THE EFFECTS OF PROBIOTICS AND *ACTINOMYCES NAESLUNDII* ON ORAL CARCINOGENESIS

BY

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ABSTRACT

Oral cancer is one of the subclassification of head and neck cancer, which is one of the most prevalent cancers globally with high incidence and mortality rate. 90% of oral cancer constitutes of oral squamous cell carcinoma (OSCC). The prognosis status of the patients after treatment is determined by the relapse of the secondary oral tumour. The relapse of the secondary oral tumour leads to poor prognosis status of patients, hence reducing its five-year survival rate. Recently, probiotics have been heavily studied due to its health benefits and its contribution to cancer prevention. This study aimed to identify the anti-cancer properties of probiotics in oral carcinogenesis. A systematic review and meta-analysis were conducted to identify the list of probiotics that have a prominent effect on oral carcinogenesis. The identified probiotics were *Acetobacter syzygii*, *Lactobacillus plantarum*, *Lactobacillus salivarius* REN and AJ2. These probiotics elicited several effects against oral cancer, including anti-proliferative effects, modulation of protein expression, and apoptosis. The meta-analysis of the present study showed that *L. salivarius* REN is associated with a lower risk of oral carcinogenesis by 95%, with ($OR=0.05$, $P<0.05$). Thus, the ability of *L. salivarius* REN as an inhibitory agent for oral carcinogenesis is identified. *Actinomyces naeslundii* is one of the early oral colonisers that is associated with oral biofilm development. However, the previous study has discovered the presence of *A. naeslundii* in polymicrobial biofilms was able to reduce the biofilm biomass and activity of the oral pathogens. Hence, this study also aimed to identify the protein similarities between *L. salivarius* REN and *A. naeslundii* using *in silico* analysis. From the analysis, elongation factor Tu (EF-Tu) protein was identified with percent similarities of 79.5% in the present study. EF-Tu is a translation factor protein that is responsible for the modulation of the immune response. In addition, *C. albicans* is an opportunistic yeast that frequently isolated from the oral cavity. The previous study has shown that *C. albicans* is associated with oral carcinogenesis. The present study aims to determine the potential of *A. naeslundii* as an oral probiotic against oral carcinogenesis. The aggregation assay of the bacterium with *C. albicans* was conducted. The polymicrobial interactions of clinical strain *C. albicans* (ALC2) with *A. naeslundii* in nutrient broth exhibited lower co-aggregation compared to the auto-aggregation indicating the ability of *A. naeslundii* to reduce colonisation of *C. albicans* by inhibiting candidal dimorphism. In conclusion, *L. salivarius* REN could be a potential inhibitory agent against oral carcinogenesis. The high percentage protein similarities between *L. salivarius* REN with *A. naeslundii* shown the potential of *A. naeslundii* as an oral probiotic which able to inhibit oral carcinogenesis.

خلاصة البحث

يعد سرطان الفم أحد التصنيفات الفرعية لسرطان الرأس والعنق، وهو أحد أكثر أنواع السرطان انتشارا على مستوى العالم بمعدل مرتفع للإصابات والوفيات. حوالي 90٪ من حالات سرطان الفم مكونة من سرطان الخلايا الحرشفية الفموي. يتم تحديد الحالة التشخيصية للمريض بعد العلاج من خلال نسبة انتكاس الأورام الفموية الثانوية. يؤدي انتكاس الأورام الفموية الثانوية إلى سوء الحالة التشخيصية للمريض، وبالتالي تقليص معدل البقاء على قيد الحياة المقدرة بخمس سنوات. في الآونة الأخيرة تمت دراسة البروبيوتيك بشكل مكثف بسبب فوائدها الصحية ومساهمتها في الوقاية من السرطان. هدفت هذه الدراسة إلى التعرف على الخصائص المضادة للسرطان الموجودة في البروبيوتيك على التسرطن الفموي. تم إجراء مراجعة منهجية وتحليل تلوي لتحديد قائمة البروبيوتيك القادرة على التأثير على التسرطن الفموي بشكل بارز. تضمنت الكائنات الحية المجهرية التي تم تحديدها كلا من: الخلية العصية القرنفلية (*Acetobacter syzygii*)، والعصية اللبنية النباتية (*Lactobacillus plantarum*)، والعصية اللبنية اللعابية (*Lactobacillus salivarius REN*)، وبكتيريا AJ2. أثارت هذه البروبيوتيك العديد من التأثيرات ضد سرطان الفم، بما في ذلك التأثير المضاد للتكاثر، وتعديل تعبير البروتين، وتنشيط موت الخلايا المبرمج. أظهر التحليل التلوي لهذه الدراسة أن العصية اللبنية اللعابية مرتبطة بانخفاض خطر الإصابة بسرطان الفم بنسبة 95٪ (OR=0.05، P<0.05). ولهذا تم تحديد قدرة العصية اللبنية اللعابية كعامل مثبط للتسرطن الفموي. الشعية الفطرية النايسلوندية (*Actinomyces naeslundii*) هي أحد أوائل المستعمرات الفموية المرتبطة بنشوء الأغشية الحيوية الفموية. ومع ذلك فقد اكتشفت الدراسات السابقة أن وجود الشعية الفطرية النايسلوندية في الأغشية الحيوية متعددة الميكروبات كان قادرًا على تقليل الكتلة الحيوية للأغشية الحيوية ونشاط مسببات الأمراض الفموية. وبالتالي فقد هدفت هذه الدراسة أيضًا إلى تحديد أوجه التشابه بين البروتينات في العصية اللبنية اللعابية والشعية الفطرية النايسلوندية باستخدام التحليل الحاسوبي. تم بعد التحليل تحديد بروتين عامل الاستطالة Tu (EF-Tu) بين العصية اللبنية اللعابية والشعية الفطرية النايسلوندية بنسبة تشابه بلغت 79.5٪. EF-Tu هو بروتين عامل ترجمي مسؤول عن تعديل الاستجابة المناعية، وكشف التحليل أيضا أن المبيضة البيضاء (*Candida albicans*) هي خميرة انتهازية يتم دائما عزلها بشكل من التجويف الفموي، وأظهرت الدراسات السابقة أن المبيضة البيضاء مرتبطة بالسرطان الفموي. هدفت الدراسة الحالية إلى تحديد إمكانات الشعية الفطرية النايسلوندية كبروبيوتيك فموي ضد التسرطن الفموي. تم إجراء اختبار تراكم البكتيريا مع المبيضة البيضاء، وأظهرت التفاعلات المتعددة الميكروبات للسلالة الإكلينيكية للمبيضة البيضاء (ALC2) مع الشعية الفطرية النايسلوندية في وسيط مغذي تراكمًا مشتركًا منخفضا مقارنة بالتجميع الذاتي مما يشير إلى قدرة الشعية الفطرية النايسلوندية على تقليل استعمار المبيضة البيضاء عن طريق تثبيط الازدواج الشكلي للمبيضات. ختاما، يتبين أنه بإمكان الشعية الفطرية النايسلوندية أن تكون بمثابة عامل مثبط واعد ضد التسرطن الفموي. أظهرت النسبة العالية للتشابه البروتيني بين العصية اللبنية اللعابية والشعية الفطرية النايسلوندية قدرة الأخير على العمل كبروبيوتيك فموي مثبط للتسرطن 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 Biobehavioural 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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LIST OF ABBREVIATIONS

HNC	Head and Neck Cancer
OSCC	Oral Squamous Cell Carcinoma
PMD	Potentially Malignant Disorder
HPV	Human Papillomavirus
WHO	World Health Organization
TNM	Tumor, nodes and metastasis classification of malignant tumors
IBS	Irritable bowel syndrome
IBD	Irritable bowel diseases
PANC-1	Pancreas cancer cell line
HT-29	Colon cancer cell line
ALC2	Clinical isolate of *Candida albicans*
SDGs	Sustainable Development Goals
HMP	Human microbiome project
ISAPP	International Scientific Association for Probiotics and Prebiotics
HCA	Heterocyclic aromatic amines
AFB 1	Aflatoxin B1
TCGA	The Cancer Genome Atlas
IAP	Inhibitor of Apoptosis protein
QS	Quorum Sensing
QSI	Quorum Sensing Inhibitory
PRISMA	Preferred Reporting Items for Systematic Review and Meta-analysis
RCT	Randomized Controlled Trial
OR	Odds Ratio
WNF	Wan Nur Fatihah
EMA	Edre Mohamad Aidid
AFI	Ahmad Faisal Ismail
NAO	Noratikah Othman
MHA	Mohd Hafiz Arzmi
NAR	Nurul Alia Risma
NAH	Noor Afifah Hanin
JBI	Joanna Briggs Institute
I^2	Heterogeneity
TCA-8113	Human tongues squamous cell carcinoma
4NQO	4-nitroquioline-1-oxide
KB	Human oral cancer cell line
KDR	Normal epithelial cell line
hu-BLT mice	Humanised BLT mice
OSCSC	Oral squamous cell stem cell
NK	Natural Killer
MAPK	Mitogen-activated protein kinase
PTEN	Phosphatase and tensin homolog
ACF	Aberrant crypt foci
DMH	1,2-dimethylhydrazine

MAUVE	Multiple Alignment of Conserved Genomic Sequences with Rearrangements
DDBJ	DNA Databank of Japan
ENA	European Nucleotide Archive
EF-Tu	Elongation factor Tu
DC	Dendritic Cells
PAMP	Pathogen associated molecular pattern
rEF-Tu	Recombinant EF-Tu
NB	Nutrient Broth
BHI	Brain Heart Infusion
OD	Optical Density
SD	Standard deviation
SAPs	Secreted aspartyl proteinases
HIV	Human immunodeficiency virus

CHAPTER ONE
INTRODUCTION

1.1 ORAL CANCER

The incidence rate of cancer has been shown to increase every year, regardless of age and gender (National Cancer Institute, 2015). Cancer is a genetic disease that occurs when there are changes to the genes (National Cancer Institute, 2017). It influences the function of the genes and may result in abnormal activities of the cells. Most people that were affected by cancer are mainly caused by carcinogen from external sources. However, certain cancers are hereditary where individuals may inherit from their parents. A carcinogen is defined as any substance that may cause changes in the human body, which may alter the normal function of human physiology. These carcinogens may come in various types such as natural carcinogens, chemical carcinogens and radionuclide carcinogens (National Cancer Institute, 2015). As mentioned by Smith et al., (2016), according to the review done by International Agency for Research on Cancer (IARC), there are 10 key characteristics accompanying carcinogens mechanisms, which are: a) electrophilic or metabolically activated, b) genotoxic, c) alters DNA repair or causing genomic instability, d) induces epigenetic alterations, e) induces oxidative stress, f) induces chronic inflammation, g) immunosuppressive, h) modulates receptor-mediated effects, i) causes immortalization, and j) alters cell proliferation, cell death or nutrient supply. These characteristics were effective in ruling out the agents with regard to the carcinogenic hazard.

Oral cancer is one of the most prevalent diseases worldwide, with approximately 354,864 new cases being reported along with 177,384 deaths reported (Bray et al., 2018). Oral cancer is a subclassification within head and neck cancer (HNC), which it

is the sixth leading mortal cancer worldwide, with 630,000 new cases reported annually, followed by 350,000 deaths every year (Vigneswaran & Williams, 2014). Oral cancer sites include the buccal mucosa (cheek), tongue, the floor of the mouth, and lip (Tsantoulis et al., 2007). Ninety percent (90%) of oral cancer arises from oral squamous cell carcinoma (OSCC). OSCC is an aggressive and lethal type of oral cancer with high incidence and mortality rate (Chi et al., 2015). The main factors that contributed to oral carcinogenesis are high consumption of alcohol and tobacco-smoking (Migueláñez-Medrán et al., 2019). Additionally, other factors that contribute to oral carcinogenesis include genetic factors, poor oral hygiene, and human papillomavirus (HPV) infection (Candotto et al., 2017). In 2005, in an epidemiological study, it was reported that HPV DNA was detected in 35.6% of oropharyngeal cancers (Kreimer et al., 2010). HPV oncogenic factors HPV16 was accounted for most HPV-positive cases, with 87% (Kreimer et al., 2010). The evaluation of the association of HPV with the risk of oropharyngeal cancer was determined through epidemiologic studies (Gröbe et al., 2013).

Besides, potentially malignant disorders (PMDs) of the oral cavity has also been proposed as the aetiological factor for the development of oral squamous cell carcinoma (Sankari et al., 2015). According to the review done by Bughsan and Farouq (2020), many PMDs can become OSCC under certain underlying factors. Contrasting to the term proposed by WHO Collaborating Centre for Oral Cancer in 2007, expressed that not all lesions and conditions described under the term of "potentially malignant disorder" may develop into cancer (Warnakulasuriya et al., 2007).

On average, the survival rate of patients with oral cancer are five years. Currently, the preferred treatment option for treating oral cancer is mainly via surgery. However, the procedure has a low success rate in advanced stage of OSCC as this cancer

tend to have a recurrence. The prognosis status of the patients after treatment was determined through the remission of the secondary oral tumour. Patients with recurrence of the secondary oral tumour have poor prognosis status. As stated by Zini et al., (2010), the recurrence of the secondary oral tumour is the causal of reduced survival rate, approximately 50%-60% among patients. The recurrence affected the five-year survival rate and disease-free survival of patients with OSCC (Lindenblatt et al., 2012). It may be due to the aggressive local invasion and metastasis that gave the poor prognosis (Wang et al., 2013).

Patients' condition might be improved if they were diagnosed at an early stage. However, two-third of the patients were diagnosed during the later stages, mainly in stage III and stage IV, thus, leading to the poor prognosis. According to Oral Cancer Foundation (2019), the mortality rate that is associated with oral cancer in 2019 is high because oral cancer is often diagnosed during their late stage. To date, there is no comprehensive program that can opportunistically screen oral cancer in the early stage. The current diagnosis method that is used to estimate the prognosis and survival of oral cancer patients is the tumour, nodes, and metastasis classification of malignant tumours (TNM classification) (Jadhav & Gusta, 2013). It also provides detailed guidance on the treatment regimen to be followed in each case of OSCC. Thus, the healthcare practitioners must provide a preventive approach against the recurrence of the secondary oral tumour in order to improve the patients' survival rate and prognosis. Consequently, improving the patients' quality of life.

1.2 PROBIOTICS AND CANCER

Probiotics is defined as 'Live microorganisms which, when administered in adequate amounts, confer a health benefit to the host' (WHO, 2001). Probiotics have sparked interest over the years due to its positive health benefits. It is widely used as therapy for the prevention and treatment of gastrointestinal disorders, including irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), pathogenic bacterial or viral infection, and antibiotic-associated bacteria. The mechanisms of probiotics were associated with health benefits, which emphasised the strengthening of the gut epithelial barrier and modulation of the immune system (Collado et al., 2010). However, the effectiveness of the probiotics in providing health benefits remains unclear. The most common bacteria strains that are used as probiotics are *Bifidobacteria* and *Lactobacillus* species. These bacteria have been added into many functional foods and dietary supplements (Fijan, 2014; Terpou et al., 2019).

Probiotics has been reported to possess anti-cancer properties. *Lactobacillus* strain, specifically *L. acidophilus* 606 and *L. casei* ATCC 393, was shown to inhibit the growth of pancreas (PANC-1) and colon (HT-29) cancer cell line (Choi et al., 2006). *L. acidophilus* 606 was found to be the most effective at inhibiting the growth of these cancer cell lines (HT-29, HeLa, and PANC-1) at 21-28% survival rate compared with control at 10^8 CFU ml^{-1}. Furthermore, *L. acidophilus* 606 was also found to be less cytotoxic to healthy cells compared to *L. casei* ATCC 393. It is also suggested that *L. acidophilus* 606 may be used as natural cancer therapeutic agents due to its lower toxicity effects (Choi et al., 2006).

Interestingly, more strains of probiotics with anticancer properties have been discovered. Hence, this study will elucidate the potential proteins/genes in probiotics strains that may have the potential to prevent oral carcinogenesis.

1.3 ORAL DISEASES

In 2016, as reported by the Global Burden of Disease Study, it is estimated that half of the world population (3.58 billion people) were affected with oral diseases. Oral diseases may contribute to the health and economic burdens, thus affected people's quality of life (Peres et al., 2019). There are a few examples of oral diseases that cause major public concern worldwide. They are dental caries, periodontal disease, oral mucosal lesions, oropharyngeal cancer, human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS)-related oral disease and orodental trauma (Petersen et al., 2005). The main factors of oral diseases were strongly associated with behavioural risk factors such as taking unhealthy diets that are high in free sugars, tobacco usage, and harmful use of alcohol. Besides, dysbiosis of the oral microbiome was suggested to play a role in the occurrence of oral disease (Sharma et al., 2018). The development of periodontitis resulted from the colonisation of anaerobic bacteria in the periodontal pocket, which caused the loss of attachment between teeth and gingivae (Darveu, 2010). Meanwhile, gingivitis is another common periodontal disease which is caused by the colonisation of gram-negative bacteria (Zijnge et al., 2010).

Dental caries or tooth decay is one of the common oral diseases that affects people of all ages (Heng, 2016). Dental caries results from the accumulation of acid production due to the fermentation of carbohydrates. The excess uptake of carbohydrates leads to the demineralisation of the tooth, due to the formation of biofilm that contains acidogenic and aciduric species (Takahashi & Nyvad, 2011). Thus, this showed that the dysbiosis of oral microbiome also leads to the development of oral diseases.

Streptococcus mutans is the primary factors that caused human dental caries as they are acidogenic and aciduric (Forssten et al., 2010). Besides, *S. mutans*,

Actinomyces naeslundii has also become the interest of researchers as it also plays a role in periodontal and caries infections. However, previous studies proved otherwise where *A. naeslundii* is also associated to good oral health where the studies found that the growth of *A. naeslundii* when co-cultured with *S. mutans* showed inhibition of *S. mutans* growth (Arzmi et al., 2016). Thus, proved the ability of *A. naeslundii* in sustaining good oral health.

1.4 PROBLEM STATEMENT

Oral cancer is a part of head and neck cancer (HNC), which is one of the most prevalent diseases worldwide with 354,864 new cases being reported along with 177,384 deaths reported (Bray et al., 2018). Most common type of oral cancers is oral squamous cell carcinoma (OSCC). OSCC patient's often get diagnosed during its late stage. The prognosis of the patient with OSCC was determined by the recurrence of the secondary oral tumour. The recurrence of the secondary oral tumour in OSCC patients indicates the poor prognosis status of the patient. It also reduces the survival rate and disease-free of the patients, thus affected the patient's quality of life. Hence, the preventive approach against the recurrence of the secondary oral tumour is needed to improve the patient's survival rate and quality of life.

1.5 RESEARCH OBJECTIVES

The study aims to achieve the following objectives:

1- To identify the probiotics that have effects on oral carcinogenesis based on the evidence collected through systematic review and meta-analysis.

2- To identify the similarities of proteins in probiotics with *Actinomyces naeslundii* by comparing genomic sequence.

3- To determine the interaction and coaggregation ability of *Actinomyces naeslundii* with clinical strain isolate of *Candida albicans* (ALC2) strain through coaggregation assay.

1.6 RESEARCH QUESTIONS

This study aims to answer the following research questions:

1. What are the probiotics that have effects on oral carcinogenesis?
2. How many proteins are similar and what are the percentage of protein similarities between genomic sequence of probiotics and *Actinomyces naeslundii*?
3. What is the percentage of coaggregation between *Actinomyces naeslundii* with the clinical strain isolate of *Candida albicans* (ALC2)?

1.7 HYPOTHESES

The hypotheses for this study are:

1. Probiotics exerts several mechanisms in inhibiting oral carcinogenesis.
2. The higher proteins similarity number and percentage between probiotics and *Actinomyces naeslundii* determined the higher possibility of *Actinomyces naeslundii* to act as potential oral probiotic against oral carcinogenesis.
3. The high coaggregation percentage between *Actinomyces naeslundii* and clinical isolate *Candida albicans* inhibits the interaction of *Candida albicans*.

1.8 SIGNIFICANCE OF THE STUDY

To our knowledge, there are still insufficient study to elucidate the role of probiotics in oral carcinogenesis. To date, the relapse of the secondary oral tumour affected the patient's quality of life as it decreases their survival rate. The relapse of the secondary oral tumour also indicates poor prognosis status, hence a proper study that can identify the preventive approach against the relapse of the secondary oral tumour is needed. This study is the first study that mainly focuses on the effects of probiotics against oral carcinogenesis. This study will also elucidate the effect of *A. naeslundii* as a preventive approach against oral carcinogenesis, mainly in focusing its activity against the development of the pre-malignant lesion of oral cancer. The preventive approach against the relapse of the secondary oral tumour may reduce the incidence of the relapse of the secondary oral tumour, hence improving the patient's survival rate. This study is aligned with the third sustainable development goals (SDGs), which aims to provide excellent health and well-being for all ages.

CHAPTER TWO

LITERATURE REVIEW

2.1 ORAL MICROBIOME

Oral microbiota or oral microbiome is defined as the collective genome of microorganisms that reside in oral cavity, which is the second largest microbial community in human after the gut (Deo & Deshmukh, 2019). Initially, these oral microbiomes eventually played an essential role in maintaining the normal oral physiological environment and are associated with human health (Gholizadeh et al., 2016). However, oral microbiome dysbiosis always occurred due to the imbalance in the oral environment. The dysbiosis of oral microbiome is associated with various local and systemic human diseases, including dental caries, periodontal disease, obesity, and cardiovascular disease (Wade, 2013).

2.1.1 Composition of oral microbiota

The human mouth is heavily colonised by microorganisms, including viruses, protozoa, fungi, archaea and bacteria (Wade, 2013). Approximately, 700 prokaryotes species have been detected in the human oral cavity, which belongs to 185 genera and 12 phyla, namely *Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Spirochaetes, SR1, Synergistetes, Saccharibacteria* (TM7), and *Gracilibacteria* (GN02) (Perera et al., 2016; Zhao et al., 2017). Based on the data provided by the National Institute of Health Research common fund Human