UNIVERSITI PUTRA MALAYSIA

MOLECULAR IDENTIFICATION, PROBIOTIC CHARACTERIZATION, ANTICANCER ASSESSMENT, AND ENCAPSULATION OF LACTIC AND ACETIC ACID BACTERIA ISOLATED FROM IRANIAN TRADITIONAL DAIRY PRODUCTS

BABAK HAGHSHENAS

IB 2015 41

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By

BABAK HAGHSHENAS

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

March 2015

DEDICATIONS

I wish to dedicate this work to my father who passed away during my study.

God bless his soul.

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

MOLECULAR IDENTIFICATION, PROBIOTIC CHARACTERIZATION, ANTICANCER ASSESSMENT, AND ENCAPSULATION OF LACTIC AND ACETIC ACID BACTERIA ISOLATED FROM IRANIAN TRADITIONAL DAIRY PRODUCTS

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March 2015

Chairman: Associate Professor Norhafizah Abdullah, PhD

Faculty: Institute of Bioscience

Lactic and acetic acid bacteria (LAB and AAB) are generally recognized as safe microorganisms. Their increasing demand in food industry has motivate this present study to perform molecular identification, *in vitro* characterization, anticancer assessment and microencapsulation of probiotics from traditional dairy products produced in the west of Iran region. Cancer epidemiology showed lower cases recorded in this region compared with other areas in Iran which can be associated with the daily dietary intake of people living in these areas. Dairy products made from the west of Iran employs traditional methods involving no antibiotics in the animal feed and often contained higher acidity compared with commercial dairy products. Probiotic bioactive compounds have fewer side effects with low-cost when use in chemotherapy. Thus, the identification of probiotic bacteria exhibiting anticancer property isolated from dairy products for medicinal purposes is highly desirable.

The isolated bacteria from 5 types of traditional dairy products were identified and clustered into 5 genera (*Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Enterococcus*, and *Acetobacter*) with 12 species, 4 subspecies, and 27 strains using combination of different molecular techniques (namely 16S rRNA sequencing, $(GTG)_5$-PCR, and ARDRA).*In vitro* characterization showed 11 isolates displaying high anti-pathogenic activity and antibiotic susceptibility. Their survival rates in the harsh gastrointestinal conditions were also high, calculated at >70%.

Cytotoxic assays indicated that extracted metabolites from *Acetobactersyzygii*38Lac, *Enterococcus durans* 39C, and *Lactococcus lactis subsp. lactis* 44Lac displayed high anticancer activity against AGS, MCF-7, HT29, and HeLa cancer cell lines but not on normal cell lines. Pronase assay revealed that proteins contained in the metabolites play a key role in anticancer properties of these isolates. Incidence of apoptosis was detected

in the isolates on the basis of the morphological assessments via fluorescent microscope and flow cytometry.
To improve delivery and prolong the survival rate of probiotic in the gastrointestinal tract, encapsulation approach was investigated using a simple extrusion method. Nine formulations using different blend of alginate with pysllium or fenugreek were designed and investigated aiming at improving the encapsulation efficiency. Results showed 2% (w/v) alginate, 1.5% (w/v) alginate +0.5% (w/v) psyllium, and 1.5% (w/v) alginate + 0.5% (w/v) fenugreek formulations, with >98% encapsulation efficiency, high stability during 28 days of storage and displayed high survival rates in harsh gastrointestinal conditions (>80 %), which were significantly ($P < 0.01$) higher than the non-encapsulated bacteria (>62%). Incorporation of 0.5% (w/v) fenugreek and psyllium into 1.5% (w/v) alginate gel resulted in enhanced encapsulation properties. For psyllium-alginate formulation, additional properties such as high growth-stimulating effects of probiotic and smaller bead sizes were observed.

Acetobacter strains were isolated for the first time from traditional dairy products, which is a new finding of this study. Their extracted metabolites displayed high cytotoxicity effects on various cancer cell lines comparable to commercial cancer drug, Taxol. Development of a new encapsulated probiotic formulation using fenugreek-alginate blend was also a success. The formulation showed significant improvement in the delivery of probiotic in gastrointestinal system.

In conclusion, probiotic bacteria were successfully isolated and characterized from Iranian traditional dairy products. These probiotics exhibited antimicrobial activity, antibiotic resistance and anticancer activity thus these make bacteria potential candidates for the development of nutraceutical products. In addition, the study demonstrates the improved survival and delivery of probiotics in the gastrointestinal conditions system by using encapsulation technique.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGENALAN MOLEKUL, PENCIRIAN PROBIOTIK, PENILAIAN ANTI KANSER, DAN PENGKAPSULAN BAKTERIA ASID LAKTIK DAN ASID ASETIK YANG DIASINGKAN DARIPADA PRODUK TENUSU TRADISIONAL IRAN

Oleh

BABAK HAGHSHENAS

Mac 2015

Pengerusi: Profesor Madya Norhafizah Abdullah, PhD

Fakulti: Institut Biosains

Bakteria asid laktik dan asid asetik (LAB dan AAB) secara umumnya dikenali sebagai mikroorganisma yang selamat. Permintaan yang semakin meningkat dalam industri makanan mendorong penyelidikan ini dijalankan bagi pengenalpastian secara molekul, pencirian *in vitro*, penilaian anti kanser dan pemikrokapsulan probiotik daripada produk tenusu tradisional yang dihasilkan di kawasan barat Iran. Epidemiologi penyakit kanser menunjukkan jumlah kes yang lebih rendah telah dicatatkan di rantau ini berbanding kawasan lain di Iran, di mana ia boleh berhubungkaitkan dengan diet pemakanan harian penduduk di kawasan ini. Produk tenusu dari kawasan ini dihasilkan dengan menggunakan kaedah tradisional yang tidak melibatkan penggunaan antibiotik dalam makanan haiwan dan mempunyai keasidan yang lebih tinggi berbanding produk-produk tenusu komersial. Sebatian bioaktif probiotik mempunyai kesan sampingan yang kurang dengan kos rendah apabila digunakan dalam kemoterapi. Oleh itu, pengenalpastian bakteria probiotik mempamerkan sifat antikanser yang dipencil dari produk tenusu untuk tujuan perubatan adalah sangat diperlukan.

Bakteria yang dipencilkan daripada lima jenis hasil tenusu tradisional telah berjaya dikenal pasti dan dikelompokkan kepada lima genera (*Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Enterococcus,* dan *Acetobacter*) dengan 12 spesis, 4 subspesis dan 27 strain dengan menggunakan kombinasi teknik molekul yang berbeza (penjujukan 16S rRNA, $(GTA)_5$-PCR, dan ARDRA). Pencirian *in* vitro telah menunjukkan 11 pencilan yang mempunyai sifat anti patogen yang tinggi dan rentangan terhadap antibiotik.

Pengasaian sitotoksik terhadap metabolit yang diekstrak dari *Acetobacter syzygii* 38Lac, *Enterococcus durans* 39C dan *Lactococcus lactis subsp. lactis* 44Lac menunjukkan aktiviti anti kanser yang tinggi terhadap sel-sel kanser AGS, *MCF-7*, HT29 dan HeLa tetapi tiada kesan ke atas sel normal. Pengasaian pronase telah menunjukkan bahawa mekanisme anti-kanser adalah berpunca daripada kandungan protin yang terkandung dalam metabolit yang dihasilkan oleh pencilan-pencilan

tersebut. Kejadian apoptosis telah dikenalpasti berlaku di dalam pencilan-pencilan ini, dengan berdasarkan ke atas penilaian morfologi melalui mikroskop pendarfluor dan teknik pengalir sitometri.

Untuk meningkatkan penyampaian dan kelangsungan hidup probiotik dalam alam persekitaran saluran pencernaan, teknik pengkapsulan telah dijalankan melalui kaedah penyemperitan yang mudah. Sebanyak 9 jenis formulasi gel menggunakan kepekatan yang berbeza antara alginat dengan psyllium atau halba telah direka dan dinilai demi menghasilkan peningkatan pengkapsulan yang efisen. Keputusan menunjukkan 2% (w/v) alginat, 1.5% (w/v) alginat + 0.5 (w/v) psyllium, dan 1.5% (w/v) alginate + 0.5% (w/v) halba telah menghasilkan pengkapsulan yang efisen sebanyak >98%, kestabilan yang tinggi semasa 28 hari penyimpanan dan memaparkan kadar hidup yang tinggi dalam alam persekiaran saluran pencernaan yang mencabar (>80%), dengan signifikan yang tinggi (P<0.01) berbanding dengan probiotik yang tidak dikapsulkan (<62%). Pengabungan 0.5% (w/v) halba dan psyllium ke dalam 1.5% (w/v) gel alginat menghasilkan penambahbaikan terhadap ciri-ciri pengkapsulan. Bagi formulasi pysllium-alginat, ciri tambahan iaitu penambahan kesan stimulasi terhadap pertumbuhan probiotik dan saiz kapsul yang kecil telah terhasil.

Penemuan baharu daripada kajian ini ialah strain *Acetobacter* telah berjaya diasingkan buat kali pertama daripada produk tenusu tradisional. Metabolit yang diekstrak daripada strain ini menunjukkan sifat sitotoksiti tinggi terhadap pelbagai sel kanser setanding dengan ubat kanser komersial, Taxol. Pengkapsulan dengan gabungan halba-alginat sebagai matriks baru telah diformulasi dengn jayanya. Formulasi produk ini menunjukkan peningkatan yang ketara dalam penghantaran probiotik di dalam sistem pencernaan.

Kesimpulannya, bakteria probiotik telah berjaya dipencilkan dan dicirikan dari produk tenusu tradisional dari negara Iran. Probiotik yang dipencilkan mempunyai sifat antimikrob, rintangan antibiotik dan anti-kanser, seterusnya menjadikan mereka calon yang berpotensi untuk dibangunkan sebagai produk nutraseutikal. Di samping itu, kajian ini telah menunjukkan penambahbaikan kelangsungan hidup dan penghantaran probiotik dalam sistem saluran pencernaan dengan mengadaptasi teknik pengkapsulan.

ACKNOWLEDGEMENTS

At the end of this step of my graduate period has allowed for a fragment of reflection, and the many dears who have contributed to both, my work, and my life during of this period of time, because this research project would not have been possible without the support of many people. First and foremost, I wish to express my full thanks and sincere gratitude to my dear supervisor, Dr. Norhafizah Abdullah for their invaluable suggestions, beneficial advices and their endless supports. My sincere gratitude also goes to Prof. Dr. Rozita Rosli and Dr. Dayang Radiah Awang Biak, who served on my thesis committee and kindly provided valuable advice and suggestions for this work and my thesis. I would like to acknowledge and have my best regards to Dr. Ahmad Yari Khosroushahi for their friendly collaboration during of my experimental research. I also would like to acknowledge Mr. Abolfazl Barzegari in Tabriz University and Mr. Yousef Nami in Universiti Putra Malaysia or their invaluable helps. Last but not least I wish to avail myself of this opportunity, express a sense of gratitude and love to my friends and my beloved parents for their support, strength, and help for everything during of my life. I wish to acknowledge my gratitude to all lecturers, officials and other staff members in Universiti Putra Malaysia.

Babak Haghshenas
March 2015

I certify that a Thesis Examination Committee has met on 11 March 2015 to conduct the final examination of Babak Haghshenas on his thesis entitled "Molecular Identification, Probiotic Characterization, Anticancer Assessment, and Encapsulation of Lactic and Acetic Acid Bacteria Isolated from Iranian Traditional Dairy Products" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percent
4T1	Mouse breast cancer cell line
AAB	Acetic acid bacteria
AFLP	Amplified Fragment Length Polymorphism
AGS	Human gastric epithelial cancer cell line
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ARE	Antibiotic-Resistant Enterococci
ATCC	America Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CAP	Cellulose Acetate Phthalate
CFU	Colony forming unit
Co_2	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diaminetetraacetic acid
EFSA	European Food Safety Authority
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus PCR
FAO	Food and Agricultural Organization of the United States
FBS	Fetal bovine serum
FISH	Fluorescent In Situ Hybridization
G+C	Guanine plus cytosine
GTG-PCR	Polymerase chain reaction by GTG primer
HeLa	Human cervix cancer cell line
HT29	Human colon cancer cell line
g	Gravity force
g	Gram
GTE	Glucose-Tris-EDTA
h	Hour
HCL	Hydrochloric acid
ITS	Intergenic spacer region
kb	Kilo base pair
kDa	Kilo Dalton
L929	Mouse fibroblast cell line
LAB	Lactic acid bacteria
Lb	Lactobacillus
Lc	Lactococcus
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
M	Molar
MgCl2	Magnesium chloride
min	Minute
Mm	Milimolar
mg	Miligram
mL	Mililiter

mm	Milimeter
MRS	Man Rogosa and Sharpe
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide
MYP	Mannitol-Egg-yolk-Polymyxine
N	Normality
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
nm	Nanometer
NTSYS-PC	Numerical Taxonomy Analysis Program Package
PBS	Potassium buffered saline
PCR	polymerase Polymerase chain reaction
PFGE	Pulsed-Field Gel Electrophoresis
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
REA	Restriction enzyme analysis
Rep-PCR	Repetitive Extragenic Palindromes-PCR
rRNA	Ribosomal ribonucleic acid
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Round per minute
sdH2O	Sterile distilled water
S	Streptococcus
SE	Standard error
sp.	Species
subsp.	Subspecies
TAE	Tris-acetate-EDTA
TGGE	Temperature Gradient Gel Electrophoresis
tRNA	Transfer RNA
TUNEL	Terminal deoxynucleotidyltransferase mediated dUTP
UPGMA	Unweighted Pair- Group Method by Arithmetic Average
V	Volt
v/v	Volume per volume
VRE	Vancomycin-Resistant Enterococci
w/v	Weight per volume
µg	Microgram
µL	Microliter
µg/mL	Microgram per milliliter
µM	Micromolar
XG	Xanthan Gum

CHAPTER 1

INTRODUCTION

1.1 Background

The term of "probiotics" was first introduced by Lilly and Stillwell as the microorganism's active factors stimulating other microorganism's growth and possessing the beneficial impacts on the host health when they are consumed in the sufficient amounts (Guarner*et al.*, 2008; Lilly and Stillwell, 1965). Probiotics significantly affect the bioavailability of nutrients in the human body (Youngand Huffman, 2003) by facilitating the absorption of magnesium and calcium from milk proteins, digesting lactose, and producing folate and B vitamins. Probiotics also prevent irritable bowel syndrome (Barouei *et al.*, 2009; Parkes, 2010) and inflammatory effects on bowel disease (Geier *et al.*, 2007). These microorganisms also control antibiotic-related diarrhea (Friedman, 2012), elicit protective effects on vaginal infections (Reid and Bocking, 2003), and decrease cholesterol levels (Guarner *et al.*, 2008). As anti-cancer agents, probiotics can inactivate pro-carcinogenic compounds, particularly in colon cancer (Serban, 2013; Zhu*et al.*, 2011).

The majority of probiotics include lactic acid bacteria (LAB),(Guarner *et al.*, 2008)but isolated acetic acid bacteria (AAB) from safe sources, such as fermented dairy products, also can be introduced as probiotics because of their strong fermentation and acidification activities and vitamin C production (González and Mas, 2011). The LAB including the genera such as *Oenococcus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*and *Pediococcus*. Among the LAB, the*Lactobacillus* and *Enterococcus*species are commonly consumed as probiotics (Biradar *et al.*, 2005; Giraffa *et al.*, 2010; Guarner *et al.*, 2008). On the other hand, *Acetobacter* is one the most common genera with commercial importance in the AAB group (Sengun and Karabiyikli, 2011). *Acetobacter* species, particularly *A. aceti* and *A. syzygii*, show the industrial importance and can be introduced as probiotic (Cleenwerck and De Vos, 2008; De Vuyst*et al.*, 2008; Gullo *et al.*, 2006; Lefeber *et al.*, 2011; Sengun and Karabiyikli, 2011).

The microbiota of traditional dairy products is supposed to be dominated by *Lactobacillus* and *Lactococcus*species(Kore *et al.*, 2012; Tabatabaee *et al.*, 2012; Tafvizi and Tajabadi, 2012; Veera Jothi *et al.*, 2012). But, the biodiversity of LAB species in fermented dairy products is variable and region specific. For example, in Armada cheese (Spanish goat's cheese); the predominant *Lactobacilli* are *L. casei ssp. casei*, *L. plantarum* and, *L. brevis* (Herreros *et al.*, 2003). But, in Batzos cheese (traditional Greek goat cheese), *L. paracasei spp. tolerans*, *L. plantarum*, and *L. sake* are the dominant species (Psoni *et al.*, 2003). Meanwhile, in Minas Frescal cheese (Brazilian fresh cheese) the predominant species is *L. acidophilus* (Lollo *et al.*, 2012).

Probiotics are an important part of the complex world of health-promoting food. The best-known example of which is yogurt. Bacteria found in traditional dairy products, such as yogurt, kefir, and fermented milk, are usually not found in the human intestines. These bacteria do not colonize the intestine, but are flushed and eliminated quickly from the body. Therefore, the introduction of potential probiotic cells from

native food sources is a crucial step to determine their efficacy. The diverse climate in Kermanshah Province, Iran particularly in rural areas, allows the villagers to produce different traditional dairy products. These products can be the ideal dairy source for the selection of new potential probiotic bacteria because of their production methods in semi-wild conditions and use of raw milk from cows and sheep.

1.2 Research Problems

Fermented dairy products such as curd, tarkhineh, shiraz, yogurt and cheese in the west of Iran (Kermanshah Province) can be explore for the ideal dairy source in the selection of new potential probiotic bacteria. Thedifferent production methods in semi-wild conditions and the use of raw milk from cows or sheep provide the advantage on the selection. The drying time and fermented processes are different among these dairy products. Moreover, they have various acidity values. Therefore, new strains with high probiotic capability can be isolated from these products.

The beneficial properties of probiotic bacteria are strain specific, and a majority of them belong to the lactic acid bacteria (LAB) group, which requires same growth conditions and nutrition (Dubernet *et al.*, 2002). Identification and differentiation up to the genus level between these closely related bacteria through traditional phenotypic and biochemical methods, such as sugar fermentation, is impossible and does not provide clear results (Gevers *et al.*, 2001). Recently, rapid, accurate, and practical molecular identification techniques, such as repetitive sequence-based PCR ($(GTG)_5$-PCR) (Gevers*et al.*, 2001), specific gene sequencing (Vaugien *et al.*, 2002), 16S rRNA sequencing (Gueimonde *et al.*, 2004), ribotyping with specific probes (Singh *et al.*, 2009), random amplified polymorphic DNA (RAPD)(Singh*et al.*, 2009), and amplified ribosomal DNA restriction analysis (ARDRA) (Satokari *et al.*, 2003), have been designed (Singh*et al.*, 2009; Ward and Roy, 2005). However, these methods individually do not create strong results for differentiation up to the strain levels. Thus, an effective combination must be considered.

A probiotic must have a number of characteristics to be effective in improving host health, such as resistance to gastrointestinal conditions, antipathogen activities, and high susceptibility to antibiotics (Biradar *et al.*, 2005; Surawicz, 2003). Most probiotics are sensitive to low acid or high bile salt conditions in the digestive system and are eliminated after consumption. Probiotics also carry antibiotic-resistant genes, which if transferred to other probiotics or pathogenic bacteria, can create high resistance to a wide variety of antibiotics (Mathur and Singh, 2005). Traditional dairy products from the west of Iran are prepared based on the traditional methods employed in this region, wherein no antibiotics are used in animal food and higher acidity of products compared with commercial dairy products. In this regard, new strains with high probiotic capability can be isolated from these products.

Although, several effects that are beneficial to health have been attributed to probiotic bacteria, the most interesting and controversial property remains to be anticancer activity (de LeBlanc *et al.*, 2010). The vast majority of studies in this area deal with protective effects against colon cancer (Rafter, 2003). A wealth of indirect evidence based largely on laboratory studies is available regarding the anticancer effects of probiotic bacteria, which may be divided into the following categories: *in vitro* studies, animal studies, epidemiological studies, and human dietary intervention studies. The

mechanisms by which probiotics may inhibit different cancer cell lines are still poorly understood. However, several potential mechanisms will be discussed in the literature. Cancer epidemiology showed lower cases recorded in rural areas of this region (Kermanshah Province), compared with other areas in Iran (Mirmomeni *et al.*, 2009; Najafi *et al.*, 2011). This difference could be related to the daily dietary intake of the areas. Through *in vitro* cytotoxic and apoptotic studies in different human cancer cell lines, effective strains can be isolated, and their anticancer mechanisms can be investigated.

Probiotic microorganisms show low survival rates in dairy products and the gastrointestinal tract (Vidhyalakshmi *et al.*, 2009). A vast variety of coating materials (e.g., starch, vegetable gums, gelatins, fats, and glyceride derivatives) can be applied using different microencapsulation methods (e.g., freeze drying (Heidebach *et al.*, 2010; Otero *et al.*, 2007), spray drying (Avila-Reyes *et al.*, 2013; Salar-Behzadi *et al.*, 2013), gel formation (Vidhyalakshmi *et al.*, 2009), co-extrusion (Shinde *et al.*, 2013), extrusion (Khan *et al.*, 2013; Lotfipour *et al.*, 2012), emulsion (Mantzouridou *et al.*, 2012), fluid bed processing (Stummer *et al.*, 2010; Stummer *et al.*, 2012), and matrix entrapment (Reyed, 2006)to increase the stability of probiotic bacteria. In addition, microencapsulation is now developed to lend protection against unwanted conditions, as well as to increase tolerance against strong acids and undesirable reactions upon storage. Therefore, this method is a novel and interesting technology in the probiotics market (Sohail *et al.*, 2011). Herbal gels, such as fenugreek and psyllium, which are cheaper than alginate and have been used for the treatment of gastrointestinal disease, have been demonstrated to be suitable candidates for encapsulation of probiotic bacteria.

1.3 Objectives

The objectives of this study are:

1) To isolate, identify and cluster the probiotic strains from five types of traditional Iranian dairy products using combination of 3 molecular methods, which are 16S rRNA sequencing, $(GTG)_5$-PCR and ARDRA.

2) To characterize and assess the acid and bile tolerance, anti-microbial potential and antibiotic susceptibility of the isolated probiotic strains.

3) To investigate the *in vitro* cytotoxic assay and morphological apoptosis assessment of probiotic metabolites on cancer and normal cell lines by MTT assay, fluorescent microscopy and flow cytometry.

4) To develop a delivery system on the best candidate of probiotic strains using microencapsulation method with alginate, psyllium, and fenugreek gels by extrusion technique and characterize its probiotics performance in acid, bile and storage time.

1.4 Scope of Work

The use of probiotic products as medical supplements has successfully spread throughout the world especially in Europe and Asia. *Lactobacillus* species are the most

famous group of probiotics which are isolated from fermented dairy products. Other probiotics isolated from dairy products are *Biiobacteria*, *Bacillus*, *Streptococci*, some strains of *E. coli*, and yeast (*Saccharomyces cerevisiae*) (Biradar *et al*., 2005; Guarner *et al*., 2008).Recent discoveries on the benefits of probiotic populations on human health motivate further interest on probiotic communities and their functions (Moore *et al.*, 2011).For this study, 200 samples were collected randomly from 5 traditional dairy products, including cheese, yogurt, curd, shiraz, and tarkhineh, from retailers in different parts of Kermanshah Province (west of Iran). Samples of traditional dairy products were obtained across five wide area of Kermanshah province, Iran. Random clustering was chosen as a suitable method for their selection. According to this method, the area was divided to five sections (North, South, East, West and center). In each section, 2 villages and in each village 4 rancher families were chosen randomly for sampling the products.

The lactic and acetic acid bacteria were isolated from traditional dairy products for the first time in the west of Iran (Kermanshah province) by the combination of three molecular techniques ($(GTG)_5$-PCR, 16S rRNA sequencing and ARDRA). *Acetobacter* species normally are isolated from ethanol riche sources such as wine and beers. But, for the first time, *Acetobacter* strains were isolated and identified from dairy products.

The most of researches and studies on probiotics have been focused on their anti-colorectal properties (Commane *et al*., 2005; de LeBlanc *et al*., 2010; Rafter, 2003).But, the extracted metabolites of isolated bacteria displayed the high cytotoxic effects on various cancer cell lines same as Taxol.

The fenugreek polymer exhibits several prebiotic effects and has been utilized in animal husbandry and nutritional supplements (Brummer *et al.*, 2003). This gum has health-promoting effects and has been prescribed by many practitioners for the treatment of diabetes and for the regulation of cholesterol level (Brummer *et al.*, 2003). In addition, its stability and emulsifying activity (Chang *et al.*, 2011; Işıklı and Karababa, 2005) make this gel a suitable stabilizer for the food industry. In this study, fenugreek for the first time was blended with the alginate polymer as a matrix for probiotic formulation. This polymer offers added advantages of being prebiotic for the enhancement of probiotic bacterial growth in the gastrointestinal environment. Hence, its incorporation with alginate can be introduced as new and unique delivery carriers for the oral administration of probiotics. The flowchart of the study is presented in Figure 1.1.

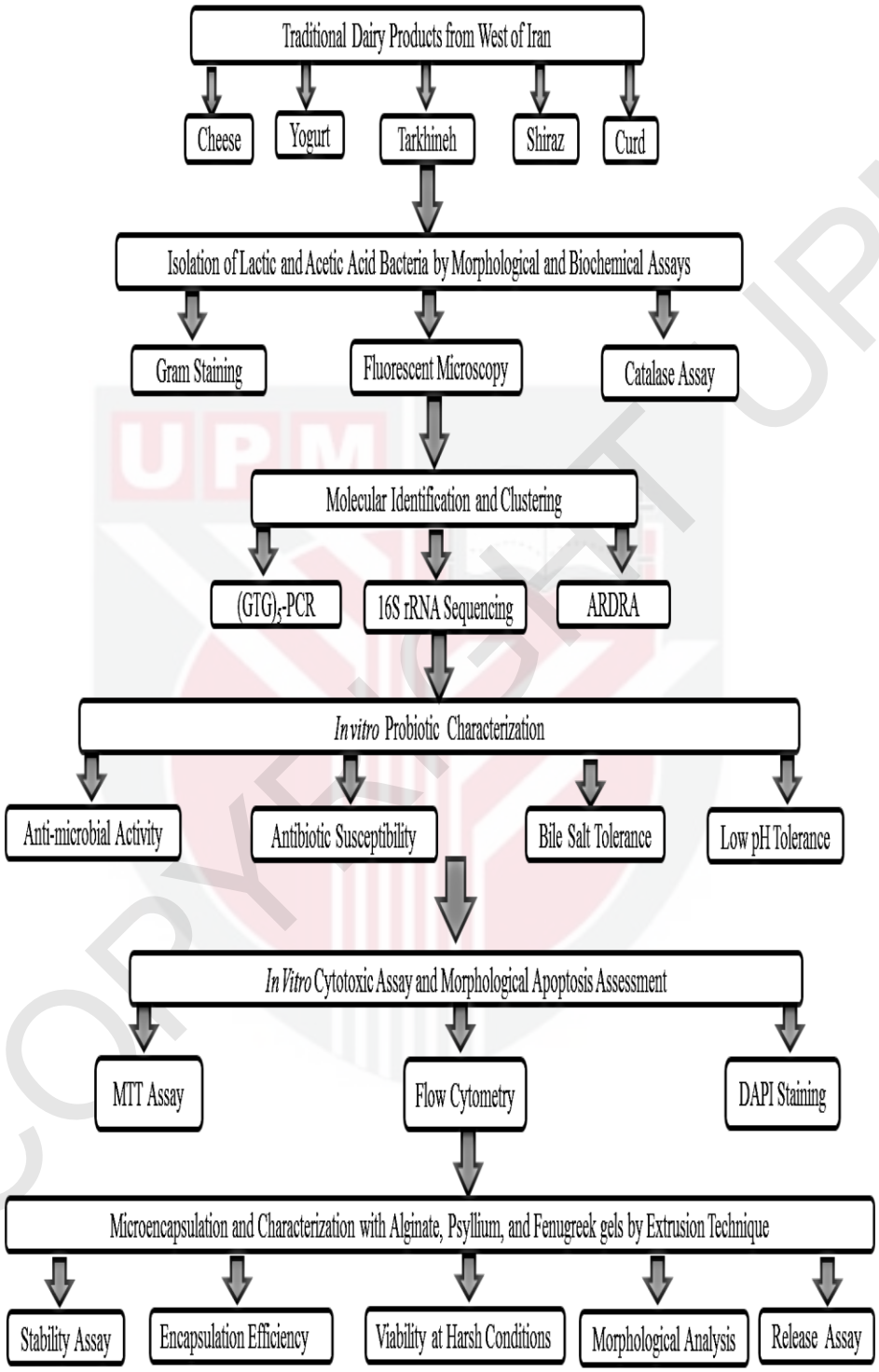

Figure1.1. Schematic diagram represented the flowchart of the study in this thesis.

1.5 Thesis Layout

In the first chapter, samples from traditional dairy products were collected to isolate probiotic bacteria belonging to LAB and AAB groups. To discover new strains, identification and discrimination of isolated bacteria were performed up to the strain levels by combining three molecular methods (i.e., 16S rRNA sequence analysis, ARDRA, and $(GTG)_5$-PCR banding patterns comparison).

In the second chapter, some probiotic characteristics of the isolated strains, such as tolerance to extra bile salts and low acidic conditions, antibiotic susceptibility, and antimicrobial activity against the human pathogens, were investigated by *in vitro* assessments.

In the third chapter, MTT assay was employed to assess the anticancer property of the extracted metabolites from candidate probiotic strains by using different human cancer and normal cell lines. The results were compared with untreated (negative control) and Taxol-treated cells lines (positive controls). The anticancer mechanisms of the probiotics were characterized by comparing the normal treated with pronase-treated, untreated, and Taxol-treated cells. In addition, some morphological apoptosis characteristics, such as membrane blebbing, disruption of membrane integrity, and DNA fragmentation, were assessed using fluorescent microscopy and flow cytometry.

In the last chapter, two effective probiotic strains (*E. durans* 39C and *L. plantarum* 15HN), which displayed low viability in harsh gastric conditions, were encapsulated through extrusion method with supporting materials including alginate, psyllium, and fenugreek. Morphological characteristics and encapsulation properties were assessed and analyzed using PSWE Statistics 18 software.

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LIST OF PUBLICATIONS

1. Babak Haghshenas, Yousef Nami, Norhafizah Abdullah, Dayang Radiah, Rozita Rosli, Ahmad Yari Khosroushahi (2014). Anti-proliferative effects of *Enterococcus* strains isolated from fermented dairy products on different cancer cell lines. Journal of Functional Foods, 11, 363–374.

2. Babak Haghshenas, Yousef Nami, Norhafizah Abdullah, Dayang Radiah, Rozita Rosli, Ahmad Yari Khosroushahi (2015). Anticancer impacts of potentially probiotic acetic acid bacteria isolated from traditional dairy microbiota. LWT - Food Science and Technology, 60, 690-697.

3. Babak Haghshenas, Yousef Nami, Norhafizah Abdullah, Dayang Radiah, Rozita Rosli, Ahmad Yari Khosroushahi (2014). Potentially probiotic acetic acid bacteria isolation and identification from traditional dairies microbiota.International Journal of Food Science and Technology, 50(4), 1056-1064.

4. Babak Haghshenas, Norhafizah Abdullah, Yousef Nami, Dayang Radiah, Rozita Rosli, Ahmad Yari Khosroushahi (2014). Different effects of two newly-isolated probiotic *Lactobacillus plantarum* 15HN and *Lactococcus lactis* subsp. *Lactis* 44Lac strains from traditional dairy products on cancer cell lines. Anaerobe, 30, 51-59.

5. Babak Haghshenas, Norhafizah Abdullah, Yousef Nami, Dayang Radiah, Rozita Rosli, Ahmad Yari Khosroushahi (2015). Microencapsulation of probiotic bacteria *Lactobacillus plantarum* 15HN using alginate-psyllium-fenugreek polymeric blends. Journal of Applied Microbiology, 118(4), 1048-1057.

6. Yousef Nami, Babak Haghshenas, Norhafizah Abdullah, Dayang Radiah, Rozita Rosli, Ahmad Yari Khosroushahi (2014). Probiotics or antibiotics: future challenges in medicine. Journal of Medical Microbiology, 64(2), 137-146.

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