11-2022

LACTIC ACID BACTERIA ISOLATED FROM FRESH VEGETABLE PRODUCTS: POTENTIAL PROBIOTIC AND POSTBIOTIC CHARACTERISTICS INCLUDING IMMUNOMODULATORY EFFECTS

Fatima Khaled Alameri

UAEU
جامعة الإمارات العربية المتحدة
United Arab Emirates University

MASTER THESIS NO. 2022: 69

College of Agriculture and Veterinary Medicine

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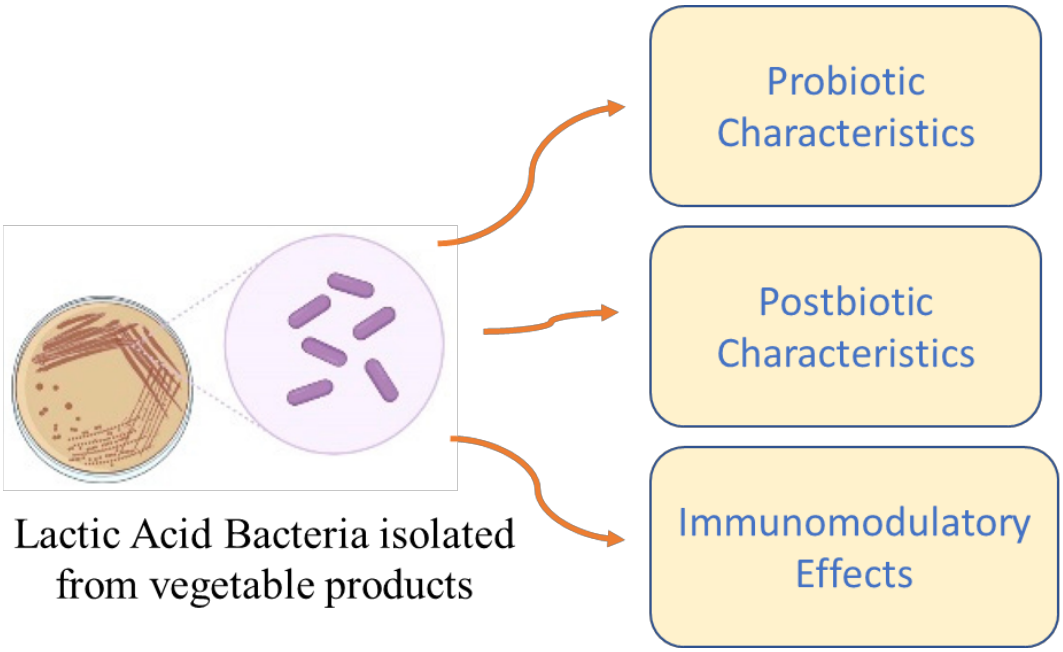

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Food Science

November 2022

United Arab Emirates University Master Thesis
2022: 69

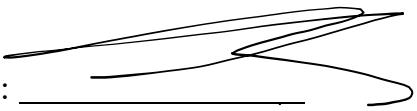
Cover: Lactic Acid Bacteria Isolated from Fresh Vegetable Products: Potential Probiotic And Postbiotic Characteristics Including Immunomodulatory Effects

(Photo: By Fatima Khaled Alameri)

Print: University Print Service, UAEU 2022

Declaration of Original Work

I, Fatima Khaled Alameri, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Lactic Acid Bacteria Isolated from Fresh Vegetable Products: Potential Probiotic and Postbiotic Characteristics Including Immunomodulatory Effects*", hereby, solemnly declare that this is the original research work done by me under the supervision of Dr. Mutamed M. Ayyash, in the College of Agriculture and Veterinary Medicine at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature: 

Date: ___25/11/2022_____

Advisory Committee

1) Advisor: Dr. Mutamed M. Ayyash

Title: Associate Professor

Department of Food Science

College of Agriculture and Veterinary Medicine

2) Co-Advisor: Dr. Raman Bhaskaracharya

Title: Associate Professor

Department of Food Science

College of Agriculture and Veterinary Medicine

3) Co-Advisor: Prof. Shao Quan Liu

Title: Professor

Department of Food Science and Technology

The National University of Singapore, Singapore

Approval of the Master Thesis

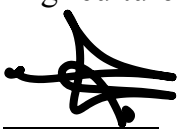
This Master Thesis is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Mutamed M. Ayyash

   Title: Associate Professor

   Department of Food Science

   College of Agriculture and Veterinary Medicine

   Signature  Date 14/11/2022

2) Member: Dawood Al-Ajmi

   Title: Associate Professor

   Department of Food Science

   College of Agriculture and Veterinary Medicine

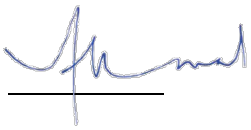
   Signature  Date 14/11/2022

3) Member (External Examiner): Harold Corke

   Title: Professor

   School of Biotechnology and Food Engineering

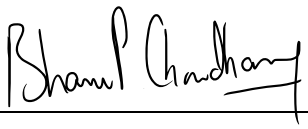
   Israel Institute of Technology, Shantou, China

   Signature  Date 28/11/2022

   Dr. Akmal Nazir
   Assistant Professor/Program Coordinator
   (On behalf of External Examiner)

This Master Thesis is accepted by:

Dean of the College of Agriculture and Veterinary Medicine: Professor Bhanu Chowdhary

Signature 

Date 05/12/2022

Dean of the College of Graduate Studies: Professor Ali Al-Marzouqi

Signature 

Date 06/12/2022

Abstract

The ability to perform effectively in the gastrointestinal system (GIT) is one of the most significant criteria for selecting the best probiotic bacteria. Thus, the present study aimed to investigate the potential probiotic characteristics of some selected lactic acid bacteria (LAB) isolated from vegetable products. Probiotic characteristics included tolerance to acid and bile, cholesterol removing ability, bile salt hydrolysis, resistance against lysozyme and antibiotics, production of exopolysaccharides (EPS), antimicrobial and hemolytic activities, and cell surface characteristics (auto-aggregation, co-aggregation, and hydrophobicity). Out of 46 isolates, 17 isolates that exhibited remarkable survivability under gastrointestinal conditions were selected for further analysis. Almost all 17 isolates tolerated bile salts, while isolates F5 and F26 exhibited the highest bile salt hydrolase activity. Isolates F1, F8, F23, and F37 were able to reduce cholesterol in the broth. The auto-aggregation, the average rate increased significantly after 24 h for all isolates, while 2 isolates showed the highest hydrophobicity values. Moreover, all isolates showed high co-aggregation values after 24 h of incubation compared to 4 h values. All isolates were resistant to lysozyme and vancomycin, and 8 out of the 17 selected isolates displayed an ability to produce EPS. Based on 16S rRNA sequencing, LAB isolates were identified as *Enterococcus faecium, E. durans, E. lactis,* and *Pediococcus acidilactici*

Keywords: Auto-aggregation, antimicrobial, cholesterol-lowering, immunomodulation.

Title and Abstract (in Arabic)

دراسة الصفات الصحية لبكتيريا حمض اللبن المعزولة من الخضروات: الخصائص المحتملة للبكتيريا النافعة ومابعد الحيوية بما في ذلك التأثيرات المناعية

الملخص

تُعد امكانية الاداء الفعال في الجهاز الهضمي من أهم المعايير التي يتم من خلالها اختيار افضل بكتيريا نافعة. تهدف هذي الدراسة إلى التعرف على الخصائص المحتملة لبعض البكتيريا المختارة من حمض اللاكتيك والتي تم عزلها من منتجات الخضروات. أظهرت الخصائص الحيوية للبكتيريا قدرتها على مقاومة الأحماض والمادة التي يفرزها الكبد، والقدرة على إزالة الكولسترول، والتحلل المائي لأملاح المرارة، ومقاومة الليزوزيم والمضادات الحيوية ، وانتاج مخلفات السكر التي يتم افرازها بواسطة الكائنات الحية الدقيقة إلى البيئة المحيطة، وأنشطة مضادات الميكروبات ومحللات الدم ، وخصائص سطح الخلية (التجميع الذاتي ، والتجمع المشترك ، ومقاومة للماء). تم اختيار 17 عينة معزولة أظهرت قدرتها الملحوظة على البقاء رغم ظروف الجهاز الهضمي من أصل 46 عينة لتخضع لمزيد من التحليل. معظم العينات المختارة تحملت أملاح المرارة، بينما أظهرت عينات F5 و F6 أعلى نشاط لانزيم الهيدرولاز لأملاح المرارة، فيما بينت عينات F1 و F8 و F23 و F37 قدرتها على تقليل الكورلسترول. زاد معدل التجميع التلقائي بشكل ملحوظ بعد 24 ساعة لجميع العينات المعزولة، في حين أظهرت عينتين معزولتين أعلى قيم لمقاومة الماء. بالإضافة إلى ذلك، جميع العينات المعزولة أظهرت قيم عالية للتجميع البكتيري المشترك خلال 24 ساعة من العزلة مقارنةً بقيم 4 ساعات. أظهرت جميع العينات مقاومتها لليزوزيم والفانكومايسين، و 8 عينات من أصل 17 عينة مختارة أظهرت قدرتها على انتاج المواد البوليمرية خارج الخلية. تم تحديد العينات المعزولة مختبرياً بناءاً على تسلسل 16S rRNA على أنها *Enterococcus faecium, E. durans, E. lactis Pediococcus acidilactici*

كلمات البحث الرئيسية: التجميع الذاتي، مضاد الميكروبات، خفض الكوليسترول، التعديل المناعي.

Acknowledgments

First, Thank God for making me cross a lot of obstacles faced in my research work to complete this paper successfully.

I would like to thank Dr. Mutamed Ayyash, my thesis advisor, for the patience, support, knowledge, and effort he spent on this work to be on the right track. His great support helped a lot in publishing this work.

I also want to thank all my lab members that assisted me during all tests performed in this study.

And finally, I want to express my gratitude and love to my mother and father for their motivation and support throughout my years of study.

Dedication

To my entire family, for their endless love, support and encouragement

Table of Contents

List of Tables

List of Figures

List of Abbreviation

ACE	Angiotensin-Converting Enzyme
ADFCA	Abu Dhabi Food Control Authority
ANOVA	Analysis of Variance
B.	*Bifidobacterium*
BHI	Brain Hear Infusion
BLAST	Basic Local Alignment Search Tool
BSH	Bile Salt Hydrolysis
CD	Crohn's Disease
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
E.	*Enterococcus*
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization
GI	Gastrointestinal
GIT	Gastrointestinal Tract
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
ICBA	International Centre for Biosaline Agriculture
L.	*Listeria*
LAB	Lactic Acid Bacteria
Lb.	*Lactobacillus*
MRS	De Man, Ragosa and Sharp
NCBI	National Center for Biotechnology Information
OPA	o-Phthalaldehyde
PBS	Phosphate Buffered Saline
PCR	Polymer Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
spp.	Species
subsp.	Subspecies
UC	Ulcerative Colitis
WHO	World Health Organization

Chapter 1: Introduction

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2002). Based on the definition, a microorganism is labeled as a probiotic only when there is scientific evidence proving its potential health benefits for the host (Mack, 2005). The International Scientific Association for Probiotics and Prebiotics (ISAPP) states that for a microorganism to be described probiotic, it should first go through a series of human or intended user trials to ensure safety, to prove at least one of the claimed health benefits that the microorganism provides for the host (Hill et al., 2014). In general, the most common microorganisms added to food products or supplements for their probiotic abilities include members of lactic acid bacteria (LAB) and bifidobacteria (Ayyash et al., 2021).

The capacity of bacteria to survive through the GI system, reach either the small or large intestine in sufficient numbers and interact with and/or attach to and colonize the host is the basis of adding probiotics to foods and supplements for their possible health advantages (Ayyash et al., 2021). Several factors have a deleterious effect on probiotics, including the stomach's high acidity (pH 1.5 – 3.0), bile salts, and digestive enzymes. Additionally, prior to consumption, the probiotics must maintain viability throughout culture manufacture and storage, product or supplement manufacture, and product shelf-life (Ayyash et al., 2021).

In point of view, one of the observations stated that the secretion of various metabolites, such as postbiotics, during the metabolic activity of microorganisms possessed beneficial effects on the host (Żółkiewicz et al., 2020). However, postbiotic precise definition is still under discussion but one study it is defined as and substance or metabolite released or produced during the metabolic activity that offer health benefits on the host (Tsilingiri & Rescigno, 2013). In addition, studies previewed that postbiotics might strengthen the intestinal microbiota (Klemashevich et al., 2014).

Currently, there are several classes of postbiotic drugs whereas each have different mechanism and provide different beneficial effect on the host. One type of the metabolites released are cell-free supernatants which are biologically containing

metabolites secreted by microorganisms that shows different activity according to the microorganism used. Indeed, *Lactobacillus acidophilus* and *Lactobacillus casei* released supernatants that showed anti-inflammatory and antioxidant on the intestinal epithelial cells that can reduce the risk of colon cancer (Żółkiewicz et al., 2020). Moreover, supernatants liberated from *Lactobacillus* and *Bifidobacterium* showed antibacterial activity against enterinvasive *E. coli* strains into enterocytes *in vitro* (Żółkiewicz et al., 2020). In addition, during microorganism growth biopolymers with different chemical properties are produced and released as exopolysaccharides outside the bacterial cell wall which showed several beneficial properties such as antimicrobial and antioxidant properties and showed great effect in inhibiting cholesterol absorption (Khalil et al., 2018; Li et al., 2014). In terms of antitumor effect, *Porpionibacterium freudenreichii* produce SCFA pronate that resulted in the apoptosis of gastric cancer cells (Cousin et al., 2012). Furthermore, some postbiotics succeeded in reducing the risk of cardiovascular disease as SCFA pronate plays a significant role in inhibiting the condensation of cholesterol precursors (Bush & Milligan, 1971). Besides, several studies displayed the therapeutic effect of postbiotic on allergic diseases by restoring the balance of Th1/Th2-mediated immune response and enhancing the maturation of the immune system (de Boer et al., 2020; Esposito et al., 2018).

Clinical studies have demonstrated various health effects of consumption of these microorganisms, such as reduction in duration and occurrences of diarrhoea, alleviation of symptoms of lactose intolerance, reduced incidences of pathogenic infection, and stimulation of the immune system and regulation of the inflammatory response (de Vrese, 2008; Hill et al., 2014). The present study aimed to investigate the potential probiotic characteristics of some selected LAB isolated from vegetable products, i.e., tolerance to acid and bile, cholesterol removing ability, bile salt hydrolysis, resistance against lysozyme and antibiotics, production of exopolysaccharides (EPS), antimicrobial and hemolytic activities, and cell surface characteristics (auto-aggregation, co-aggregation, and hydrophobicity).

Fruits and vegetables are one of the main dietary requirements in an adult's diet, and according to The Dietary Guidelines for Americans, it is recommended to consume half section of the plate as fruits or vegetables in all-day meals (Slavin & Lloyd, 2012).

In terms of nutritional composition, fruits and vegetables are considered highly nutritious foods as they provide high amounts of vitamins, such as vitamin C and A, minerals, specifically electrolytes, and phytochemicals in specific antioxidants that fight free radicals in the body (Slavin & Lloyd, 2012). In terms of the selection of the source of probiotics, this study used fresh vegetables as a source of probiotics due to their beneficial properties, nutritional value, flavor enhancement and reduced toxicity that is granted from the process of lactic acid fermentation that is usually performed to increase the shelf life of fresh fruits and vegetables. In addition, the low sugar content, enrich mineral and vitamin content, and neutral pH enhance the process of LA fermentation. Several studies showed the great health benefit offered from LAB that were isolated from fresh vegetables. Indeed, studies proved that the consumption of LA fermented vegetables and fruits provided balanced nutrition value in terms of vitamins, minerals, and carbohydrates that plays a role in the prevention of several diseases such as diarrhea and liver cirrhosis. Moreover, colored pigments found in some fruits and vegetables such as flavonoids, lycopene, anthocyanin, β- carotene, and glycosylates acts as antioxidants that fights free radicals that might result in reducing the risk of several disease such as cancer, arthritis, and ageing. However, the current study aimed to isolate LAB from fruits and vegetables, describe, and discover new probiotics with different properties, including 1) gastrointestinal. Tolerance by a) *in-vitro* digestion, b) bile salts, and c) lysozyme; 2) physiological properties such as a) auto-aggregation, b) co-aggregation, c) hydrophobicity, d) adhesion to HT-29 cells, and e) cholesterol reduction; 3) production of desirable substances such as a) bile salt hydrolase, b) antimicrobials, and c) EPS; 4) bioactivities such as a) immunomodulation and sensitivity to antibiotics. It was expected to discover new probiotic strains in the end of the study.

Chapter 2: Literature Review

Probiotics are defined as living microorganisms that are found naturally in the body that provide beneficial health effects when consumed in adequate amount (Floch, 2010). During illness, harmful microorganism level increase in the body affecting the balance of the microorganisms in the body. However, probiotics help in eliminating excess harmful microorganisms, and this will eventually help in restoring equilibrium in the body (Floch, 2010). Moreover, probiotics are found in different parts of the body but mainly in the gut, especially in the large intestine, due to their favorable environment (Floch, 2010). From the definition, it is concluded that the great number of microorganism species are classified as probiotics, whereas LAB was widely used in food and nutritional fields. Generally, LABs are a group of anaerobic microorganisms which produce lactic acid as a by-product of sugar fermentation (Teuber, 2001). Moreover, LABS are Gram-positive, catalase-negative, non-spore-forming, can tolerate acidic environments, and are fastidious as it requires sufficient levels of carbohydrates (specifically monosaccharide and disaccharide), protein by-products, vitamin, low oxygen tension in the process of fermentation (Teuber, 2001). Based on the characteristic of LAB, probiotics belong to the lactic acid bacteria group, which belongs to different genera, including *Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, and Streptococcus* (Teuber, 2001). However, studies have shown that the impacts of probiotics are considered strain-specific, meaning that each strain can be screened individually to prove certain health effects (Kekkonen et al., 2008). In terms of probiotics' beneficial capabilities, studies have proven that LAB plays a significant role in changing the intestinal environment by blocking pathogenic bacteria in the gastrointestinal system, which will reduce pathogen adhesion activity (Zommiti et al., 2020). In addition, probiotics helped in reducing mucosal inflammation (Heller & Duchmann, 2003), lactose intolerance, flatulence, and bowel symptoms of infants' food (O'Mahony et al., 2005). Based on countless studies, it has been shown that the significant number of species were classified as probiotics as they belong to *Lactobacillus*, *Enterococcus*, and *Bifidobacteria* (Ankaiah et al., 2021). According to some studies, newly discovered probiotics are expected to offer huge health benefits in terms of human health and food manufacturing. Indeed, a study was conducted on

fermented dates in Saudi Arabia that isolated *L. Paraplantarum* D-3, however Araus and Al-Dhabi succeeded in proving that fermented dates are considered a vulnerable source of novel probiotics with antifungal and antioxidant functional properties (2017).

For this reason, most researchers are motivated to discover new novel probiotics or fermented food products due to the huge health benefits behind it. Moreover, a study was conducted on wheat bran sourdough, whereas 13 LABs were isolated and categorized as: 7 were identified as *Lactobacillus*, 4 *Leuconostoc* spp., and 2 *Pediococcus* spp (Manini et al., 2016). However, the authors found out that wheat bran is considered a rich source of new LAB with its favorable characteristic (Manini et al., 2016). Regarding the isolates, 9 of them can be utilized in the food industry due to their EPS production and antifungal activity (Manini et al., 2016). This study aimed to isolate LAB from fruits and vegetables, describe, and discover new probiotics with different properties, including 1) gastrointestinal tolerance by a) in-vitro digestion, b) bile salts, and c) lysozyme; 2) physiological properties such as a) auto-aggregation, b) co-aggregation, c) hydrophobicity, d) adhesion to HT-29 cells, and e) cholesterol reduction; 3) production of desirable substances such as a) bile salt hydrolase, b) antimicrobials, and c) EPS; 4) bioactivities such as a) immunomodulation and sensitivity to antibiotics.

2.1 Probiotic Definition

Before defining probiotics, probiotics were first named as a synonymy word for "antibiotic," afterward it has been discovered that the word probiotics is divided into two parts where 'pro' belongs to a Latin word and 'bios' to a Greek word which stands for "for life," which is the opposite of 'antibiotics' that stands for 'against life' (Hamilton-Miller, 2004; Schepper et al., 2017). Kollath, which is a German scientist, was the first one who discovered probiotics that were used for malnourished patients to enhance and restore their health status through various organic and inorganic supplements (Schepper et al., 2017). In the next year, the definition of probiotics was modified into substances that are formed by other microorganisms to enhance the growth of other microorganisms (Azizpour et al., 2009). Later on, in the 1970s, the definition of probiotics was updated into compounds that provide the host resistance against infection without affecting the

growth of microorganisms in vitro (O'Sullivan et al., 1992; Azizpouret et al., 2009). From 1980 to 1990, there were various changes in the definition of probiotics, in 1990 a scientist called Parker defined probiotics as organisms or substances found in food supplements that provide microbial balance in the intestine (O'Sullivan et al., 1992; Gogineni et al., 2013). However, Parker's definition was not supported as he included the word 'substances' in his definition, which includes other chemical substances such as antibiotics that differs from probiotics (O'Sullivan et al., 1992; Salminen et al., 1999). At last, most researchers agreed with Fuller's definition, which declared that probiotic benefits the host by providing intestinal microbial equilibrium (McFarland, 2015). Nevertheless, his definition works more on animals than humans (Azizpour et al., 2009). The final definition was proposed by FAO/WHO ((Food and Agricultural Organization/World Health Organization) to be as 'live microorganisms that offer a health impact on the host when consumed in adequate amount" (FAO/WHO, 2002).

2.2 The History of Probiotics

Probiotic usage is not new in the process of discovering new microorganisms, as it was found over 10,000 years ago (Ozen & Dinleyici, 2015). Indeed, Egyptian hieroglyphs identified some sort of fermented milk, such as Labna Rayeb and Laban Kha,d in the early 7000 BCE (Gogineni et al., 2013; Ozen & Dinleyici, 2015). In addition, Tibetan nomads used to apply a traditional method to Yak milk to preserve it for longer periods (Azizpour et al., 2009). In the 1800s, Branett (2000) discovered the health effect of consuming the number of fermented milk products, but unfortunately, it remained unknown; although it was approved by Louis Pasteur that bacteria and yeast are considered the byproducts of the fermentation process, it still did not relate these byproducts to any health benefits.

A Russian Zoologist Elie Metchnikoff worked with Pasteur in 1905 and concluded that the reason behind the long life in most Bulgarian farmers' population is *Lactobacilli* that ferment the yogurt they used to consume (McFarland, 2015). In 1922 health improvement was seen in patients with diarrhea, chronic constipation, and eczema as a result of *Lactobacillus acidophilus*, which was included in all three cases' treatment plans (Rettger et al., 1938). In addition, *Lactobacillus acidophilus* showed a health

enchantment in volunteers with mental disease and constipation (Kopeloff et al., 1932). The belief that yogurt is the best and the main type of probiotic led to a debate about using lactic acid bacteria as yogurt starters (*Streptococcus thermophiles and Lb. bulgaricus*), but these starters failed to colonize human intestine (Kopeloff et al., 1932). Therefore, *Lactobacillus acidophilus* was injected into milk which succeeded in colonizing the human colon without increasing the acid level (Ozen & Dinleyici, 2015). Due to this colonization, researchers termed 'this complicated interaction of normal flora, and its ability to withstand the attack of pathogenic bacteria' as colonization resistance (McFarland, 2015).

In terms of probiotic description, in 1965, two researchers presented a new form to the previous description. Indeed, they were the first to categorize compounds that are secreted by another microorganism that promote the growth of another microorganism considered as probiotics (Lilly & Stillwell, 1965). Nevertheless, the widespread myth that yogurt is the superior form of probiotics was rejected by the global guidelines on probiotics and postbiotics of the World Gastroenterology Organization in 2013, as probiotics efficiency depends on specific doses and strain (McFarland, 2015). A year later, according to the International Scientific Association for probiotics and prebiotics, probiotics are classified into three categories: 1) group that are considered safe and do not require efficient evidence, 2) probiotics that offer health benefits and are mainly used in food supplements, 3) probiotic drug which undergoes clinical strain procedures as well as risk and benefits evaluation to confirm that the drug is safe to use according to the regulatory standards for drug production (Hill et al., 2014).

2.3 Probiotic Classification and Taxonomy

The various number of microorganisms are classified as probiotics based on their favorable characteristics (Khalighi et al., 2016). In terms of naming, bacteria are usually named according to their description, the place of classification, the name of the scientists classified, or an organization (Schepper et al., 2017). However, based on the countless studies conducted, it has been found that bacteria that are classified as LAB demonstrated great interest in the field of food and nutrition. According to LABs' characteristics, they are anaerobic, Gram-positive, catalase-negative, non-spore-forming,

acid-tolerant, and fastidious (Schepper et al., 2017). Moreover, in the process of fermentation, L ABs form lactic acid as byproducts when fermenting sugar (Felis & Dellaglio, 2009). Orlajensen divided LAB genera into 7 groups based on their morphologic and phenotypic characteristics, which include1) *Mycobacterium*, 2) *Betabacterium,* 3) *Thermocautery*, 4*) Streptococcus,* 5) Tetracoccus*,* 6) *Streptobacterium*, 7) *Betacoccus* (Holzapfel et al., 2001; Tindall, 2008). Having general knowledge about the bacteria's classification and taxonomy is confedered significant as it determines the strain's source, physiology, and habitat that will help in selecting new strains that might either be used in food application or be utilized as probiotics (Holzapfel et al., 2001). Probiotics belong to lactic acid bacteria that are composed of *Lactobacillus, Streptococcus, Bifidobacteria,* or *Enterococcus* (Remacle & Reusens, 2004). As mentioned previously, these genera produce lactic acid as byproducts in the process of fermentation after fermenting glucose (Mayo et al., 2008).

Firstly, *Lactobacillus* belongs *Lactobacillaceae* family, which is characterized as rod-shaped, phylum Firmicutes (Pfeiler & Klaenhammer, 2013). Lactobacillus bacteria plays a significate role in the field of the food industry, specifically in the production of fermented products such as yogurt, cheese, and fermented sausages (Felis & Dellaglio, 2007). Indeed, *Lactobacillus delbrueckii* subsp. *bulgaricus,* which belongs to the Lactobacillus genera, is considered the superior vehicle in yogurt and cheese production (Felis & Dellaglio, 2007).

Secondly, *Bifdobacteira* is a well-known genus that is classified as a probiotic microorganism that is characterized by a branched shape (Pfeiler & Klaenhammer, 2013). This genus is considered one of the members of the phylum *Actinobacteria* and *Bifidobateriacae* family (Pfeiler & Klaenhammer, 2013). It has been proven that *Bifidobacteria* members, such as *B. longum* and *B. animalis*, demonstrated health-promoting benefits both *in-vivo* and *in-vitro* (Ventura et al., 2004).

Thirdly, *Enterococcus*, which belongs to the family *Enterococcaceae*, that exists in groups, in short chains, in pairs, or in single and is classified as cocci-shape (Holzapfel & Wood, 2014). *Enterococcus* species favor acidic environments; indeed, their main habitat is in the gastrointestinal tract (GI) (Zhong et al., 2017). Like

Lactobacillus, Enterococcus also plays a great role in the field of food production, mainly in dairy products (Foulquié Moreno et al., 2006). In addition, *Enterococcus* produces bacteriocins to suppress the growth of foodborne pathogens (Giraffa, 2003). According to some studies conducted, which stated that *Enterococcus* species are correlated with some types of infectious diseases (Gaet et al., 2014).

And finally, *Streptococcus* are gram-positive bacteria that belong to the phylum *Firmicutes*, and the family *Streptococcaceae* exists in chains and pairs (Gao et al., 2014). Moreover, Streptococci are considered homofermentative since they do not form CO_2 as byproducts in the process of glucose fermentation (Gao et al., 2014). In addition, their favorable growth temperature is 37°C (Holzapfel & Wood, 2014). In terms of food production, the specie *Streptococcus thermophilus* is included in the production of yogurt, cheese and other types of cheese as it is used as a starter culture in the process of production.

2.4 Health Benefits of Probiotic

Several studies have been conducted to determine the health-promoting benefits of probiotics, which stated that their impacts are considered strain-specific (Kekkonen et al., 2008). For this reason, screening strain efficacy is required to determine each health effect and to determine the capability of some strains to provide microbiota equilibrium within the GI of the host (Hertzleret et al., 1997). Furthermore, it has been reported that probiotics play a significant role in reducing mucosal inflammations (McCarthyet et al., 2003). Besides, probiotics minimize the effects of lactose intolerance, abnormal colonic fermentation, flatulence, and symptoms of infant food (O'Mahony et al., 2005). In addition, probiotics helped in inhibiting the growth of some disease-induced pathogens by blocking the attachment of pathogens in the digestive epithelium (Ariful et al., 2010). Moreover, probiotics reduce the risk of diarrhea through their antimicrobial activity that kills or inhibits the growth of the pathogens behind it (Ariful et al., 2010).

2.4.1 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease is a chronic disease that causes inflammation in the tissues of the digestive tract (Baumgart & Carding, 2007). There are two main types,

which are Crohn's disease (CD) and Ulcerative colitis (UC) (Baumgart & Carding, 2007). Crohn's disease is caused by inflammation in the lining of the digestive tract (Baumgart & Carding, 2007). While Ulcerative colitis results in ulcers in the lining of the colon and rectum (Baumgart & Carding, 2007). Both types have the same symptoms, which include diarrhea, rectal bleeding, abdominal pain, and fatigue (Baumgart & Carding, 2007). However, studies showed that probiotics could reduce the effect of both types of IBD (Mowat & Bloom, 2013). According to some studies, it has been noticed that the composition of fecal microbiota in IBD patients differs from the composition of healthy controls (Huttenhower & Xavier, 2014). Under experimental conditions, it has been seen that Lactobacillus strains succeeded in reducing symptoms of inflammation (Liang et al., 2014).

According to the screening of *Lactobacillus* GG strains and after using it in patients with IBS, including both types of UC and CD, it has been found that those strains gave effect as mesalazine medication, which is an anti-inflammatory drug (Zocco et al., 2006). This study was conducted on 187 patients that were randomized to three open-label arms, including 1) *Lactobacillus rhamnosus* GG (*Lb. rhamnosus* GG) added with mesalazine, 2) *Lb. rhamnosus* GG strains only, 3) and mesalazine only (Zocco et al., 2006). In conclusion, the two trials showed that *Lactobacillus* displayed the same effect as the medication drug (Zocco et al., 2006). Another study was performed on 21 UC patients that were given *Bifidobacterium* fermented milk supplements; however, those patients exhibited fewer relapses throughout the whole duration of the study (12 months) (Ishikawa et al., 2003). To sum up, probiotic treatment succeeded in giving the same effect as most drugs used to treat UC.

2.4.2 Irritable Bowel Syndrome (IBS)

Irritable bowel syndrome (IBS) is a common condition that disturbs the digestive system due to the fermentations that occur in the colon, which affects the gas volume in the body (Defrees & Bailey, 2017). Changes in the gas volume in the body might result in some symptoms, such as flatulence, abdominal pain, and bloating (Defrees & Bailey, 2017). Some strains of probiotics can be used in IBS patients to minimize gas accumulation (Charalampopoulos & Eds, 2009). Indeed, some studies have reported that

including *Lactobacillus* and *Bifidobacterium* in the treatment plan of IBS patients resulted in strengthening immune response, enhancing digestive permeability, and changing colon fermentation to avoid gas accumulation in the body (Sartor, 2004). IBS symptoms can be reduced by using probiotic supplements, which work in chaning the path of the gut-brain axis (Cryan & O'Mahony, 2011). Probiotics that showed great effects on reducing IBS symptoms include *Lb. paracasei, B. infantis, B. breve*, and *B. longum* (Cremon et al., 2017; Giannetti et al., 2017).

2.4.3 Acute Diarrhea

Acute Diarrhea can be due to a wide variety of reasons, either bacterial, viral, or parasitic (Drancourt, 2017). However, rotavirus is the main cause of acute diarrhea among children (Drancourt, 2017). During infection, proteins get destroyed due to the elevation of the permeability in gut cells (Shah, 2007). The duration of rotavirus diarrhea can be shortened by using some strains of probiotics such as *B. animalis* Bb-12 and *Lb. acidophilus* (Park et al., 2017a). In the case of antibiotic-associated diarrhea, a strain with an inhibitory effect against entero-pathogens can be used to reduce or prevent symptoms (Shah, 2007). In point of fact, yogurt mixed with *B. longum* showed a great effect in reducing the diarrheic effect of the antibiotic erythromycin (Shah, 2007).

2.4.4 Allergic Diseases

The causes leading to allergic diseases are still unclear as the method that explains the effect of the bacteria on the growth and intensity of allergic diseases requires more investigation. In general, modification in the balance of Th1/Th2 cytokines might trigger the activation of Th2 cytokines, which will release interleukin-4(IL-4), IL-,5, and IL13 and will produce IgE (Michail, 2009). However, in this case, probiotics might lead to enhancing the immune tolerance in the gut in the first year of life. Indeed, a study was conducted on infants and stated that infants who were supplemented with probiotics had less ratio of eczema compared to the control group (Zuccotti et al., 2015).

2.4.5 Colon Cancer

Several studies were performed both *in-vivo* and *in-vitro* and demonstrated the impact of probiotics, including *Bifidobacteria* and *Lactobacillus* strains, or a mixture of

probiotics and probiotics, on the growth of transplantation metastasis and chemically-induced tumor such as colon cancer (Charalampopoulos & Eds, 2009).

However, their accurate prevention method for the growth of colon cancer is still unclear, but several studies were investigated to clear it out, which include: 1) changes in the metabolic actions of intestinal microflora, 2) physiological or chemical modifications within the colon, 3) killing and destroying carcinogens in the body, 4) qualitative and quantitative change in the intestinal microflora to prevent the production of promoters and expected carcinogens, 5) production of anti-mutagenic or anti-tumorigenic substances, 6) enhancing immune response of the host, 7) applying some physiology changes on the host (Rafter, 2004). There are specific strains of probiotics that are well known to perform anticancer activities against Cao-2 cells; those probiotics include *Lactobacillus pentosus* B281 and *Lb. plantarum* B282 (Saxami et al., 2016).

2.5 Health Benefits of Food Products Fermented by Probiotics

Fermented food products are described as the end products of bacterial activity on a specific product that resulted in changes in their chemical, physical, and biological properties (Pihlanto & Korhonen, 2015). Fermented food products are composed of microbial metabolites, including lactic acid, acetic acid, alcohol, CO_2, propionic acid, exopolysaccharides, and bioactive peptides that are liberated in the process of fermentation (Gan et al., 2017). The production of those bioactive peptides plays a significant role in performing various bioactivities such as antihypertension, cholesterol-reduction, antioxidant, and anticancer activities, and each will be explained more separately.

2.5.1 Antihypertension Property

Peptides that exhibit antihypertensive activities are the most tested peptide in the process of food fermentation by probiotic bacteria (Fujita et al., 2017). Those peptides can result in lowering blood pressure by preventing the conversion of angiotensin I to angiotensin II (Zhang et al., 2017). For this reason,angiotensin-converting enzyme (ACE) inhibitory peptides acquired from food proteins, efficacy in lowering blood pressure is much higher than hypertension medication (Haque & Chand, 2008).

Moreover, one of the studies showed that soybean meal that is fermented by *Bacillus Subtilis* exhibited antihypertensive activity (Wang et al., 2017).

2.5.2 Cholesterol-Reduction Property

According to some documentation, bioactive peptides with hypocholesterolemic activities are liberated as a result of the proteolysis of casein, β-lactoglobulin, soy protein, and fish protein (Kudaet et al., 2016). Ataie-Jafari and his collogues reported that serum total cholesterol level was reduced due to the consumption of fermented yogurt by *Lb. acidophilus* and *B. lactis* (2009). Another study stated that consuming Kimichi, which is Korean traditional food, is fermented by different strains of Lb. plantarum showed a great role in removing cholesterol (Park et al., 2017b).

2.5.3 Antioxidant Property

Body cells can be destroyed with the presence of free radicals, as these free radicals are composed of oxygen molecules with an uneven number of electrons which can easily bind with molecules and cause destruction. This process is known as oxidation (Lobo et al., 2010). Moreover, this cellular destruction due to free radicals might lead to different diseases such as diabetes, cancer, arthritis, and atherosclerosis (D'Souzaet et al., 2002). Pessione and Cirrincione (2016) stated that the consumption of fermented with probiotics could reduce the effect of these free radicals. There are several peptides that provide antioxidant activities in various fermented food. However, their efficacy can be tested by determining its inhibiting lipid peroxidation and scavenging free radicals (Tamanet et al., 2016). In terms of pedetids composition, peptides with antioxidant activities are enriched in aromatic and/or hydrophobic amino acids (Sarmadi & Ismail, 2010). Nevertheless, antioxidant activity has been described in the vegetable-fruit beverage that was fermented by *L. plantarum* as results indicated significant antioxidant activity that played a role in forming high-quality fermented products (Yang et al., 2018).

2.5.4 Anticancer Properties

Several studies have described the anticancer activity in peptides of fermented food products by probiotic bacteria. In point of view, one of the studies observed their

activity on fermented goat milk by Lb. plantarum and Lb. paracasei; however, results showed that with higher concertation of goat milk hydrolysate, there was a reduction in Hela cells level, which are cervical cancer cells (Nandhini & Palaniswamy, 2013). In addition, it has been documented that fermented camel milk injected with Lb. reuteri and Lb. Plantarum displayed anticancer activity against colon cancer cells (Caco-2), breast cancer cells (MCF-7), and Hela cells (Ayyash et al., 2018b).

2.6 Importance of Isolating New Probiotics

LABs are considered the most abundant microorganisms that are usually isolated in the field of probiotics. Indeed, various studies discovered several numbers of species that belong to a group of probiotic bacteria, including *Lactobacillus*, *Enterococcus,* and *Bifidobacteria* (Ankaiah et al., 2017; Liuet et al., 2017; Quattrinet et al., 2018). Identifying new probiotic bacteria will expand their beneficial effect on both human health and the food industry.

As mentioned previously, new probiotics were isolated from fermented date in Saudi Arabia, which is Lb. paraplantarum D-3 has also been found that dates are considered a rich source of new probiotic strains due to their antioxidant and antifungal activities (Arasu & Al-Dhabi, 2017).

In addition, 21 new strains of LAB were isolated from ham, such as Lb. paraplantarum-GS54, Lb. plantarum GS16, which were classified as bacteriocin-like substance producers (Anacarso et al., 2017).

2.7 Characterization of Probiotics

For microorganisms to be classified as probiotics, they should show certain probiotics favorable characteristics. According to the guidelines of FAO/WHO (2002), the in-vitro test should be performed on each probiotic strain to test their efficiency. For this reason, in-vitro teste has been applied to various expected probiotic strains for the initial selection (Morelli, 2000).

2.7.1 Tolerances to the Gastrointestinal Tract (GI) Conditions

The guidelines stated by FAO/WHO (2002) emphasize the importance for probiotics to withstand and survive GI conditions to ensure their viability within the GIT, which is their main habitat. There are some conditions that might affect their survival, such as low pH (2.0) and pepsin activity. Moreover, probiotics should pass by the stomach within less than 1 to 4 hours (Ruiz-Moyano et al., 2010). Selected probiotic strains should succeed in passing by the GIT to provide their beneficial health effect on the host (Shokryazdan et al., 2017). In terms of probiotic survival, there are some obstacles that might prevent them from surviving within the GIT, like the presence of trypsin and bile salts. However, probiotics should be able to defeat and withstand these obstacles.

2.7.2 Probiotics Cell Surface Properties

In order to apply the beneficial effect of probiotics on the host, their population should be more than 6.0 log CFU/g (Shah, 2000). For probiotics colonization to take place, probiotics should first attach to the intestinal epithelial cells (Lee & Salminen, 1995). Thus, probiotics attachment to the host's GIT is considered a significate criterion to provide its beneficial effects. Auto-aggregation, hydrophobicity, and co-aggregation act as indicators to evaluate probiotics attachment (Hernández-Alcántara et al., 2018).

2.7.2.1 Auto-Aggregation

One of the indicators for probiotics' ability to attach to the GIT is auto-aggregation, which is performed to determine the ability of bacterial strains to aggregate with each other (Del et al., 2000).

2.7.2.2 Hydrophobicity

Hydrophobicity is usually conducted to identify the ability of probiotics to attach to hydrocarbons that result in a strong link (Piwat et al., 2015). Since hydrophobicity is considered a parameter for cellular attachment, it means that probiotic strain with high hydrophobicity will be more attached to GIT walls (Shokryazdanet et al., 2017). Indeed, countless numbers of studies stated the correction between hydrophobicity and auto-

aggregation test with probiotic attachment to the host's GIT (Boteset et al., 2008; Tarebet et al., 2013; Felipet et al., 2017).

2.7.3 Co-aggregation

Co-aggregation is performed to describe the aggregation ability among bacteria of various species (Piwat et al., 2015). One of the main defensive barriers that prevent pathogens from attaching to the host's mucosa is the direct aggregation of probiotics with pathogens (Vidhyasagar & Jeevaratnam, 2013). Moreover, studies proved that the presence of gut pathogens enables co-aggregation to enhance probiotics properties as well as their attachment to gut cells (Peres et al., 2014; Amaral et al., 2017).

2.7.4 Antimicrobial Activity

One of the significant characteristics of probiotics that should be present in expected bacterial strains is antimicrobial activity. The efficiency of antimicrobial activity of probiotic strain is usually tested by the production of organic acid, metabolite, and bacteriocins during probiotic growth. Bacteriocins produced by these bacteria are significate as they are utilized as food bio-preservatives. In addition, bacteriocins are heat-stable peptides that defeat pathogenic bacteria via their antimicrobial activity (Nami et al., 2015). Besides, it has been reported that *Enterococcus spp.* Isolated from fresh shrimps exhibited antimicrobial activity against various indicator strains (Ben Braiek et al., 2017).

2.7.5 Antibiotic Resistance

All selected probiotics should go through a safety assessment before selection which is performed by testing their resistance against antibiotics. Probiotics' antibiotic resistance can be affected by membrane impermeability and cell wall structure (İspirli et al., 2017). However, one of the main criteria for potential probiotics is to exhibit sensitivity toward antimicrobial and antibiotic components (Peres et al., 2014). One of the studies was conducted on Feta cheese which isolated *Lactobacillus* spp. that showed sensitivity toward some antibiotics (Plessas et al., 2017).

2.7.6 Hemolytic Activity

The safety of the potential probiotic strains should be investigated before using it in food products through hemolytic activity, as the epithelial layers might be broken down by the strains that shows positive hemolytic activity. Isolates that didn't exhibit hemolytic activity are considered as non-virulent strains making them safe since they don't cause infections (Tejero-Sariñena et al., 2012). Indeed, one of the studies conducted on *Enterococcus faeclais* and *Weilissela* sp. that was isolated form fish which showed negative results in the hemolytic activity test (Shahid et al., 2017). However, due to unavoidable circumstances this test was not performed in this study.

2.7.7 Bile Salt Hydrolysis

Conjugated bile salts are usually hydrolyzed by a specific type of enzyme known as bile salt hydrolase (BSH), which is formed by potential probiotics. One of the end products of these conjugated bile salts is micelle with cholesterol, which to enhances the absorption of cholesterol. Thus, the hydrolysis of conjugated bile salt to deconjugated is significant as it plays a role in reducing the absorption of cholesterol in the host's intestine. This reduction occurs by minimizing cholesterol solubility resulting in elevated levels of cholesterol excreted with fecal (Shokryazdan et al., 2017). The impact of deconjugated bile salts was documented in vitro by Lactobacillus isolates from chicken (Ramasamy et al., 2010).

2.7.8 Cholesterol Removal

Cholesterol removal can be performed through various methods, either via assimilation, binding to the cell surface, embedding in the cell wall, or co-perception with deconjugated bile (Kumar et al., 2013; Noh et al., 1997). It has been reported that LAB with active BSH worked in lowering cholesterol levels by interacting with the metabolism of the host bile salt (De Smet et al.,1998). Point of view, a study was performed on Kimchi that isolated *Lactobacillus plantarum*, which showed significant removal of cholesterol (Choi & Chang, 2015).

2.7.9 Heat Tolerances

Potential probiotic strains might get exposed to heat during the processing and storage phase in food processes (Aakko et al., 2014). Moreover, probiotics that were not able to tolerate heat showed less progress in most food production phases. Heat might result in several effects,s such as altered membrane fluidity, which might lead to the destabilization of several macromolecules such as RNA and ribosomes (Guchte & Serror, 2002). For this reason, heat tolerance is one of the main criteria that should be present in potential probiotics to enhance their efficiency during fermented food production. As an example of good heat-resistant probiotics, *Lactobacillus kefiranofeciens* MI, which was isolated from Taiwanese kefir grains, showed great heat tolerance (Chen & Chiang, 2017).

2.7.10 Lysozyme Tolerances

Lysozyme is an antimicrobial enzyme (EC 3.2.2.17) that is usually formed in tears, human milk, saliva, neutrophil granules, mucus, and egg white. However, potential probiotic strains should tolerate the effect of lysozyme. Lysozymes target gram-positive bacteria more than gram-negative as they might result in the destruction of gram-positive bacterial cell walls (Rada et al., 2010). In terms of lysozyme tolerance, the dairy product requires probiotics with a range of lysozyme tolerance between 25-35 mg/L (Guglielm et al., 2007). Ladakh isolated LAB strains exhibited tolerance toward lysozyme activates (Angmo et al., 2016).

2.7.11 Exopolysaccharides (ESP) Production

Exopolysaccharides provide cellular protection against toxic metals, bacteriophage attacks, and the innate immune factors of the host (Zannini et al., 2016). Besides, the dairy products industry requires probiotics that are able to produce ESP due to their improving impact on the rheological properties, texture, and taste of their products (Caggianiello et al., 2016). It has been found that ESP results in several health impacts, including 1) cholesterol reduction, 2) exhibiting antihypertensive activity, 3) amending fecal microbiota, and 4) protecting epithelium cells against intestinal pathogens (Bengoa et al., 2018). Moreover, a study reported that Turkish wheat

sourdough isolated probiotics strains that were able to produce ESP, such as *Lb. sanfranciscensis* ED5, *Lb. rossiae* ED1, *Lb. brevis* ED25 and *Lb. plantrarum* ED10 (Dertli et al., 2016).

2.8 Isolating Novel Probiotics from Traditional Foods

Most researchers are motivated to discover new probiotic strains from fermented food products due to the countless beneficial health impacts they offer. Manini and his colleagues discovered 13 LABs isolated from wheat bran sourdough, which were composed of 7 isolates of the *Lactobacillus* group, 4 *Leuconostoc* spp., and 2 *Pediococcus* spp (2016). Besides, all authors agreed that wheat bran sourdough is considered a wealthy source of new LAB with all favorable probiotic characteristics (Manini et al., 2016). In addition, 9 of the total isolated exhibited antifungal activity and were able to produce ESP, which will enable them to work efficiently in food industries (Manini et al., 2016). Regarding the limitations of the study, which was in the bile salt tolerance test, it was tested against oxgall only, and the antimicrobial activity assessment was performed against *Listeria* spp., only.

Another study was conducted on fermented cereals that isolated two strains of *Lb. plantarum* ULAG11 and ULAG24 in which both showed great resistance against acids and salt, while ULAG24 produced bacteriocins that inhibited the growth of pathogens (Oguntoyinbo & Narbad, 2015). In addition, ULAG24 was able to attach the HT29 cell line and BALB/C gut. However, the limitation of this study was in the acid tolerance assessment as pepsin was not added to the potential strains which affected LAB survival.

New probiotics were identified by Abbasiliasi (2017) and his colleagues, which isolated *Pediococcus acidilactici* kp10 from dried crude. In terms of characteristics, *Pediococcus acidilactici* kp10 displayed tolerance against phenol. Their antimicrobial activity played a role in defeating food-borne pathogens and produced peptidase and esterase-lipase (Abbasiliasi et al., 2017). Due to these characteristics, this probiotic is expected to work efficiently in the food industry. In terms of limitation, this study did not determine tolerance toward gastric and bile conditions.

2.9 Vegetables Production in UAE

The production and agriculture of most vegetables and crops are one of the main challenges faced in the UAE due to various land, water, and management challenges (Fathelrahman et al., 2017). However, vegetable production can occur under greenhouse conditions which enable crops to be produced in the offseason (Fathelrahman et al., 2017). According to the Ministry of Water and Environment (2022), in 2012, the vegetable production rate in the UAE reached 364 million AED, which is equivalent to $100 million USD. Most of the vegetable agriculture was carried out in Abu Dhabi. Indeed, statistics held by the UAE Bureau of Statistics displayed that 40% of the total agriculture of vegetables took place in Abu Dhabi. Farming is considered one of the emerging practices in the UAE as a large group of the population owns noncommercial small farms to raise their own fresh crops. Point of view in 2012, the International Center for Biosaline Agriculture ICBA reported that 70% of the total farms in the UAE were created to provide fresh vegetable and meat animals for home use purposes (Degefa et al., 2021).

2.10 Thesis Objective

As mentioned earlier, raising fresh vegetables is one of the common practices spread in the UAE, in which these vegetable products can be used to isolate potential probiotics that can be later used in food industries. However, this article was performed to improve the health benefits of vegetable products and to identify new LAB with all favorable characteristics. In terms of the thesis of objectives were to isolate LAB from fresh fruits and vegetables and new potential probiotics characteristics, including:

1) tolerance to the gastrointestinal conditions by a) *in-vitro* digestion, b) bile salts, and c) lysozyme;
2) physiological properties such as a) auto-aggregation, b) co-aggregation, c) hydrophobicity, d) adhesion to HT-29 cells, and e) cholesterol reduction;
3) production of desirable substances such as a) bile salt hydrolase, b) antimicrobials, and c) EPS production;
4) Immunomodulatory activities such as a) Immunomodulation and sensitivity to antibiotics.

Chapter 3: Methodology

3.1 Sample Collection

Samples (140) of fresh vegetables (various types, namely tomato, cucumber, strawberry, peach, lettuce, parsley, cabbage) were collected from local markets (Sharjah, UAE) and transported in an icebox to the food microbiology lab of the University of Sharjah, Sharjah, UAE, where the isolation on MRS agar was carried out. Characterization of the LAB isolates as potential probiotics was carried out in the food microbiology lab of United Arab Emirates University (UAEU). Unless otherwise mentioned, the isolation and characterization were completed using Sigma-Aldrich chemicals (St. Louis, MO, USA).

3.2 Isolation of Lactic Acid Bacteria

The pour-plate technique was performed using MRS agar (de Man, Rogosa and Sharpe; LAB-M, Heywood, UK) by mixing the sample with 99 mL MRS broth that is supplemented with 2% NaCl which was then blended for 2min, the mixture was then incubated for 24h at 37°C. After incubation, spread plate method on MRS agar was performed to the isolates from the incubated mixture, then the plates were incubated at 37°C for 24 h anaerobically in a CO_2 incubator (Binder C 170, Germany) for MRS. The Gram-positive and catalase-negative isolates were sub-cultured in MRS broth, and then the working stocks were prepared using 50 mL:50 mL glycerol: water. The stocks were stored at -80°C. The overnight activation at 37°C was carried out to investigate the potential probiotic characteristics of the isolates.

3.3 Tolerance to Stimulated Digestion Condition using INFOGEST2.0

The tolerance of the potential probiotic strains against *In-vitro* digestion conditions were performed according to the method of INFOGEST2.0 as described by Brodkorb and his colleges (2019). However, the 46 isolates were activated in MRS broth then was kept for 18hrs at 37℃. Later, overnight grown isolates were centrifuged at 5000x g at 4°C for about 10 min, and then the pellets were re-suspended in 0.1 mM sodium phosphate buffer (pH 7.0). After each digestion phase, 1 ml sample of the digest was aseptically taken, and serial dilution was made before being spread out on MRS and

M17 agar. Isolates to be classified as probiotics should survive several stresses while in GIT transit, including the low pH of the stomach, bile salts, and digestive enzymes (Ayyash et al., 2021). Thus, at this stage IN-120, isolates with the higher survival rates were selected for further investigations. After incubation at 37°C for 48 h, the plates were counted using a colony counter (Interscience San 1200; NY, USA). As a result of IN-120, 17 out of the 46 isolates had a significant reduction compared to their average in G0, which were excluded from further investigations.

3.4 Bile Salts Tolerance

As described by Li and Huo (2020), bile salt tolerance was carried out by adding cholic acid (0.30%), taurocholic acid (1.0%), and ox gall (1.0%) separately to the overnight activated isolates. After addition, plates were then incubated at 37°C in temperature-controlled Epoch™ microplate spectrophotometer (Epoch-2, BioTek; VT, USA). The absorbance was then measured at 620 nm at three different incubation times 0, 3, and 6 hrs. before each absorbance time, microplate instrument was used to shake each isolate for 5 sec.

3.5 Identification of the Probiotics

The 16S rDNA of the selected isolates was amplified according to AlKalbani et al. (2019). using PCR primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- TACGGYTACCTTGTTACGACTT-3'), and 16S rDNA sequence of the PCR product was done by Macrogen Sequencing Facilities (Macrogen, Seoul, Korea). The BLAST algorithm in the NCBI database was used to align the sequences and retrieve the accession number for each isolate from the GenBank. An online tool developed by Lemonie et al. (2019) was used to determine the most closely related bacterial species to the isolates by using MEGA software 7.0, and to create the dendrogram.

3.6 Safety Assessment of Selected LAB Isolated

3.6.1 Antibiotic Susceptibility Test

Antibiotic resistance test was performed according to Shivangi et al. (2018) with slight modifications as MRS and M17 agar plates were used for the respective isolates. The susceptibility of the isolates was tested against penicillin (PEN, 10 mg), clindamycin

(CLI, 2 mg), vancomycin (VAN, 30 mg), and erythromycin (ERY, 15 mg). 4 antibiotic discs were disturbed on MRS agar spread by selected LAB isolates. However, caliber was used to measure the diameter of the inhibition zone (mm).

3.7 Bile Salt Hydrolase (BSH) Activity

BSH activity can be observed by measuring the number of amino acids released from conjugated bile salts (6mM)) by LAB isolates as reported by Ayyash et al. (2018a). MRS broth was used to culture bacterial cells for 20 h at 37℃, which is then centrifuged at 4000x g for 15 min at 4°C. after washing the pellet with sterilized distilled water, they were suspended in 5mL of 0.1M PBS (pH6.0). Cell suspension was sonicated four time of 1 min interval by sonicater bath 2510 (Branson, Danbury, CT, USA), which is then cooled for 2 min in ice bath between each interval. Following by, these cell suspension is then centrifuges at 1000 x g for 5 min at 4°C. however, 100 µL of cell suspension was added with 1. mL of 0.1M PBS (pH 6.0) and 100 µL of tested bile salts that is a mix of 6mM sodium glycocholate, 6mM sodium taurocholate or 6 mM conjugated bile salt mixture (sodium glycochenodeoxycholic, taurocholic, taurochenodeoxycholic, and taurodeoxycholic acids). The mixture was then incubated for 30 min in the water bath at 37℃. Trichloroacetic acid (15%w/v) was added to inhibit the enzymatic reaction. After this addition, the mixture was centrifuged for 15 min at 4°C, and then 500 µL of the supernatant was mixed with 1 mL of distilled water, 1mL of ninhydrin reagent (500 µL of % ninhydrin in 0.5 M citrate buffer pH 5.5), 2 mL of 30% glycerol and 0.2 mL of 0.5M citrate buffer pH 5.5. this whole mixture was then vortexed for 30 s, then boiled at 100°C for 15 min, which was kept later to cool at room temperature. The mixture's absorbance was measure at 570 nm using a Epoch-2 Microplate Spectrophotometer (BioTek, CA, USA).

3.8 Cholesterol Removal

The ability of the isolates to remove cholesterol was tested based on the method of Shivangi et al. in order to produce cholesterol stock solution, 30mg of polyoxyethancyl-cholesterol sebacate (water-soluble cholesterol) was dissolved in 10ml of distilled water. Furthermore, final cholesterol concentration 100 µL/mL was formed by mixing 100 µL of cholesterol stock with 9.9 mL of MRS broth that is added with

0.3% oxgall. After producing the final cholesterol concentration 100 µL, centrifugation at 4000 x g for 14 min at 4°C was performed to remove bacterial cells. Afterwards, 1 mL of the supernatant was mixed and vortexed for 1 min with 2 mL of 96% ethanol and 1 mL of KOH (33%w/v). The mixture was then incubated in a water bath (ESB-18; Wisd-Witeg Labortechnik) for 15 min at 37°C and was then kept to cool down at room temperature. After this step, 2mL of distilled water and 3 mL of hexane was added to the mixture and vortexed for 1 min. However, the mixture was kept a side for a while till the two phases separate. After the two layers form, 1 milliliter of the upper hexane layer was placed in dried and sterile tubes that was then evaporated under nitrogen gas. The other dried tubes were used to add 2 milliliters of o-phthalaldehyde (OPA) reagent, which is 50 mg of OPA in 100 mL glacial acetic acid, that is then mixed and vortexed for 1 min with 0.5 ml of concentrated sulphuric acid. The mixture is then kept for 10 min at room temperature. The absorbance was measured at 550 nm using Epoch-2 Microplate Spectrophotometer.

3.9 Auto-Aggregation

Auto-aggregation of the activated cultures was performed according to the method described by Gao et al. (2021). The 17 selected isolates were cultured 16-8hrs at 37°C in MRS broth, which is then centrifuged at 5000 x g for 5min at 5°C. Followed by this step, the pellets were washed by 0.1 M phosphate buffer solution (PBS) at pH 6.8-7.0 to achieve (10^7-10^8 CFU/mL) and absorbance to 0.25. The absorbance was then measured at three timings 0, 4, and 24 h at 600 nm. Auto-aggregation was estimated according to the following equation: 37°C

$$\text{Auto-aggregation (\%)} = \left[1 - \frac{\text{At}}{\text{A0}}\right] \times 100$$

3.10 Hydrophobicity

Hydrophobicity of the isolates to various hydrocarbons, namely xylene, hexadecane, and octane was evaluated according to Ayyash et al. (2018a). Firstly, selected LAB isolates were activated and cultured in MRS broth for 16-16h at 37°C. afterwards, cells were collected by centrifugation at 5000 x g for 5min at 5°C, which was then washed by 0.1M phosphate buffer PBS at pH 6.8-7.0. then, the cell suspension was

mixed with 1mL of the hydrocarbon (xylene, hexadecane, and octane); each was added separately in dry sterilized culture tube that was then vortexed for 2 min. The mixture was kept on the side for 1h at 37°C until the separation phase occurred. After separation, the lower aqueous phase was placed to the UC cuvette (3 mL) by micropipette. The final absorbance was measured at 600 nm using a Epoch-2 Microplate Spectrophotometer.

3.11 Adhesion to HT-29 Cells

For determining the adhesion ability, the overnight activated isolates were centrifuged and washed twice with Dulbecco's phosphate-buffered saline (DBPS). Then adhesion percentage was estimated as per Oh et al. (2015) using the following equation

$$\text{Adhesion ability (\%)} = \left[\frac{\text{At}}{\text{A0}}\right] \times 100$$

3.12 Co-Aggregation

Co-aggregation was examined according to Ayyash et al. (2018a) using four pathogenic bacteria, namely *Escherichia coli* 0157:H7 1934, Staphylococcus *aureus* ATCC 25923, *Salmonella* Typhimurium 02-8423, and *Listeria monocytogenes* DSM 20649. Firstly, Bran heart infusion (BHI) broth was used to activate cell suspensions and each cell suspension of the four pathogens at 37°C. The absorbance was then measured at 0h (A_0) at 600 nm, and then without mixing the mixture was incubated for 4h at 37°C. After incubation, the absorbance was recorded at 2h and 4h (A_t). The results were expressed as co-aggregation percentages utilizing the equation below:

$$\text{Co-aggregation (\%)} = \left[1 - \frac{\text{At}}{\text{A0}}\right] \times 100$$

3.13 Antimicrobial Production

Antimicrobial activity was determined by using a cell-free supernatant as per Ayyash et al. (2018a). As a start, selected LAB isolates and indicator pathogens were activated in MRS and BHI broth overnight at 37°C. BHI agar injected with the indicator pathogen was placed in petri dish and kept for 2h under laminar flow to solidify. In BHI agar plate, a six 5-mm well was performed in each plate. The cell-free supernatant pH of

the selected LAB was modified to pH 6.5 ± 0.1 by 1 M NaOH. After this modification, 50 μL was added into the 5-mm well plates and was incubated aerobically for 1 whole day at 37℃. however, 1mm ≤ of clear inhibition zone (mm) in the well of each plate indicated positive inhibition.

3.14 Lysozyme activity

Evaluation of LAB isolates tolerance to lysozyme during 90 minutes of incubation at 37℃ was carried out as per Ayyash et al. (2018a). Overnight activated isolates were centrifuged at 4000 x g for 10 min at 4°C and washed with 0.1M PBS (pH 6.5) twice. To reach final concentration of 0.1mg/mL, washed pellets were added to 0.1M PBS (pH 6.5) inoculated with lysozyme. However, survival cells were then counted on MRS agar that was incubated for two days anaerobically at 37℃.

3.15 Exopolysaccharides (EPS) Production

EPS production indication test (-ve/+ve) was examined as described by Abushelaib (2017), using milk-ruthenium media. Sucrose, which is composed of 1.0% (w/v), skim milk powder 10% (w/v), agar 1.5% (w/v) and ruthenium red 0.08g/L, was added to prepare the ruthenium red milk agar. The activated LAB isolates were then marked on ruthenium red milk agar. However, isolates showing white ropy colony indicated positive ESP.

3.16 Statistical Analysis

One-way ANOVA was applied to determine if the differences between LAB isolates had a significant influence on the quantitative parameters (p <0.05). Tukey's test was used to detect differences between mean values with a *p-value* of <0.05. To calculate the mean values and standard deviations, all tests were performed at least three times. Minitab version 21.0 (Minitab, Ltd., Coventry, UK) was used for all statistical analyses for non-cell line studies. For the immunomodulatory effects, statistical significance between control and LAB isolate-stimulated cultures was analyzed by the unpaired two-tailed Student's **t**-test. The statistical analyses were performed using GraphPad PRISM 8 software (GraphPad Software, San Diego, CA), and differences with a *p-value* ≤ 0.05 were considered significant.

Chapter 4: Results and Discussion

4.1 Tolerance to the Gastrointestinal Conditions

4.1.1 Tolerance to In-Vitro Digestive Condition

One of the main criteria for any microorganism to be classified as probiotics, they must be able to endure gastrointestinal conditions like low pH, digestive enzymes, and bile salts (FAO/WHO, 2002). *In vitro* digestion test INFOGEST 2.0 is used, which measures microbial survival after each phase of contact with different simulated fluids. The viability of the microbes is determined by investigating the survival rate of LAB isolates before and after each phase. The survival rate of LAB isolates after in vitro digestion using INFOGEST2.0 is shown in Table 1. The salivary phase (G0) of in vitro digestion begins with the isolates reacting to salivary fluids containing salivary amylase, followed by the gastric phase (G120). The isolates' survival averaged 8.3 to 9.0 Log10 CFU/mL after the first stage G0 of the INFOGEST, which is similar to the beginning of the digestion (data not shown); no viability loss was observed in LAB isolates at the end of the oral phase implying that the LAB isolated survived salivary simulated fluid containing enzymes. This finding is consistent with previous research, which found the same behavior (Melchior et al., 2020; Reuben, 2020).

Almost all the bacterial isolates showed a slight decrease in viable numbers following the gastric phase as the pH in the simulated gastric fluid is maintained at <3, with varying levels depending on the isolate. Probiotic viability is generally determined by quantifying its resistance to simulated gastric juice or simulated intestinal fluid (Grispoldi et al., 2020). Isolates survived after G120 at an average rate of 8.0 to 8.6 Log10 CFU/mL, observing not much significant change in the bacterial number as seen in G0. The survival of LAB isolates at low pH <3 containing strong gastric enzymes like pepsin and lipases shows that it can resist the harsh stomach environment. This study aligns with the previous studies on camel milk isolates (Reuben, 2020; Yasmin et al., 2020).

After the intestinal phase (IN-120), the bacterial count ranged from 7.3 to 8.5 Log_{10} CFU/mL in simulated fluid containing bile as well as pancreatin at pH 7. Our

findings are consistent with those reported (Reuben, 2020; Yasmin et al., 2020; Tarique et al., 2022) after in vitro digestion. The overall survival rate from the beginning of the INFOGEST was higher than ~ 90% (Table 1), indicating the resistance to the salivary, gastric, and intestinal fluid containing different enzymes at different pH.

According to the (FAO/WHO, 2002), probiotics must have the ability to survive in the low pH, different enzymes, and salts that are seen in our isolates. The mechanism of resistance to GIT conditions differs depending on the strain and species (Ayyash et al., 2021). Survival in the harsh environment was used as preliminary criteria to select the LAB for continuing investigation of Probiotics. Thus, at this IN-120 stage, isolates with the highest survival rates were selected for further investigations. Out of the 46 isolates only 17 isolates (F1, F5, F8, F13, F15, F18, F21, F23, F25, F26, F28, F31, F37, F40, F41, F43, F46) with outstanding survival rate were selected for further assessment.

Table 1: In vitro digestive system tolerance (Log10 CFU/mL) of LBA isolated from vegetables

Isolates	G0	G120	In120
F1	8.8±0.42	8.3±0.67	8.2±0.59
F2	8.6±0.26	8.5±0.76	8.2±0.61
F3	8.6±0.28	8.4±0.77	8.1±0.50
F4	8.8±0.52	8.5±0.85	8.3±0.70
F5	8.9±0.45	8.5±0.85	8.1±0.70
F6	8.8±0.37	8.4±0.72	8.2±0.53
F7	8.8±0.49	8.4±0.84	8.3±0.73
F8	8.9±0.44	8.4±0.79	8.3±0.65
F9	8.9±0.54	8.2±0.90	8.3±0.75
F10	8.7±0.44	8.4±0.78	8.4±0.73
F11	8.8±0.57	8.1±0.87	8.3±0.67
F12	8.7±0.53	8.5±0.86	8.4±0.69
F13	8.7±0.41	8.4±0.63	8.3±0.74
F14	8.7±0.43	8.4±0.77	8.2±0.78
F15	8.8±0.38	8.4±0.72	8.5±0.75
F16	8.8±0.31	8.4±0.77	8.4±0.68
F17	8.8±0.30	8.4±0.69	8.3±0.73
F18	8.9±0.46	8.4±0.71	8.4±0.75
F19	8.3±0.09	8.1±0.62	7.9±0.58
F20	8.4±0.18	8.3±0.68	7.6±0.57
F21	8.9±0.49	8.6±0.83	8.4±0.66
F22	8.4±0.32	8.3±0.61	8.3±0.64
F23	8.6±0.31	8.5±0.88	8.2±0.61
F24	8.8±0.46	8.4±0.77	7.9±0.35
F25	8.7±0.39	8.3±0.78	8.2±0.59
F26	8.8±0.53	8.5±0.87	8.1±0.47
F27	8.4±0.68	8.4±0.85	8.1±0.50
F28	9.0±0.57	8.5±0.93	8.4±0.81
F29	8.8±0.48	8.4±0.90	7.6±0.38
F30	8.8±0.51	8.6±1.00	8.1±0.51
F31	8.7±0.54	8.4±0.78	8.2±0.50
F32	8.9±0.61	8.2±0.63	7.9±0.44
F33	8.6±0.57	8.4±0.74	7.7±0.46
F34	8.3±0.48	8.0±0.69	8.0±0.40
F35	8.6±0.50	8.2±0.59	7.9±0.37
F36	8.8±0.43	8.4±0.68	7.7±0.30
F37	8.9±0.52	8.4±0.84	8.2±0.50
F38	8.7±0.40	8.3±0.66	7.4±0.20
F39	8.8±0.54	8.2±0.71	8.1±0.56
F40	8.8±0.58	8.5±0.81	8.2±0.52
F41	8.9±0.57	8.4±0.72	8.5±0.85
F42	8.8±0.51	8.5±0.93	8.1±0.53
F43	8.9±0.78	8.5±0.79	8.5±0.74
F44	8.8±0.52	8.5±0.87	8.1±0.53
F45	9.0±0.52	8.4±0.72	8.4±0.62
F46	8.6±0.38	8.4±0.71	8.2±0.49

Values are expressed as the mean ± standard deviation of triplicate. G0 = Salivary phase, G120 = Gastric phase, In120 = Intestinal phase

4.1.2 Identification by 16S DNA

Using the 16S rRNA, each of the 17 isolates was able to be positively identified, aligned, and classified into one of five groups of Lactic Acid Bacteria that are *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus lactis, Enterococcus faecalis,* and *Pediococcus acidilactici* and listed with their accession number, as can be seen in Table 2. To identify Lactic Acid Bacteria at the species level, a molecular phylogeny analysis and the phylogenic tree was constructed by an online tool "https://ngphylogeny.fr/." The analysis was based on 16S rRNA sequences, and the evolutionary distances were calculated using the neighbor-joining method. In Figure 1, we see an illustration of the phylogenetic tree that includes all 17 isolates. According to the results of the sequence analysis, 7 out of 17 isolates grouped together with the 16S rRNA sequences of *Enterococcus faecium*, 6 out of 17 isolates grouped together with the sequences of *Enterococcus durans*, 2 out of 17 isolates grouped together with the sequences of *Pediococcus acidilactici* and remaining were *Enterococcus lactis* and *Enterococcus faecalis* each.

Table 2: Identification of LAB isolates using 16S rDNA gene sequencing and their accession number from GenBank

Sample	Organism	Accession No
F1	*Enterococcus faecium*	MW721241
F5	*Enterococcus durans*	MW721242
F8	*Enterococcus lactis*	MW721243
F13	*Enterococcus faecium*	MW721244
F15	*Enterococcus faecium*	MW721245
F18	*Enterococcus faecium*	MW721246
F21	*Pediococcus acidilactici*	MW721247
F23	*Enterococcus durans*	MW721248
F25	*Enterococcus faecium*	MW721249
F26	*Enterococcus durans*	MW721250
F28	*Pediococcus acidilactici*	MW721251
F31	*Enterococcus faecium*	MW721252
F37	*Enterococcus faecium*	MW721253
F40	*Enterococcus durans*	MW721254
F41	*Enterococcus durans*	MW721255
F43	*Enterococcus durans*	MW721256
F46	*Enterococcus faecalis*	MW721257

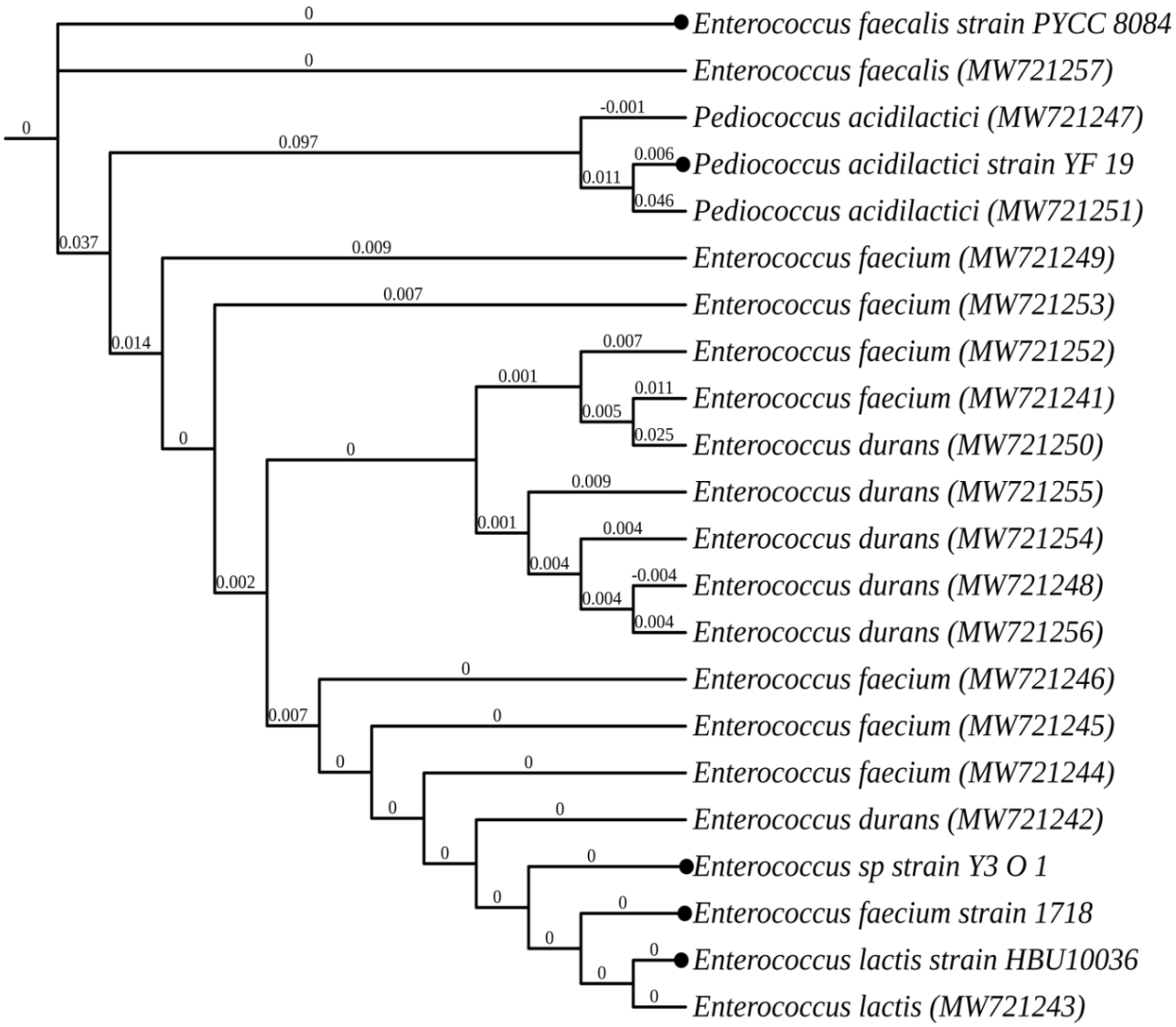


Figure 1: Polygenetic tree based on 16S rRNA sequences. Numbers in parentheses are accession numbers of identified sequences from GenBank. Filled circles are the reference strains from NCBI.

4.1.3 Bile Salts Tolerance

Bile tolerance is one of the most important characteristics of probiotic strains, and it should possess good resistance toward bile salts to survive in the human gastrointestinal tract (Abdalla et al., 2021). The selected 17 isolates were exposed to different bile salts (cholic acid (CA), oxgall (OX), and taurocholic acid (TA)), and their growth percentages are displayed in Table 2. Therefore, high survival percentages indicate good bile salts tolerance (Reuben, 2020; Stasiak-Różańska et al., 2021). As illustrated in Table 3, the survival rates ranged from 22.5 to 53.6%, 32.5 to 51.5%, and 41.8 to 60.9% in MRS supplemented with CA, OX, and TA, respectively, after 3 h of incubation. After 6 h, the survival rates ranged from 17.8 to 51.1%, 33.6 to 63.9%, and 55.9 to 72.5% for CA, OX, and TA, respectively. As a result, the survival rates generally increased against OX and TA and decreased against CA. This implies that CA has more inhibitory effects on the 17 isolates compared with OX and TA. These results are in accordance with those reported by (Abushelaibi, 2017). In conclusion, most isolates have reasonable resistance to taurocholic acid and oxgall compared to cholic acid. Bile salts have a destructive role on the membrane lipids of bacterial cells (Abdalla et al., 2021). Because of strain or species differences, the bile tolerances of all isolates differed dramatically. Bile salts are commonly used as detergents, which can harm the lipid membrane of bacteria. It may cause membrane damage and protein misfolding in the small intestine, resulting in DNA damage from oxidative stress and low intracellular pH. The presence of polysaccharides on the outer cell membrane has been suggested as a possible cause of bile salt resistance (Yerlikaya & Akbulut, 2019; Stasiak-Różańska et al., 2021; Melchior et al., 2020).

Table 3: Bile tolerances (%) for 17 selected LAB isolates *after 3h and 6h*

Isolates	3h			6h		
	CA	OX	TA	CA	OX	TA
E. faecium MW721241	43.9	42.7	56.1	39.3	39.9	58.8
E. durans MW721242	29.1	38.9	48.7	27.6	52.4	63.0
E. lactis MW721243	53.7	40.2	60.9	51.1	39.0	60.7
E. faecium MW721244	46.2	37.4	59.5	45.3	33.6	60.0
E. faecium MW721245	45.0	39.9	59.0	41.5	35.2	57.0
E. faecium MW721246	41.4	40.7	60.0	36.4	38.8	58.1
P. acidilactici MW721247	22.5	39.7	49.8	20.5	51.1	63.8
E. durans MW721248	26.7	33.5	44.9	23.9	49.9	59.6
E. faecium MW721249	32.3	40.7	55.3	35.4	55.7	66.9
E. durans MW721250	33.0	42.5	41.8	29.7	60.6	56.0
P. acidilactici MW721251	26.3	42.1	51.2	17.8	56.0	63.5
E. faecium MW721252	28.0	35.6	47.5	25.2	52.4	64.0
E. faecium MW721253	33.0	32.5	45.4	37.3	55.8	62.7
E. durans MW721254	36.1	50.1	48.3	34.5	62.2	62.2
E. durans MW721255	35.3	40.7	45.5	29.3	42.9	63.6
E. durans MW721256	32.3	45.1	56.3	37.8	50.4	72.5
E. faecalis MW721257	37.6	51.5	54.8	44.4	63.9	68.6

Values are expressed as the mean ± standard deviation of triplets. CA= cholic acid, OX = oxgall TA= taurocholic acid

4.1.4 Lysozyme Tolerance

In the oral cavity, probiotic bacteria are subjected to saliva, which contains lysozyme as well as electrolytes; consequently, lysozyme tolerance is one of the criteria that is used in the selection process for probiotic bacteria. Lysozyme is an antibacterial enzyme that may be found in tears, egg white, human milk, neutrophil granules, and saliva, as well as mucus and mucus membranes. Lysozyme can cause damage to the bacterial cell wall in certain bacteria. When compared to Gram-negative bacteria, Gram-positive bacteria are more prone to the hydrolysis process induced by lysozyme. The initial average growth of each of the isolates was 8.3 Log10 CFU/ml, and after 90 minutes of incubation with lysozyme, it was 8.4 Log10 CFU/ml. All of the isolates exhibited good tolerance to lysozyme, as shown in Table 4. Other authors have noted that *Lactobacillus* strains are able to withstand concentrations of lysozyme with a high level of resistance and our findings are comparable to those observed from isolates derived from fermented idli, Rabaadi, pickles, camel milk, and sausages (Yadav et al., 2016; Abushelaibi et al., 2017; Jia et al., 2021). Studies suggest that the presence of peptidoglycan in probiotics can be a reason for the resistance of the lysozyme (Ferraboschi et al., 2021).

Table 4: Lysozyme tolerance (Log10 CFU/mL) of LAB isolated from vegetables *at 0 and 90min*

	Lysozyme tolerance	
Isolates	0 min	90 min
E. faecium MW721241	8.1±0.47^{c}	8.3±0.27^{bc}
E. durans MW721242	8.1±0.45^{c}	8.1±0.04^{d}
E. lactis MW721243	8.8±0.57^{a}	8.4±0.49^{b}
E. faecium MW721244	8.5±0.49^{b}	8.2±0.25^{c}
E. faecium MW721245	8.5±0.31^{b}	8.3±0.34^{bc}
E. faecium MW721246	8.1±0.35^{c}	8.2±0.32^{c}
P. acidilactici MW721247	8.0±0.43^{c}	8.2±0.27^{c}
E. durans MW721248	8.8±0.36^{a}	8.2±0.25^{c}
E. faecium MW721249	8.1±0.59^{c}	8.3±0.25^{bc}
E. durans MW721250	8.8±0.40^{a}	8.4±0.32^{b}
P. acidilactici MW721251	8.0±0.52^{c}	8.5±0.47^{a}
E. faecium MW721252	8.6±0.27^{ab}	8.2±0.23^{c}
E. faecium MW721253	8.4±0.33^{bc}	8.3±0.29^{bc}
E. durans MW721254	8.4±0.32^{bc}	8.4±0.42^{b}
E. durans MW721255	8.4±0.33^{bc}	8.4±0.31^{b}
E. durans MW721256	8.5±0.46^{b}	8.3±0.35^{bc}
E. faecalis MW721257	8.4±0.37^{bc}	8.6±0.45^{a}

$^{a–d}$ Mean values in the same column with different lowercases differ significantly ($p < 0.05$).

4.2 Auto-Aggregation, Co-Aggregation, Hydrophobicity, Adhesion to HT-29 Cells, and Cholesterol-Lowering

4.2.1 Auto Aggregation

Auto-aggregation is a desirable trait for probiotic strains since it is thought to aid the colonization of the human gut, prevent pathogen infections, and alter the mucosa of the colon. When choosing a probiotic bacterium, the ability to adhere to the digestive tract walls is critical.

Table 5 shows that after 4 hours, the auto-aggregation of the isolates varied from 1.8 to 26.2%; after 24 hours, it rose considerably ($p < 0.05$) from 42.4 to 73.2%, with an average of 59.6%, with a smaller difference than after 4 hours. These findings are greater than some of the probiotic's studies (Abushelaibi et al., 2017; Vasiee, 2020) but were lower than that of *L. plantarum* and *Pediococcus pentosaceus* (Gao et al., 2021; Sui et al., 2021). The production of biofilms, which prevent pathogens from attaching to the gut, is indicated by auto-aggregation and improves gut colonization and the effectiveness of other probiotics (Gao et al., 2021; Ladha & Jeevaratnam, 2018).

Table 5: Auto-aggregation (%) of potential probiotic LAB isolates *at 4h and 24h*

Isolate	Auto-aggregation (%)	
	4 h	24 h
E. faecium MW721241	26.1±0.87[a]	66.5±2.83[c]
E. durans MW721242	9.8±2.27[d]	63.6±2.57[d]
E. lactis MW721243	15.6±1.90[c]	45.0±2.19[f]
E. faecium MW721244	26.2±0.02[a]	56.7±0.13[e]
E. faecium MW721245	18.9±0.31[b]	47.2±0.60[f]
E. faecium MW721246	17.4±0.58[b]	42.9±0.29[g]
P. acidilactici MW721247	2.0±0.16[h]	70.3±0.84[b]
E. durans MW721248	3.8±1.15[f]	73.2±2.90[a]
E. faecium MW721249	2.3±1.06[g]	63.6±1.44[d]
E. durans MW721250	3.6±0.30[f]	70.6±2.61[b]
P. acidilactici MW721251	1.8±0.13[h]	70.5±1.99[b]
E. faecium MW721252	2.4±0.16[g]	72.0±1.17[a]
E. faecium MW721253	8.2±0.34[d]	70.5±2.73[b]
E. durans MW721254	8.0±0.53[e]	63.4±1.02[d]
E. durans MW721255	11.1±1.04[c]	35.2±0.35[h]
E. durans MW721256	9.4±1.91[d]	42.4±1.61[g]
E. faecalis MW721257	-	-

Values are the mean ± standard deviation of triplicates. [a–h] Mean values in the same column with different lowercases differ significantly ($p < 0.05$).

4.2.2 Hydrophobicity

The hydrophobicity of the cell surface of the investigated strains varied greatly, with the hydrophobic spectrum ranging from 6.9% to 77.1, 17.3% to 86.7%, and 29.3% to 84.3% for xylene, octane, and hexadecane, respectively (Table 6). *E. durans* MW721250 and *E. durans* MW721254 had the lowest hydrophobicity for all hydrocarbons. The fact that various hydrocarbons have varied hydrophobicity patterns demonstrates that hydrophobicity is linked to strain-specific cell surface proteins. The hydrophobic components of the outer membrane are responsible for this capacity (Vasiee, 2020; Gao, 2021). In adhesion and biofilm development, bacteria's hydrophobic contacts are crucial. The content of the surrounding media, the stage of bacteria's development, and the shape of cell surface components all affect how bacteria behave as hydrophobic particles (Rokana et al., 2018). The findings were better than those reported in investigations on isolates from dairy, sausages, and other sources (Ayyash et al., 2018b; Gao et al., 2021; Reuben et al., 2020). When tested with n-hexadecane alone, however, *P. pentosaceus* and *Latilactobacillus sakei* demonstrated better hydrophobicity (Ladha & Jeevaratnam, 2018; Sharma et al., 2021) showed lactic acid bacteria isolated from camel milk had hydrophobicity > 95% to various hydrocarbons.

Table 6: Hydrophobicity (%) of potential probiotic LAB isolates

Isolate	Hydrophobicity (%)		
	Xylene	Octane	Hexadecane
E. faecium MW721241	61.4±3.07^{b}	68.7±2.06^{c}	76.1±3.05^{b}
E. durans MW721242	35.9±1.79^{cd}	40.7±1.22^{de}	47.3±1.89^{c}
E. lactis MW721243	77.1±3.86^{a}	79.0±2.37^{b}	84.3±3.37^{a}
E. faecium MW721244	71.0±3.55^{a}	86.7±2.60^{a}	82.0±3.28^{ab}
E. faecium MW721245	66.0±3.30^{b}	79.0±2.37^{b}	80.7±3.23^{b}
E. faecium MW721246	56.9±2.84^{bc}	69.4±2.08^{c}	73.3±2.93^{bc}
P. acidilactici MW721247	13.3±0.66^{f}	36.6±1.10^{de}	39.6±1.58^{d}
E. durans MW721248	32.7±1.64^{d}	38.3±1.15^{de}	45.7±1.83^{c}
E. faecium MW721249	13.7±0.69^{f}	28.6±0.86^{e}	32.1±1.29^{e}
E. durans MW721250	13.0±0.65^{f}	17.3±0.52^{g}	29.3±1.17^{f}
P. acidilactici MW721251	11.4±0.57^{g}	29.9±0.90^{e}	34.9±1.39^{de}
E. faecium MW721252	18.9±0.94^{e}	23.7±0.71^{ef}	42.3±1.69^{c}
E. faecium MW721253	33.4±1.67^{cd}	21.7±0.65^{f}	34.1±1.37^{de}
E. durans MW721254	6.9±0.34^{h}	30.3±0.91^{e}	35.3±1.41^{de}
E. durans MW721255	34.0±1.70^{cd}	44.6±1.34^{d}	30.7±1.23^{e}
E. durans MW721256	40.1±2.01^{c}	56.1±1.68^{cd}	46.9±1.87^{c}
E. faecalis MW721257	31.0±1.55^{d}	45.7±1.37^{d}	39.9±1.59^{d}

Values are the mean ± standard deviation of triplicates. $^{a–f}$ Mean values in the same column with different lowercases differ significantly ($p < 0.05$).

4.2.3 Attachment to HT-29 Cells

The potential of probiotics is linked to their ability to survive in the gastrointestinal system. As a result, attachment ability is used as a criterion for choosing a suitable probiotic. The interaction of lipids, peptidoglycan, and surface proteins on the bacterial cell wall is involved in the attachment. Many *Lactobacillus* species have been shown to have protein elements connected to their cell walls that facilitate bacterial attachment to intestinal epithelial cells.

After allowing isolates to attach to HT-29 cells for 2 hours, they displayed similar attachment capacities, with an average of 8.03 Log10 CFU/mL. The growth rate varied between 7.5 and 8.3 Log10 CFU/mL. (Table 7). These findings are in agreement with those of (Oh & Jung, 2015; Vasiee, 2020; Gao, 2021), who recovered *Lactobacillus*, *Pediococcus*, and *Lactiplantibacillus* bacteria from kimchi, alcoholic drinks, and other sources, but the camel milk isolates from (Sharma et al., 2021) showed 99% attachment ability which is much better than our findings. The ability of probiotics to adhere to epithelial cells is thought to be strain dependent as well as different cell lines showed different attachment properties; hence the rates of adhesion vary between isolates (Oh & Jung, 2015; Domingos-Lopes et al., 2020).

Table 7: Attachment to HT-29 cells (Log10 CFU/well) of potential probiotic LAB isolates

Isolate	Attach to HT-29
	Log_{10} CFU
E. faecium MW721241	8.0±0.08[c]
E. durans MW721242	8.0±0.00[c]
E. lactis MW721243	8.1±0.02[b]
E. faecium MW721244	8.0±0.10[c]
E. faecium MW721245	8.1±0.07[b]
E. faecium MW721246	8.1±0.04[b]
P. acidilactici MW721247	8.1±0.04[b]
E. durans MW721248	8.1±0.03[b]
E. faecium MW721249	8.1±0.04[b]
E. durans MW721250	7.9±0.03[d]
P. acidilactici MW721251	8.0±0.07[c]
E. faecium MW721252	8.1±0.06[b]
E. faecium MW721253	8.0±0.05[c]
E. durans MW721254	7.5±0.01[d]
E. durans MW721255	8.0±0.05[c]
E. durans MW721256	8.1±0.02[b]
E. faecalis MW721257	8.3±0.08[a]

Values are the mean ± standard deviation of triplicates. [a–d] Mean values in the same column with different lowercases differ significantly ($p < 0.05$).

4.2.4 Co-Aggregation

Coaggregation plays a significant role in the elimination of pathogens from the gastrointestinal system. The coaggregation of different strains of *Lactobacillus* has the potential to create a barrier that impedes the colonization of harmful bacteria. Table 8 presents the findings of a co-aggregation test conducted against four well-known foodborne pathogens (*E. coli* O157:H7, *S. Typhimurium*, *L. monocytogenes*, and *S. aureus*) while the bacteria were incubated at 37°C for 4 and 24 hours. As observed in Table 8A, after 4 hours, the co-aggregation rates ranged from a low of 3.44% to a high of 10.43%, but after 24 hours, as shown in Table 8B, the range increased to a high of 21.38%to 42.61%. This indicates that the ability to co-aggregate is directly correlated with the passage of time (Abushelaibi, 2017). After 24 hours, the coaggregation rates of *E. faecium* MW721252 with all pathogens were the highest, followed by *E. durans* MW721255, which had the lowest rate (Table 8B). The results of the analysis of variance did not show any significant differences in the co-aggregation of the four foodborne pathogens when they were found in the same isolate at the same time. Our results show lesser coaggregation than isolates obtained from raw milk and rumen liquor of goats, but similar results to that of probiotics obtained from sausages and dairy products reported (AlKalbani et al., 2019; Ladha & Jeevaratnam, 2018; Tatsaporn & Kornkanok, 2020). The capacity of the probiotic strains to co-aggregate is one of the most important factors in biofilm formation and the competition with pathogens for binding sites and is considered an essential characteristic of probiotics.

Table 8A: Coaggregation (%) of LAB isolates with four pathogens after 4h

Isolates	4h			
	E. coli	*S. Typhi*	*S. aureus*	*L. monocytogenes*
E. faecium MW721241	10.1±0.50^{a}	10.6±0.63^{a}	10.0±0.70^{a}	10.2±0.71^{a}
E. durans MW721242	7.6±0.38^{c}	8.4±0.51^{bc}	9.7±0.68^{b}	8.0±0.56^{b}
E. lactis MW721243	9.4±0.47^{ab}	6.7±0.40^{d}	10.0±0.70^{a}	6.8±0.47^{d}
E. faecium MW721244	6.8±0.34^{ed}	9.6±0.58^{b}	9.3±0.65^{b}	10.3±0.72^{a}
E. faecium MW721245	8.4±0.42^{b}	9.4±0.57^{b}	10.8±0.76^{a}	8.7±0.61^{b}
E. faecium MW721246	7.8±0.39^{c}	5.9±0.35^{e}	8.2±0.58^{c}	6.7±0.47^{d}
P. acidilactici MW721247	5.5±0.27^{d}	3.8±0.23^{g}	4.4±0.31^{d}	3.9±0.27^{f}
E. durans MW721248	4.0±0.20^{e}	6.7±0.40^{d}	6.3±0.44^{de}	6.1±0.43^{d}
E. faecium MW721249	4.1±0.21^{e}	5.5±0.33^{e}	5.1±0.36^{e}	3.8±0.27^{f}
E. durans MW721250	4.9±0.25^{e}	7.7±0.46^{c}	4.9±0.34^{f}	6.2±0.43^{d}
P. acidilactici MW721251	3.2±0.16^{f}	4.4±0.26^{f}	3.2±0.23^{g}	5.1±0.36^{e}
E. faecium MW721252	2.9±0.15^{g}	5.5±0.33^{e}	4.7±0.33^{g}	3.9±0.27^{f}
E. faecium MW721253	3.4±0.17^{f}	6.1±0.37^{d}	9.1±0.64^{b}	5.7±0.40^{e}
E. durans MW721254	5.7±0.28^{d}	7.7±0.46^{c}	7.1±0.50^{cd}	6.7±0.47^{d}
E. durans MW721255	5.7±0.28^{d}	6.4±0.38^{d}	7.5±0.53^{cd}	7.1±0.49^{c}
E. durans MW721256	5.1±0.26^{de}	5.2±0.31^{e}	7.0±0.49^{d}	6.1±0.43^{d}
E. faecalis MW721257				

Values are the mean ± standard deviation of triplicates. $^{a–g}$ Mean values in the same column with different lowercases differ significantly ($p < 0.05$)

Table 8B: Coaggregation (%) of LAB isolates with four pathogens after 24h

Isolates	24h			
	E. coli	*S.* Typhi	*S. aureus*	*L. monocytogenes*
E. faecium MW721241	25.5±1.78^{d}	32.7±1.31^{b}	34.8±0.70^{d}	32.6±1.63^{b}
E. durans MW721242	23.8±1.67^{d}	24.4±0.98^{c}	27.1±0.54^{e}	25.0±1.25^{de}
E. lactis MW721243	21.7±1.52^{e}	23.8±0.95^{cd}	27.4±0.55^{e}	23.9±1.19^{de}
E. faecium MW721244	22.1±1.55^{e}	28.0±1.12^{bc}	31.3±0.63^{d}	28.6±1.43^{c}
E. faecium MW721245	22.4±1.57^{e}	26.7±1.07^{c}	30.7±0.61^{d}	26.2±1.31^{d}
E. faecium MW721246	20.5±1.44^{e}	22.5±0.90^{d}	29.7±0.59^{e}	24.8±1.24d^{e}
P. acidilactici MW721247	38.6±2.70^{b}	40.0±1.60	38.6±0.77^{b}	35.6±1.78^{b}
E. durans MW721248	43.8±3.07^{a}	40.8±1.63^{b}	39.2±0.78^{b}	28.0±1.40^{c}
E. faecium MW721249	33.7±2.36^{c}	42.3±1.69^{aa}	44.0±0.88^{a}	36.6±1.83^{ab}
E. durans MW721250	43.5±3.05^{a}	39.1±1.56^{ab}	39.4±0.79^{b}	38.7±1.94^{a}
P. acidilactici MW721251	42.4±2.97^{a}	33.8±1.35^{b}	37.4±0.75^{c}	31.8±1.59^{b}
E. faecium MW721252	45.5±3.18^{a}	40.7±1.63^{ab}	40.7±0.81^{b}	36.7±1.83^{ab}
E. faecium MW721253	30.5±2.14^{c}	24.2±0.97^{c}	27.4±0.55	26.4±1.32^{d}
E. durans MW721254	26.6±1.86^{d}	25.7±1.03^{c}	28.5±0.57^{e}	26.5±1.32^{d}
E. durans MW721255	18.2±1.28^{f}	21.0±0.84^{d}	25.2±0.50^{f}	21.2±1.06^{e}
E. durans MW721256	21.5±1.50^{e}	22.7±0.91^{d}	27.8±0.56^{e}	21.1±1.05^{e}
E. faecalis MW721257				

Values are the mean ± standard deviation of triplicates. $^{a–f}$ Mean values in the same column with different lowercases differ significantly ($p < 0.05$)

4.2.5 Cholesterol Removal

The deconjugation of bile salts by the enzyme bile salt hydrolase (BSH), the production of short-chain fatty acids, the assimilation of cholesterol into bacterial cell membranes, and the conversion of cholesterol by hydrogenation to the poorly absorbed sterol coprostanol have all been demonstrated as mechanisms for cholesterol removal by probiotics (Hernández-Gómez et al., 2021). From Table 9, it is seen that all the isolates have the ability to reduce the cholesterol in the broth, and the reduction percentage ranged from 17% to 35%, and *E. faecium* MW721241, *E.lactis* MW721243, *P. acidilactici* MW721247, *E. faecium* MW721249, *E. faecium* MW721252, *E. faecium* MW721253, *E. durans* MW721254, and *E. faecalis* MW721257 showed reduction more than 30%, which coincided with the lactic acid bacteria isolated from traditional Italian cheeses (Albano et al., 2018). Several studies showed the reducing potential of cholesterol using LAB and its role in controlling hypercholesterolemia (Albano et al., 2018; Tsai et al., 2014).

Table 9: Cholesterol removal (%) ability of LAB isolates

Isolate	CR (%)
E. faecium MW721241	35.0±1.41[a]
E. durans MW721242	27.5±2.12[e]
E. lactis MW721243	34.5±0.71[a]
E. faecium MW721244	17.5±0.71[g]
E. faecium MW721245	17.0±1.41[g]
E. faecium MW721246	29.5±0.71[d]
P. acidilactici MW721247	30.0±0.23[c]
E. durans MW721248	33.0±1.41[b]
E. faecium MW721249	30.5±3.54[c]
E. durans MW721250	24.5±2.12[f]
P. acidilactici MW721251	29.0±0.99[d]
E. faecium MW721252	30.0±2.83[c]
E. faecium MW721253	35.0±0.98[a]
E. durans MW721254	30.0±2.83[c]
E. durans MW721255	27.5±2.12[e]
E. durans MW721256	24.5±2.12[f]
E. faecalis MW721257	30.0±2.83[c]

Values are the mean ± standard deviation of triplicates. [a–g] Mean values in the same column with different lowercases differ significantly ($p < 0.05$)

4.3 Clinical and Industrial Benefits (Bile Salt Hydrolase, Antimicrobials, and Exopolysaccharides (EPS)), Immunomodulatory Effects and Susceptibility to Antibiotics

4.3.1 Bile Salt Hydrolase (BSH) Activity

Bile salt hydrolase is an enzyme that probiotic bacteria can produce to hydrolyze conjugated bile salts, and these deconjugated bile salts or acids trigger the removal of cholesterol by helping in the absorption of cholesterol into the human gut. Deconjugated bile salts consequently co-precipitate with cholesterol and damage its solubility, resulting in the ejection of cholesterol in the stool (Begley et al., 2006). Deconjugation is a gateway reaction in the metabolism of bile acids in the small intestine, which overall impacts in reduction of the blood cholesterol level in the individual with hypercholesterolemia (Hernández-Gómez et al., 2021; Xu et al., 2019). Table 10 shows that nearly all the LAB showed the ability to hydrolyze the bile salts mixture by releasing the amino acids in the medium. As shown in Table 10, *E. faecium* MW721249 and *Enterococcus durans* MW721254 had the lowest activity compared to *E. lactis* MW721243 and *E. faecium* MW721244 which had the highest. BSH activity plays a significant role in inhibiting cholesterol absorption/uptake in the human intestine. The ability to hydrolyze bile salt leads to disrupting the formation of the cholesterol micelle in the human intestine (Abdalla et al., 2021). Our BSH with those reported by (Ayyash et al., 2018a; Tarique et al., 2022). BSH activity, along with cholesterol removal, has become one of the criteria for probiotics (Amiri et al., 2020).

Table 10: Bile salt hydrolysis activity (U/mg) of LAB isolates

Isolate	BSH(U/mg)
E. faecium MW721241	0.83 ± 0.03^{f}
E. durans MW721242	0.95 ± 0.05^{b}
E. lactis MW721243	1.03 ± 0.07^{a}
E. faecium MW721244	1.00 ± 0.08^{a}
E. faecium MW721245	0.93 ± 0.05^{b}
E. faecium MW721246	0.83 ± 0.03^{f}
P. acidilactici MW721247	0.91 ± 0.06^{c}
E. durans MW721248	0.87 ± 0.07^{d}
E. faecium MW721249	0.80 ± 0.02^{g}
E. durans MW721250	0.84 ± 0.04^{e}
P. acidilactici MW721251	0.84 ± 0.04^{e}
E. faecium MW721252	0.84 ± 0.05^{e}
E. faecium MW721253	0.82 ± 0.04^{f}
E. durans MW721254	0.80 ± 0.03^{g}
E. durans MW721255	0.81 ± 0.06^{g}
E. durans MW721256	0.97 ± 0.04^{ab}
E. faecalis MW721257	0.91 ± 0.07^{c}

Values are the mean ± standard deviation of triplicates. $^{a–g}$ Mean values in the same column with different lowercases differ significantly ($p < 0.05$)

4.3.2 Antimicrobial Activities

One of the most remarkable effects that have been observed is the antimicrobial activity against various pathogens. To be taken into consideration for the selection of potential probiotic strains. LAB isolates exhibited a wide range of inhibitory levels against all of the foodborne pathogens tested, despite having a relatively broad spectrum of activity. Lactic Acid Bacteria have the ability to produce antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide, ethanol, reuterin, bacteriocins, and proteins that kill bacteria. It has been discovered that LABs are able to inhibit microorganisms that cause spoilage and pathogens by producing anti-microbial metabolites. According to the work that was done, it would appear that the antimicrobial activities of all of the isolated LAB resulted not from the acidity of the crude extracts but rather from the active compounds that were produced by the probiotics, as noticed in Table 11B, postbiotic, which are heat-killed bacteria, had a better antimicrobial effect against *E. coli* than probiotics, while probiotics Table 11A had a better effect against *S. aureus* and *L. monocytogenes* when compared with postbiotic. It has been reported that metabolites produced by LAB isolates, such as bacteriocins, peptides, organic acids, and volatile compounds, are highly associated with antimicrobial activity (Abushelaibi, 2017; Reuben, 2020; Vasiee, 2020). The antimicrobial activity of the killed cells suggests that the cell membrane and cytoplasm possess antimicrobial activities against foodborne pathogens. Our results coincide with those reported by (Miremadi et al., 2014).

Table 11A: Antimicrobial activity of potential probiotics (live bacteria) LAB isolates against four foodborne pathogens

Isolates	Probiotic [a]			
	E. coli	*S. aureus*	*S.* Typhi	*L. monocytogenes*
E. faecium MW721241	+	++	+++	+++
E. durans MW721242	+	++	++	+++
E. lactis MW721243	+	++	++	++
E. faecium MW721244	+	+++	+++	+++
E. faecium MW721245	+	+++	+++	+++
E. faecium MW721246	+	++	+++	+++
P. acidilactici MW721247	+	+++	+++	+++
E. durans MW721248	+	+++	+++	++
E. faecium MW721249	+	++	++	++
E. durans MW721250	+	++	++	++
P. acidilactici MW721251	+	++	++	+++
E. faecium MW721252	+	+++	++	++
E. faecium MW721253	+	++	++	++
E. durans MW721254	+	+	+	+
E. durans MW721255	+	++	+	+
E. durans MW721256	+	+	++	+
E. faecalis MW721257	+	++	++	+

(+) log reduction was <1.0; (++) log reduction was less than 2.0 and higher than 1.0; (+++) log reduction was greater than 2.1

Table 11B: Antimicrobial activity of potential postbiotics (heat killed bacteria) LAB isolates against four foodborne pathogens

Isolates	Postbiotic [b]			
	E. coli	*S. aureus*	*S.* Typhi	*L. monocytogenes*
E. faecium MW721241	+++	+++	+++	+++
E. durans MW721242	++	++	++	++
E. lactis MW721243	++	++	++	++
E. faecium MW721244	++	++	++	++
E. faecium MW721245	+	+	+	+
E. faecium MW721246	++	++	++	++
P. acidilactici MW721247	++	++	++	++
E. durans MW721248	++	++	++	++
E. faecium MW721249	++	++	++	++
E. durans MW721250	++	++	++	++
P. acidilactici MW721251	++	++	++	++
E. faecium MW721252	++	++	++	++
E. faecium MW721253	+	+	+	+
E. durans MW721254	+	+	+	+
E. durans MW721255	+	+	+	+
E. durans MW721256	+	+	+	+
E. faecalis MW721257	+	+	+	+

(+) log reduction was <1.0; (++) log reduction was less than 2.0 and higher than 1.0; (+++) log reduction was greater than 2.1

4.3.3 Antibiotic Resistance

In order to be considered probiotics, microbial strains should not serve as a reservoir for antibiotic resistance genes, as these genes could potentially be passed on to intestinal pathogens via transposons and plasmids (Goh et al., 2021). Table 12 presents the findings regarding the levels of antibiotic resistance and susceptibility discovered throughout the course of this investigation. However, the majority of probiotic bacteria were either susceptible to the majority of antibiotics or were only moderately susceptible but were resistant to the vancomycin, as it's known that most of the *Lactobacillus* strains are resistant naturally to some of the antibiotics (Wong et al., 2015). Some other studies showed similar behavior where they found the probiotics were either resistant or partially susceptible, which suggests that it can be used in combination with antibiotics for the treatment of certain infections (Neut et al., 2017). Because there is a risk that antibiotic-resistant bacteria could be horizontally transmitted to non-resistant bacteria, including pathogens, the antibiotic resistance of potential probiotic bacteria is an important safety consideration when selecting these bacteria for use as probiotic organisms and starter culture (Vieco-Saiz et al., 2019).

Table 12: Antibiotics susceptibility of LAB isolated from vegetables

Isolates	Antibiotics susceptibility			
	Vancomycin	Erythromycin	Penicillin	Clindamycin
E. faecium MW721241	R	S	S	S
E. durans MW721242	R	S	S	S
E. lactis MW721243	R	R	S	R
E. faecium MW721244	R	MS	S	R
E. faecium MW721245	R	S	S	MS
E. faecium MW721246	R	S	S	R
P. acidilactici MW721247	R	S	S	S
E. durans MW721248	R	S	S	S
E. faecium MW721249	R	S	S	S
E. durans MW721250	R	S	S	S
P. acidilactici MW721251	R	S	S	S
E. faecium MW721252	R	MS	S	S
E. faecium MW721253	R	MS	MS	MS
E. durans MW721254	R	S	S	S
E. durans MW721255	R	S	S	MS
E. durans MW721256	R	S	S	S
E. faecalis MW721257	R	S	S	S

R = resistant; MS = moderately resistant; S = susceptible.

4.3.4 EPS Production

Fermented foods, which are associated with probiotic microorganisms, create substances that are significant from a technological perspective, such as exopolysaccharides (EPS). EPS are extracellular macromolecules that are excreted by microorganisms either in the form of a tightly bound capsule or a loosely attached slime layer (Angelin & Kavitha, 2020; Tarique et al., 2022). Desiccation, phagocytosis, cell recognition, phage attack, antibiotics or toxic compounds, and osmotic stress are some of the things that they are most effective at defending against (Angelin & Kavitha, 2020). Table 13 provides a summary of the findings from the production of exopolysaccharides (EPS). *E. faecium* MW721241*, E. durans* MW721242*, P. acidilactici* MW721247*, E. durans* MW721248, *E. durans* MW721250, *E. durans* MW721256, and *E. faecalis* MW721257 did not demonstrate the ability to produce EPS, whereas the remaining demonstrated the ability to produce EPS. EPS can be produced in bacteria either freely in the medium or in the form of capsules. Nevertheless, EPSs have been shown to have a significant correlation with the formation of biofilms, attachment to the intestinal cell wall, reduction in cholesterol levels, and protection against harsh environmental conditions (Abdalla et al., 2021). In comparison to other naturally occurring agents, exopolysaccharides that are produced by microorganisms offer a number of advantages, both in terms of their potential for use in industrial and therapeutic applications (Angelin & Kavitha, 2020). EPS obtained from lactic acid bacteria possess remarkable and valuable properties that can be used in place of polysaccharides derived from either plants or animals.

Table 13: EPS production of LAB isolated from vegetables

Isolate	EPS production
E. faecium MW721241	-
E. durans MW721242	-
E. lactis MW721243	+
E. faecium MW721244	+
E. faecium MW721245	+
E. faecium MW721246	+
P. acidilactici MW721247	-
E. durans MW721248	-
E. faecium MW721249	-
E. durans MW721250	-
P. acidilactici MW721251	+
E. faecium MW721252	+
E. faecium MW721253	+
E. durans MW721254	+
E. durans MW721255	+
E. durans MW721256	-
E. faecalis MW721257	-

(-) EPS negatives; (+) EPS positive

4.3.5 Immunomodulatory Effect

The literature provides a detailed account of the interaction that occurs between the microbiota in the gut and the immune system. Probiotics have been shown to have anti-allergic properties in a number of studies, primarily through the induction of a predominant Th1 cytokine response (Ai et al., 2016; Vinderola et al., 2005; Cuffia et al., 2019). One of these studies claimed that probiotics could lessen the damage that is caused by allergic reactions to the host and identified three types of lactic acid bacteria (La, Lp, and Lc) that have these characteristics (Ai et al., 2016). In light of the findings we've obtained thus far, the potential probiotics *E. lactis* MW721243 and *P. acidilactici* MW721251 have been chosen for additional immunological investigation.

The capability of *E. lactis* MW721243 and *P. acidilactici* MW721251 as probiotics (live bacteria) and postbiotics (killed bacteria) to induce the secretion of IFN- and IL-4 cytokines in ex vivo cultured murine spleen cells was used to evaluate the immunomodulatory effects of these two strains of bacteria as probiotics and postbiotics, respectively. These studies were conducted using spleen cells obtained from two different inbred mouse strains, namely C57BL/6 and BALB/c mice, which are genetically distinct from one another (Figure 2). This was done so that the immunological profiles of the LAB strains would be applicable across a wide variety of host genetic backgrounds (Mills et al., 2000). The findings show that *E. lactis* MW721243 and *P. acidilactici* MW721251 were responsible for stimulating the production of IFN-g by spleen cells (Figure 1A-D). The fact that the higher LAB concentration of 107/ml was toxic to splenocytes most likely explains why the detectable IFN-g levels were higher when *E. lactis* MW721243 and *P. acidilactici* MW721251 were used at the 106/ml concentration. In addition, when the *E. lactis* MW721243 was used as a postbiotic (a preparation in which the bacteria have been killed) rather than a probiotic (live bacteria), a significantly higher amount of IFN- was secreted (Figure 2A, C). In contrast, the use of *P. acidilactici* MW721251 as a probiotic consistently resulted in higher levels of IFN- production, and this was true regardless of the mouse strain (Figure 2B, D). In addition, there was no discernible increase in the amount of IL-4 that was produced by spleen cells when they were cultured with either LAB strain (Figure 2E-H). It is interesting to note that both *E. lactis* MW721243 and *P. acidilactici*

MW721251 appear to induce higher levels of IFN- production by BALB/c splenocytes compared to C57BL/6 cells. This finding highlights the powerful pro-Th1-inducing capacity of both isolates. Given what is already known about the tendency of BALB/c mice to develop Th2 immune responses, this is a striking finding (Mills et al., 2000). These findings demonstrate that *E. lactis* MW721243 and *P. acidilactici* MW721251 are capable of inducing IFN- production in spleen cells that have been cultured in vitro, which suggests that they have the potential to inhibit Th2 responses in vivo. These findings need to be confirmed in additional experiments before they can be applied to a preclinical allergy model.

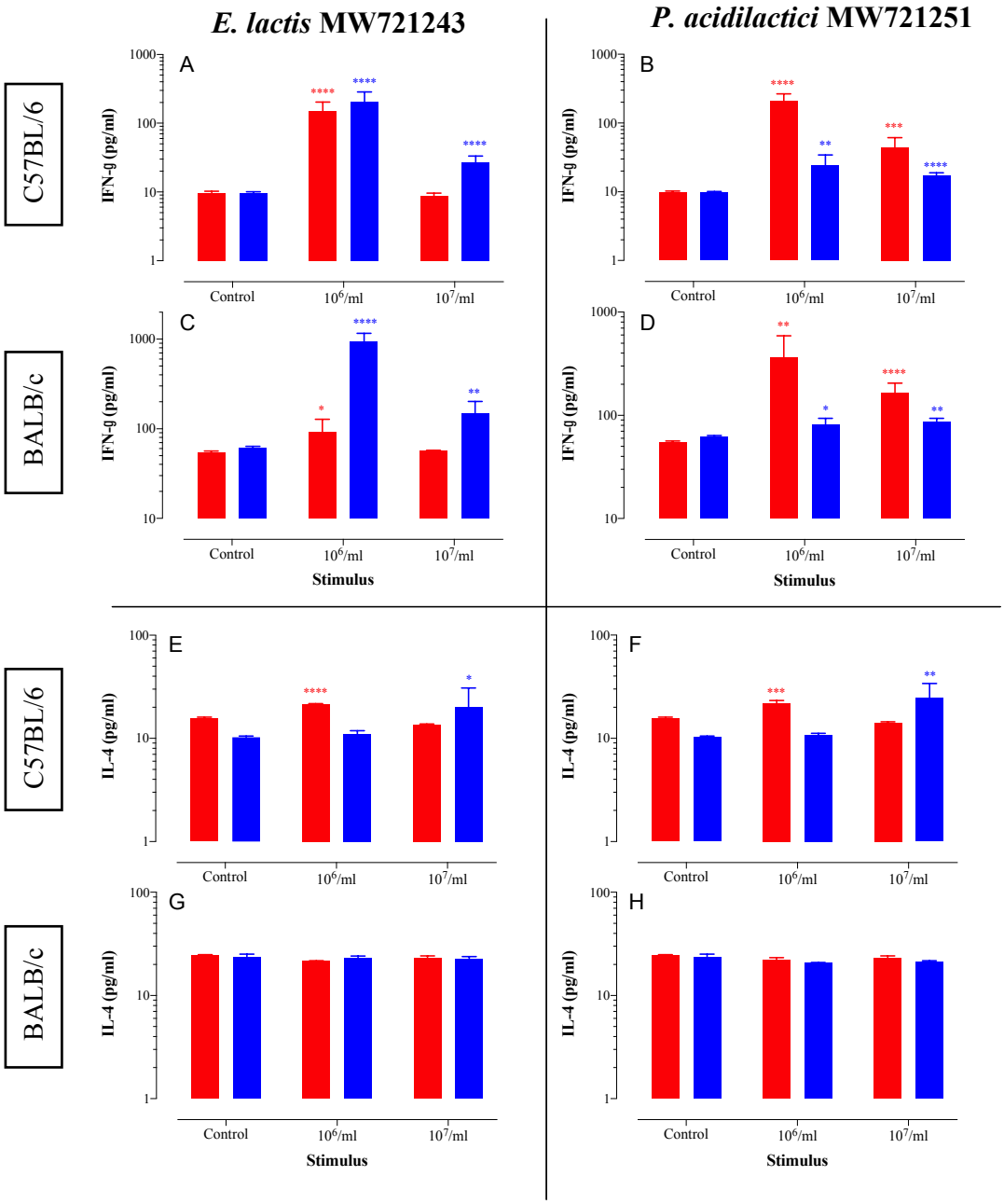


Figure 2: Immunomodulatory effect of two potential probiotics (live, red bar) and postbiotics (killed, blue bar) of *E. lactis* MW721243 (A,C,E,G) and *P. acidilactici* MW721251 (B,D,F,H) isolates against spleen cells C57BL/6 and BALB/c. where (A) and (B) are IFN-γ response of C57BL/6, (E) and (F) are IFN-γ response of BALB/c, (C) and (D) are IL-4 response of C57BL/6, (G) and (H) IL-4 response of BALB/c against MBL3 and MBL10, respectively. Asterisks denote statistical significantly differences between the indicated groups and the corresponding control groups.
(*p <0.05; **p<0.01; ***p<0.001; **** p < 0.0001).

Chapter 5: Conclusions, Limitation, and Future Directions

Vegetables were used as a source to isolate Lactic Acid Bacteria with desirable probiotic characteristics. This study chose fresh fruits and vegetables due to their low sugar content, enrich mineral and vitamin content, and neutral pH that enhanced the process of LAB fermentation. In addition, consuming LAB fermented fruits and vegetables provided healthy balanced diet in terms of vitamins, minerals and carbohydrates content which played a significant role in the reduction of diarrhea and liver cirrhosis. Moreover, the colored pigments found in several fruits and vegetables acts a antioxidant that fights free radicals which reduce the risk of several diseases including cancer, arthritis, and ageing.

In terms of result of the tests performed in this study, almost all the isolates were able to tolerate simulated oral, gastric, and intestinal conditions. They also showed resistance to bile and lysozyme, reduced cholesterol in the media, and showed outstanding adhesion to the intestine, which includes hydrophobicity study on hydrocolloids, auto-aggregation, co-aggregation, attachment to HT-29 cells, and some selected isolates showed promising immunomodulatory effects. Moreover, the selected isolates displayed antimicrobial and bile salt hydrolysis activity and were susceptible to antibiotics which erases the concern for the gene transfer in the non-resistant pathogens. However, EPS was produced by all isolates except *E. faecium* MW721241*, E. durans* MW721242*, P. acidilactici* MW721247*, E. durans* MW721248, *E. durans* MW721250, *E. durans* MW721256, and *E. faecalis* MW721257. All 17 isolates belonged to *Enterococcus faecium, Enterococcus durans, Enterococcus lactis, Pediococcus acidilactici,* and *Enterococcus faecalis*. The isolates showed exceptional probiotics properties *in vitro* and can be used to exploit industrial and clinical purposes. Regarding the limitations of this study, the hemolytic activity of the selected LAB isolates was not performed. Further studies are required to test whether the new probiotics discovered exhibited any of antimicrobial, anticancer, antihypertensive, and antioxidant features. In addition, more investigation is needed to identify the industrial properties of these new isolates.

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List of Publications

Alameri, F., Tarique, M., Osaili, T., Obaid, R., Abdalla, A., Masad, R., Al-Sbiei, A., Fernandez-Cabezudo, M., Liu, S. Q., Al-Ramadi, B., & Ayyash*, M. (2022). Lactic Acid Bacteria Isolated from Fresh Vegetable Products: Potential Probiotic and Postbiotic Characteristics Including Immunomodulatory Effects. Microorganisms, 10(2), 389-397.

UAE UNIVERSITY MASTER THESIS NO. 2022:69

The ability to perform effectively in the gastrointestinal system (GIT) is one of the most significant criteria for selecting the best probiotic bacteria. Thus, the present study aimed to investigate the potential probiotic characteristics of some selected lactic acid bacteria (LAB) isolated from vegetable products.

Fatima Khaled Alameri received her MSc in Food Science from the Department of Food Science, College of Agriculture & Veterinary Medicine at UAE University, UAE. She received her Bachelor in Public Health and Nutrition from the College of Natural and Health Sciences, Zayed University, UAE.