THE EFFECT OF *STREPTOCOCCUS SALIVARIUS* AND *ACTINOMYCES NAESLUNDII* IN COMBINATION WITH *MUSA ACUMINATA* EXTRACT ON THE BIOLOGICAL PROPERTIES OF *CANDIDA ALBICANS*

BY

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A thesis submitted in fulfilment of the requirement for the degree of Masters of Biobehavioral Health Sciences

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ABSTRACT

The association between *Candida albicans* infection with oral carcinogenesis has been suggested in the previous studies. Recent studies showed that *Streptococcus salivarius* K12 and *Actinomyces naeslundii* were able to inhibit cariogenic pathogen growth, including *Streptococcus mutans*. The hypotheses of the present study are that *A. naeslundii* possesses oral probiotic properties as in *S. salivarius* K12 and that probiotic and synbiotic of *S. salivarius* K12 *or A. naeslundii* and *Musa acuminata* inhibit the aggregation and biofilm of *C. albicans.* The objectives of the study are to identify oral probiotic properties of *S. salivarius* and *A. naeslundii* using *in-silico* analysis, to determine the effect of *S. salivarius* and *A. naeslundii* on the co-aggregation of *C. albicans*, to determine the antimicrobial effect of *M. acuminata* on *C. albicans* and to evaluate the effect of *S. salivarius, A. naeslundii* and *M. acuminata* formulation on the biofilm-forming ability of *C. albicans.* The present study showed that *A. naeslundii* possessed the probiotic protein Uvrb that involves stress response, which is similar to *S. salivarius* K12. In addition, the aggregation assay showed that *S. salivarius* K12 and *A. naeslundii* co-aggregated with the yeast and hyphal form of *C. albicans.* However, a significant decreased co-aggregation of oral cancer isolated *C. albicans* (ALC3) was observed in the presence of *S. salivarius* K12 or *A. naeslundii* ($P<0.05$). The static biofilm showed that the biofilm biomass was found to decrease when *C. albicans* was co-cultured with the bacteria and *M. acuminata*. In conclusion, *A. naeslundii* possesses oral probiotic properties as in *S. salivarius* K12, and that probiotic and synbiotic of *S. salivarius* K12 *or A. naeslundii* and *Musa acuminata* inhibit the aggregation and biofilm of *C. albicans.* Therefore, it has the potential for the development of the potential positive influence that can be used as an adjuvant for the prevention of oral carcinogenesis.

خلاصة البحث

اقترحت الدراسات السابقة أن هناك علاقة بين عدوى المبيضة البيضاء والسرطان الفموي، وأظهرت الدراسات الحديثة أن المكورات العقدية اللعابية (*Streptococcus salivarius* K12) والشعية الفطرية النايسلودنية (*Actinomyces naeslundii*) كانتا قادرتين على تثبيط نمو مسببات السرطان بما في ذلك العقدية الطافرة (*Streptococcus mutans*). فرضت هذه الدراسة أن لدى الشعية الفطرية النايسلودنية خصائص بروبيوتيكية فموية كما في المكورات العقدية اللعابية، وأن بروبيوتيكية وسنبيوتيكية المكورات العقدية اللعابية أو الشعية الفطرية النايسلودنية ونبتة الموز المستدق (*Musa acuminata*) لديها القدرة على تثبيط التجميع المشترك وتكون الغشاء الحيوي للمبيضة البيضاء. هدفت الدراسة على التعرف على الخصائص البروبيوتيكية للمكورات العقدية اللعابية والشعية الفطرية النايسلودنية باستخدام التحليل الحاسوبي، لتحديد تأثيرهما على التجمع المشترك للمبيضة البيضاء، ولتحديد التأثير المضاد للميكروبات للموز المستدق على المبيضة البيضاء، ولتقييم تأثير تركيبة الشعية الفطرية النايسلودنية والموز المستدق والمكورات العقدية اللعابية على قدرة المبيضة البيضاء على تكوين الأغشية الحيوية. أظهرت الدراسة الحالية أن الشعية الفطرية النايسلودنية تمتلك البروتين البروبيوتيكي Uvrb الذي يلعب دورا في الاستجابة للضغط، والتي تشبه تلك في المكورات العقدية اللعابية. بالإضافة إلى ذلك أظهر اختبار التجميع أن الشعية الفطرية النايسلودنية والمكورات العقدية اللعابية تجمعا اشتراكيا مع الخميرة والشكل الخباطي للمبيضة البيضاء. ومع ذلك لوحظ انخفاض ملحوظ في التجميع المشترك للمبيضة البيضاء المسببة لسرطان الفم (ALC3) وذلك في وجود المكورات العقدية اللعابية أو الشعيات الفطرية النايسلودنية ($P<0.05$). أظهر الغشاء الحيوي الساكن انخفاض الكتلة الحيوية للأغشية الحيوية عند زراعة المبيضة البيضاء اشتراكيا مع البكتيريا والمكورات العقدية اللعابية. في الختام، يظهر أن لدى الشعيات الفطرية النايسلودنية خصائص بروبيوتيكية فموية كما هو الحال في المكورات العقدية اللعابية، وأنه بإمكان سنبيوتيكية وبروبيوتيكية الشعية الفطرية النايسلودنية والموز المستدق والمكورات العقدية اللعابية تثبيط التجميع المشترك والغشاء الحيوي للمبيضة البيضاء، ولذلك فإن لديها قدرة واعدة على تطوير التأثير الإيجابي المحتمل الذي من الممكن استخدامه كمساعد للوقاية من التسرطن الفموي.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Biobehavioral Health Sciences.

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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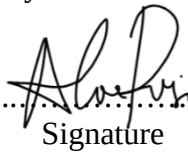

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ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
OSCC	Oral squamous cell carcinoma
DS	Denture stomatitis
MRG	Median rhomboid glossitis
SECC	Severe early childhood caries
h	Hour
µg	Microgram
µl	Microlitre
mg	Milligram
g	Gram
min	Minute
mL	Millilitre
mm	Millimetre
nm	Nanometre
CV	Crystal violet
OD	Optical density
PBS	Phosphate buffer saline
rpm	Revolution per minute

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Oral cancer is malignant neoplasia, ranked the 18th most common cancers worldwide, with a 4.6% incidence reported annually (GLOBOCAN, 2020). Oral cancer emerges as a health problem in Malaysia, with an incidence rate of 2.2% (GLOBOCAN, 2020). The incidence rate of oral cancer was found to be more common in men compared to women. According to the National Cancer Registry, Ministry of Health (Malaysia), oral cancer is most likely to happen among Indian ethnicity (2019).

Many risk factors lead to oral squamous cell carcinoma (OSCC), the most common type of oral cancer. There are alcohol consumption, the use of tobacco smoking, unbalanced diet, poor oral hygiene, immune deficiency, and microbial infection, including *Candida albicans* (Khajuria & Metgud, 2015; de Mattos Camargo Grossmann et al., 2020). In addition, the correlation of tobacco chewing with pre-cancerous and oral cancer has been reported, including erythroplakia, oral submucous fibrosis and leukoplakia (Mortazavi et al., 2014). The study has also shown the association of betel quid chewing with oral carcinogenesis (Wang et al., 2018). The betel quid has psychotropic and antihelminthic properties due to the presence of betel alkaloid known as arecoline. This active metabolite was suggested to involves in fibroblast stimulation (Anand et al., 2014). Betel quid has been described to affect the oral epithelium and cause molecular alterations. These modifications contribute to oral carcinogenesis in the oral mucosa eventually (Islam et al., 2019).

The association between *Candida* to oral cancer has been reported (Mortazavi et al., 2014). Candida's virulence, such as its ability to produce hydrolytic enzymes and metabolise acetaldehyde from ethanol by oxidation, which later promotes oral carcinogenesis (Mortazavi et al., 2014). *Candida* can cause several oral mucosal lesions leukoplakia, which has a high incidence of malignant transformation (Kang et al., 2016).

The key treatments for the treatment of oral cancer are chemotherapy and immunotherapy. Recently, probiotic has become the focus of research due to its anti-cancer properties. Probiotics are living microorganisms that confer a health benefit on the host when given sufficient quantities (WHO, 2001). The underlying mechanisms for its anti-cancer effects include the suppression of microbial growth involved in mutagenesis, alteration of carcinogenic metabolism, oxide preservation of DNA, and regulation of the immune system (Abedin-Do et al., 2015). Moreover, several clinical trials focused on the health effects of oral probiotics. A previous study showed that probiotics' benefits are dependent on the probiotic strains; thus, more research is required to explain the anti-cancer properties of oral cancer probiotics (Yu & Li, 2016).

Prebiotic is a food that provides the host with a nutritional advantage. It increases the benefits of probiotics in the host systems (Hutkins et al., 2016). The use of prebiotic was developed in 1995 to improve the beneficial commensal and probiotic gut microbes, *Lactobacillus* and *Bifidobacterium*. (Gibson & Roberfroid, 1995). It has been suggested to enhance the growth of beneficial microorganisms that resides in the gut microbiome (Twetman et al., 2015). Prebiotics can modify the microbial composition or activity, aimed at stimulating growth in resident gut microbiota health-promoting bacteria, thus bringing local and systemic health benefits (Slomka et al.,

2018). The prebiotic ingredients currently identified are lactulose, β-galactooligosaccharides (GOSs), inulin, and fructooligosaccharides (FOSs) (Figueroa-González et al., 2019). Selective stimulation of *Lactobacilli*, *Bacillus* and *Bifidobacteria* by these non-digestible oligosaccharides were recorded well (Silva et al., 2017; Thongaram et al., 2017; Figueroa-González et al., 2019).

Synbiotics are dietary supplements that incorporate the synergistic process of probiotics and prebiotics. The concept of "synbiotics" was developed in 1995 by Gibson and Roberfroid which they characterised synbiotics as "a mixture of probiotics and prebiotics that are benefiting the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract through selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria" (Gibson & Roberfroid, 1995). Synbiotics have been shown to exhibit a better effect than the administration of probiotics alone by increasing the survival and activity of the beneficial microorganisms (Hemarajata & Versalovic, 2013). A previous study has also demonstrated synbiotics' action in suppressing oral pathogens without interfering with a safe oral environment where it neutralises the growth of oral pathogenic microorganisms (Kojima et al., 2016). Therefore, probiotic, prebiotic and synbiotic effects in oral health are suggested in the current research, potentially preventing oral carcinogenesis.

1.2 STATEMENT OF THE PROBLEM

Among the oral pathogenic yeast, *Candida albicans* are known as the etiological factors for various oral diseases, including oral candidiasis, denture-associated stomatitis, rhomboid glossitis and OSCC. There are many ways to treat outbreaks in the last decades. Infectious illnesses have been treated with antibiotics. However,

overuse of these drugs over a long time causing infectious organisms to become resistance to antibiotics. Hence, making the drugs ineffectiveness. To rectify this situation, the use of chemicals in disease management is discouraged and greater emphasis on safe treatment has been progressively developed. Thus, probiotics and synbiotic treatments are particularly desired as a wide range of positive health effects have been identified. These effects have been strain-dependent. A previous study has shown that *Streptococcus salivarius* and *Actinomyces naeslundii* inhibit the growth of other oral pathogens. Therefore, it is suggested that these bacteria may possess probiotic properties that can inhibit the colonisation of *C. albicans*, which leads to a healthy oral cavity.

On the other hand, banana peel was selected in this study due to the phytocompounds present in the natural conditions. Several parts of the banana such as leaf, pseudostem, flowers, fruit or peel have long been used in traditional medicine in America, Asia and Africa. It has also been previously reported for numerous biological applications such as antimicrobial and antioxidant.

1.3 HYPOTHESIS

The overall hypothesis is that *A. naeslundii* possesses oral probiotic properties as in *S. salivarius* K12 and that probiotic and synbiotic of *S. salivarius* K12 *or A. naeslundii* and *Musa acuminata* inhibit the aggregation and biofilm of *C. albicans.* The specific hypothesis of the first study is that *A. naeslundii* possesses oral probiotic properties as in *S. salivarius* K12. The null hypothesis is *A. naeslundii* does not possess oral probiotic properties as in *S. salivarius* K12. The hypothesis of the second study is that probiotic *S. salivarius* K12 and *A. naeslundii* inhibit the aggregation of *C. albicans.* The null hypothesis is that probiotic *S. salivarius* K12 and *A. naeslundii* do not inhibit

the aggregation of *C. albicans*. The hypothesis of the third study is that Prebiotic *M. acuminata* have an antifungal effect on *C. albicans.* The null hypothesis is that Prebiotic *M. acuminata* does not have an antifungal effect on *C. albicans*. Finally, the hypothesis of the last study is that probiotics and synbiotic of *S. salivarius* K12 *or A. naeslundii* and *Musa acuminata* inhibit the biofilm formation of *C. albicans.* The null hypothesis is that probiotics and synbiotic of *S. salivarius* K12 *or A. naeslundii* and *Musa acuminata* does not inhibit the biofilm formation of *C. albicans.*

1.4 RESEARCH OBJECTIVES

The study aimed to achieve the following objectives:

1. To identify the oral probiotic properties of *S. salivarius* and *A. naeslundii* using *in-silico* analysis.
2. To determine the effect of *S. salivarius* and *A. naeslundii* on the co-aggregation of *C. albicans*.
3. To determine the antimicrobial effect of *Musa acuminata* on *C. albicans*.
4. To evaluate the effect of *S. salivarius, A. naeslundii* and *M. acuminata* formulation on the biofilm-forming ability of *C. albicans.*

1.5 RESEARCH QUESTIONS

1. Do *S. salivarius* and *A. naeslundii* possess probiotic properties?
2. What is the effect of *S. salivarius* and *A. naeslundii* on *C. albicans* co-aggregation?
3. Does *M. acuminata* possess antimicrobial activity against *C. albicans*?
4. Does the combination of *S. salivarius* and *A. naeslundii* with *M. acuminata* inhibit the biofilm-forming ability of *C. albicans*?

1.6 SIGNIFICANCE OF STUDY

This study is significant as can elucidate the potential of *S. salivarius* or *A. naeslundii* with *M. acuminata* as an alternative mouthwash that is based on oral probiotic and prebiotic, which is significant in maintaining a healthy oral cavity through modulation of the oral microbiome with a reduced side effect. This is also in-line to the Sustainable Development Goal (SDG) No. 3: Good health and well-being by United Nation.

CHAPTER TWO

LITERATURE REVIEW

2.1 ORAL DISEASES AND THE INVOLVEMENT OF *CANDIDA* SPECIES

According to the World Dental Federation, a healthy oral cavity is a chronic-mouth-and-face pain-free condition, oral infection and sores, periodontal infections, tooth decay, teeth loss, and other conditions that impair the capacity of the person to bite, chew, smile, and psychosocial well-being (Glick et al., 2016). At least 3.58 billion individuals worldwide are the most frequently affected by oral diseases of chronic caries (GBD, 2017).

The worldwide rise of oral diseases has created alarm among health professionals. Multiple causes, primarily due to insufficient oral treatment, lead to an increase in oral diseases. According to the World Health Survey (WHS) (2002-2004), in low- and middle-income countries, demand for oral health services goes beyond the health care system's potential (Hosseinpoor et al., 2012). Oral diseases impact impoverished and economically vulnerable in society (Peres et al., 2019).

2.1.1 Dental caries

The dental cavity or tooth decay is a bacterial infectious disease, contributing to partial demineralisation and tooth loss. It is among the most prevalent human diseases, second only to the common cold. Around 2.4 billion people have permanent teeth caries, and 486 million children possess primary teeth caries (GBD, 2017). The homeostasis of this environment will then turn into acidophilic bacteria colonisation that is proven to demineralise the teeth through excessive glucose intake, contributing to dental caries (Lin et al., 2017).

S. mutans is the main pathogen that involves in caries development. When the colonisation by the *S. mutans* occurred, fermentable carbohydrates that provide a substratum for bacterial growth, organic acids like lactic, formic and acetic acids that may trigger the pH-decrease contributing to demineralisation of the tooth (Metwalli et al., 2013). *S. mutans* colonisation is proposed to occur after tooth eruption, and colonisation of the fissures at their depths will eventually lead to decay (Wan et al., 2003). However, this colonisation is postponed until other bacteria reach the fissure depths, it can be stopped from decaying.

Additionally, *C. albicans* seemed to be aligned with children's caries, with the *C. albicans* genotypes subgroup A being the dominant strain of the children plaque with severe early childhood caries (SECC) (Qiu et al., 2015; Wu et al., 2015). Previous experiments in vitro and in vivo confirmed that *C. albicans* can contribute to a complex combination of bacterial and fungal substances contributing to the development of the cariogenic polymicrobial biofilms ecosystem (O'Donnell et al., 2015; Bowen et al., 2018). The systematic research results and meta-analysis suggest that the ECC level of children with C. *albicans* is considerably greater than children without *C. albicans* (Xiao et al., 2018).

2.1.2 Periodontal diseases

Periodontal disease has been shown to increase its severity when *Candida* spp. are present mainly in HIV-infected patients (Lourenço et al., 2017). Furthermore, the study found that the quantity of *C. albicans* was higher in the periodontal pockets of patients with severe chronic periodontitis compared to non-chronic periodontitis (De-La-Torre et al., 2018; Pl et al., 2017). It is believed that sites affected by advanced periodontal lesions may favour colonisation by *Candida* spp., since some bacterial

species, such as *P. gingivalis*, when altering the host's response, create favourable conditions for the development of hyphae (Rocha & Dias, 2018). These microorganisms have also been reported to degrade hydroxyapatite structure which worsen the periodontitis of the oral cavity (Kaminishi et al., 1986).

2.1.3 Oral candidiasis

Candida albicans, though other species such as the *Candida glabrata* and the *Candida tropicalis* are known, are the most important cause-effect agent for oral candidiasis (OC) (Singh et al., 2014). Persons with poor oral hygiene and HIV infection are more vulnerable to oral candidiasis (Pedersen et al., 2015). Among important clinical manifestations of oral candidiasis include denture associated stomatitis, rhomboid glossitis, leukoplakia, angular cheilitis, and Chronic mucocutaneous candidiasis.

2.1.3.1 Denture Associated stomatitis

Denture-related stomatitis refers to an inflammatory state of the denture-bearing mucosa (Ohshima et al., 2016). It has a multifactorial etiology; however, the following causes of predisposition are significant: poorly fitting denture, excessive use of the denture, inappropriate cleanliness of teeth, nutritional factors, underlying systemic conditions and *Candida* infections (Hasan & Kuldeep, 2015). The most common *Candida* isolates from denture-associated stomatitis site is *C. albicans* (Tobouti et al., 2016).

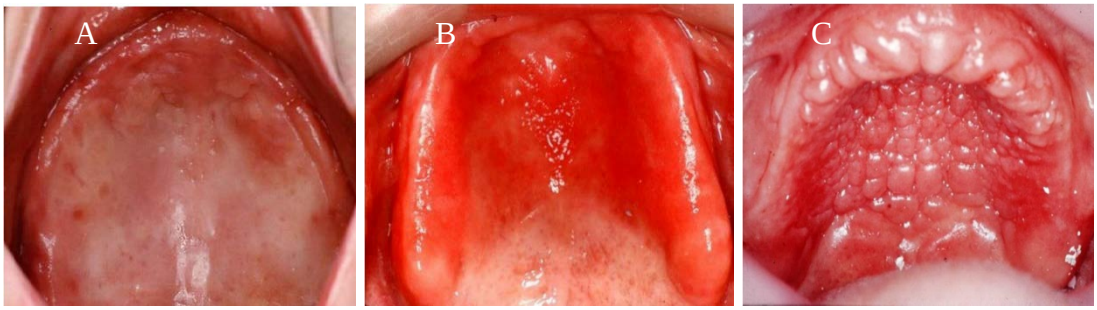

Figure 2. 1 Newton's Type I II and III denture stomatitis showing (A) areas of localised inflammation, (B) generalised erythema covering the denture bearing area and (C) inflammatory papillary hyperplasia (Puryer, 2016)

2.1.3.2 Rhomboid glossitis

Candida has been associated with median rhomboid glossitis (MRG) (Terai et al., 2016; Kaur et al., 2017). MRG is a disorder distinguished by papillary atrophy of the tongue's dorsum typically situated in an area anterior to the circumvallate papillae. It takes the form of an elliptical or rhomboid, well-marked area of depapilation in the middle line of the tongue. Although the lesion surface usually is flat, it often tends to be hyperplastic, exophytic, or even lobed (Song et al., 2015). MRG first occurred in <1% of the general population with a high incidence in men than women and adults more than children (Peleg & Shvartzman, 2001). Tobacco smoking has been stated to play a crucial function in increasing the *Candida*l colonisation that leads to MRG (Bojan et al., 2012).

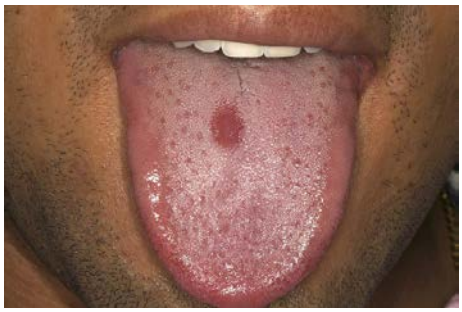

Figure 2. 2 Presentation of Central papillary atrophy (median rhomboid glossitis)

(Muzyka & Epifanio, 2013)