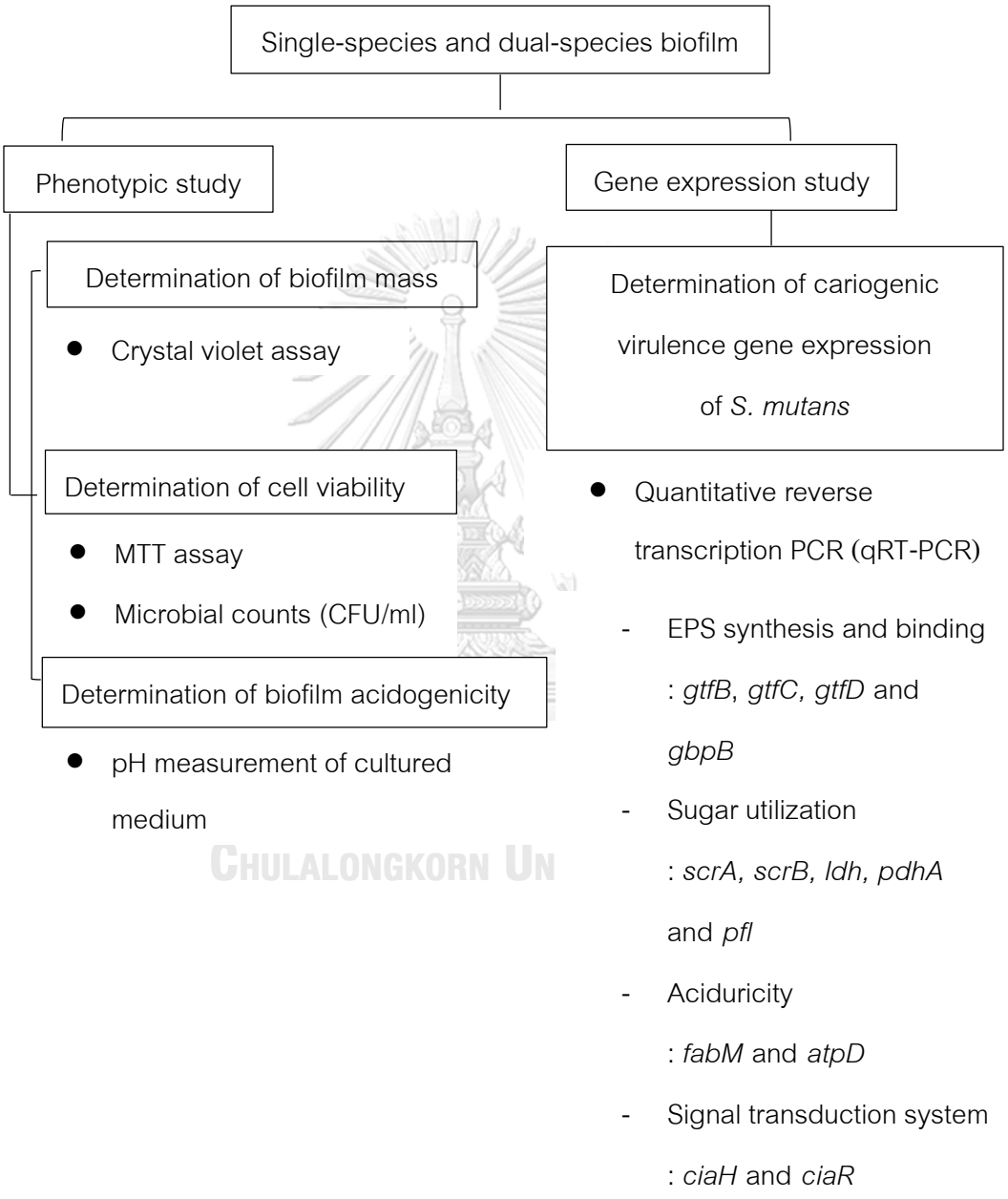
Structure of Candida spp. cell wall mannan. Candida albicans (A ,reproduced from (49), CC-BY 3.0), Candida tropicalis (B), Candida glabrata (C) and Candida parapsilosis (D) (15, 47, 48, 55).

Candida cell wall mannan has been reported to play an important role in the interaction between *C. albicans* and *S. mutans* in dual-species biofilms. Thus, the differences in the structure and composition of cell wall mannan among distinct *Candida* species may affect the binding of GTFs and biofilm formation. This, and other properties of these *Candida* species, may influence cariogenic virulence factors of *S. mutans* and of dual-species biofilms. Thus, this study aims to determine the effects of NACs on biofilm formation and acidogenicity in dual-species biofilms, and cariogenic virulence gene expression of *S. mutans*.

CHAPTER III

MATERIALS AND METHODS

Methodology scheme



3.1 Microorganism strains and growth conditions

Streptococcus mutans UA159, *Candida albicans* SC5314, *Candida dubliniensis* NCPF3949, *Candida glabrata* ATCC2001, *Candida tropicalis* ATCC750, *Candida parapsilosis* ATCC90018 and *Candida krusei* (*Issatschenki orientialis*) ATCC6258 were used in this study.

An inoculum from -80°C bacterial glycerol stocks was streaked on Brain Heart Infusion (BHI) agar and was incubated for 36 hours at 37°C with 5% CO_2. A single colony was inoculated into 7 ml of BHI broth and was incubated for 18 hours at 37°C with 5% CO_2. The optical density of overnight culture was measured at the wavelength 600 nm. (OD_{600}) and adjusted to OD_{600} of 0.1. The culture was incubated until it reached log phase (OD_{600} = 0.4 - 0.6; approximately 4 hours).

An inoculum from -80°C fungal glycerol stocks was streaked onto Yeast Extract-Peptone-Dextrose (YPD) agar and was incubated for 36 hours at 30°C. A single colony was inoculated into 7 ml of BHI broth and was incubated for 18 hours at 30°C. The optical density of overnight culture was measured at the wavelength 600 nm. (OD_{600}) and adjusted to OD_{600} of 0.1. The culture was incubated until it reached log phase (OD_{600} = 0.4 - 0.6; approximately 4 hours).

3.2 Preparation of biofilm

For single-species biofilm formation, log phase culture of *S. mutans* UA159 was adjusted to OD_{600} of 0.4 (approximately 10^8 CFU/ml). *Candida* spp. cultures were also

adjusted to OD_{600} of 0.1 (approximately 10^6 CFU/ml). One ml of each adjusted culture was centrifuged at 12,000 g, 25°C for 15 minutes. The cell pellets were re-suspended in 500 µl of BHI broth supplemented with 5% sucrose and transferred to separate wells in a 24-well plate. The plate was incubated for 36 hours in 37°C with 5% CO2 to form biofilm.

To prepare dual-species biofilms, the protocol was similar to the single-species biofilm but the microbes were mixed with a ratio 10^8 CFU/ml of *S. mutans* to 10^6 CFU/ml of *Candida* spp. to simulate the proportion found in the saliva samples of children with ECC (15), and resuspended in BHI broth supplemented with 5% sucrose before transferring to a well in a 24-well plate.

3.3 Determination of biofilm mass using crystal violet assay

After biofilm formation, the plate was washed three times with 1 ml of sterile 1xPhosphate Buffered Saline (1xPBS, pH 7.4). The biofilm of each well was fixed with 500 µl of 95% ethyl alcohol for 20 minutes at room temperature and then stained with 500 µl of 0.1% (w/v) crystal violet (CV) for 15 minutes. After that, the stained-biofilm was washed three times with distilled water and the plate was dry. The biofilm then was de-stained with 1 ml of 33% acetic acid. The optical density of the de-stained solution was measured at 520 nm (56).The sterile BHI broth supplemented with 5% sucrose was used as a blank.The experiments were performed three times independently. Biofilm mass was calculated using the following formula:

$$\text{Ratio of biofilm mass} = \frac{OD_{520} \text{ of dual species biofilm}}{OD_{520} \text{ of } \textit{S. mutans} \text{ biofilm} + OD_{520} \text{ of } \textit{Candida} \text{ spp. biofilm}}$$

3.4 Determination of cell viability

3.4.1 MTT assay

After biofilm formation, the plates were washed three times with 1 ml of sterile 1xPhosphate Buffered Saline (1xPBS, pH7.4). Next, 500 µl of MTT ((3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) solution was added into each well and then incubated for 3 hours. After incubation, 500 µl of 100% dimethyl sulfoxide (DMSO) was added to dissolve the formazan. The solution was transferred to a new plate and the OD_{570} and OD_{690} (background) were measured (57). The sterile BHI broth supplemented with 5% sucrose was used as a blank. The experiments were performed three times independently. The cell viability was calculated using the following formula:

$$\text{Ratio of cell viability} = \frac{OD_{570,690} \text{ of dual species biofilm}}{OD_{570,690} \text{ of } \textit{S. mutans} \text{ biofilm} + OD_{570,690} \text{ of } \textit{Candida} \text{ spp. biofilm}}$$

3.4.2 Microbial counts

After biofilm formation, the plates were washed three times with 1 ml of sterile 1xPhosphate Buffered Saline (1xPBS, pH 7.4) to remove non-adherent cells. Biofilms were scraped and re-suspended into 1 ml of PBS. The cell clumps were disrupted by an ultrasonic homogenizer with amplitude of 25% for 30 seconds twice (16). The homogenized suspension was serially diluted and plated on 1) Mitis-Salivarius agar (MS

agar: selective media for streptococci) and 2) Sabouraud dextrose agar (SDA) for *Candida* spp. MS agar plates were incubated at 37°C with 5% CO_2 whereas SDA plates were incubated at 30°C (16). After 36 hours of incubation, the number of colony-forming unit (CFU) was determined. The experiments were performed three times independently. The number of microbes were presented as Log_{10} CFU/ml.

3.5 Determination of biofilm acidogenicity

There is no difference between pH value of within biofilm and those of culture medium (17); therefore, biofilm acidogenicity was determined by measuring the pH of the culture medium.

After 36 hours of biofilm formation, the pH value of culture medium was measured by a pH meter (LAQUAtwin, HORIBA, and Singapore). The experiments were performed three times independently.

To investigate dynamic change of biofilm acidogenicity, the pH value of culture medium was measured at the beginning (0 min), 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, 8 h, 12 h, 24 h and 36 h. The experiments were performed three times independently.

3.6 Determination of virulence gene expression of *S. mutans*

3.6.1 RNA extraction and purification

The protocol described by Cury JA and Koo H was employed with minor modifications (58). After 36 hours of biofilm formation, the biofilms were scraped from 24-well plates and kept in RNAlater at 4°C until use. The biofilms were suspended in

cold PBS, sonicated by an ultrasonic homogenizer (Sonics, USA) with amplitude of 25% for 30 seconds and then centrifuged at 5,500 g, 4°C, for 10 minutes. This sonication-washing step was repeated 2 times in order to break cell clumps. After the final washing step, the cells were re-suspended in 0.25 ml of NAES buffer (50mM sodium acetate buffer, 10 mM EDTA and 1% SDS, pH 5.0) and transferred to new 2.0 ml microcentrifuge tubes. Glass beads (0.1 mm diameter) were added into the tubes until the final volume reached 0.5 ml, and an equal volume of acid phenol/chloroform (5:1, pH 4.5) was added and mixed by vortexing with maximum speed at 40 seconds in order to break the cells. This mixing step was repeated 2 times and then the cell extract was centrifuged at 14,000 g, 25°C for 15 minutes. To isolate the nucleic acid, the aqueous layer was collected and mixed with equal volume of acid phenol/chloroform (5:1, pH 4.5) for 10 seconds and then centrifuged at 14,000 g, 4°C for 15 minutes. The aqueous layer was then mixed with equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 14,000 g, 4°C for 5 minutes. The aqueous phase was transferred to a new 1.5 ml microcentrifuge tube. Nucleic acid (RNA/DNA) was precipitated using a 1/10 volume of 3M sodium acetate (pH 5.0) and 1 volume of cold isopropanol and stored at -20°C for at least 30 minutes. The precipitates were collected by centrifugation at 14,000 g, 4°C for 15 minutes and washed with 0.5 ml of cold 75% (v/v) ethanol three times followed by 0.5 ml of 99% cold ethanol. The pellets were dried at 65°C for 3 minutes and was dissolved in 20 µl of sterile distilled-DEPC water and stored in -80°C until use.

The samples were treated with DNase I (Thermo Fisher Scientific, USA) for 30 minutes at 37°C to remove DNA, and purified using ethanol precipitation to remove the DNase. The RNA concentration was quantified using the NanoDrop™ 2000/2000c spectrophotometers (Thermo Fisher Scientific, USA).

3.6.2 Quantitative reverse transcription PCR (qRT-PCR)

The bacterial mRNA was converted to cDNA by using Random Hexamer primer (Macrogen, Korea) and ImProm-II™ Reverse Transcriptase (Promega, USA) according to manufacturer's instruction. Subsequently, quantitative real-time PCR (qPCR) with Luna Universal qPCR master mix (New England Biolabs, USA) was performed using primers shown in **Table 1**. The sequences of *pfl* primers were designed in this study using Primer3Plus software (online access: https://primer3plus.com).

Table 1 List of primer sequences of cariogenic virulence genes of *Streptococcus mutans*.

Genes	Primer sequences	Descriptions	Ref.
EPS synthesis and glucan binding			
gtfB	F: 5'- CACTATCGGCGGTTACGAAT-3' R: 5'- CAATTTGGAGCAAGTCAGCA-3'	Glucosyltransferase GTF-I	(59)
gtfC	F: 5'- GATGCTGCAAACTTCGAACA -3' R: 5'- TATTGACGCTGCGTTTCTTG -3'	Glucosyltransferase GTF-SI	(59)
gtfD	F: 5'- TTGACGGTGTTCGTGTTGAT -3' R: 5'- AAAGCGATAGGCGCAGTTTA -3'	Glucosyltransferase-S	(59)
gbpB	F: 5'- CGTGTTTCGGCTATTCGTGAAG -3' R: 5'- TGCTGCTTGATTTTCTTGTTGC -3'	Glucan-binding protein B	(60)
Sugar utilization			

scrA	F: 5'- GATTGCCCTCAGCAGTTGACAT -3' R: 5'- GCTGGGAAACTTTGATGGAGAC -3'	PTS system sucrose-specific transporter subunit IIABC	(61)
scrB	F: 5'- ACAGCCTGTCCTGATTTATAGTC -3' R: 5'- CTGGTAACCCAATCCATGAGAC -3'	Sucrose-6-phosphate hydrolase	(61)
ldh	F: 5'- AAAAACCAGGCGAAACTCGC -3' R: 5'- CTGAACGCGCATCAACATCA -3'	Lactate dehydrogenase	(59)
pdhA	F: 5'- ATGCCAAACTATAAAGATTTAC -3' R: 5'- TCTTGGGCTTCAATATCT -3'	Pyruvate dehydrogenase, TPP-dependent E1 component alpha-subunit	(33)
pfl	F: 5'- ACGACCTTGGATACCTGTGC -3' R: 5'- AAGACATGGCGAATCCAAAC-3'	Pyruvate formate-lyase	In this study
Aciduricity			
atpD	F: 5'- TGTTGATGGTCTGGGTGAAA -3' R: 5'- TTTGACGGTCTCCGATAACC -3'	F_1F_0-ATP synthase subunit beta	(35)
fabM	F: 5'- ACTGATTAATGCCAATGGGAAAGTC -3' R: 5'- TGCGAACAAGAGATTGTACATCATC -3'	Enoyl-CoA hydratase	(35)
Signal transduction system			
ciaH	F: 5'- CGTCATCAATAATGTCAATGCCTTC -3' R: 5'- TACCTTAACTGTCACTGTCCGATAC -3'	Histidine kinase sensor CiaH	(62)
ciaR	F: 5'- GAAGCAGAGTGGCGTTTATG -3' R: 5'- TGTCATCCAAACCTTCCTTAGC -3'	Response regulator CiaR	(33)
Housekeeping genes			
16sRNA	F: 5'- AGCGTTGTCCGGATTTATTG -3' R: 5'- CTACGCATTTCACCGCTACA -3'	16S Ribosomal RNA	(59)
rpoB	F: 5'- GCAGTCAAGGGGTGGAAATCG -3' R: 5'- TGGACGGCTTGTTGCAGGAATAC -3'	DNA-dependent RNA polymerase beta-subunit	(63)

A reaction mixture was prepared by mixing 5 µl of qPCR master mix, 0.25 µl of each (10 µM) primer, 3 µl of cDNA template and sterile MilliQ water up to 10 µl. The

qRT-PCR was performed using the following cycling parameters: 1 cycle of initial denaturing at 95°C for 10 minutes and amplified for 40 cycles with denaturing at 95°C for 15 seconds, followed by annealing and extension at 60°C for 30 seconds. Melting curve analysis was subsequently performed from 60-95°C. *16sRNA* and *rpoB* genes were used as housekeeping genes. Levels of gene expression were calculated using the double delta CT method. The experiments were performed three times independently.

3.7 Statistical analysis

All data were analyzed using IBM SPSS Statistics version 22 (SPSS; Chicago, IL, USA). The differences of biofilm mass (crystal violet assay) and cell viability (MTT assay and microbial counts) between single- and the respective dual-species biofilms were evaluated with Mann-Whitney U test. Moreover, the differences of *S. mutans* gene expression in single- and dual-species biofilms co-cultured with each *Candida* spp. (two-variables) were examined using Mann-Whitney U test. The differences of biofilm mass, cell viability and *S. mutans* gene expression among dual-species biofilm of all NACs comparing to dual-species biofilms of *C. albicans* were analyzed using Kruskal-Wallis test followed by Mann-Whitney U test with Bonferroni correction. The differences of pH at 36 hours among all dual-species biofilms and *S. mutans* single-species biofilm

were also evaluated with Kruskal-Wallis test followed by Mann-Whitney U test with Bonferroni correction. Statistical significance level was set at α = 0.05.

CHAPTER IV

RESULTS

4.1 Biofilm mass of single- and dual-species biofilm

We investigated the effects of non-*albicans Candida* species (NACs) on biofilm mass, cell viability, and biofilm acidogenicity in co-culture with *Streptococcus mutans* as dual-species biofilm. First, we determined the biofilm mass of *S. mutans* and each *Candida* spp. in single-species and dual-species biofilm. In this experiment, single-species and dual-species biofilms were placed in 24-well plates containing BHI broth supplement with 5% sucrose. The plates were incubated for 36 h at 37˚C with 5% CO_2. Total biofilm mass was measured using crystal violet assay, which stains both live and dead cells as well as exopolysaccharides in the biofilm matrix.

The ratio of biofilm mass of dual-species biofilm to the sum of each single-species biofilm is shown in **Figure 4**. When comparing between the 2 species in single-species culture, *S. mutans* (dark gray bars) can form biofilm better than *Candida* spp. (light gray bars). The ratio of *S. mutans* and *C. albicans* dual species biofilm mass tended to be greater than the ratio of the sum of the two single-species biofilm (P = 0.05). Similar results were observed in the dual species of *S. mutans* and each NACs. When comparing dual species biofilm mass among different *Candida* spp., the dual species biofilm of *S. mutans* and *C. tropicalis* was likely to be the highest relative biofilm mass (P = 0.05)

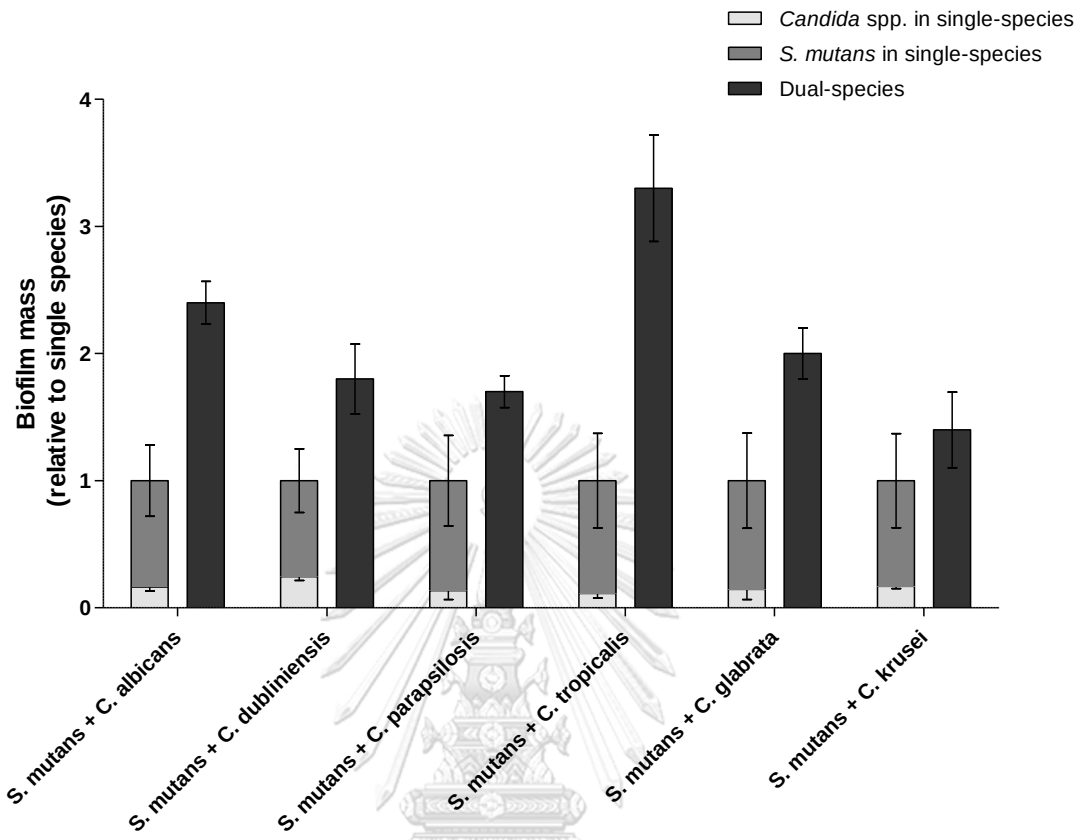


Figure 4 Determination of biofilm mass of single-species and dual-species biofilm via crystal violet assay.

The bar chart showed the average ratios of relative biofilm mass of the sum of single-species biofilm (light gray bars and dark gray bars), and dual-species biofilms (black bars), to the sum of biofilm mass of respective single-species biofilm. Error bars represent standard deviations from three biological replicates.

4.2 Cell viability of *S. mutans* and *Candida* spp. in single- and dual- species biofilm

To determine cell viability of *S. mutans* and *Candida* spp. in single-species and dual-species biofilm, MTT assay and microbial counts methods (CFU/ml) were performed.

MTT assay determines cell viability based on enzymatic activity in the cells. The ratios of cell viability of dual-species biofilm were calculated relative to the sum of respective single species biofilm (**Figure 5**). Similar levels of viability to single-species biofilm were observed in dual-species biofilm of *S. mutans* and all *Candida* spp., except *S. mutans* and *C. tropicalis* dual-species biofilm, which had a tendency to be higher than the sum of single species biofilm (P = 0.05).

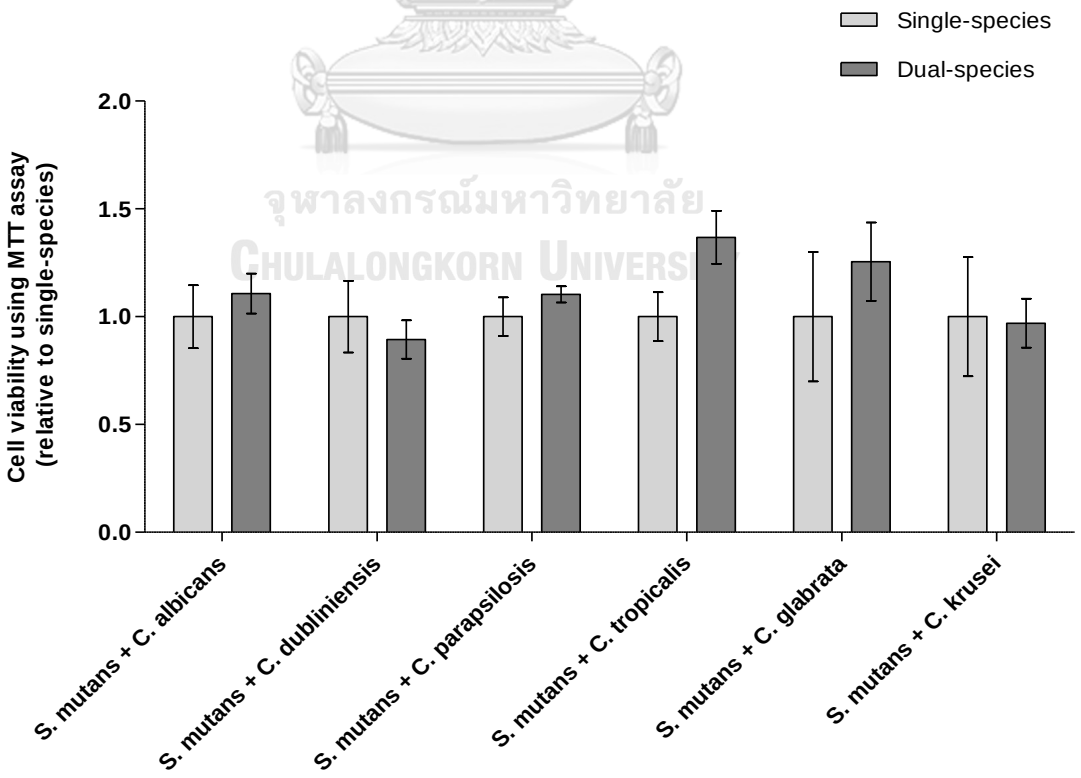


Figure 5 Determination of cell viability using MTT assay in single-species and dual-species biofilms.

The bar chart shows the average ratio of cell viability of the sum of single-species biofilm (*light gray bars*) *and dual-species biofilms, relative to the sum of single-species biofilm* (*dark gray bars*). *Error bars represent standard deviations from three biological replicates.*

To assess cell viability of each species in the dual species biofilm, the microbial count method was performed. The results showed that when *S. mutans* were cultured with *Candida* spp., they showed higher viability than when they were grown alone (Fig. 6A). On the other hand, the viability of *C. albicans, C. dubliniensis* and *C. glabrata* when co-cultured with *S. mutans* showed similar viability as when they were grown alone whereas *C. parapsilosis, C. tropicalis* and *C. krusei* in dual-species biofilm showed an increase in CFU (Fig. 6B).

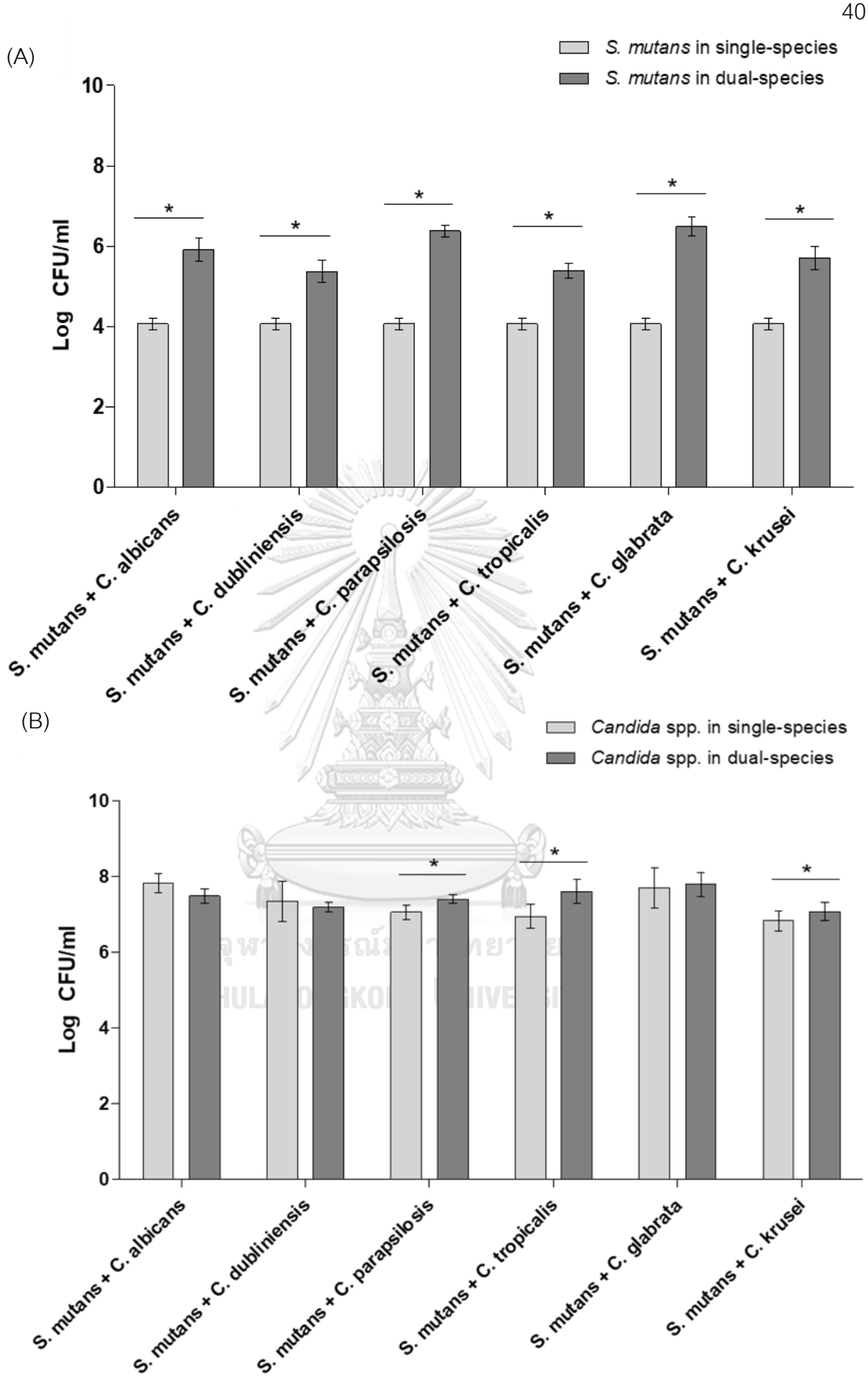


Figure 6 Determination of cell viability using microbial count method *(CFU/ml)*.

Bar graphs showed the number of S. mutans (Log CFU/ml) (Fig. 6A) and Candida spp. (Log CFU/ml) (Fig. 6B) in single-species biofilm (light gray bars) and dual-species biofilm (dark gray bars). Data are presented as means and standard deviation from three biological replicates. Asterisk (*) represents statistically significant difference (P < 0.05) by Mann-Whitney U test.

4.3 pH of single-species and dual-species biofilm

After 36 hours of biofilm formation, pH values of *S. mutans* single species biofilm and dual species biofilm of all tested strains were approximately 4, which was lower than the critical pH of enamel (pH=5.5, **Figure 7**). However, the average pH of all *Candida* single species biofilm was higher than pH 5.5.

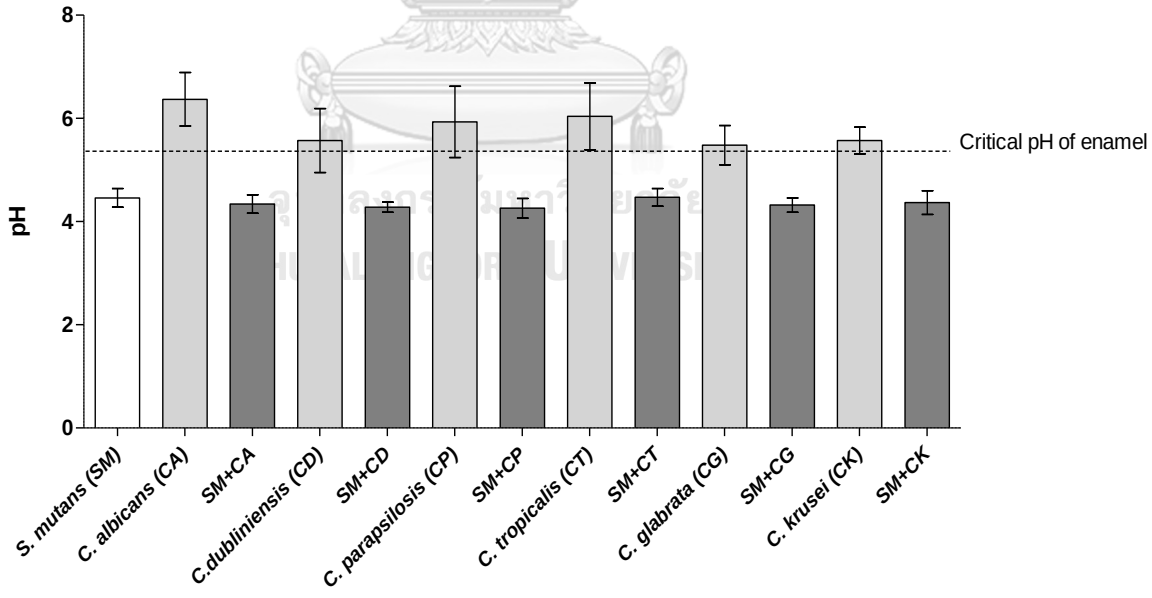


Figure 7. Determination of pH after 36 h of biofilm formation.

Data were presented as mean and standard deviation of single-species biofilm (S. mutans, white bar; Candida spp., light gray bars) and dual-species biofilms (dark gray bars). Three biological replicates were performed.

Acid is a byproduct of sugar fermentation, which occurs throughout the process of biofilm formation. To monitor the change of pH during the formation of biofilm, the kinetics of pH production was performed. The results showed that the pattern of pH change in dual-species biofilms (black color symbols and lines) was similar to that of *S. mutans* in single-species biofilm (red dots and red line), but was different from *Candida* spp. in single-species biofilm at all stages of development as shown in **Figure 8**.

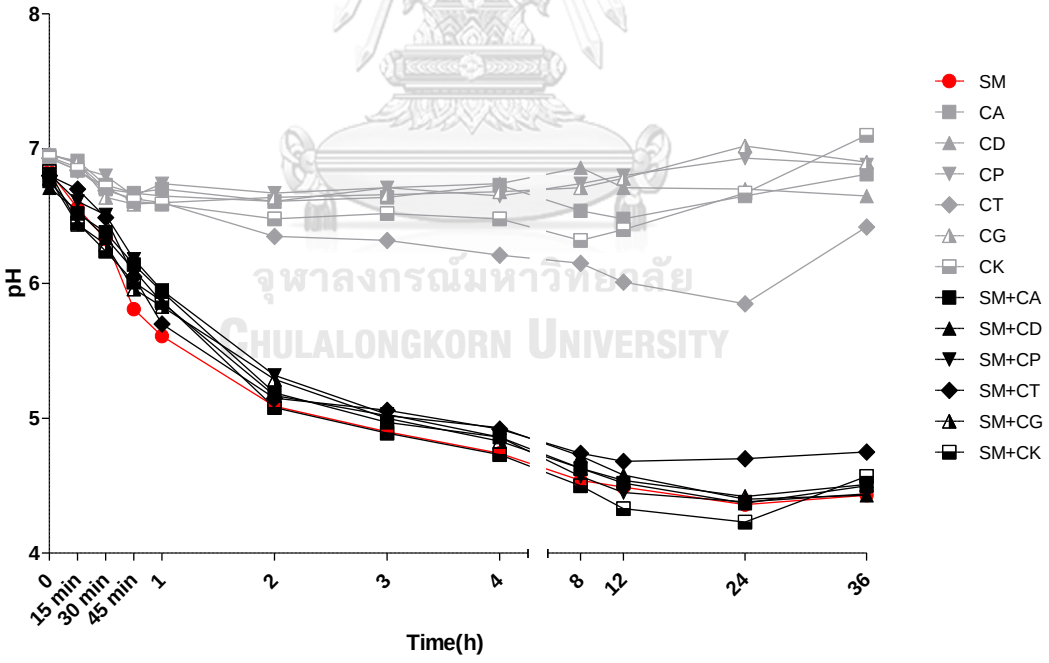


Figure 8. Determination of the dynamics of pH change over time during biofilm formation.

Data are presented as means from three biological replicates. ***SM:*** *S. mutans;* ***CA:*** *C. albicans;* ***CD:*** *C. dubliniensis;* ***CP:*** *C. parapsilosis;* ***CT:*** *C. tropicalis;* ***CG:*** *C. glabrata;* ***CK:*** *C. krusei.*

4.4 Effects of *Candida* spp. on the expression cariogenic virulence genes of *S. mutans* in dual-species biofilm

To investigate the effects of non-*albicans Candida* species (NACs) on cariogenic virulence gene expression of *S. mutans* in dual-species biofilm, we measured the levels of gene expression using quantitative reverse transcription PCR (qRT-PCR). We examined the levels of expression of genes related to exopolysaccharides (EPS) synthesis (*gtfB, gtfC* and *gtfD*), glucan binding (*gbpB*), sugar utilization (*scrA, scrB, ldh, pfl* and *pdhA*), acid stress tolerance (*atpD* and *fabM*) and signal transduction system (*ciaR* and *ciaH*). These genes were selected based on previous studies that showed *S. mutans* virulence gene upregulations when co-cultured with *C. albicans* (17, 33, 35, 60).

The expression levels of genes related to exopolysaccharides (EPS) synthesis genes (*gtfB*, *gtfC* and *gtfD*) are shown in **Figure 9.** The expression of *gtfB* were up-regulated in dual species biofilm especially when co-culture with *C. albicans* (3.7 folds), *C. tropicalis* (6.1 folds) and *C. krusei* (6.5 folds). Similar results were observed in *gtfD* whereas the expression of *gtfC* of *S. mutans* with *C. albicans* and *C. parapsilosis* were

similar to the expression of *S. mutans* single-species biofilm (Fig.9). In addition, the expression levels of *gbpB* in dual-species biofilms were lower than or similar to that of single species biofilm (Fig. 9).

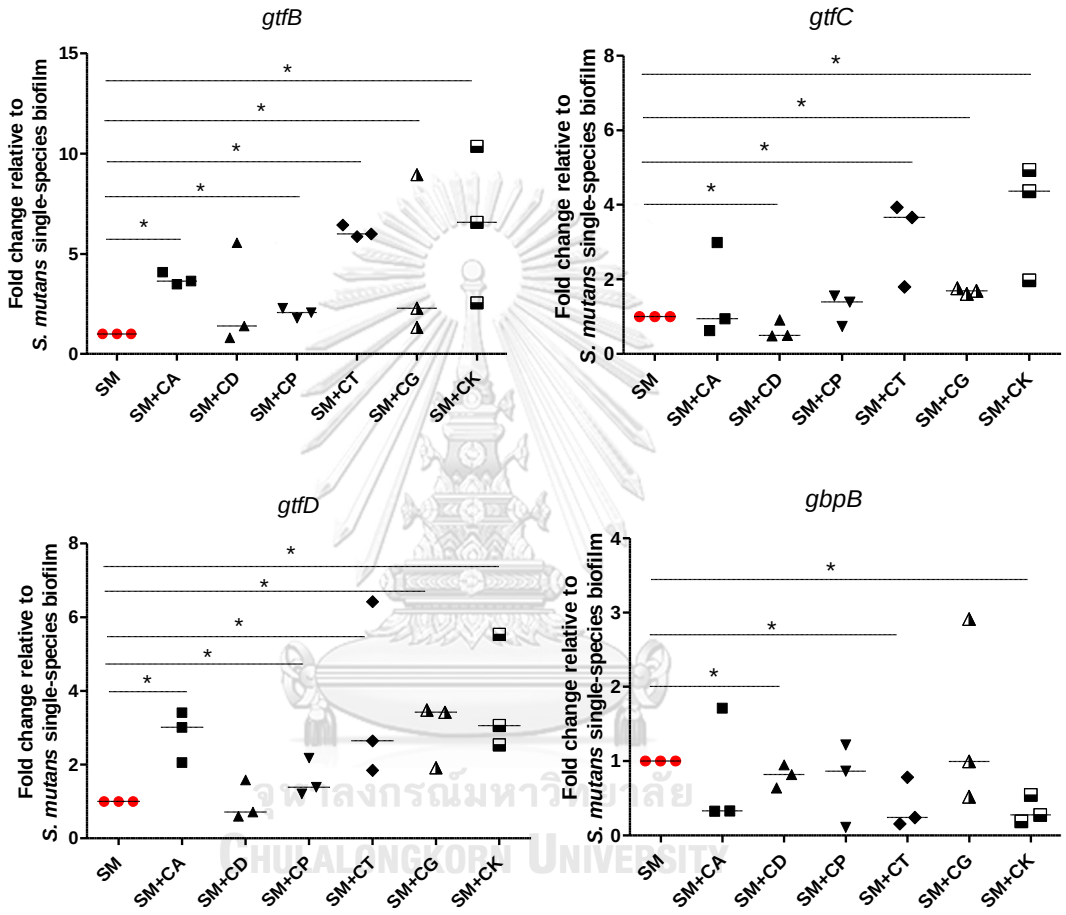


Figure 9. The relative levels of expression of genes involved in EPS synthesis and glucan binding.

The relative levels of expression of genes involved in EPS synthesis and glucan binding were calculated using the double delta CT method and normalized to S. mutans single-species biofilm (represented by the red dots). The data points

showed data from three independent experiments and the horizontal line represented the median. Asterisk (*) represented statistically significant difference ($P < 0.05$) by Mann-Whitney U test. ***SM:*** *S. mutans;* ***CA:*** *C. albicans;* ***CD:*** *C. dubliniensis;* ***CP:*** *C. parapsilosis;* ***CT:*** *C. tropicalis;* ***CG:*** *C. glabrata;* ***CK:*** *C. krusei.*

The expression levels of genes related to sugar utilization (Fig. 1) were shown in **Figure 10.** The expression of *S. mutans scrA* and *scrB* was upregulated when co-cultured with *C. albicans, C. tropicalis, C. glabrata* and *C. krusei,* but not with *C. dubliniensis* and *C. parapsilosis* (Fig. 10). The levels of *ldh* expression of *S. mutans* in dual species biofilm showed similar to or lower than that of *S. mutans* single species biofilm. The expression levels of *pdhA* of *S. mutans* in dual species biofilm were higher than in single-species biofilm but not significantly different (Fig. 10). For *pfl* gene, the expression levels were increased in dual-species biofilms of *S. mutans* with *C. tropicalis,* but were downregulated with *C. dubliniensis* (Fig 10).

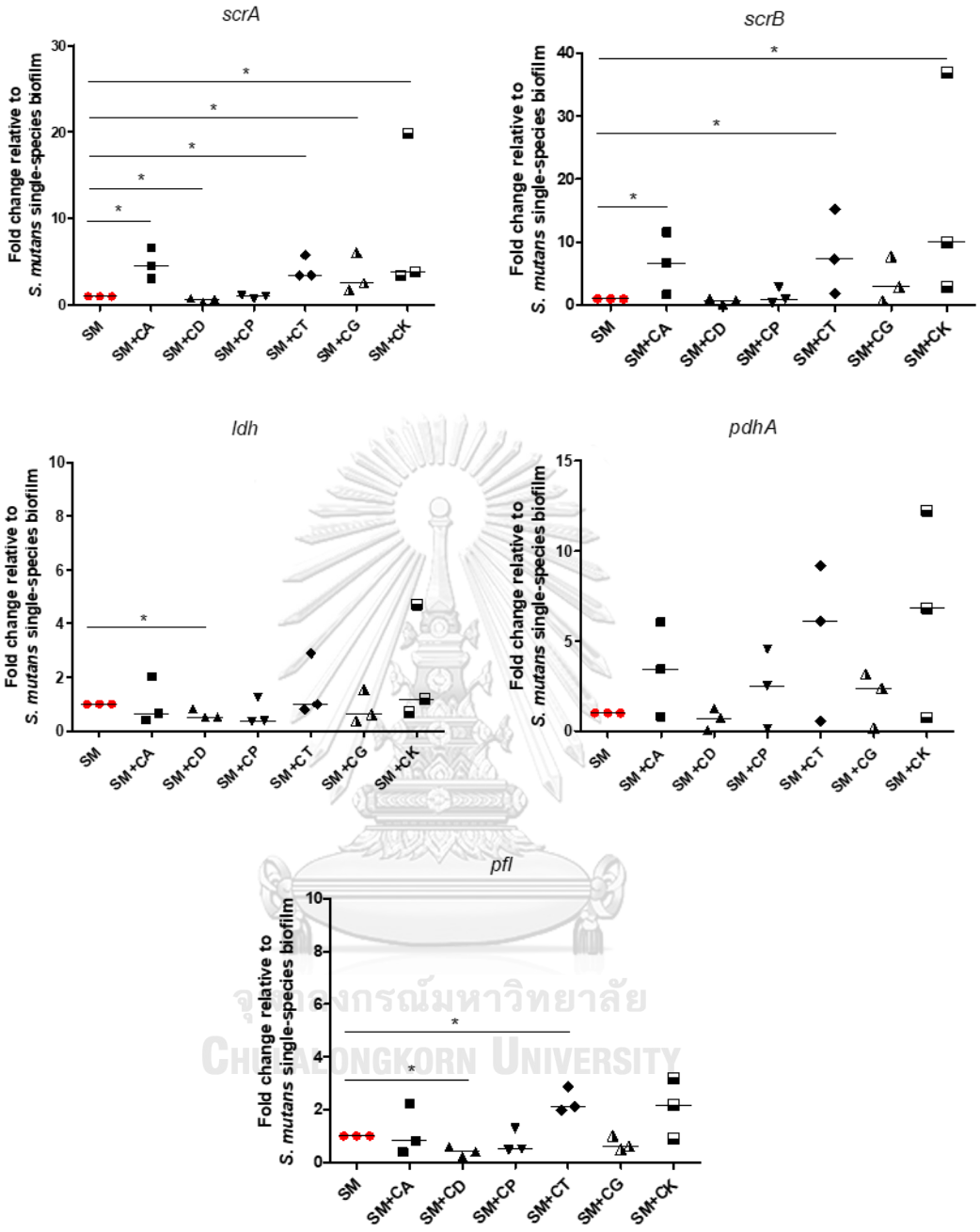


Figure 10. The relative levels of expression of genes involved in sugar utilization.

The relative levels of expression of genes involved in sugar utilization were calculated using the double delta CT method and normalized to S. mutans single-species biofilm (represented by the red dots). The data points showed data from three independent

experiments and the horizontal line represented the median. Asterisk (*) represented statistically significant difference (P < 0.05) by Mann-Whitney U test. **SM:** *S. mutans;* **CA:** *C. albicans;* **CD:** *C. dubliniensis;* **CP:** *C. parapsilosis;* **CT:** *C. tropicalis;* **CG:** *C. glabrata;* **CK:** *C. krusei.*

The relative expression levels of genes related to acid stress tolerance are presented in **Figure 11**. The expression level of *atpD* and *fabM* of *S. mutans* were downregulated in the presence of *C. dubliniensis*. For other NACs, the relative expression of *S. mutans atpD* and *fabM* in dual-species biofilm (except *C. dubliniensis*) were similar to *S. mutans* in single-species biofilm.

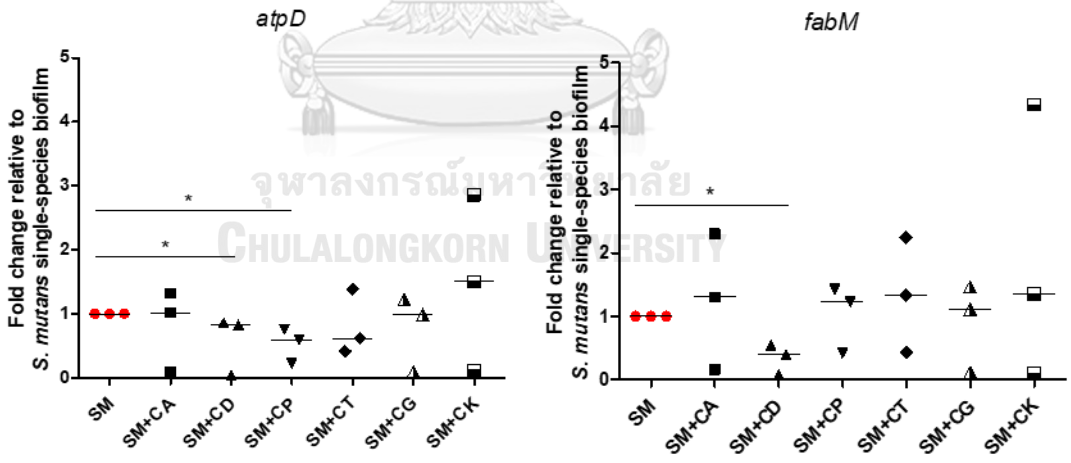


Figure 11. The relative levels of expression of genes involved in acid stress tolerance.

The relative levels of expression of genes involved in acid stress tolerance were calculated using the double delta CT method and normalized to S. mutans single-species biofilm (represented by the red dots). The data points showed data from three

independent experiments and the horizontal line represented the median. Asterisk (*) represented statistically significant difference (P < 0.05) by Mann-Whitney U test. ***SM:*** *S. mutans;* ***CA:*** *C. albicans;* ***CD:*** *C. dubliniensis;* ***CP:*** *C. parapsilosis;* ***CT:*** *C. tropicalis;* ***CG:*** *C. glabrata;* ***CK:*** *C. krusei.*

The signal transduction system is related to biofilm formation (EPS synthesis and glucan binding), sucrose-dependent adherence and acid tolerance. The expression levels of *ciaR* and *ciaH* genes of the signal transduction system are presented in **Figure 12.** The expression of *ciaR* was upregulated in the presence of *C. tropicalis* only, whereas the expression of *ciaH* were upregulated in the presence of all *Candida* spp. , except *C. dubliniensis*.

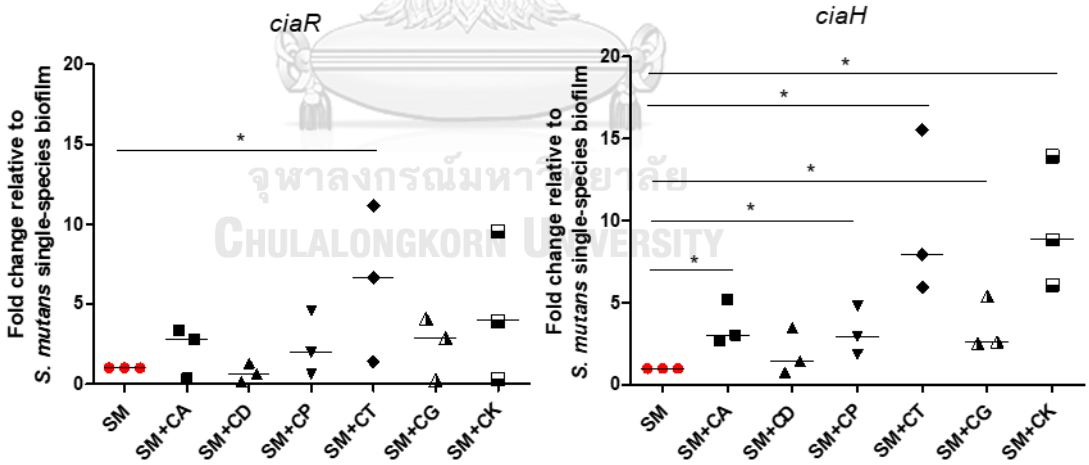


Figure 12. The relative levels of expression of genes involved in signal transduction system.

The relative levels of expression of genes involved in signal transduction system were calculated using the double delta CT method and normalized to S. mutans single species biofilm (represented by the red dots). The data points showed data from three independent experiments and the horizontal line represented the median. Asterisk (*) represented statistically significant difference (P < 0.05) by Mann-Whitney U test. ***SM:*** *S. mutans;* ***CA:*** *C. albicans;* ***CD:*** *C. dubliniensis;* ***CP:*** *C. parapsilosis;* ***CT:*** *C. tropicalis;* ***CG:*** *C. glabrata;* ***CK:*** *C. krusei.*

CHAPTER V

DISCUSSION AND CONCLUSION

This study investigated the effects of co-culturing of non-*albicans Candida* species (NACs) and *S. mutans* on virulence-related phenotypes, including biofilm mass, cell viability and biofilm acidogenicity of the dual species biofilms, and on cariogenic virulence gene expression of *S. mutans*. Our results revealed that *S. mutans* and NACs dual species biofilm exhibited increased biofilm mass compared to the combined mass of the 2 respective single species biofilm. This is similar to *S. mutans* and *C. albicans* dual species biofilm, which has been previously reported (16, 17, 64, 65) (Fig. 4). Interestingly, the highest level of biofilm mass was observed in *S. mutans* and *C. tropicalis* dual species and followed by dual species biofilm of *S. mutans* and *C. albicans* (Fig. 4). We also observed changes in virulence gene expression of *S. mutans* that may contribute to the increase in biofilm mass and cariogenicity in dual species biofilm.

Most NACs dual species biofilm showed increased biofilm mass, but *C. krusei* showed the least increase. We hypothesize that this was due to the weaker adherence to plastic surfaces (66). The thickness of dual species biofilms of *S. mutans* and *C. krusei* was visible during the biofilm formation process. However, the biofilm often became partially dislodged during the washing step before CV staining. This may result in the lower level of measured biofilm mass than the actual biofilm formed.

Based on previous studies, the increase in biofilm mass of dual species culture of *S. mutans* and *C. albicans* resulted from the interactions between glucans and *C. albicans* (16, 17, 64, 65). The presence of *C. albicans* up-regulates the *gtfBCD* expression of *S. mutans* (33, 67). The different *gtf* genes encode glucosyltransferases (Gtfs) with different characteristics. While GtfB produces insoluble glucans, which are the main component of EPS, GtfC produces both insoluble and soluble glucans and GtfD creates only soluble glucans (10). Furthermore, the Gtf exoenzymes, especially GtfB, bind to *C. albicans* cell wall mannan and turn the fungus into a glucan producer resulting in the large production of glucans, which promote the binding between microbial cells and to tooth surface (17).

Our results on the expression of genes related to exopolysaccharides (EPS) synthesis suggested that the presence of *C. albicans* enhance the expression of *gtfBD* but not *gtfC* (Fig. 9). This result was different from previous reports, which showed increased *gtfBCD* expression in dual species biofilm (33, 67). This variation of *gtf* expression may be a result of the differences in growth conditions among studies. The expression of *gtf* genes was shown to depend on growth phase (68). Moreover, the growth of microbes as biofilm appears to be heterogeneous; this may cause variation in *gtf* gene expression. Additionally, the variation of *gtf* expression may also be strain-dependent (68).

The presence of NACs, especially *C. tropicalis* and *C. krusei,* upregulated the *gtfBCD* expression (Fig. 9). The expression of both *gtfBC,* which produce insoluble glucans, may provide the large amount of glucans leading to the higher biofilm mass of *S. mutans* and *C. tropicalis* than *S. mutans* and *C. albicans*.

Moreover, a previous study using *C. albicans* with defects in mannoprotein synthesis showed that *O*- and *N*-mannan are parts of *C. albicans* cell wall where GtfB binds (15). They also showed that α-mannan, which is found in *O*- and *N*- mannan, plays an important role in the binding strength (Fig. 3) (15). *C. tropicalis* also possess *O*- and *N*- mannan, similarly to *C. albicans,* but the composition and arrangement of cell wall mannans are different (Fig. 3) (47). The *N*-mannans of *C. tropicalis* are more abundant of phosphomannan and β 1,2-mannose residues than those of *C. albicans;* this difference may affect GtfB binding (47, 69). In addition, a proteomics study revealed that the presence of *S. mutans* upregulated genes related to cell wall components of *C. albicans,* such as mannan and glucan (70). We hypothesize that similar responses may happen in *C. tropicalis,* but may be to a different degree. Further analysis is needed to prove this hypothesis. These differences may contribute to the higher biofilm mass of *S. mutans* and *C. tropicalis* dual species biofilm than those of *S. mutans* and *C. albicans* dual species biofilm.

Based on the results from MTT assays, the viability of *S. mutans* and *C. albicans* in dual-species biofilm was similar to the sum of single species biofilm. On the other

hand, the dual species biofilm of *S. mutans* and *C. tropicalis* clearly exhibited higher number of viable cells comparing to the sum of single species biofilm (Fig. 5). Several previous studies reported the viability using microbial count method which can exhibit the number of each microorganism in dual species biofilm (16, 17, 64, 65). Our microbial count results showed that when *S. mutans* was co-cultured with *C. albicans* or NACs, the numbers of viable *S. mutans* cells were higher than in single species biofilm (Fig. 6a). On the other hand, in the presence of *S. mutans*, the number of viable *Candida* cells was similar to the number of *Candida* in single species biofilm (Fig. 6b). Previous studies reported different results on the number of *S. mutans* in dual species biofilm. While some studies reported no change, others found a higher number of *S.mutans* in dual species biofilm than single species biofilm (16, 17, 65). However, the results on the number of *C. albicans* were consistently higher in dual species biofilm compared to single species biofilm (16, 17, 65). The differences between previous studies and ours may come from the difference in the culture conditions. In our study, biofilm was formed in BHI containing 5% sucrose, which is favorable for *S. mutans* growth, whereas other studies used a yeast based medium and glucose which may favor *Candida* growth (16, 17, 65). Moreover, in previous studies, the culture media were changed several times during the growth of biofilm and the cultures were incubated for a longer period of time than in our study. These factors may affect the growth of microorganisms in the biofilm.

We also determined the level of acid production by measuring the pH of the medium. We found similar results to previous studies (17, 64, 65, 70) that the pH values of all dual species biofilm were similar to those of *S. mutans* single species biofilm (pH = approximately 4.0, which is lower than the critical pH of enamel (pH = 5.5)). On the other hand, the pH values of *C. albicans* and NACs single species biofilm ranged from 5.5 to 6.4 (Fig. 7). The high pH value found in *Candida* single species biofilm may result from their poor metabolism of sucrose comparing to glucose (65). In dual species biofilm, *S. mutans* metabolize sucrose and release glucose which can be further metabolized by *Candida* spp. (65, 70). Nevertheless, because *S. mutans* is the major acid producer, the final pH in the dual-species biofilm is similar to that of *S.mutans* single species culture.

Our results on the expression of genes related to sugar metabolism of *S. mutans* revealed that the presence of *C. albicans* upregulated *scrA/B* (sugar transportation), *pdhA* and *pfl* (pyruvate metabolism) genes of *S. mutans,* consistently with the previous transcriptome study (33). In dual species biofilm, *S. mutans* and *C. albicans* consumed sugar for their growth causing the sugar limitation. In this sugar limitation condition, *S. mutans* upregulated *scrA* gene, which encodes an enzyme that transport sucrose into the cells, and increased *ScrB,* which produces glucose-6-phosephate for pyruvate production. Moreover, this condition may push *S. mutans* to metabolize pyruvate to formate and acetyl CoA (acetate and ethanol) via a pyruvate dehydrogenase (encoded by *pdh*) and a pyruvate-formate lyase (encoded by *pfl*). The formate and acetate are

acid end-product causing the acidic pH in dual species biofilm (33). We found the same result in the presence of *C. tropicalis* and *C. krusei* (Fig. 10).

A previous study showed that the expression levels of genes involved in acid stress tolerance (*atpD* and *fabM*) of *S. mutans* in dual species biofilms were increased (17). *S. mutans* has an ability to survive, grow and can adapt well to low pH condition via the increase of proton extrusion by F_1F_0- ATPase system (encoded by *atpD*) (35, 36). The system prevents proton influx to the cell by increasing the production of monosaturated fatty acid at the cell membrane (encoded by *fabM*) (34) and passing the acid end product out of the cells (71). In contrast, we observed similar levels of expression of these acid stress tolerance genes in *S. mutans* in single species biofilm and *S. mutans* co-cultured with *Candida* spp. (Fig. 11). The different results may be due to the differences in the period of measurement; the previous study found the upregulation of the genes expression at 42 hours of biofilm formation, while we measured the gene expression at 36 hours (17).

Our and previous studies found that the presence of *C. albicans* upregulated *ciaRH* of *S. mutans* (33). Moreover, we found that *C. tropicalis* and *C. krusei* also enhanced the expression of these two genes in *S. mutans* (Fig. 12). The CiaRH, a two-component signal transduction system, plays a role in biofilm formation, sucrose-dependent adherence and acid tolerance of *S. mutans* (71). Therefore, the presence of

C. albicans and NACs increase the expression of genes related to cariogenicity of *S. mutans*.

Several previous studies observed the presence of NACs, including *C. tropicalis, C. krusei, C. parapsilosis, C. glabrata* and *C. dubliniensis,* in children with dental caries (12, 22, 23, 53) and *C. tropicalis* was the most commonly reported species among NACs (12, 53, 72, 73). Moreover, some studies showed the prevalence of NACs such as *C. tropicalis, C. krusei* and *C. dubliniensis* in the caries-active group were higher than in the caries-free group (12, 22). Although there is no strong evidences on the cause and effect relation between *Candida* spp. and ECC, a retrospective study in a large cohort suggested that oral thrush and other *Candida*-related conditions may increase the risk of caries (74). We also found that several *Candida* species can enhance cariogenic potentials of *S. mutans*. These altogether imply that the presence of NACs may contribute to the development of ECC. However, further longitudinal and experimental studies are needed to prove this hypothesis.

Our study carries certain limitations. First, our experiments were performed *in vitro* with a small sample size (n=3), thus statistical analysis may not be appropriate (75) This may explain why we could observe only a marginally significant difference (P = 0.05) (Fig. 4 and 5). Furthermore, we used standard laboratory strains of microorganisms, which may behave differently from clinical isolates. The cultures were performed under 1 condition which may not simulate conditions in the oral cavity. In

addition, we only investigated the interactions in dual-species biofilm, but dental plaque is composed of a multitude of microorganisms. Whether our observations occur in the complex context of multispecies dental plaque remains unknown.

In conclusion, the presence of NACs, especially *C. tropicalis* and *C. krusei,* can promote *S. mutans* virulence in the same way as *C. albicans,* but to different degrees. In term of biofilm mass, the greater upregulation of *gtfBCD* and *ciaRH* of *C. tropicalis* and *C. krusei* than *C. albicans,* together with the difference of cell wall mannan structures, may lead to the higher biofilm mass. Furthermore, in our culture condition, we found that the glucan binding protein (Gbp) may not contribute to biofilm formation of dual species biofilm, which is consistent with the study by Falsetta *et al*. (17). However, the pH of culture media of dual species biofilm of *S. mutans* and *C. albicans* and of *S. mutans* and NACs were similar although the expression of gene related to sugar metabolism of NACs group were greater than *C. albicans*. Interestingly, the presence of *C. dubliniensis*, which have similar characteristics to *C. albicans*, in dual species biofilms did not affect *S. mutans* gene expression.

The results of this study provide basic information regarding the interactions between *S. mutans* and non- *albicans Candida* species (NACs). The better understanding of the cariogenic process will lead to future development of effective prevention and treatment for dental caries.

APPENDIX A

REAGENTS AND INSTRUMENTS

Reagents

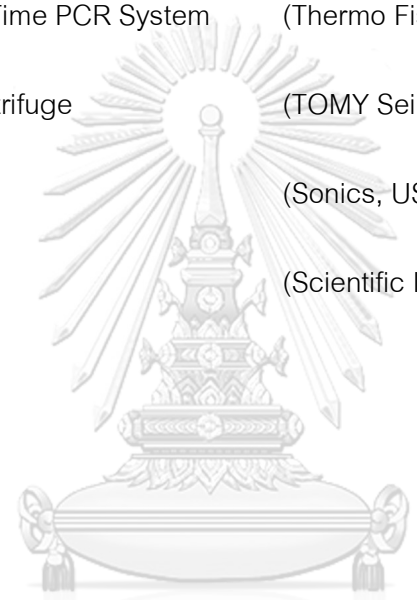
Absolute ethanol	(Merck, Germany)
Acetic acid	(Merck, Germany)
Agarose	(Vivantis, Malaysia)
Brain Heart Infusion (BHI) agar	(HiMedia, India)
Brain Heart Infusion (BHI) broth	(HiMedia, India)
Chloroform	(RCI LABSCAN, Thailand)
Crystal violet	(PanReac AppliChem, USA)
DEPC	(Sigma, USA)
DMSO	(Sigma, USA)
DNase I	(Thermo Fisher Scientific, USA)
DNA ladder 100 bp	(GeneDireX, USA)
EDTA	(USB, USA)
ImProm-II™ Reverse Transcriptase	(Promega, USA)
Isopropanol	(Merck, Germany)
KCl	(Merck, Germany)
KH2PO4	(Merck, Germany)
Luna® Universal qPCR Master Mix	(NEB, USA)

MTT	(Sigma, USA)
Mitis-Salivarius (MS) agar	(Difco, USA)
NaCl	(Thermo Fisher Scientific, USA)
Na2HPO42H2O	(CARLO ERBA Reagents, USA)
Nucleic acid gel stained	(Thermo Fisher Scientific, USA)
PCR mastermix	(Qiagen, Germany)
Primers	(Macrogen, Korea)
Phenol	(Merck, Germany)
RNAlater™ Stabilization Solution	(Thermo Fisher Scientific, USA)
Sabouraud dextrose agar (SDA)	(HiMedia, India)
Sucrose	(Thermo Fisher Scientific, USA)
Taq DNA polymerase	(Thermo Fisher Scientific, USA)

Instruments

15 ml falcon tube	(Corning, USA)
24-well plates	(Sigma-Aldrich, USA)
Autopipette 10, 200 and 1,000 µl	(Mettler-Toledo Rainin, USA)
Glass beads (0.1 mm diameter)	(Kepler, Thailand)
Gel Documentation	(Bio-rad, USA)
Incubator	(Thermo Fisher Scientific, USA)

Microplate Spectrophotometer Epoch II	(Bio-Tek, USA)
Microcentrifuge tubes	(KIRGEN, China)
NanoDrop™ 2000/2000c Spectrophotometer	(Thermo Fisher Scientific, USA)
PCR tubes	(Extra Gene, Taiwan)
pH meter	(HORIBA, Singapore)
QuantStudio™ 5 Real-Time PCR System	(Thermo Fisher Scientific, USA)
Refrigerated micro centrifuge	(TOMY Seiko, Japan)
Sonicator	(Sonics, USA)
Vortex mixer	(Scientific Industries, USA)



APPENDIX B

REAGENTS PREPARATION

1. 0.1% of crystal violet

Crystal violet powder	0.1	g

Dissolve in Milli Q water 100 ml and stored at room temperature until use.

2. 33% of acetic acid

Acetic acid	33	ml

Dissolve in Milli Q water 67 ml and stored at room temperature until use and must be used in a fume hood.

3. MTT

MTT powder	5	mg/ml

Dissolve in sterile milli Q water and must be do the experiment under the darkened place.

4. 1XPhosphate Buffered Saline (1xPBS, pH 7.4)

NaCl	8	g
KCl	0.2	g
Na2HPO42H2O	1.78	g
KH2PO4	0.24	g

Dissolve in Milli Q water 1 L and adjust pH to 7.4. Sterilize solution by autoclaving at 121 ˚C for 15 min and stored at 4 ˚C until use.

5. DEPC water

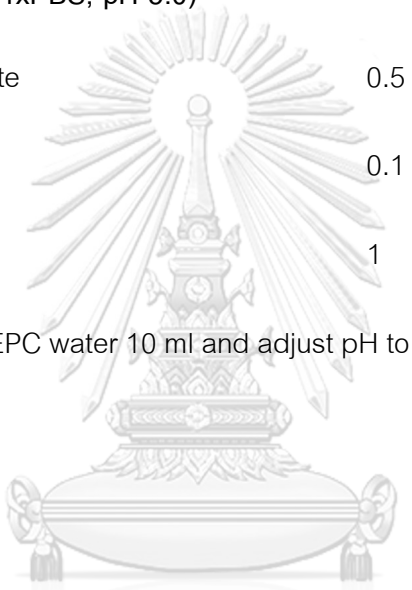
DEPC solution	1	ml/L

Dissolve in Milli Q water 1 L using a magnetic stirrer overnight. After that, sterilize solution by autoclaving at 121 ˚C for 15 min and stored at 4 ˚C until use.

6. 1XNAES buffer (1xPBS, pH 5.0)

50mM sodium acetate	0.5	ml
10 mM EDTA	0.1	ml
1% SDS	1	ml

Dissolve in sterile DEPC water 10 ml and adjust pH to 5.0 ± 0.2. Stored at 4 ˚C until use.



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