UNIVERSITI PUTRA MALAYSIA

MOLECULAR IDENTIFICATION, PROBIOTIC CHARACTERIZATION, ANTICANCER ASSESSMENT AND ENCAPSULATION OF LACTIC ACID BACTERIA ISOLATED FROM VAGINAS OF IRANIAN FERTILE WOMEN

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By

YOUSEF NAMI

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

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This thesis dedicates to:

My family for their love, endless support and encouragement…

Abstract of thesis presented to the Senate of Universiti Putra Malaysia, in fulfilment of the requirements for the degree of Doctor of Philosophy

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Chairman: Associate Professor Norhafizah Abdullah, Ph.D
Faculty: Institute of Bioscience

Probiotics are non-pathogenic microorganisms that positively influence their hosts when ingested in adequate amounts. Research on probiotics has increased over the past years. This trend has resulted in the commercialization of probiotic supplements and functional food. Numerous investigations have evaluated the potential of probiotic bacteria for cancer prevention or treatment. Most previous works isolated probiotic bacteria from dairy products or other fermented foods. This study isolated and characterized lactic acid bacteria (LAB) from a healthy vaginal ecosystem. LAB that exhibited probiotic properties were screened for their antimicrobial activity, antibiotic resistance, and anticancer activity. Isolation was performed using preliminary phenotypic screening based on Gram-staining and catalase reaction, followed by 16S rRNA gene sequencing, amplified ribosomal DNA restriction analysis (ARDRA), and repetitive sequence–based PCR fingerprinting. The bacteria isolated from 40 samples were classified into three genera with 13 species, three subspecies, and 45 strains. The combined use of molecular techniques was proven effective for the identification and classification of vaginal LAB, particularly *Lactobacillus*, *Lactococcus*, and *Enterococcus*.

The 45 vaginal LAB strains were characterized for their probiotic and therapeutic potentials. Probiotic characterization included in vitro assays of acid and bile salt tolerance, antimicrobial activity, and antibiotic resistance. Results showed that the survival rate of the isolates ranged from 18 to 88% and from 31 to 93% under acidic (pH 3.0) and 0.3% bile salt conditions in a simulated gastrointestinal environment, respectively. The strains from each of the 13 species that demonstrated the highest acid and bile salt tolerance were further evaluated for their antibiotic susceptibility and antimicrobial activity. Results showed that the survival rates of these 13 isolates under acidic and bile salt conditions were higher than 58 and 78%, respectively, qualifying them to be probiotic candidates. All 13 isolates exhibited antimicrobial activity against different pathogenic bacteria. Among the LAB, *Lactococcus lactis* 2BL showed the strongest antimicrobial activity against 15 pathogenic bacteria. The 13 isolates showed

different levels of antibiotic resistance depending on antibiotic used. Among the LAB, *Enterococcus avium* 7BL and *Enterococcus durans* 6HL were resistant and sensitive to all nine antibiotics used, respectively.

The anticancer effects of the 13 isolates on some human cancer cell lines, such as HeLa, AGS, HT-29, and MCF-7, were also assessed. The human normal cell line HUVEC was used as a control. The cytotoxicity of these strains and the occurrence of apoptotic cells were evaluated using DNA fragmentation, fluorescent microscopy, and flow cytometry. The metabolites produced by *Lactobacillus plantarum* 5BL, *Lactobacillus acidophilus* 36YL, *Enterococcus faecalis* 16H, and *Enterococcus lactis* 2BL exhibited remarkable anticancer activity against the tested human carcinoma cell lines with no significant cytotoxicity on the HUVEC normal cells. Apoptotic cells were also observed in the cancer cell lines.

Finally, the isolates were subjected to encapsulation to improve their bioavailability and survival rate under harsh gastrointestinal conditions. Herbal-based biopolymer matrices, such as alginate, gum arabic, and psyllium, were prepared through extrusion. Three gel formulations, namely, 1, 1.5 and 2% (w/v) alginate, 2% (w/v) alginate + 0.1 and 0.3% (w/v) gum arabic + psyllium, 1.5% (w/v) alginate + 0.3 and 0.5% (w/v) gum arabic + psyllium, and 1% (w/v) alginate + 0.4 and 0.6% (w/v) gum arabic/psyllium were prepared to improve the encapsulation efficiency, gastrointestinal survival, and colonic release rates of the isolates. All encapsulated bacteria exhibited significantly improved ($P < 0.05$) encapsulation efficiency (>98%) and survival rate (>75%) compared with the un-encapsulated bacteria (>45%) under harsh gastrointestinal conditions. The incorporation of gum arabic and psyllium into the alginate gel enhanced encapsulation properties. The use of combination gels for encapsulation improved the survival rate and storage stability of the probiotic strains under gastrointestinal conditions.

In conclusion, probiotic bacteria were successfully isolated and characterized from a healthy vaginal ecosystem. These probiotics exhibited antimicrobial activity, antibiotic resistance, and anticancer activity, making these bacteria potential candidates for the development of nutraceutical products.

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PENGENALAN MOLEKUL, PROBIOTIK PENCIRIAN, ANTIKANSER PENILAIAN DAN PENGKAPSULAN ASID LAKTIK BAKTERIA RIBUT DARI VAGINA IRAN WANITA SUBUR

Oleh

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Probiotik adalah mikroorganisma bukan patogen yang boleh memberi kesan positif terhadap hos mereka apabila dimakan dalam jumlah yang mencukupi. Penyelidikan probiotik meningkat sejak beberapa tahun kebelakangan ini. Kecenderungan ini telah menghasilkan pengkomersialan makanan tambahan probiotik dan makanan berfungsi. Selain dari itu, semakin banyak kajian dilakukan bagi mengkaji potensi bakteria probiotik untuk pencegahan atau rawatan kanser. Kebanyakan kajian sebelum ini telah menggunakan probiotik yang dipencil daripada produk tenusu atau makanan diperam yang lain. Pada kajian ini, kelainan sumber bakteria asid laktik (LAB) yang dipencil daripada ekosistem faraj yang sihat dan subur telah diperkenalkan. LAB yang mempamerkan sifat probiotik seterusnya disaring untuk aktiviti anti-mikrob, rintangan antibiotik, dan aktiviti anti kanser. Pengasingan dilakukan dengan menggunakan saringan awal fenotip berdasarkan teknik pewarnaan-Gram dan reaksi katalase, diikuti pula dengan teknik penjujukan gen 16S rRNA, teknik penguatan sekatan ribosoma DNA (ARDRA), dan analisa pengecaman *'finger-printing'* turutan berasaskan PCR. Bakteria yang diasingkan daripada 40 sampel telah diklasifikasikan kepada tiga genus dengan 13 spesies, tiga subspesies, dan 45 jenis. Penggunaan gabungan teknik molekul telah terbukti berkesan bagi mengenalpasti dan mengklasifikasi LAB yang dipencil dari ekosistem faraj, terutamanya *Lactobacillus*, *Lactococcus*, dan *Enterococcus*.

45 strain LAB faraj telah dicirikan mempunyai potensi probiotik dan terapeutik. Pencirian probiotik termasuk ujian toleransi *'in vitro'* terhadap asid dan garam hempedu, aktiviti antimikrob, dan rintangan antibiotik. Hasil kajian mendapati kadar kelangsungan hidup daripada strain yang dipencil adalah dari 18 kepada 88% dan dari 31% kepada 93% di dalam persekitaran berasid (pH 3.0) dan 0.3% garam hempedu yang mana masing-masing adalah mewakili persekitaran sistem percernaan. Strain dari setiap satu daripada 13 spesies yang menunjukkan toleransi terhadap asid dan garam hempedu selanjutnya dinilai untuk kerentanan terhadap antibiotik dan aktiviti anti-mikrob. Keputusan menunjukkan bahawa kadar kelangsungan hidup dari 13 pencilan LAB di bawah keadaan berasid dan garam hempedu adalah lebih tinggi daripada 58%

dan 78% masing-masing, melayakkan mereka untuk menjadi calon probiotik. Kesemua 13 pencilan menunjukkan aktiviti antimikrob terhadap bakteria patogenik yang berbeza. Antara LAB itu, *Lactococcus lactis* 2BL menunjukkan aktiviti antimikrob yang kuat terhadap 15 bakteria patogenik. 13 pencilan menunjukkan tahap yang berbeza terhadap rintangan antibiotik, bergantung kepada jenis antibiotik digunakan. Antara LAB itu, *Enterococcus avium* 7BL dan *Enterococcus durans* 6HL adalah tahan dan sensitif kepada kesemua sembilan antibiotik digunakan, masing-masing.

Kesan anti-kanser daripada 13 pencilan pada beberapa jenis sel kanser manusia, seperti HeLa, AGS, HT-29, dan MCF-7, juga dinilai. Sel normal manusia HUVEC digunakan sebagai kawalan. Sitotoksiti strain ini menunjukkan berlakunya *'apoptosis'* sel, seperti yang dinilai oleh teknik pemecahan DNA, mikroskop pendarfluor, dan pengalir sitometri. Metabolit yang dihasilkan oleh *Lactobacillus Plantarum* 5BL, *Lactobacillus acidophilus* 36YL, *Enterococcus faecalis* 16H, dan *Enterococcus lactis* 2BL menunjukkan aktiviti anti-kanser yang luar biasa terhadap sel-sel karsinoma manusia yang diuji, tetapi tiada kesan sitotoksiti yang ditunjukkan ke atas sel-sel normal HUVEC.

Akhirnya, pencilan tertakluk kepada pengkapsulan bagi meningkatkan *"bioavailability"* mereka dan kadar kelangsungan hidup di bawah persekitaran sistem pencernaan. Matriks biopolimer berasaskan herba, seperti alginat, gam arabik dan helba telah disediakan melalui teknik penyemperitan. Tiga formulasi gel, iaitu, 1, 1.5 dan 2% (w/v) alginat, 2% (w/v) alginat + 0.1 dan 0.3% (w/v) gam arabik / helba, 1.5% (w/v) alginat + 0.3 dan 0.5% (w/v) gam arabik / helba, dan 1% (w/v) alginat + 0.4 dan 0.6% (w/v) gam arabik / helba telah disediakan untuk meningkatkan kecekapan pengkapsulan ini, kelangsungan hidup dalam sistem pencernaan, dan kadar pembebasan kolon daripada pencilan. Semua bakteria yang terkandung mempamerkan penambahbaikan yang ketara ($P < 0.05$) dengan kecekapan pengkapsulan yang tinggi (>98%) dan kadar kelangsungan hidup (>75%) berbanding dengan bacteria yang tidak dikandung dalam gel matrik (>45%). Pemerbadanan polimer gam arab dan helba ke dalam pengkapsulan gel alginat turut juga dipertingkatkan. Penggunaan kombinasi gel-gel ini menghasilkan pengkapsulan baik dengan kadar kelangsungan hidup dan kestabilan penyimpanan strain probiotik dalam keadaan sistem pencernaan yang memuaskan.

Kesimpulannya, bakteria probiotik telah berjaya dipencilkan dan dicirikan dari ekosistem vagina yang sihat. Probiotik ini mempunyai sifat antimikrob, rintangan antibiotik dan anti-kanser, menjadikan mereka calon yang berpotensi untuk dibangunkan sebagai produk nutraseutikal.

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CHAPTER 1

INTRODUCTION

Lactic acid bacteria (LAB) are heterogeneous, Gram-positive, and non-spore-forming bacteria that display fermentative metabolism. These organisms naturally thrive on humans, animals, and plants. LAB are commonly utilized in the dairy and food industries because of their health-promoting and well-documented fermentative properties (Temmerman *et al.*, 2004). LAB are normal flora in the mouth, intestine, and other parts of mammals. LAB also dominate the vaginal microbiota in normal post-pubertal and pre-menopausal women (Nam *et al.*, 2007; Ehrstrom *et al.*, 2010). They share a mutual relationship with their hosts by promoting health and preventing diseases (Turnbaugh *et al.*, 2007; Ma *et al.*, 2012a). Most LAB can inhibit the growth of other bacteria by producing different metabolites, such as lactic acid, acetic acid, H_2O_2, bacteriocin, and nitric oxide (Ehrstrom *et al.*, 2010). These metabolites help prevent bacterial vaginosis, yeast infections, urinary tract infections, and sexually transmitted diseases (Ma *et al.*, 2012a).

However, a major health concern regarding LAB supplementation is the transmission of antibiotic resistance genes from LAB to unrelated pathogenic or potentially pathogenic bacteria in the gut. LAB strains that harbor antibiotic resistance plasmids are unsuitable for use as human or animal probiotics (Hart and Powell, 1990; Palmer *et al.*, 2010). Some strains of *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* carry potentially transmissible plasmid–encoded antibiotic resistance genes (Begovic *et al.*, 2009).

Among the LAB, lactobacilli are important members of vaginal bacterial communities in women of reproductive age (Ma *et al.*, 2012a). Lactobacilli maintain the vaginal ecological equilibrium by protecting against pathogenic microorganisms. They also assist in changing the physiology of the urogenital tract by modifying the normal microbial flora (Vintini *et al.*, 2004; Pascual *et al.*, 2010). They are considered non-pathogenic because of their long-standing reputation as health-promoting organisms in many fermented food products. Lactobacilli are probiotics that have been used to treat different bacterial vaginal infections (Mastromarino *et al.*, 2009). The ecological functions and effects of other LAB on the overall dynamics and functions of vaginal bacterial communities remain unclear. Recent evolutions in biotechnological and biochemical techniques have been directed to the use of LAB in the production of bulk enzymes and biopolymers or as oral delivery vehicles (Steidler, 2002; Nam *et al.*, 2007).

Probiotics are live non-pathogenic microorganisms that confer health benefits to the host when consumed in adequate amounts (Barrons and Tassone, 2008b). Probiotics, which exist in the human body as microflora, promote the intestinal microbial balance

(Zhang *et al.*, 2012). Probiotics can adhere to epithelial cells and tolerate high pH and bile levels in the gastrointestinal tract. Acid tolerance is one of the desirable properties used during the screening of potential probiotic strains. The competition of commensal and probiotic bacteria with pathogens to adhere and colonize is an important mechanism in resisting intestinal pathogens and preventing diseases. Probiotic bacteria produce various compounds or metabolites that inhibit pathogen growth. These metabolites include organic acids (lactic and acetic acids), bacteriocin, and reuterin (Alvarez-Olmos and Oberhelman, 2001). Organic acids affect pathogen growth and elicit toxic effects on microbes by reducing pH. Not all LAB have probiotic properties that control or prevent infectious diseases (Colodner *et al.*, 2003; Reid and Bruce, 2003). Disruption and damage of the normal genital microflora, particularly *Lactobacillus* species, lead to the increase of genital infections (Reid, 2002; Pascual *et al.*, 2008a; Pascual *et al.*, 2008b). The *in vitro* attachment of pathogenic bacteria can be blocked by the normal vaginal flora of healthy females (Pascual *et al.*, 2010). Lactobacilli produce bacteriocins, such as acidophilin, acidolin, lactocidin, bulgarican, lactolin, lactobacillin, and lactobrevin (Dimitonova *et al.*, 2007; Pan *et al.*, 2009). Many *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* species are safe to use for the prevention or treatment of various infectious diseases (Lee *et al.*, 2003).

Probiotic strains should not carry transmissible antibiotic resistance genes. Probiotics may become hosts of antibiotic resistance genes that can be transferred to pathogenic bacteria. Ingestion of bacteria carrying such genes is undesirable because horizontal gene transfer to recipient bacteria in the gut can lead to the development of new antibiotic-resistant pathogens (Zhou *et al.*, 2005).

Cancer is one of the main causes of human deaths. Chemotherapy and chemoprevention are applied to control metastasis and decrease human mortality (Miranda *et al.*, 1999). Probiotics also exhibit anticancer activities against different carcinoma cells, such as colon, bladder, and breast (de Blanc and Bonet, 2010). Most studies focused on the anticancer effect of probiotics on colon carcinoma cells (Rafter, 2004). Studies have discovered that some specific strains of LAB can induce the production of pro-inflammatory and anti-inflammatory cytokines in animal or human body. Evaluation of the toxicity of bacterial cytotoxic agents to different cell types is performed by *in vitro* cytotoxicity tests and by analyzing the mechanism of apoptosis in treated cancer cells (Hsu *et al.*, 2005). Induction of apoptosis, which can be applied to control cancer development, involves complex anticancer activity of several therapeutic substances (Li *et al.*, 2005). Although various methods are available for identification, quantification, and characterization of apoptosis, flow cytometry is the most common choice to study apoptosis because of its applicability to an extensive range of cell types, stimulants, and time. Evaluating apoptosis by flow cytometry provides information on symptoms and incidence of apoptosis that is interpreted as a sign. Besides, cellular morphological characteristic is another method to determine the cell death mode (apoptosis or necrosis).

In the present study, effective probiotic strains were isolated from the vaginas of Iranian women. The anticancer effects of the isolates on different human cancer cell lines were evaluated through *in vitro* cytotoxic and apoptotic analyses.

Encapsulation is the process of entrapping bioactive molecules (e.g., vitamins, minerals, antioxidants, fatty acid, lutein, and lycopene) and living cells (e.g., probiotics) within carrier materials (Nedovic *et al*. 2011). Encapsulation is the most effective technique to protect probiotic bacteria during processing and storage (Kanmani *et al*. 2011). Improving the delivery of these active agents into foods and medicines is important and many substances can be used for encapsulation. Encapsulation materials are selected based on the following criteria: functionality, stability, type of release, encapsulates concentration, and cost. In addition, carrier materials must be biodegradable, biocompatible, food grade, and capable of barrier formation (Nedovic *et al*. 2011).

Encapsulation causes the production of a physical barrier between the internal phase and its surrounding to protect them against pH alterations, moisture variations, and oxidation; thus, this process controls the release of active molecules and increases their bioavailability (Dubey *et al*. 2009). The most significant aim for the encapsulation of active agents is to make available improved stability in final products and during processing.

The most common encapsulation material is sodium alginate because of its simplicity, biocompatibility, non-toxicity and low cost (Krasaekoopt et al., 2003). In addition, alginate microspheres with porous structure allow diffusion of acid in and out of microspheres easily. These disadvantages can be effectively overcomed by blending alginate with other polymers or coating one polymer layer on alginate microspheres (Krasaekoopt *et al*., 2003; Burgain *et al*., 2011). New biopolymers for encapsulation proposes have been recently reported (Dickinson 2003; Mahfoudhi *et al*. 2014). Gum exudates are predominantly composed of polysaccharides that function as stabilizing and emulsifying agents (Orozco-Villafuerte *et al*. 2003). Gum Arabic (GA) has the highest commercial value among the gum exudates because of its extensive application in the pharmaceutical, food, and cosmetic industries (Mahfoudhi et al. 2014).

Psyllium (PSY), an arabinoxylan herbal-based biopolymer, extracts from Plantago species. PSY can stimulate the growth of probiotic bacteria in the gastrointestinal tract (GIT) and treat several gut disorders, including ulcerative colitis, chronic kidney, constipation, and diarrhea (Guo *et al*. 2008; Rishniw and Wynn, 2011).

Studies on the isolation of probiotic bacteria from the vaginas of healthy Iranian women and on the evaluation of their efficacy on pathogenic bacteria and different cancers are lacking. In addition, the probiotic activities of LAB isolated from the vagina have yet to be assessed. Accordingly, the principal objectives of this study were:

1. To isolate and identify LAB from the vaginas of healthy Iranian women using different molecular fingerprinting methods and evaluation of the isolated LAB for probiotic characteristics: low pH resistance, high bile salt tolerance, antimicrobial activity and antibiotic susceptibility.

2. To evaluate the *in vitro* cytotoxic assay and morphological apoptosis assessment of probiotic secretions on different cancerous and normal cell lines using MTT, fluorescent microscopy, DAPI staining and flow cytometry assays.

3. To enhance the survival and bioavailability of probiotic bacteria under gastrointestinal conditions by using microencapsulation with alginate, psyllium and Gum Arabic by extrusion method.

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