UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERISATION OF LACTOBACILLI FROM THE FEMALE ANOGENITAL TRACT IN HEALTHY MALAYSIAN WOMEN AND ITS PROBIOTICS POTENTIAL

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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December 2017

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The female urogenital tract has long been recognised as a reservoir for a great diversity of microorganisms. The most prominent species isolated from this anatomical site among healthy women has been identified as lactobacilli. Previous studies conducted on these species indicate that they function to maintain a healthy reproductive system and protect the host from harm. In this study, the characteristics and properties of several lactobacilli strains isolated from the urogenital tract and perianal region of healthy female participants were evaluated to identify potential probiotic strains. The lactobacilli strains were isolated from twenty out of fifty-two Malaysian women wherein a total of sixty-two species of lactobacilli were identified using 16S rDNA sequencing. The most common species isolated in the present study were identified as *L. reuteri* (n = 14), *L. mucosae* (n = 12), *L. fermentum* (n = 10) and *L. oris* (n = 9). The least common species were *L. vaginalis* (n = 5), *L. gasseri* (n = 4), *L. delbrueckii* (n = 3), *L. jensenii* (n = 2) followed by *L. salivarius*, *L. plantarum* and *L. rhamnosus* (all with n = 1 isolate each). All lactobacilli strains demonstrated the ability to reduce the growth of several reference and clinical strains of *Candida albicans* and *Candida glabrata*, as observed in the broth microdilution assay. Five of the lactobacilli strains with potent anti-*Candida* effects were selected for subsequent assays based on the characteristics exhibited. These five lactobacilli strains exhibited fairly strong antimicrobial effects against ten of the pathogens tested as observed from the agar plug diffusion assay. All five lactobacilli strains could produce varying degrees of hydrogen peroxide, H_2O_2 as evaluated using 3'3, 5'5 tetramethylbenzidine (TMB) agar. Meanwhile, the differences in lactic acid production among the five strains indicate that the growth inhibitory mechanisms may differ for each strain. Besides that, the findings from the autoaggregation and coaggregation assays indicate that these strains have a high percentage of autoaggregation (50 – 80%) among themselves and coaggregation abilities with the fungal pathogens (75 – 90%). Only one of the lactobacilli strains, *Lactobacillus reuteri* 29B was also able to withstand highly acidic environment and high concentration of bile

for up to four hours as indicated in the pH and bile tolerance test. The five lactobacilli strains isolated showed the ability to tolerate the presence of 10% of nonoxynol-9 (N-9) with no or only a slight reduction in growth for most of the strains. The results obtained from this study indicates that whilst the five strains have promising characteristics, *L. reuteri* 29B is the ideal strain to be considered as a probiotic. Further studies are required to prove the suitability of *L. reuteri* 29B as a probiotic.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

ISOLASI DAN KARAKTERISASI LACTOBACILLI DARIPADA SALURAN *ANOGENITAL* WANITA MALAYSIA YANG SIHAT DAN POTENSI PROBIOTIKNYA

Oleh

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Saluran anogenital wanita telah lama diiktirafkan sebagai takungan pelbagai mikroorganisma. Spesies yang paling dikenali diasingkan daripada bahagian anatomi ini dalam wanita sihat telah dikenalpasti sebagai lactobacilli. Kajian terdahulu yang dijalankan ke atas spesies ini menunjukkan fungsinya ialah untuk mengekalkan sistem reproduktif yang sihat serta melindungi hos daripada bahaya. Dalam kajian ini, ciri-ciri dan sifat beberapa strain lactobacilli yang diasingkan daripada saluran urogenital dan bahagian perianal peserta wanita sihat telah dinilai untuk mengenalpasti strain probiotik berpotensi. Di kalangan lima puluh dua orang wanita Malaysia, strain lactobacilli telah diasingkan daripada dua puluh orang di mana sejumlah enam puluh dua spesies lactobacilli telah dikenalpasti melalui 16S rDNA *sequencing*. Spesies yang paling biasa diasingkan dalam kajian semasa dikenalpasti sebagai *L. reuteri* (n=14), *L. mucosae* (n=12), *L. fermentum* (n=10) dan *L. oris* (n=9). Spesies yang paling jarang diasingkan adalah *L. vaginalis* (n=5), *L. gasseri* (n=4), *L. delbrueckii* (n=3), *L. jensenii* (n=2) diikuti oleh *L. salivarius, L. plantarum* dan *L. rhamnosus* (kesemua dengan n=1 asingan setiap satu). Kesemua strain lactobacilli mendemonstrasi keupayaan untuk mengurangkan pertumbuhan beberapa strain *reference* dan klinikal *Candida albicans* dan *Candida glabrata*, seperti yang diperhatikan dalam *broth microdilution assay*. Lima strain lactobacilli yang mempunyai kesan anti-*Candida* yang poten telah disiasat selanjutnya berdasarkan ciri-ciri yang dipamirkan. Lima strain lactobacilli tersebut telah mempamerkan ciri-ciri antimicrobial tinggi terhadap sepuluh patogen yang dipilih menurut pemerhatian *agar plug diffusion assay*. Kesemua lima strain lactobacilli dapat menghasilkan pelbagai peringkat hydrogen peroksida, H_2O_2 seperti yang dinilai dengan menggunakan 3'3, 5'5 tetramethylbenzidine (TMB) agar. Tambahan lagi, perbezaan dalam penghasilan asid laktik antara lima strain tersebut menunjukkan bahawa mekanisme inhibitori pertumbuhan berkemungkinan berbeza untuk setiap strain. Sementara itu, hasil daripada *autoaggregation and coaggregation assays* menunjukkan bahawa strain ini mempunyai peratusan autoagregasi yang tinggi (50-80%) antara mereka dan keupayaan coaggregation dengan patogen fungal (75-90%). Hanya satu

strain lactobacilli, *Lactobacillus reuteri* 29B juga mampu menahan persekitaran yang sangat berasid dan konsentrasi hempedu yang tinggi sehingga 4 jam seperti yang ditunjukkan dalam pH dan ujian tolerans hempedu. Selain itu, kelima-lima strain lactobacilli mempamerkan kebolehan untuk menahan tahap nonoxynol-9 (N-9) yang setinggi 10% dengan tiada ataupun sedikit kesan terhadap pertumbuhan mereka. Hasil daripada kajian ini menunjukkan walaupun lima strain tersebut mempunyai ciri-ciri yang menjanjikan, *L. reuteri* 29B adalah strain yang paling sesuai untuk dipertimbangkan sebagai probiotik. Kajian selanjutnya diperlukan untuk membuktikan kesesuaian *L. reuteri* 29B sebagai probiotik.

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I certify that a Thesis Examination Committee has met on 26 January 2018 to conduct the final examination of Choo Sulin on her thesis entitled "Isolation and Characterisation of Lactobacilli From the Female Anogenital Tract in Healthy Malaysian Women and its Probiotics Potential" in accordance with the Universities Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
Apf	Aggregation-promoting factor
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BV	Bacterial vaginosis
CARB	Combating Antibiotic-Resistant Bacteria
CDC	Centers for Disease Control and Prevention
CFS	Cell-free supernatants
CFU	Colony-forming units
DNA	Deoxyribonucleic acid
EPC	Extracellular molecular weight proteins
FDA	Food and Drug Administration
FAO	Food and Agriculture Organization of the United Nations
GCMS	Gas chromatography mass spectrometry
GDP	Gross Domestic Product
GIT	Gastrointestinal tract
H_2O_2	Hydrogen peroxide
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
HMP	Human Microbiome Project
HRT	Hormone replacement therapy
HSV2	Herpes simplex virus type 2
HPV	Human papillomavirus
HRP	Horseradish peroxidase

IBS	Irritable bowel syndrome
JKEUPM	Ethics Committee for Research Involving Human Subjects
LAB	Lactic acid bacteria
LCMS	Liquid chromatography mass spectrometry
MATH	Microbial adhesion to hydrocarbons
MH agar	Mueller-Hinton agar
MMP-8	Matrix-metalloproteinase-8
MREC	Medical Research Ethical Committee
MRS agar	De Man, Rogosa, Sharpe agar
MRS broth	De Man, Rogosa, Sharpe broth
MRSA	Methicillin-resistant *Staphylococcus aureus*
N-9	Nonoxynol-9
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phophate buffered saline
PCR	Polymerase chain reaction
RP-HPLC	Reverse phase high-performance liquid chromatography
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SD	Standard deviation
TBE	Tri-borate EDTA
TMB	3,3',5,5'-tetramethylbenzidine
UTI	Urinary tract infections

UV	Ultraviolet
VVC	Vulvovaginal candidiasis
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Introduction

There are currently many different types of commercialised probiotics supplements out in the market. Most of these commercialised clinically isolated probiotic strains are of Western origin such as *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14. Based on the comparison of the extensive reports from the Western hemisphere and the few studies conducted in Asia, there is an observable difference between the common lactobacilli strains isolated. The common strains reported among the Western countries were *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jensenii* and *Lactobacillus iners* whereas, the limited data in Asia indicated that *L. reuteri, Lactobacillus fermentum* and *Lactobacillus salivarius* were the most common strains. As more findings indicate that the strains present may differ based on our geographical location and as the focus of probiotics shift towards a more personalised approach, it is preferable to find strains that are more common among individuals within Asia.

The human body is reported to house more microorganisms than mammalian cells by a ratio of 10 to 1 trillion as discovered by the Human Microbiome Project (HMP) that was completed in 2007. Another fact noted from the HMP study indicate that various anatomical sites such as the gut and vagina are found with reservoirs for a vast diversity of microorganisms which are as different as continents. These microorganisms play an important role in aiding some of our essential functions. An example of this is observed with the microbiota in the vaginal cavity which serves to protect the reproductive health of women through a complex relationship with the host epithelial cells and by preventing the overgrowth of opportunistic microorganisms. The protective effect associated with the microbiota highlights the possibility of discovering potential substitutes for antibiotics from this site.

According to the Food and Drug Administration (FDA) in 2001, probiotics are defined as, 'live microorganisms that when administered in adequate amount confers a health benefit on the host'. Lactobacilli have shown to exert many positive effects on their host. Lactobacilli are able to produce antimicrobial substances such as lactic acid, hydrogen peroxide and bacteriocin that enable them to inhibit the proliferation of both pathogenic and opportunistic microorganisms. This has been described as the main mechanism behind its ability to maintain a healthy environment in the host. Besides that, lactobacilli are capable of preventing the adherence of pathogens by steric hindrance. Likewise, in the body, when lactobacilli are found in the right proportion, they are able to attach to surface to prevent the adherence of pathogenic microorganisms. This ability is probably the mechanism behind its ability to restore the normal healthy microbiota as observed in cases of prolonged antibiotics administration.

However, out of the many positive effects that lactobacilli have exhibited the most highly favoured property is the fact that most of these strains do not have any adverse side effects when consumed by healthy individuals. This attractive property sets it apart from the likes of most antibiotic agents as it implies that there is a lesser chance that pathogens would develop a resistance towards it.

Most probiotic research seek to find potential probiotic strains from the gut. There is a growing trend of upcoming research that are looking for other regions of isolating probiotics from the human body. One of the sites which has gained much interest is the vagina, due to the many positive effects that the species isolated from this site has exhibited. The vaginal cavity has been reported to harbour a great diversity of lactic acid bacteria (LABs) primarily lactobacilli. Although the vagina as a source for probiotics has been studied extensively in Western countries, this site remains relatively undiscovered in Asia.

Another site that could potentially harbour probiotics is the perianal which due to its close proximity to the rectum should be rich with microorganisms such as lactobacilli from the gut. Unlike most regions in the body, the perianal has not been reported as a potential site for the isolation of probiotics. Due to the anatomical location of this site, it is possible that this site serves as a reservoir for probiotics as well.

The emergence of antimicrobial resistance (AMR) was foreseen soon after the discovery of antibiotics. The number of AMR cases has rapidly escalated since then and the never-ending battle between human and microorganisms has now shifted towards the latter. This form of resistance is not exclusive to bacteria but includes viruses and parasites as well. Factors that have contributed to the global spread of AMR are mainly due to the irresponsible prescription of antibiotics and the increase in globalisation such as travelling and the transportation industry. This threat against our most effective form of treatment for infections has raised the awareness that an alternative approach is required.

1.2 Objectives

General objective:

To identify and characterise the lactobacilli strains isolated from Malaysian women.

Specific objectives:

1. To isolate and profile the lactobacilli strains from healthy Malaysian women.

2. To assess the antimicrobial inhibitory effects of the isolated lactobacilli on vaginal pathogens.

3. To isolate hydrogen peroxide- and lactic acid producing lactobacilli.

4. To evaluate the probiotic properties of the lactobacilli isolated.

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