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INVESTIGATION OF PROBIOTIC POTENTIAL OF HUMAN-DERIVED *LACTOBACILLUS PLANTARUM* B14

MASTER OF SCIENCE

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ABSTRACT

INVESTIGATION OF PROBIOTIC POTENTIAL OF HUMAN-DERIVED *LACTOBACILLUS PLANTARUM* B14
MSC THESIS
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(xiii + 69)

Lactic acid bacteria (LAB) which are major probiotic microorganisms and essential components of the gut microbiota of humans and other mammals, have significant positive impact on the host's health. Therefore, discovering new probiotics with novel probiotic properties would be immensely appreciated by the medical, scientific, and food industries. Despite the fact that probiotic strains can be obtained from a variety of sources, one of the most important criteria for probiotics to be applied for humans is being human originated. The aim of this study is in-vitro characterization of probiotic potential of *Lactobacillus Plantarum* B14 originated from human gastro intestinal system (GIS). Probiotic features of this strain was examined using several tests, including acid, bile salts and phenol tolerance, antibiotic susceptibility, anti microbial activity, acidification capacity and aggregation ability. Molecular identification by PCR and gene cloning was also used for bacteriocin gene secreening. The results showed *L. Plantarum* B14 had a high tolerance to varying acid and bile conditions, and high tolerance to different phenol concentration (0.1%, 0.2%, 0.3%, 0.4%), susceptibility to ampicillin, and showed antimicrobial activity against three pathogenic bacteria which are *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* RSKK. *L. Plantarum* B14 was found to be slow acidifier also showed high auto aggregation reaching 100% after 5 h of incubation and high coaggregation ability with all five tested pathogens. In-silico analysis for cloned PCR product showed that bacteriocin coding gene was not detected and further investigation is required to determine bacteriocins coding genes in this strain. The results of these tests indicate that *L. Plantarum* B14 possess desirable probiotic properties and can be a candidate for further in vivo research aimed in developing a novel probiotic starter cultures.

KEYWORDS: Probiotic, Lactic acid bacteria, *L. plantarum*, PCR, antimicrobial activity.

ÖZET

İNSAN KAYNAKLI *LACTOBACİLLUS PLANTARUM* B14'ÜN PROBİYOTİK POTANSİYELİNİN ARAŞTIRILMASI
YÜKSEK LİSANS TEZİ
LINA ZEYAD AL-HAKEEM
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ
BİYOLOJİ ANABİLİM DALI

(TEZ DANIŞMANI: PROF DR. MEHMET ÖZTÜRK)

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Başlıca probiyotik mikroorganizmalar ve insan ve diğer memelilerin bağırsak mikrobiyotasının temel bileşenleri olan laktik asit bakterilerinin (LAB), konakçının sağlığı üzerinde önemli olumlu etkileri vardır. Bu nedenle, yeni probiyotik özelliklere sahip yeni probiyotiklerin keşfedilmesi, tıp, bilim ve gıda endüstrileri tarafından son derece takdir edilecektir. Probiyotik suşları çok çeşitli kaynaklardan elde edilebilmesine rağmen, insanlar için uygulanacak probiyotikler için en önemli kriterlerden biri insan kaynaklı olmasıdır. Bu çalışmanın amacı, insan gastro-intestinal sisteminden (GIS) kaynaklanan *Lactobacillus Plantarum* B14'ün probiyotik potansiyelinin in vitro karakterizasyonudur. Bu suşun probiyotik özellikleri, asit, safra tuzları ve fenol toleransı, antibiyotik duyarlılığı, anti mikrobiyal aktivite, asitleştirme kapasitesi ve agregasyon yeteneği dahil olmak üzere çeşitli testler kullanılarak incelenmiştir. Bakteriyosin geninin taranması için PCR ve gen klonlaması ile moleküler tanımlama da kullanılmıştır Sonuçlar, *L. Plantarum* B14'ün değişen asit ve safra koşullarına karşı yüksek bir toleransa ve farklı fenol konsantrasyonlarına (%0,1, %0,2, %0,3, %0,4) yüksek toleransa sahip olduğunu gösterdi, ve ampisiline duyarlılık, *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes* RSKK olan üç patojenik bakteriye karşı antimikrobiyal aktivite göstermiştir. *L. Plantarum* B14'ün yavaş asitleştirici olduğu ayrıca 5 saatlik inkübasyondan sonra %100'e ulaşan yüksek otomatik agregasyon ve test edilen beş patojenin tümü ile yüksek pıhtılaşma yeteneği gösterdiği bulundu. Klonlanmış PCR ürünü için yapılan in-silico analiz, bakteriyosin kodlayan genin saptanmadığını ve bu suştaki bakteriyosin kodlayan genlerin belirlenmesi için daha fazla araştırma yapılması gerektiğini göstermiştir. Bu testlerin sonuçları, *L. Plantarum* B14'ün istenen probiyotik özelliklere sahip olduğunu ve yeni bir probiyotik başlatıcı kültür geliştirmeyi amaçlayan daha fazla in vivo araştırma için aday olabileceğini göstermektedir.

ANAHTAR KELİMELER: Probiyotik, Laktik asit bakterileri, *L. plantarum*, PCR, , antimikrobiyal aktivite**.**

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LIST OF ABBREVIATIONS AND SYMBOLS

LAB **:** Lactic acid bacteria

GIS **:** Gastrointestinal system

WHO **:** World health organization

FAO **:** Food and agriculture organization

GIT **:** Gastrointestinal tract

GI **:** Gastrointestinal

AAD **:** Antibiotic-associated diarrhea

CDAD **:** *Clostridium difficile*-associated diarrhea

IBS **:** Irritable bowel syndrome

FGID **:** Functional gastrointestinal disorder

G+C **:** Guanine plus cytosine

DNA **:** Deoxyribonucleic acid

rDNA **:** Ribosomal deoxyribonucleic acid

CO2 **:** Carbon dioxide

GRAS **:** Generally Recognized as Safe

LA **:** Lactic acid

EMP **:** Embden-Meyerhof-Parnas pathway

PP **:** Pentose Phosphate

µm **:** Micrometre

AMPs **:** Antimicrobial proteins

Da **:** Dalton

MW **:** Molecular weight

°C **:** Celsius

BSH **:** Bile salt hydrolayse

PCR **:** Polymerase chain reaction

bp **:** Base Pair

rpm **:** Rotation per minute

IPTG **:** Isopropyl β-D-1-thiogalactopyranoside

OD : Optical density

PBS **:** phosphate buffer saline

EtBr **:** Ethidium Bromide

UV **:** Ultra violate

Amp^R **:** Ampicilline resistance

µl **:** Mikroliter

ml **:** Mililiter

SD **:** Standard deviation

GCA **:** Glycocholic acid

GDCA : Glycodeoxycholic acid

GCDCA **:** Glycochenodeoxycholic acid

TCA **:** Taurocholic acid

TDCA **:** Taurodeoxycholic acid

TCDCA **:** Taurochenodeoxycholic acid

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1. INTRODUCTION

1.1 History of probiotics

German scientist Werner Kollath in 1953, used the word ″Probiotika″ to describe active substance which are major to a proper healthy life development. It comes from the Latin pro and the Greek bios, which literally means ″for life″ (1). The history of probiotics dates to thousands of years which was proven by discovering wall paintings from 2500 B.C. which indicate that the Sumerians used to inoculate milk to cause fermentation, as well as the Egyptian hieroglyphs showed that the ancient people used fermentation to preserve milk for longer periods as early as 3500 B.C. (1,2).

The importance of studying the bacteria present in healthy feces and the digestive system to understand the physiological process of digestion as well as the pathology and treatment of intestinal microbial originated disorders was first recognized by Escherich in 1885 (3).

The modern concept of probiotics beginnings in the early 1900s with Elie Metchnikoff, a Russian scientist working at the Pasteur institute in Paris, which performed fundamental experiments on the aging process and its potential connection to intestinal autointoxication and probiotics that earn him the Nobel prize in 1908 (4). He claimed that the long-life spans of Bulgarian peasant people is not related to the yogurt product they ingested, but to the Lactobacillus species that is used in the fermentation process and reach the colon as mentioned in his book *The Prolongation of Life* (5). Metchnikoff established the hypothesis that the presence of LAB in the intestine may prevent illnesses caused by pathogenic microorganisms as the PH is lowered by LAB as a result of lactose fermentation and assist in controlling toxin producing bacteria (6,7).

In the 1920s, studies conducted by Leo F. Rettger showed that various bacteria found naturally in the gut could work as probiotics when introduced to the human digestive system. One of these bacteria is *Lactobacillus acidophilus* which can be an effective treatment for constipation (7,8).

In the 1930s, they were able to isolate Lactobacillus strain that could survive in the GIT of humans. This was *Lactobacillus casei* strain Shirota which utilized in the manufacturing of fermented milk products. In the late 1950s and early 1960s, there was a resurgence of interest in the human gut microbiota, which gave rise to the probiotic concept (3).

The scientific community worldwide for prebiotic and probiotic released a consensus statement on the three primary probiotic categories in 2014, which include: (i) probiotics without health claims, which are generally thought to be safe and don't need to provide proof of efficiency, (ii) probiotics with health claims, and (iii) probiotic medication that complies with regulatory requirements for pharmaceuticals by using a clinical strain (7).

1.2 Probiotic

Probiotics is a Latin word which means ″forever″. The definition of probiotic continuously evolved over the years. It was described firstly by Kollath in 1953 as means that vegetarian foods frequently contain probiotics in the form of vitamins, aromatic compounds, enzymes, and maybe other chemicals involved in critical functions. Then, Vergin in 1954 describe probiotic as opposite to antibiotics (3). Lilly and Stillwell in 1965 identified probiotics as compounds generated by one microorganism that promote the development of another microorganism in a paper they published in Science. According to Parker in 1974 probiotics are organisms and substances that support the equilibrium of bacteria in the gut (9). Fuller in 1989 identified the probiotic as ″a live microbial feed supplement which beneficially affects the host animal by improving microbial balance″. Today the current universal definition of probiotic was proposed by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) (2001) as ″live microorganism which when administered in adequate amounts confer a health benefit on the host″.

Probiotics are now more clearly described as single or mix cultures of living microorganisms that when administered to an animal or man, have a positive impact on the microbiota. According to probiotic definition, many different species and genera of microorganisms are classified as probiotic. The most frequently utilized probiotics are *Lactobacillus* and *Bifidobacterium*. Due to

their advantages for human health, the majority of these strains are employed in dairy products as well as commercial produtcs. Also, some *Escherichia coli*, *Enterococcus*, *Streptococcus thermophilus* and *Bacillus* species in edition to yeast like *Saccharomyces cerevisiae*, and others are used as probiotics (Table 1.1) (10,11).

Table 1.1. The microorganisms considered as probiotics (11).

***Lactobacillus* species**	***Bifidobacterium* species**	**Other lactic acid bacteria**	**Non-lactic acid bacteria**
L. casei	*B. bifidum*	*Enterococcus faecium*	*Escherichia coli strain nissle*
L. amylovorus	*B. breve*	*Enterococcus faecalis*	*Bacillus cereus var. toyoi*
L. crispatus	*B. infantis*	*Lactococcus lactis*	*Propionibacterium freudenreichi*
L. gallinarum	*B. lactis*	*Leuconstac mesenteroides*	*Saccharomyces boulardii*
L. delbrueckii	*B. animalis*	*Streptococcus thermophilus*	*Sarccharomyces cerevisiae*
L. acidophilus	*B. longum*	*Sporolactobacillus inulinus*	
L. gasseri	*B. adolescentis*	*Pediococcus acidilactici*	
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			
Subsp. Bulgaricus			

1.3 Human microbiota

The microbiota of humans is one of the most intricate microbial ecosystems. It's colonized by different microorganisms including bacteria, fungi, archaea, viruses, and protozoans which create a mutual benefit relationship with the host (12). Many of the current microbiota have been demonstrated to colonize different areas of the human body, with microbial diversity varying depending on the colonization site which can include the skin, oral cavity, GIT, and urogenital tract (13). The degree of colonization and the composition of the residing microbial communities vary significantly between anatomical sites and are influenced by host secretions, environmental factors, substrate availability, and

the composition of the surface of the wall (14). However, there are tens of billions of microorganisms in the human GIT which forms the majority of the human microbiota (15). The bacteria in the gastrointestinal tract are either commensal microorganisms that are colonizing or transient microorganisms that are just fleeting. Probiotics are regarded as transient microorganisms even though some of them are commensal species. Certain probiotics can reproduce and remain in the gut for a few days before disappearing when their consumption is stopped.

1.3.1 Human gut microbiota

The GI tract's microbiota is not thought to be homogenous. Moving down the GI tract toward the colon, which has around 10^1 cells per gram of contents in the esophagus, 10^0-10^2 per gram of contents in stomach and around 10^{11}- 10^{12} per gram of contents in the colon, both cell density and microbial diversity increase (16).upper and lower portions of the human gastrointestinal tract can be distinguished. After meals, transitory bacteria pass through the upper intestinal tract in the stomach, duodenum, jejunum, and ileum, among other areas. The human stomach contains low amounts of bacteria due to its low pH and digestive enzymes, although some microorganisms such as *Lactobacillus, Candida*, *Streptococcus*, and *Helicobacter pylori* are able to reside on the stomach mucosal layer (7). The small intestine has the lowest bacterial counts since the proximal portion connected to the bile duct, which secretes bile acids into the tract and may cause bactericidal effect to some species and therefore limiting the bacterial growth. The predominant bacterial genus in this region includes *Lactobacillus* and *Streptococcus*. In contrast, the large intestine's environment is typically more favorable for the development of an extremely dense microbial population, resulting in microbial diversity in the caecum and colon greater than that in the small intestine. These organisms mostly obtain energy by fermenting non-digested food particles. Information regarding the composition of large intestine microbiota is obtained mainly from fecal samples analysis. The predominant organisms in this environment are *Bacteroides*, *Clostridium*, *Bifidobacterium* and *Enterobacteriaceae* (Figure 1) (14). Specialized epithelial cells called goblet cells secrete mucus throught the GI tract, which produces two layers in the colon and one layer in the small intestine. It is separated between an outer and an inner

mucus layer in the small intestine. The outer layer of mucus is home to microorganisms whereas the inner layer is essentially sterile. (18).

A healthy human's intestinal microbiota's composition is influnced by a variety of variables, including host age, genetics, and environmental factors, particularly nutrition and continuous treatment, with the latter two aspects playing a significant effect. Diet thought to be the major factor influencing the gut microbiota of different people (19). According to research, the human gut microbiota is closely related with the health of the host. It performs several important functions, including acting as a physical barrier against pathogenic invasion and colonization, stimulating the immune system, producing the vitamins B and K as well as carrying out important metabolic processes, such as obtaining energy from breaking down of non-digestible fibers (20).

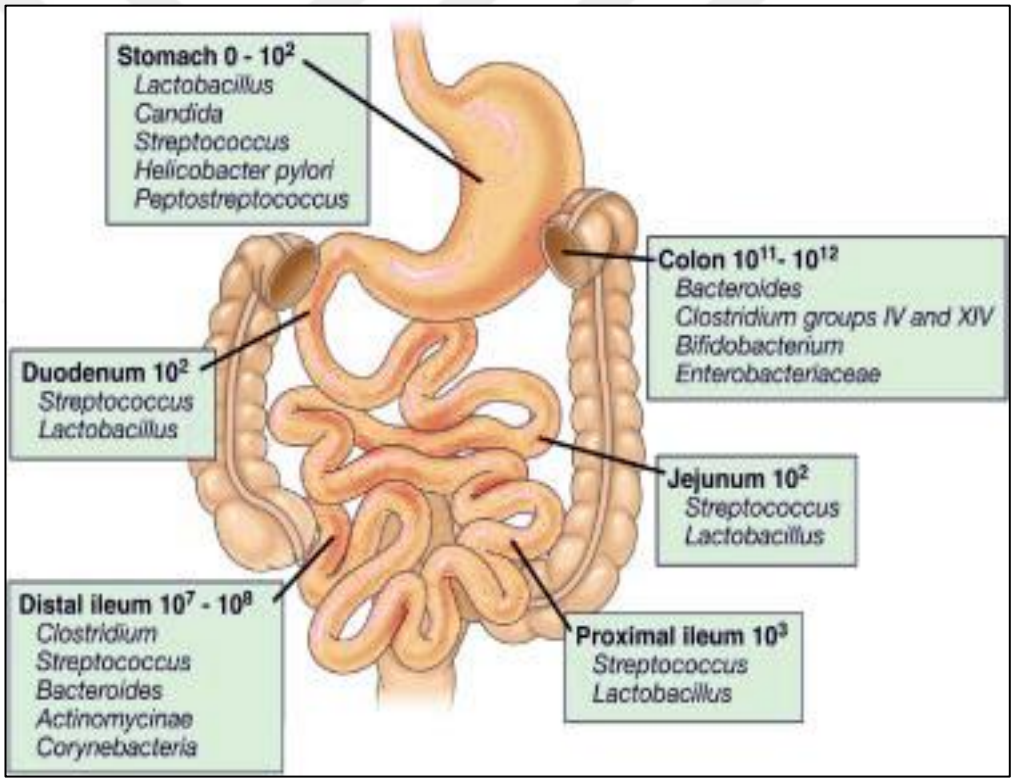

Figure 1.1. Bacteria's distribution and quantity in human GI. Obtained from http://www.wright.edu/~oleg.paliy/research.html

1.3.2 Development of the human gut microbiota

Most experts agree that human microbiota development starts at birth, but recent research indicates that maternal bacteria may also be transmitted to the fetus during pregnancy by placenta (21). It has been suggested that many factors

can affect the diversity and patterns of colonization in the microbiota of the gut including: the style of delivery, having siblings, gestational age, birth weight, the antibiotic's usage during the first year of life, the presence of furry pets in the house and diet. The microbial colonization of babies' guts is strongly influenced by the mode of delivery. During the first days after birth, the microbiota of vaginally delivered newborns contains a high abundance of *Lactobacillus* and *Prevotella* species, indicating the high load of lactobacilli in the flora of vagina. In contrast, the microbiotas of newborns delivered by cesarean section contain a high abundance of *Streptococcus*, *Corynebacterium*, and *Propionibacterium* species similar to that of mother's skin. Moreover, the feeding type is greatly affecting the colonization of microbial species in the gut. In fact, compared to breast-fed infants, formula-fed newborns had lower concentration of *Lactobacillus* and *Bifidobacterium* and greater concentration of *Escherichia*, *Enterococcus*, *Enterobacter*, and *veillonella*. The predominance of these genera may be related to the colons of breastfed infants having a higher acidic pH (22). In the first year of life, the variety of microorganisms rises, and the microbiota composition converges towards a distinctive microbiota profile that is similar to that of adults, with temporal patterns that are unique to each newborn. By the time a child reaches the age of 2.5, the baby microbiota resembles the microbiota of adults in terms of composition, variety, and functional skills (21).

1.4 Health benefits of probiotics

Studies that focus on the health advantages provided by probiotic bacteria have increased in recent years. Probiotic use has been shown to have several health benefits, particularly in the treatment of GI disorders, and is crucial for defining the digestive system and overall health of all animals and humans. Probiotic's positive effects can change according to different variables, such as the strain used, the quantity consumed, the frequency and length of exposue, and the individual's physiological state (23). Research has established that the effects of probioric are strain-specific therefore, it is critical to evaluate each strain's efficiency separately in providing certain health effects. There are some strains that can provide more than one benefit; however, there is no universal strain that can deliver all the suggested benefits (3). The potential benefits range from the avoidance of major life-threatining conditions such inflammatory bowel disease,

tumors, and cardiovascular issues to the treatment of diarrhoea. While some of these claims, like the reduction in time of intestinal transiting or relief of lactose intolerance by probiotics, are regarded as well-established, others, like the prevention of cancer or the impact onlevels of blood cholesterol, require further research to support them (24).

1.4.1 Lactose intolerance

Lactose intolerance is a genetic disorder resulted from the lack of lactose cleaving enzyme β-galactosidase catalyst, people with this case are unable to breakdown of lactose into the monosaccharide's galactose and glucose. Those who are lactose intolerant have swelling, diarrhea, discomfort in the abdomen, and gases after consuming milk or dairy products. The most extensively researched probiotics' health benefits are the enhancement of lactose digestion and reduction of intolerance symptoms, and it seems to be strain specific, which means certain strains can give better results than others. When probiotic bacteria are lysed by bile secretion, large amounts of lactase enzyme are released into the intestinal lumen, which acts on lactose and decrease the maldigestion symptoms (17,25). The usage of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* have been reported to help in lactose break down since it has higher β-galactosidase activity (26,27).

1.4.2 Antibiotic associated diarrhea

Antibiotic-associated diarrhea (AAD) is one of the most known side effects of antibiotic treatment that disrupts the gastrointestinal flora. It affects between 5% to 39% of people depending on their individual health, exposure to infections, and antibiotic type. There is no universal agreement over which antibiotics has the most danger, and any antibiotic may disturb the colonic flora and cause diarrhea but the risk of AAD increases with the overuse of the second-line antibiotics, in particular clindamycin, cephalosporins and fluoroquinolones. The symptoms range from minor inconvenience to severe diarrhea, electrolyte disruption and dehydration, and it can start in the same day of starting antibiotics intake or a few weeks later. There are several mechanisms that antibiotics might cause diarrhea. Firstly, the concentration of anaerobes in the intestine is reduced by antibiotics that are effective against anaerobic bacteria, which slow down the metabolism of carbohydrates and cause osmotic diarrhea. Secondly, the usage of

antibiotics may alter the gut micobiota, leading to the proliferation of pathogenic bacteria like *Clostridium difficile* leading to *Clostridium difficile*-associated diarrhea (CDAD) formation. Finally, some antibiotics have a direct impact on the gastrointestinal tract's motility. Probiotics applicatios for the prevention and treatment of AAD are thought to be the most important health benefit. Probiotics may maintain or restore equilibrium of the gut microbiota while or following the antibiotic treatment by several mechanisms including: nutrient competition with other bacteria, inhibition of pathogen epithelial and mucosal adhesion, introduction of lower pH in the colon favoring the growth of nonpathogenic species, immune system stimulation, or antimicrobial substances production, supporting carbohydrate fermentation, and reestablish the gut microbiota during or after antibiotic treatment (28–30).

1.4.3 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is the most frequent chronic functional gastrointestinal disorder (FGID), its affects 12%-20% of world's population, with the frequency for women being 2-3 times higher than men (31). It characterized by discomfort or pain in the abdomen that is reduced by the passage of gas or by defecation, bloating, and changes in bowel habits (32). Based on the type of bowel movement disruption, there are four different forms of IBS: IBS with predominant diarrhea, IBS with predominant constipation, alteration between the two, and undefined IBS. The pathophysiology of IBS, which is associated to alteration in GI motility, microbiota of the intestine, visceral hypersensitivity, gut epithelium, and immunological activity, as well as dysfunction of the brain-gut signaling pathways or specific psychosocial factors, it is thought to have a multifactorial etiopathogenesis. Probiotic use is associated with improvement in overall IBS symptoms and decrease abdominal pain, and it's effective in both adults and children. Studies have shown that it is possible to administer chosen and suitable bacterial strains to IBS patients in order to reduce gas accumulation in the colon and induce symptom improvement. Moreover, there are various factors related to how probiotic work to reduce IBS symptoms include: restoring the motility and visceral sensitivity of the digestive tract, raising the number of beneficial bacteria in the gastrointestinal system, redusing small bowel bacterial overgrowth, correcting the imbalance between pro- and anti-inflammatory cytokines. Several

studies showd that the usage of different strains of *Lactobacillus* and *Bifidobacterium* to treat IBS is highly effective (7,31,33).

1.4.4 Cancer

Probiotic bacteria have been linked to a veriaty of health benefits, but their anticancer properties are possibly the most investigated. Probiotics like *Bifidobacteria* and *Lactobacillus* strains or combination of them have been shown in both in vivo and in vitro experimental studies to have a preventive effect against colon cancer. Those combinations work together to prevent the development, growth, metastasis following transplantation, and chemically induced cancer (7). According to epidemiological studies, colon cancer is more common in the western world as saturated fat consumption rises in the diet. Probiotics are believed to reduce cancer risk by decreasing the activity of bacterial enzymes that change pre-carcinogens into active carcinogens (34). Despite the fact that the exact mechanism underlying the antitumor activity is unknown, different potential protection mechanisms have been described: changing the physio-chemical conditions in the colon, binding, and degrading possible carcinogens, altering the intestinal microflora's metabolic process, modifying colonic transit time to eliminate fecal mutagens more effectively, and enhancing the host's immune system (7,35). A number of research has shown that *Lactobacillus casei* Shirota has positive effects on preserving the gut microbiome's equilibrium and provides defense against gastrointestinal diseases. It shows an inhibiting impact on bladder cancer and colorectal cancer, and immunomodulatory effect which can enhance the host's immune system by encouraging phagocytes to produce IL-12 (interleukin). It aids in the treatment of colon cancer, decreases fecal enzymes, and protects against food-borne mutagens (36). According to other investigations, *Lactobacillus plantarum* B282 and *Lactobacillus pentosus* B281 exhibited noteworthy anticancer and attachment properties against Caco-2 cells. (37). According to the results of numerous clinical investigations, probiotics are believed to be effective in individuals with colorectal, liver, breast, bladder, and colon cancer, in prevention, treatment, and slowing the disease progression (38).

1.4.5 Other health benefits

Probiotics can have a number of positive health effects, in addition to those already listed (Table 1.2). Nevertheless, further human research is needed to confirm a number of these potential effects.

Table 1.2. Probiotic health benefits and estimated mechanisms.

Health benefit	**Proposed mechanism(s)**
Atopic disease treatment and prevention	Modification of the host's immune system response.
Inflammatory bowel diseases (Crohn's disease, ulcerative colitis, pouchitis) manegmant	Modulation of both gut microbiota and immune response.
Blood lipid/ heart disease	Bacterial cells assimilate cholesterol, change in BSH enzyme activity, and antioxidant effect.
Urogenital tract disorder treatment	Antimicrobial substances production, adhesion sites competition, competitive exclusion of pathogens
Treatment of *Helicobacter pylori* infection	Antimicrobial substances production, mucus secretion stimulation, adhesion sites competition, specific and non-specific immune response stimulation
Colonic transit time reduction	Influence of bacterial metabolite synthesis on peristalsis
Relief of lactose indigestion	Lactose is affected by bacterial β-galactosidase

1.5 Lactic acid bacteria (LAB)

They are considered as the main category of probiotic bacteria. Historically, the first recorded dates back to 1857, when Louis Pasteur examined lactic acid fermentation and found that lactic acid plays a part in wine spoilage (39). In 1873, Louis Pasteur student Joseph Lister successfully isolated a pure lactic acid bacterium known as *Bacterium lactis* from milk. The lactic acid bacteria *Lactococcus lactis* is currently used to ferment milk to create a wide variety of dairy products. Later, various commercial starting culture were discovered and studied, but most early study on LAB was concentrated on those related to dairy products (40).

LAB are characterized as gram positive cocci or rods shapes, facultatively anaerobic, catalase negative, and fastidious organisms with a high tolerance for low pH as well as non-spore forming bacteria with some exceptions on some occasions. Also, low G+C content can be seen in the DNA of LAB. They are mostly non-motile, and their cells divide in a single plane, except in pediococci. Although phenotypic approaches have been utilized traditionally to identify LAB, more recent developments in molecular techniques, including 16S rDNA sequencing, have made it possible to identify particular strains of LAB with more consistency and accuracy. They can be identified by the production of lactic acid, which is a key consequence of the breakdown of glucose, and growth-inhibiting substances like hydrogen peroxide, diacyls, and bacteriocins, etc. that prevent the spread of bacteria and pathogens that lead to sickness and food degradation (41). Besides their capability to produce lactic acid, however, LAB may also influence the flavor, texture, and nutrition of the product that is added to it.

Based on the results of fermenting carbohydrates' end products, they are divided into homofermentative, heterofermentative bacteria. When glucose is fermented by LAB that are homofermentative, the main by-product that is produced is lactic acid. They split a glucose molecule into two molecules of lactic acid. Other than lactic acid, which is the major result of heterofermentative bacteria, a significant amount of one or more metabolites such as ethanol, acetic acid, CO_2, are also formed during the fermentation of glucose (41). Moreover, starter cultures for homofermentative bacteria are frequently utilized in dairy products, while heterofermentative bacteria are rarely used in dairy products.

Currently, the LAB groups are categorized in the phylum *Firmicutes,* class *Bacilli*, and order *Lactobacillales*. Taxonomy revisions state that there are 17 genera in the LAB, including *Lactobacillus*, *Lactococcus*, *Alloiococcus, Aerococcus*, *Dolosigranulum, Globicatella*, *Carnobacterium, Enterococcus, Lactosphaera, Leuconostoc, Leuconstoc, Mlissococcus, Streptococcus, Oenococcus, Tetragenococcus, Pediococcus, Vagococcus,* and *Weissella,* the biggest genus containing more than 100 species, many of which are abundant in compounds rich in carbohydrates, is lactobacillus (11,41).

LAB members are non-pathogenic organisms with the exception of a few species. They are usually acknowledged as safe food-grade bacteria and have the status ″Generally Recognized as Safe″ (GRAS), so they are utilized as fermentative agent in food. LABs are found in rotting fruit and plant matter, dairy products, beets, pickled vegtables, fermented meat, sewage, sourdough, silages, juices, and in the cavities of both humans and animals. They predominate in the vagina, ileum, colon, and oral cavity of humans (41).

1.5.1 *Lactobacillus* genus

Lactobacillus is the biggest genus of lactic acid bacteria (LAB), which contains groups of very varied microorganisms with over 67 known species (42). Many *Lactobacillus* species are used as starter culutres or food preservatives, and they are crucial in the production of fermented foods. Additionally, some strains with human origins serve as probiotic or vaccination carriers (43). They are gram-positive, non-motile, catalase-negative, non-spore-forming bacilli, and have low GC content (guanine and cytosine) in a percentage of 33%-55% mol in their DNA. They can grow in a temperature extending from 2 to 53°C, and they may grow in a pH range of 3 to 8. PH and temperature for optimal growth are typically 5.5-6.2 and 30-40 C, respectively. Many industrial uses for Lactobacilli exist, including the manufacture of LA and as a probiotic. There are several uses for LA in the food, pharmaceutical, and chemical industries, all of which have high demand for the manufacturing of poly-L-LA, a biodegradable polymer (42).

They are a typical part of the GI and oral flora and can be found in large numbers in situations where substrates containing carbohydrates are present, such

as food, water, soil, and sewage. They require a variety of nutrients, including fatty acids, vitamis, minerals, and amino acids (43).

Initially, Lactobacilli were categorized based on their capacity to ferment hexoses and their growth temperature and then, based on their homo/heterofermentative potential. Pot and colleagues reviewed the subdivision of the genus Lactobacillus, but the generally accepted ″modern″ definition is that provided by Hammes and Vogel and Hammes and Hertel, which categorizes Lactobacilli as facultative heterofermentative, obligate heterofermentative, and obligate homofermentative based on the kind of sugers and fermentation byproducts that have been fermented. Hexoses are converted to lactic acid by lactobacilli that homoferment, commonly known as metabolic group A; neither pentose nor gluconate are fermented. This process uses the Embden-Meyerhof-Parnas pathway (EMP) or glycolysis. When glucose is scarce, facultative heterofermentative species (metabolic group B) can degrade pentoses and gluconate through an inducible phosphoketolase, an enzyme of the pentose phosphate (PP) pathway, producing acetic acid, ethanol, and formic acid as a result. They can also ferment hexoses to lactic acid via EMP. Pentoses and hexoses, which correspond to the first half of the PP, are solely metabolized by the obligatory heterofermentative Lactobacilli (metabolic group C) via the phosphogluconate pathway, which yields lactic acid, ethanol (or acetic acid), and CO2. They lack phosphoketolase but have FDB aldolase (43).

1.5.2 *Lactobacillus plantarum*

One of the most prevalent and beneficial LAB is *Lactobacillus plantarum*. *L. plantarum* is a ubiquitous member of the genus Lactiplantibacillus that is commonly present in both anaerobic plant debris and numerous fermented food products. *L. plantarum* was first discovered in saliva. It belongs to the lactobacilli hetero-fermentative group, which is a gram positive, short rod, anaerobic, micro-aerophilic, acid tolerant, non-motile group of lactobacilli. It is employed as a starter culture and as a preservative in the food industry. The cell size is approximately 0.9-1.2×3.0-8.0 μm, straight rods with round ends, and can be found singly, in pairs, or in small chains. It is a non-spore bacterium that produces acetic acid, succinic acid, and lactic acid as its main products. The chromosome

has a G-C content of 44.5% while the plasmid has a lower G-C content that ranges from 34.3 to 40.8% (44).

The lactic acid bacterium *Lactobacillus plantarum* is quite common and adaptable. It forms a significant portion of the microbiota of numerous organisms. It also colonizes the mucosa of both humans and animals (oral cavity, GIT, vagina, etc.) (45). The impressive ecological adaptation to such diverse ecological niches is due to its capacity to consume a wide variety of carbohydrates, from which the main end metabolite, lactic acid is formed. Also, *L. plantarum* has a multiple functional genome which permits metabolic flexibility, enabling it to colonize a veriety of habitats (46).

L. plantarum as probiotic candidate has been shown to have numerous significant traits including adaptability, successfully colonizing the human GIT, and shows a wide range of health benefits. *L. plantarum* possesses notable antioxidant properties and promotes intestinal permeability. It can prevent the formation of bacteria that cause gas in the intestine, which may help some people with IBS. It promotes microbial balance and maintains the digestive enzymes stability. Also, experimental tests showed that *L. plantarum* increased hippocampus brain derived neurotrophic factor, indicating that these bacteria may be helpful in the treatment of depression (45). *L. plantarum* has recently been used in the treatment of variety of chronic and cardiovascular diseases, such as Parkinson's, diabetes, cancer, Alzheimer's, obesity, hypertension, and urogenital infections (46).

In addition to organic acids, *L. plantarum* can produce several chemicals that have antibacterial activity, such as peptides, fatty acids, phenyllactic acid and bacteriocins. Plantaricins are the name given to the diverse class of bacteriocins produced by *L. plantarum*, which typically belongs to class II. Plantaricins can be chromosomally, or plasmid expressed, and they are often arranged in operon clusters containing a structural gene or genes, and immunity gene, and ABC transporter, and an auxiliary protein that aids in export (44).

Table 1.3. Scientific classification of *Lactobacillus plantarum* (retrieved from https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=962794#null)

Taxonomic Hierarchy	
Domain	Prokaryote
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	Lactobacillus
Species	*Lactobacillus plantarum*

1.6 Antimicrobial activity of LAB

Antagonistic effects of lactobacilli on other species have been discovered since the publication of Metchnikoff's article in 1908 (47). Living organisms produce a range of antimicrobial compounds as components of their immune response or defense system, which can improve their capacity to compete for resources and survivability. All living organisms generate antimicrobial protein (AMPs), many of which are referred to as antimicrobial peptides due to their small size. Particularly, the creation of antimicrobial compounds by LAB, such as bacteriocins, fatty acids, organic acids, ethanol, lytic enzymes, and hydrogen peroxide, is clear evidence of their antagonistic activity (48). This inhibitory effect created by lactobacillus strains may be crucial in preserving the right microbial balance in the intestine and in food industry as bio preservative. Use of antimicrobial peptides-producing lactic acid bacteria during the manufacturing of industrial food products suggests an advantage to enhance the safety and quality of foods, offering an alternative method to lower the danger of developing food-borne diseases. There are two primary categories of antimicrobial compounds produced by LAB: low molecular weight compounds with a molecular mass less than 1000 Da and high molecular weight compounds with a molecular mass greater than 1000 Da such as bacteriocins (49). Recent studies have progressively shown the importance of antimicrobial substances as a defense mechanism against

pathogens and some strains may have positive effect on both food and intestinal pathogens (49)

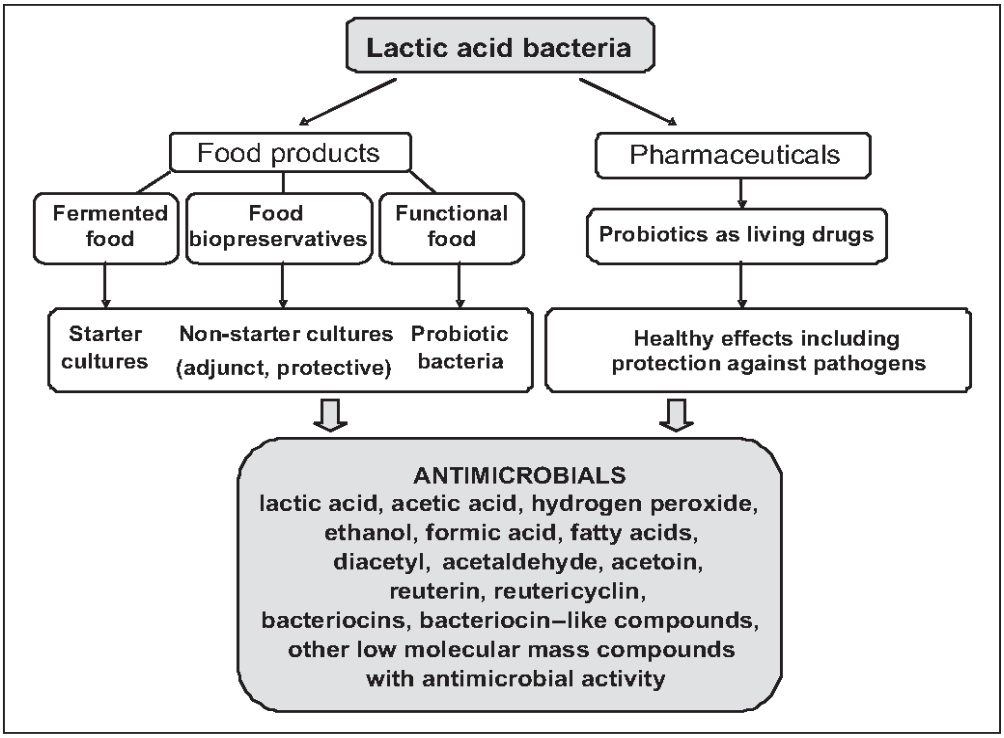

Figure 1.2. Lactic acid bacteria application and their antimicrobial activity (49).

1.6.1 Bacteriocin

Bacteriocins are antimicrobial peptides created by microorganisms, particularly LABs, to gain a competitive edge over similar organisms, mainly Gram-positive bacteria, in their environment. These peptides are ribosomally produced and are typically small, cationic molecules consisting of 30 to 60 amino acids. They may adopt amphiphilic helical structures and exhibit remarkable stability, even at high temperatures. Bacteriocins differ in their molecular weights, mechanisms of action, range of effectiveness, genetic composition, and other biochemical traits. By producing a particular immune protein that is encoded in the bacteriocin operons, LAB strains that make bacteriocin defend themselves against their own toxins (41). Because bacteriocins undergo posttranslational modification and are easily broken down by proteolytic enzymes, particularly by the proteases of the mammalian's GIT, they are safe for human intake.

Generally, bacteriocins can tolerate a wide range of temperatures without degrading. Moreover, they continue to exhibit antimicrobial properties throughout a wide pH range. As bacteriocins have no flavor, color, or odor, they are ideal for food related applications (50). There are no reports of bacterial resistance of bacteriocins developing despite the long history of their use. A bacterium's bacteriocins are typically referred to as narrow-spectrum bacteriocins if they are capable of inhibiting only bacteria of the same species. In contrast, they are considered to be broad-spectrum bacteriocins if they also inhibit bacteria from a different genus. The following characteristic reduce the possibility of bacteriocins and bacteria interacting to cause the emergence of antibiotic resistance, they have mechanisms that quickly create pores, are easily broken down by proteolytic enzymes, and have a shorter biological half-life in the body or in natural surroundings (48).

Bacteriocins from lactobacilli are being studied for two main reasons. Firstly, starter cultures that produce bacteriocin may lead to a fermentation process that is more reliable and inhibiting spoilage bacteria growth. Secondly, bacteriocin production and resistance genes have huge potential for future application as genetic markers in rDNA technology for inclusion in the synthesis of food additives or supplements from microrganisms in the future (49).

1.6.1.1 Mode of action of bacteriocin

Bacteriocin's mode of actions can be categorized according to whether they have a bacteriostatic effect, which stops cell development, or whether they have a bactericidal impact, whether or not cell lysis occurs. Although some bacteriocins have been demonstrated to have bacteriostatic effect, the majority of bacteriocins work agains sensitive microorganisms in a bactericidal manner. Most bacteriocins produced by LAB, especially those that target Gram-positive bacteria, work by affecting the cell envelope mechanisms. Among these are lantibiotics and certain class II bacteriocins, which specifically target Lipid II. Lipid II is an intermediate in the peptidoglycan synthesis pathway within the bacterial cell envelope. By targeting Lipid II, these bacteriocins inhibit the production of peptidoglycan, leading to the disruption of the bacterial cell wall and subsequent inhibition of bacterial growth. In order to enhance pore formation, lipid II is used by other bacteriocins as a docking molecule. This modifies the

potential of the cytoplasmic membrane which ultimately results in cell death. The most researched lantibiotic, nisin, has both mechanisms. The ″barrel-stave″ mechanism explains how bundle of peptides create transmembrane channels or pores. The size of the pore gradually increased as more peptide monomers are gradually attracted. Cell death results from intracellular components leaking via these pores. Moreover, other bacteriocins can destroy their target cells by preventing the expressions of genes and protein synthesis (49,53).

1.7 Selection criteria

To be considered as a probiotic, each possible probiotic stain must have certain properties. According to the recommendations by Fao\Who (2002) each possible probiotic strain needs to undergo a number of in-vitro experiments to determine its effectiveness. Although the in-vivo tests are significant, for preliminary selection, various probiotic selection criteria have been offered as in-vitro testing (7).

Some of the most important selection criteria will be discussed below.

1.7.1 Acids and bile tolerance

For probiotic bacteria to demonstrate their health benefits, it is essential for them to reach and persist in the lower gastrointestinal (GI) tract. Probiotic strains are commonly evaluated through in vitro tests that simulate the conditions of the GI tract during transit. These assessments involve subjecting the bacteria to different pH levels and the presence of bile salts. The ability to tolerate bile salts and acidic conditions, with a pH as low as 2, is a critical factor for bacteria to be considered as probiotics, as it allows them to thrive and survive in the GI tract. This tolerance to bile and acid varies depending on the bacterial strain. Although most bacteria are sensitive to the low pH levels in the stomach, certain LAB species can survive in this environment due to their unique system that concurrently transports lactic acids and protons out of the cell. This mechanism enables their survival and potential to provide health benefits once they reach the lower GI tract (6).

1.7.2 Antimicrobial activities of probiotics

The probiotic bacteria capacity to demonstrate antimicrobial activity against pathogens is one of the most crucial selection criterions. Probiotics create extracellular antimicrobial components by converting carbohydrates, proteins, and other insignificant molecules into essential compounds that can destroy pathogenic bacteria, such as bacteriocins, enzymes, organic acids, and H_2O_2. These bacteria have an impressive capacity for producing bacteriocins, which can be used as bio preservatives in food. Other probiotic antagonistic strategies include competition for resources, coaggregation with pathogens, and immune system activation. These antagonistic activities differ depending on the bacterial strain (7,54).

1.7.3 Bile salt hydrolase activity (BSH)

Potential probiotics may produce the bile salt hydrolase (BSH) enzyme that can hydrolyze conjugated bile salts. Micelles formed by cholesterol and conjugated bile salt enhance the absorption of cholesterol. When the process of hydrolyzing conjugated bile salts to deconjugated bile salts occurs, it leads to a reduction in cholesterol absorption through the host's gut. Furthermore, these deconjugated bile salts can combine with cholesterol and lower its solubility, resulting in a greater amount of cholesterol being excreted in the feces. It has been proposed that probiotic bacteria with BSH activities have a selection advantage in conditions high in bile salts (55).

1.7.4 Cell surface properties of probiotics

A key factor in selecting potential probiotic strains is microbial adherence to biological surfaces. This phenomenon controls the probiotics' survival and colonization in lumen of the gut, pathogens exclusion, and interaction of the bacteria with the host cells to carry out their beneficial effects. For this, the cell surface properties of a proposed probiotic strain are taken into consideration as an essential component for the evaluation of its possible positive effects on the host. Probiotic adhesion property is measured using the parameters auto-aggregation, hydrophobicity, and co-aggregation (56).

1.7.4.1 Auto-aggregation

Auto-aggregation refers to a bacterial strain's ability for non-specific cellular aggregation manner, which is regarded as an sign of colonization capacities in the host's GIT to exert their beneficial effect. According to reports, cell surface proteins and a small number of soluble proteins mediate the auto-aggregation of probiotic cells. This type of contact is beneficial for probiotics' efficient colonization and communication with the host tissue. Furthermore, it enhances the probiotics' ability to compete against pathogens and remove them out of the gut mucosa by enhancing their antibacterial properties (56).

1.7.4.2 Co-aggregation

Coaggregation is a term that describes the ability of some probiotic bacteria to create inter-species aggregates with bacteria from different species, for example pathogens. The coaggregation of probiotics with pathogens can hinder or reduce the pathogens' ability to adhere to the host's tissue. This occurs by promoting the clearance of co-aggregates or creating a competitive environment for adhesion to the mucosal tissue. Essentially, the presence of probiotics in co-aggregates with pathogens helps prevent the pathogens from firmly attaching to the host's tissues, thus potentially reducing their harmful effects (57). The process of bacterial coaggregation is typically controlled by specific proteins on the surfaces of the bacteria. However, the formation and properties of these aggregates can be influenced by bacterial surface hydrophobicity and environmental factors such as ions, oxygen, and carbohydrates. In essence, the presence of certain proteins and the interplay of various external elements can impact the formation and stability of bacterial coaggregates (58). It was proposed that co-aggregation of probiotic bacterial strains enables them to create a physical-chemical barrier against the colonization of the pathogenic bacteria in the host (5). In summary, co-aggregation strains are one of the GIT defense mechanisms and have a competitive advantage against non-aggregating planktonic bacteria.

1.7.5 Antibiotic resistance

An essential consideration in determining the chosen probiotic safety is antibiotic resistance. Probiotic bacteria do not present a safety concern even when they possess intrinsic antibiotic resistance or acquire it through mutations in their

chromosomes. In such cases, these resistant probiotic strains can be safely administered either concurrently with or following antibiotic treatment to help restore the gut microbiota. However, it is essential to note that the rapid emergence of antibiotic-resistant strains can be attributed to probiotics' ability to engage in horizontal gene transfer facilitated by mobile elements such as plasmids, conjugative transposons, integrons, and bacteriophages. This process can contribute to the spread of antibiotic resistance among bacteria. Therefore, distinguishing intrinsic resistance from acquired resistance is essential for understanding antibiotic resistance in probiotic bacteria. Probiotic bacteria must show sensitivity to antibiotic components (54).

2. THE AIM OF THIS STUDY

The relation between health and the gut microbiota composition has drawn more attention to the administration of probiotics for the treatment of some diseases in both humans and animals. The emergence of multidrug-resistant bacteria necessitates the development of efficient alternative antibacterial peptides. Furthermore, the increased consumer attention for natural products over synthetic additives in food preservatives demands the search for alternative molecules. Lactic acid bacteria (LAB) are significant microorganisms utilized as probiotics and are essential components of the gut microbiota of humans and other mammals. Probiotic bacteria can produce antimicrobial peptides like bacteriocin, have positive impacts on the host's health and have essential bio-preservative agents. To ensure the efficiency and benefits of these probiotics, some quality requirements have been established.

Therefore, the aim of this study is in vitro investigation the potential probiotic properties of human originated *Lactobacillus plantarum* B14 and screening for bacteriocin encoding gene.

Based on this aim, *L. plantarum* B14 was subjected to several tests to examine the probiotic characteristics including acid and bile tolerance, antipathogenic activity, phenol tolerance, acid production capacity, cell surface properties (auto-aggregation, co-aggregation), and antibiotic susceptibility. Polymerase chain reaction (PCR) is applied for the detection of bacteriocin encoding gene with special designed primers, then the detected gene was cloned into a cloning vector and conducting in-silico analysis.

3. MATERIALS AND METHOD

3.1 MATERIALS

3.1.1 Bacterial strains and growth conditions

All bacterial strains and plasmid DNA used in this study were obtained from microbial stocks of biology department at Bolu Abant Izzet Baysal University (BAIBU), stored at - 80°C with 30% glycerol. Bacterial strains, plasmid DNA, and their growth conditions are shown in Table 3.1. *Lactobacillus plantarum* B14 strain used in this work is human originated.

Table 3.1. Bacterial strains and plasmid.

Bacteria	**Strain**	**Growth media**	**Growth conditions**	
			Temperature	**Time**
Lactobacillus plantarum	B14	MRS	37°C	24-48h
Listeria monocytogenes	RSKK	NA/PCA	37°C	24h
Staphylococcus aureus	Foodborne	NA/PCA	37°C	24h
Escherichia coli	Foodborne	NA/PCA	37°C	24h
Salmonella typhimurium	ATCC13311	NA/PCA	37°C	24h
Yerisinia enterocolitica	ATCC23715	NA/PCA	30°C	24h
Escherichia coli	XL1-Blue[1]	LB	37°C	16-18h

Plasmid	**Genotype**	**Phynotype**	**Growth media**	**Temperature**	**Time**
pBluescript[2]	SKII+	Amp^r	LB	37°C	16-18h

[1]: This strain has the genotype: rec A end A 1 gyr A986 thi-1hsdr17supE44 rel A1 lac. (Stratagene USA)

[2]: pBluescript was obtained from Thermo Scientific (Fermantas, Europe).

3.1.2 Chemicals and enzymes

All chemicals and enzymes that were used in this study and their suppliers were listed in appendix A.

3.1.3 Growth media and buffers

The preparation of growth media of de Man, Rogosa, and Sharpe (MRS), Luria-Bertani (LB), Plate Count Agar (PCA), Nutrient Agar (NA) and buffers like TAE, Tris-HCL, EDTA and PBS used in this study were listed in appendix B and C.

3.2 METHOD:

3.2.1 Characterization of *L. plantarum* B14

3.2.1.1 Acid tolerance assay

The most crucial criteria for probiotic bacteria are their survivability to exert beneficial effects. Probiotic bacteria must survive and pass the stomach conditions before reaching the intestinal tract to colonize. Therefore, the acid tolerance potential of *L. plantarum* B14 was tested by acid tolerance assay described by Li et al., with some modifications (59). *Lactobacillus plantarum* B14 was grown on MRS plate anaerobically at 37°C for 18-24 h, then a colony was inoculated into 5 ml MRS broth and incubated anaerobically at 37°C for 18 h. 3% (150 µl) of overnight grown broth culture was inoculated into 5 ml of previously prepared MRS broth with different pH values and incubated for 6 h at 37°C. The pH values of MRS broth were adjusted using 1M HCl and 1M NaOH to obtain MRS broth with pH values of pH 2, pH 3, pH 4, pH 5, pH 6 and a normal MRS used as a control with a pH value of 5.47. Then, the growth was measured by spectrophotometer at OD_{600nm} every two hours. After 6 hours all the bacteria were resuspended in normal MRS (2%) to control the survival rate. The obtained results were calculated as Log_{10} (cfu/ml).

3.2.1.2 Bile tolerance

To assess LAB's potential as effective probiotics, it is typically thought important to assess their ability to resist the effects of bile salts in the environment. The average concentration of bile acids in the intestine is estimated to be 0.3 % (w/v), and the time for passage through the intestine is expected to be 4 hours (34). Because of that, this test was conducted with bile concentrations of 0.1% and 0.3%, with incubation period pf 5 h. The bile tolerance of *L. plantarum* B14 was tested according to Li et al., with some modifications (59). The bacterial strain was grown anaerobically in 4 ml MRS broth at 37°C for 14-16 h, then 2%

(100 µl) of overnight grown broth culture was inoculated into 5 ml of previously prepared MRS broth supplemented with 0.1%, 0.3 % bile povine (Oxgall, Mereck) and normal MRS (without bile salts) as control and incubated for 5 h at 37°C. The growth was measured by spectrophotometer at OD_{600nm} at each hour of incubation. The growth rate was calculated as follow:

$$\text{Growth rate \%} = \left(\frac{\text{Growth } in \text{ bile salts medium}}{Growth\ in \text{ control medium}}\right) \times 100.$$

3.2.1.3 Antimicrobial activity

The antimicrobial activity of *L. plantarum* B14 strain was evaluated against five pathogenic bacteria using the Disk Diffusion method as described by Karimi et al., with some diffrences (60). To collect the bacterial pallet and cell free supernatant (CFS), *L. plantarum* B14 bacteria was grown anaerobically in 4 ml MRS broth at 37°C for 24 hours, then the CFS and cell pallet was collected separately by centrifuging at 6000 rpm for 5 min. The CFS was filtered using 0.22 µm micron filter. To detect the antimicrobial potential of the *L. plantarum* B14 strain, the pellet of this strain was lysed by adding 200 µl lysis buffer (lysozyme 30mg\ml, Tris-Hcl 1mM, EDTA 50 mM, Triton 100X 1.2%) then incubated at 37°C for 45 min, and the mixture was centrifuged for 5 min at 13000 rpm. The antipathogenic activity of *L. plantarum* B14 was assessed against five pathogenic bacteria which are *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* (ATCC13311*), Listeria monocytogenes* RSKK, and *Yersinia enterocolitica* (ATCC23715). These pathogenic bacteria were grown on nutrient agar plate then the bacteria were inoculated into 5 ml nutrient broth (Neogen, UK) and the concentration was adjusted to 0.5-0.7 OD_{570} then spread completely on NA plate using cotton swab. The blank disks were impregnated with the supernatant and with lysed pellet and placed on the plate. After incubation at 37°C for 24 h (except *Yersinia enterocolittica* at 30°C) inhibition zone was measured using millimetric ruler.

3.2.1.4 Phenol tolerance

The degradation of some aromatic amino acids by gut bacteria results in phenol formation, which can have a bacteriostatic activity against some strains of Lactobacillus. Phenol tolerance testing can provide additional information on the

survival potential of lactobacilli in GI condition. The ability of phenol tolerance was determined as described by Reuben et al., (61). 100 µl of fresh overnight broth culture of *L. plantarum* B14 strain was inoculated into 5ml of MRS broth containing different concentration of phenol (0.1%, 0.2%, 0.3%, and 0.4%) and incubated at 37°C for 24h. Then, the bacteria viability was determined by measuring the absorbance by spectrophotometer at OD_{600nm} before and after incubation.

3.2.1.5 Acidifying activity

To detect the acidifying activity of *L. plantarum* B14 strain, the pH measurement was used as described by Mousaa et al., (62). The *L. plantarum* B14 was grown in MRS broth culture for 24 h at 37°C then the bacteria were inoculated into sterile skimmed milk at a level of 50 µl/ 5 ml. The acidity was determined by measuring the pH values after 8, 10, 24, 48 hours of incubation at 37°C.

3.2.1.6 Cell surface properties

3.2.1.6.1 Autoaggregation assay

The autoaggregation capacity of *L. plantarum* B14 was evaluated following the method described by Sharma et al., (63). The bacterial culture was grown anaerobically in MRS broth for 18 hours at 37°C. After centrifugation at 4000 rpm and 4°C for 15 minutes, the pellet was collected, washed twice in sterile phosphate buffer saline (PBS) with a pH of 7.4, and then resuspended in PBS. The bacterial suspensions were mixed gently using a vortex, and 100 µl of the suspension was added to 3.9 ml of PBS buffer. The optical density (OD) was measured at OD_{600nm} from the upper layer of the suspension and then incubated at 37°C for 5 hours. The absorbance of the upper suspension was measured after each hour. To calculate the autoaggregation percentage, an aliquot of 0.1 ml from the upper suspension was taken and added to 3.9 ml of PBS buffer. The formula used for autoaggregation was:

$$\text{Autoaggregation \%} = \left[1 - \frac{\text{At}}{\text{A0}}\right] \times 100$$

where At represents the absorbance at specific time points (t = 1, 2, 3, 4, 5 hours) and A0 represents the initial absorbance at t = 0.

3.2.1.6.2 Co-aggregation assay

The co-aggregation property was examined according to the method described by Li et al., with some modification (59). The lactobacilli bacteria were grown and suspended as described in Auto-aggregation assay section. The five pathogenic bacteria, *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* ATCC13311, *Listeria monocytogenes* RSKK, and *Yersinia enterocolitica* ATCC23715, were grown overnight in nutrient broth at 37°C except *Yersinia enterocolitica* at 30°C. 100 µl of *L. plantarum* B14 and 100 µl of pathogenic bacteria were added to 3.8 ml PBS buffer and incubated at room temperature without agitation. The absorbance of the samples was measured at time 0 for both the mixture and individual bacterial broth and at time 2, 4, 6, 24 h for the mixture by using spectrophotometer at OD_{600nm} from the upper layer of the suspensions. After every measurement, 100 µl of the upper suspension was taken and added to 3.9 ml fresh PBS buffer. The co-aggregation percentage was calculated as follow:

Coaggregation % = $[((Apro + Apat) - Amix) \div (Apro + Apat)] \times 100$

where Apro + Apat represents the absorbance of the Lactobacillus and the pathogens at the initial time point (0 hours), and Amix represents the absorbance of the mixture of the Lactobacillus strain and the pathogens at various time points.

3.2.1.7 Antibiotic susceptibility

The antibiotic susceptibility of *L. plantarum* B14 was tested using Kirby-Bauer disk diffusion method as described by Reuben et al., (61). The bacterial strain was grown anaerobically overnight in MRS broth culture at 37°C. Then the bacterial concentration was measured using spectrophotometer at OD_{570nm} reaching the concentration (0.6-0.8), then the bacterial suspension was streaked on the surface of NA plates by using sterile cotton swab. Commercially available discs of antibiotic (Oxoid, UK) including ampicillin (10µg), erythromycin (15µg), kanamycin (30µg), and Neomycin (30µg) were placed on the surface of the dried streaked agar plates, and then incubated at 37°C for 24 h. The diameter (mm) of zone of inhibition of bacterial growth around each disc was measured using millimeter ruler. The results were described as susceptible (S), intermediate (I), and resistant (R) according to Clinical and Laboratory Standards Institute CLSI (2022) interpretive category.

3.2.1.8 Bile Salt Hydrolysis (BSH) activity

BSH enzyme which is commonly found in probiotic bacteria helps in decreasing cholesterol levels and allows bacterial colonization by catalyzing the hydrolysis of conjugated bile salts. In a previous study in BAIBU labs (64,65), the BSH gene was detected in *L. plantarum* B14 and expressed. The activity of BSH enzyme was tested qualitatively by direct plate assay and quantitatively using six different bile salts by ninhydrin assay which are glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA).

3.2.1.9 Statistical analysis

For *L. plantarum* B14 characterization tests, one way ANOVA was preformed to compare the results using Turkey's HSD test. The statistical significance was determined as $p<0.05$. All the tests were repeated at least three times to determine the means and standard division. The test results of characterization were represented as means ±SD.

3.2.2 Screening for bacteriocin gene

3.2.2.1 Genomic DNA isolation of *L. plantarum* B14

A single colony taken from a streaked plate of *L. plantarum* B14; was inoculated into 4ml MRS broth and incubated anaerobically at 37°C for 24h, then 2% from the first broth culture was inoculated into 1.5 ml MRS broth and incubated anaerobically at 37°C for 18h. The cell pellet was collected by centrifugation at 6000 rpm for 5 min and the supernatant was removed. Then the cell pellet was used for genomic DNA isolation.

To start genomic DNA isolation, 2×10^9 (Cell/ml) bacterial cell from the pellet was washed with MRS broth then the cell harvested by centrifugation at 5000 rpm for 10 min. Then, the genomic DNA isolation was preformed following the protocol described by GeneJET™ genomic DNA purification kit (Thermo Scientific).

3.2.2.2 DNA amplification by PCR

Screening for bacteriocin coding gene in *L. plantarum* B14 was preformed using three specific primers supplied by Oligomer were shown in Table 3.2 (59).

The PCR reaction and the conditions for the reaction were optimized for each of the primers as shown in Table 3.3 with final volume of 50 µl, containing dNTP mix (5 mM), the foreword and reverse for each of the primers (5 µM) and template DNA of *L. plantarum* B14, AQ90 High Fidelity DNA polymerase enzyme (AMPLIQON, Denmark) and its supplemented buffer (1x). To visualize the result, the PCR results were loaded into agarose gel (0.8 %) and separated by electrophoresis for 1 h at 75 V, and then the gel stained with Ethidium Bromide (EtBr).

Table 3.2 Primers used for PCR reaction to detect bacteriocin gene (66)

Bacteriocin name	Primers	Necleiotide sequance (5'-3')	Tm (°C)	Primer length(bp)
Plantaricin S	PlanS-F PlanS-R	GCCTTACCAGCGTAATGCCC CTGGTGATGCAATCGTTAGTTT	61 57	20 bp 22 bp
Plantaricin NC8	PlanNC8-F PlanNC8R	GGTCTGCGTATAAGCATCGC AAATTGAACATATGGGTGCTTTAAATTCC	59 60	20 bp 29 bp
Plantaricin W	Plan W-F Plan W-R	TCACACGAAATATTCCA GGCAAGCGTAAGAAATAAATGAG	46 57	17 bp 23bp

Table 3.3 Reactant for PCR reaction.

Reactant	**Volume**
UPH_2O	34µl
dNTP mix (5mM)	2µl
Primer F (5µM)	2µl
Primer R (5µM)	2µl
Template DNA (5ng/µl)	4µl
AQ90 HiFi Polymerase Buffer (1x)	5µl
AQ90 HiFi DNA Polymerase enzyme (2u/µl)	1µl
Total volume	**50µl**

Table 3.4 PCR conditions optimized for each primer.

Primers	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Plan S	95˚C, 5 min	95˚C 30 sec.	54˚C, 40sec.	72˚C,2 min	72˚C,5 min
Plan NC8	95˚C, 5 min	95˚C 30 sec.	55˚C, 40sec	72˚C,1min	72˚C,5 min
Plan W	95˚C, 5 min	95˚C 30 sec.	41°C, 40sec	72˚C, 40sec	72˚C ,5min

Number of cycles 30×

3.2.2.3 Purification of PCR product

To remove unwanted non-specific PCR products, the PCR products were loaded into agarose gel (1%) (Sigma, USA) and separated by electrophoresis using 1× TAE buffer at 75 volts for 1.15 h. Then the PCR product was purified individually from agarose gel using the GeneJET gel extraction kit (Thermo Scientific) by following the instructions.

3.2.2.4 pBluescript plasmid isolation

pBluescript cloning vector was streaked from stock into LB plate with 30µg/ml ampicillin added and incubated at 37°C for 18h. A liquid culture of LB+amp was started using one colony from the streaked plate and incubated at 37°, 16h, and 175 rpm. After 16-18 h of incubation the pellet was collected by first centrifugation the tubes at 4000 rpm, 4min, 25°C. Then the pellets are washed with 1ml LB and centrifuge at 6000 rpm, 3min. Then the DNA plasmid was isolated from the pellets following the instructions of GeneJET plasmid Miniprep-Kit (Thermo Scientific).

3.2.2.5 pBluescript digestion with *Sma*I fast digest restriction enzyme

*Sma*I fast digest enzyme (Thermo Scientific, EU) was used to digest the circular pBluescript vector to obtain linear vector with blunt end since the PCR product has a blunt end. The digestion reaction components are shown in Table 3.5. The incubation in the PCR machine was for 23min at 37° C. Then the linear pBluescript vector was purified from 1% agarose gel by using GeneJET gel extraction kit (Thermo Scientific) in compliance with the protocol.

Table 3.5 Reaction conditions for pBluescript digestion by *Sma*I fast restriction enzyme.

Reactants	**Volume**	
UpH_2O	21µl	**At 37° C**
pBluescript DNA plasmid	30µl	
Fast digest green buffer (10X)	6µl	**For 23 min**
*Sma*I fast digest enzyme (10U/µl)	3µl	
Total volume	**60µl**	

3.2.2.6 Insertion of *L. plantarum* plan S and plan W genes into linear pBluescript vector

The *L. plantarum* plan S and plan W genes and pBluescript linear plasmid vector were ligated by T4 DNA ligase enzyme (Thermo scientific, EU) at 22°C for 2 h with appropriate buffer in a reaction condition shown in (Table 3.6). The new constructs were givin the name pLAS and pLAW. Then the T4 ligase enzyme was inactivated after ligation reaction by incubating at high temperature (65°C) for 10 min. The proper amounts of insert and vector DNA were calculated using the formula below for an effective ligation reaction:

Insert mass (ng) = Ration $\times \left[\frac{\text{Insert lenght (Kb)}}{\text{Vector lenght(Kb)}}\right] \times$ Vector mass (ng).

Table 3.6 Ligation reaction for plan S and plan W into pBluescript vector

Reactants	***L. plantarum* plan S**	***L. plantarum* plan W**
UpH_2O	12 µl	14 µl
pBluescript vector	1 µl	1 µl
***L. plantarum* DNA**	4 µl	2 µl
T4 Ligase Buffer (10X)	2 µl	2 µl
T4 DNA Ligase enzyme (5U/µL)	1 µl	1 µl
Total volume	**20µl**	**20µl**

3.2.2.7 Preparation of XL1-Blue competent cell

The preparation of E. coli XL1-Blue (Stratagene, USA) competent cells for the transformation process involved several steps. Initially, XL1-Blue was streaked on a solid LB plate and incubated at 37°C for 18 hours. A single colony was then inoculated into 3 ml of LB broth and incubated at 37°C with shaking at 175 rpm for 16 hours. Next, a second culture was prepared by transferring 1% of the first culture into 50 ml of LB broth. This culture was incubated at 37°C with shaking at 125 rpm for 2-2.5 hours until the desired cell concentration of 0.04-0.06 OD at 600nm was achieved. The broth culture was then divided into two 20 ml portions and centrifuged at 4000 rpm and 4°C for 5 minutes. The resulting pellets were resuspended in 10 ml of CaCl2 solution (100 mM) and placed on ice for 30 minutes. After that, another centrifugation step at 4000 rpm and 4°C for 10 minutes was performed.

The final pellet was dissolved in 1 ml of 10% glycerol CaCl2, and 100 µl aliquots were transferred to individual Eppendorf tubes. These tubes were stored at -80°C for future use as competent cells for transformation experiments.

3.2.2.8 Transformation of pLAS and pLAW into *E. coli* XL1-Blue

The heat shock transformation procedure involved transferring 10 µl of the pBluescript ligation product (pLAS and pLAW) into an Eppendorf tube containing 100 µl of previously prepared competent cells of XL1-Blue. The heat shock process began with a 45-minute incubation on ice, followed by a 5-minute incubation at 37°C, and then another 5-minute incubation on ice. After that, the transformed cells were transferred into a glass tube containing 1 ml of LB broth and incubated for 1 hour at 37°C with shaking at 175 rpm. To collect the cell pellet, the sample was centrifuged at 6000 rpm for 3 minutes, and the resulting pellet was resuspended in 100 µl of LB broth. The cell suspension was streaked on an LB plate containing Ampicillin (25 mg/ml), IPTG (Isopropyl β-D-1-thiogalactopyranoside), and Xgal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). The plate was then incubated at 37°C for 14-16 hours.

The following day, four white colonies were randomly selected from the plate and inoculated into a 5 ml LB+Amp medium. The culture was incubated at 37°C with shaking at 175 rpm for 14-16 hours. After collecting the cell pellet through centrifugation, plasmid DNA isolation was performed using the GeneJET Plasmid

Miniprep Kit from Thermo Scientific, EU. Finally, to detect the presence of the inserted gene inside the plasmid, the pLAS and pLAW were digested with *Bam*H and *Pst*I fast restriction enzyme respectively and visualized using agarose gel electrophoresis.

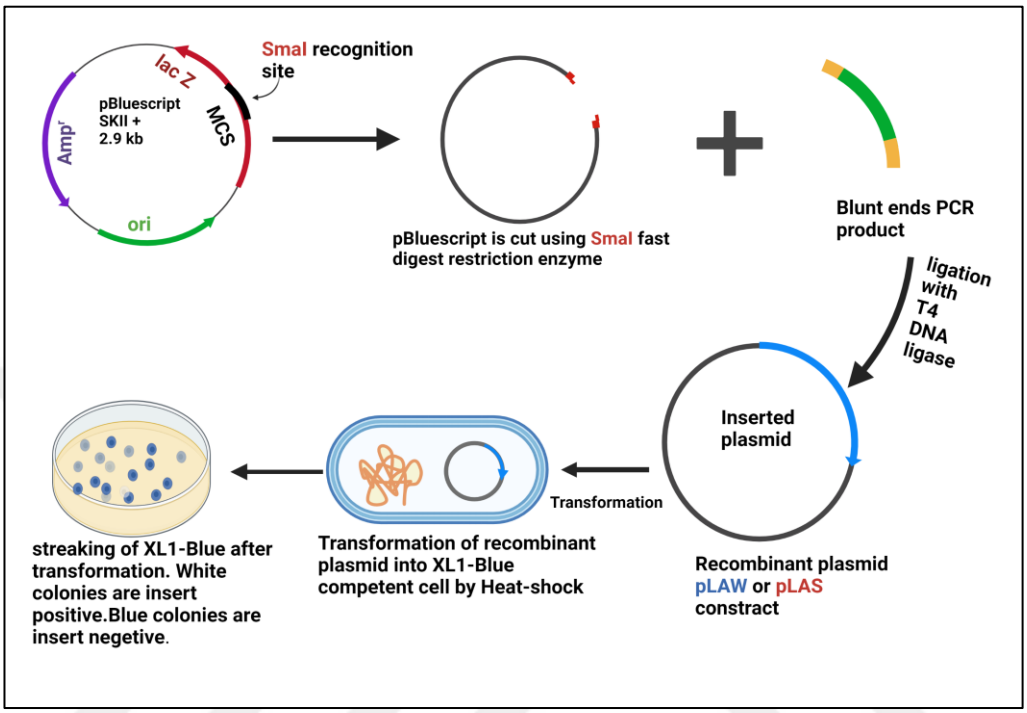

Figure 3.1 Preparation of pLAW and pLAS construct and transformation into XL1-Blue competent cell.

3.2.2.9 DNA sequencing

The recombinant DNA sequence was obtained from Macrogen Inc. (Seoul, Korea) by using the universal primers M13/pUC Foreword (5´-GTAAAACGACGGCCAGT-3´) and M13/pUC Reverse (5´-CAGGAAACAGCTATGAC-3´).

3.2.2.10 In-silico analysis

After obtaining the nucleotide sequence result, it was verified using BioEdit program. In silico analysis was performed. First, the nucleotide sequence was translated into amino acid sequence by using ExPASy translation tool (https://web.expasy.org/translate/), the amino acid sequence was then aligned against those present in a selected data base using BLAST tool in National Center

for Biotechnology Information (NCBI) https://blast.ncbi.nlm.nih.gov/Blast.cgi, and Molecular Evolutionary Genetic Analysis (MEGA5) software.

4. RESULTS

4.1 Characterization of *Lactobacillus plantarum* B14

4.1.1 Acid tolerance assay

An essential quality required for probiotics is their capacity to survive in various pH levels. According to the results in Table 4.1, *L. plantarum* B14 was able to surivive and grow in all different pH values in duration of six hours which indicates a tolerance to different acid levels. Log_{10} (cfu/ml) results suggest that the growth rate was slower in pH 2, pH 3, and pH 5 in comparssion to the control group with a change in Log values of 0.37, 0.42, and 0.05 respectivly. On the other hand, the control group (MRS broth with normal pH value of 5.47) had an increase in the log value by 0.69 after six hours of incubation. Although *L. plantarum* B14 showed growth in all pH values, no significant change was detected by statistical analysis. The viability of bacteria was controlled by re-inoculation into a control MRS medium with a normal pH value for two hours, the results showed increase in the bacterial growth for all pH values.

Table 4.1. Acid tolerance assay results of *L. plantarum* B14 to five different pH values.

Log $_{10}$ (cfu/ml)	**Time (h)**	**pH values**					
		pH:2	**pH:3**	**pH:4**	**pH:5**	**pH:6**	**control**
	0:00	7.662±0.49	7.823±0.15	7.495±0.54	8.218±0.62	8.219±0.79	8.171±0.18
	2:00	8.035±0.49	7.958±0.32	7.963±0.46	8.862±0.07	8.639±0.51	8.310±0.58
	4:00	7.840±0.32	7.962±0.34	7.845±0.39	8.154±0.52	8.689±0.43	8.202±0.97
	6:00	8.038±0.35	8.244±0.44	8.019±0.34	8.261±0.50	8.877±0.41	8.795±0.57
	8:00	8.292	9.283	9.279	9.313	9.285	

Data are expressed as mean ± SD (n=3) with the exception for time 8:00 which is repeated once as a control. Values are not significant different ($P > 0.05$).

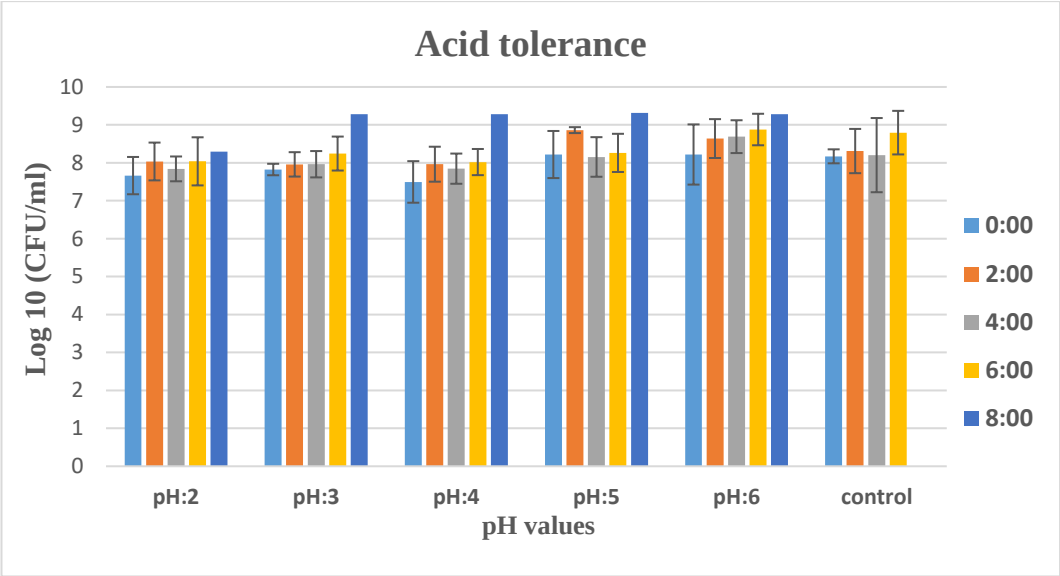

Figure 4.1 Acid tolerance assay of *L. plantarum* B14.

4.1.2 Bile tolerance

Bile in the intestine is a crucial element that influences the viability of LAB cells since bile is toxic and disrupts the cell membrane structure (6). To test the bile tolerance of *L. plantarum* B14, oxgall (0.1%, 0.3%) was used as a replacement to the human bile salts due to its resemblance. The cell growth was calculated as $\log_{10}$ (cfu/ml). According to the results shown in Table 4.2, *L. plantarum* B14 was able to tolerate bile salts with 94.4% growth rate in 0.1% bile concentration, and 74.5% growth rate in 0.3 % bile concentration after 5 hours of incubation in comparison to bacterial growth in normal MRS. According to the statistical analysis, the bacteria showed significant ($p<0.05$) growth in 0.3% after three and four hours of incubation. These results indicate a good ability of this strain to tolerate these bile concentrations.

Table 4.2 Bile tolerance results of *L. plantarum* B14 to 0.1%, 0.3% bile concentration.

Log $_{10}$ (cfu/ml)	Time (h)	Bile concentration		
		Control 0%	**0.1%**	**0.3%**
	0:00	8.268±0.05	8.337±0.17	8.395±0.10
	1:00	8.294±0.02	8.471±0.01	8.420±0.08
	2:00	8.304±0.06	8.487±0.08	8.416±0.15
	3:00	8.434±0.13	8.475±0.06	8.590±0.06*
	4:00	8.483±0.06*	8.553±0.05	8.624±0.06*
	5:00	8.670±0.06**	8.644±0.11	8.543±0.05

Data are expressed as mean ± SD (n=3). $^{**}p< 0.01$, $^{*}p<0.05$, the significance is expressed in comparison to time 0:00.

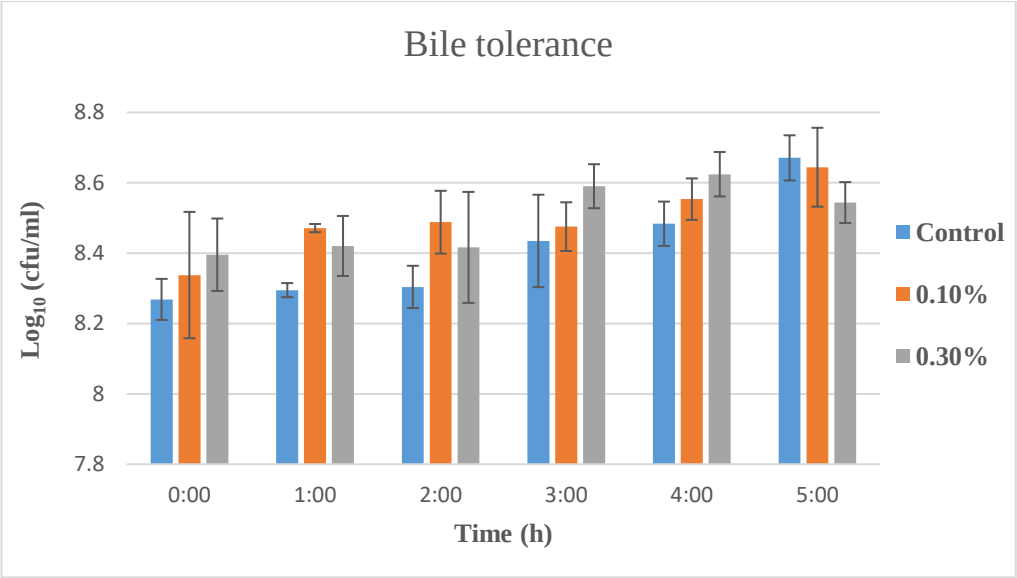

Figure 4.2 Bile tolerance of *L. plantarum* B14 to different bile concentrations.

4.1.3 Antimicrobial activity

One of the major criteria of probiotics is the antagonistic activity against pathogens. The antibacterial activities of both cell lysate and CFS of the *L. plantarum* B14 strains were tested against five different pathogenic bacteria including *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* ATCC13311, *Listeria monocytogenes* RSKK, and *Yersinia enterocolitica* ATCC23715 by applying Disk diffusion method. The antimicrobial action was shown as a halo of pathogens growth suppression on NA plates shown in Table 4.3. According to the test result, the cell lysate has shown strong antimicrobial activity against *Staphylococcus aureus* and pathogenic *E. coli* with inhibition zones of 13 mm and 15 mm respectively. The CFS has shown inhibition zone of 8 mm against *Listeria monocytogenes*.

Table 4.3. The diameter of inhibition zone for antimicrobial activity of *Lactobacillus plantarum* B14 against pathogenic bacteria**.**

Pathogens	***L. plantarum* B14 cell lysate**	***L. plantarum* B14 CFS**
Staphylococcus aureus	15 mm	--
Escherichia coli	15 mm	--
Salmonella typhimurium ATCC13311	--	--
Listeria monocytogenes RSKK	15 mm	8 mm
Yersinia enterocolitica ATCC23715	--	--

4.1.4 Phenol tolerance

Resistance to phenol was evaluated as an additional survival indicator in the intestinal conditions. The test results showed *L. plantarum* B14 strain has high tolerance ability to different phenol concentration. *L. plantarum* B14 grew significantly after 24 h in the presence of 0.1%, 0.2%, and 0.3% phenol in comparison to the bacterial growth at time 0. At 0.4% phenol the bacteria were able to grow with no significant value with an increase in the log value was 0.6. 0% phenol used as positive control for bacterial growth,

Table 4.4 Phenol tolerance of *L. plantarum* B14.

		Phenol concentration %				
	Time	**0%**	**0.1%**	**0.2%**	**0.3%**	**0.4%**
Log_{10} (cfu/ml)	**0:00**	7.539±0.21	7.873±0.66	7.271±0.10	7.425±0.27	7.380±0.48
	24:00	9.061±0.04**	9.060±0.01*	8.797±0.11**	8.238±0.39*	8.017±0.21

Data are expressed as mean ± SD (n=3). $^{**}p< 0.01$, $^{*}p<0.05$, the significance is expressed in comparison to time 0:00.

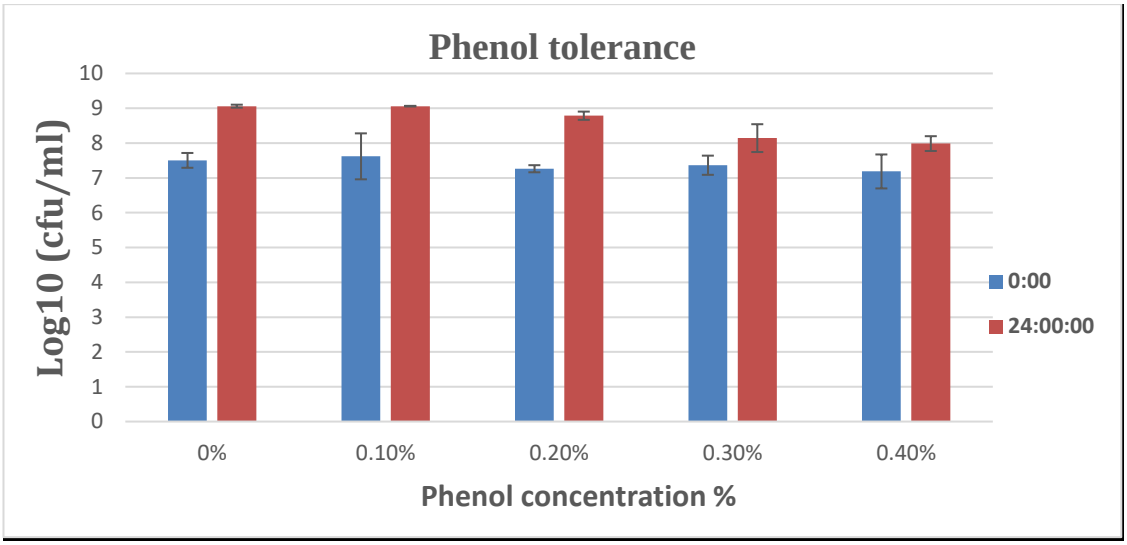

Figure 4.3. Phenol tolerance of *L. plantarum* B14 to phenol with different concentrations (0.1%, 0.2%, 0.3%, 0.4%). 0% indicate MRS broth without phenol used as positive control for growth.

4.1.5 Acidifying activity

The acidifying activity of *L. plantarum* B14 was measured using a pH meter. The pH measurements of skimmed milk inoculated with bacteria showed that *L. plantarum* B14 had a significant role in decreasing acidity. The results indicated a significant ($p<0.01$) decrease in pH values at time 8:00, 10:00, 24:00, and 48:00 in comparison to the pH value at time 0:00. The decrease in pH value for a duration of 48 h was 2.1.

Table 4.5. pH values of the acidifying activity of *L. plantarum* B14.

	Acidifying activity				
Time (hours)	**0:00**	**8:00**	**10:00**	**24:00**	**48:00**
pH values	6.33±0.10	5.82±0.07**	5.66±0.08**	4.86±0.11**	4.37±0.13**

Data are expressed as mean ± SD (n=3).

**p< 0.01, the significance is expressed in comparison to time 0:00

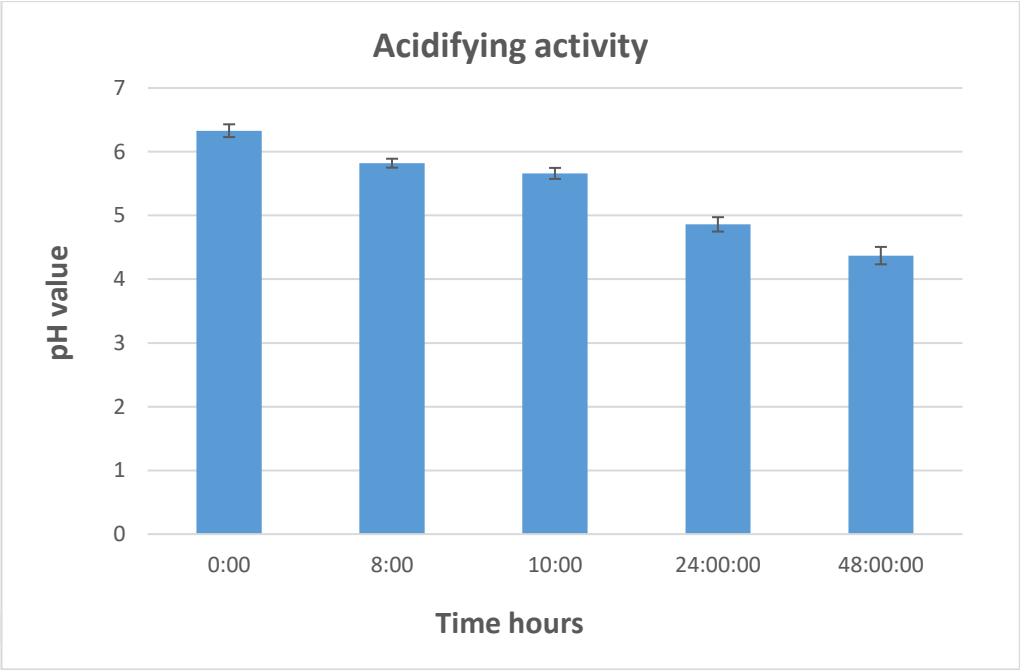

Figure 4.4. The acidifying activity of *L. plantarum* B14.

4.1.6 Autoaggregation assay

One of the most significant characteristics of a probiotic bacteria is its ability to aggregate, also known as autoaggregation or self-aggregation which is cell clumping from the same strain. This ability affects the probiotic's ability to attach to mucosal epithelia to colonize and exert health benefits (7). Auto-aggregation ability in terms of sedimentation rate *of L. plantarum* B14 was measured using spectrophotometer (OD_{600nm}) and calculated as autoaggregation % over a period of 5 h. As shown in Table 4.4. *L. plantarum* B14 demonstrated a good autoaggregation percentage ranging from 22.51% to reach 100 % throughout 5 h of incubation. In present study, autoaggregation results had a significant ($p<0.01$) increase during each hour of incubation time in comparison to first hour value.

Table 4.6. Autoaggregation rate of *L. plantarum* B14 in 5:00 h of incubation.

Autoaggregation rate %	**Time**				
	1:00	**2:00**	**3:00**	**4:00**	**5:00**
	22.51±12.25	81.63±9.60**	88.70±12.01**	90.92±3.63**	100±0**

Data are expressed as mean ± SD (n=3).

**p< 0.01, the significance is expressed in comparison to time 1:00.

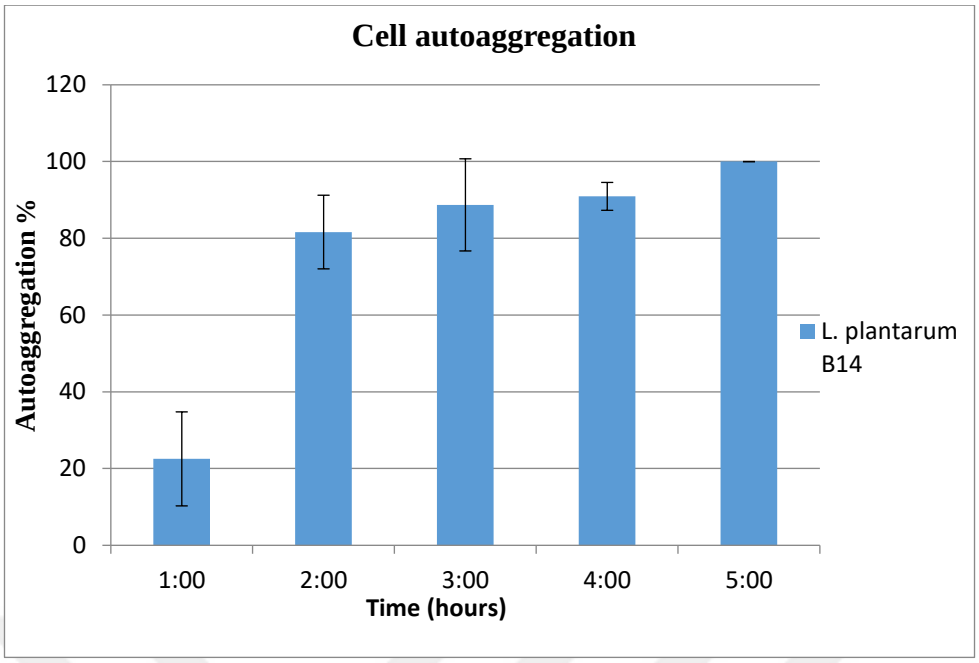

Figure 4.5. Autoaggregation rate of *L. plantarum* B14 during 5:00 h

4.1.7 Co-aggregation assay

Coaggregation is the term used to describe the aggregation of bacteria from different species and/or strains. Probiotic strains' ability for coaggregation may help in creating a barrier that inhibits pathogenic bacteria from colonization in the GIT (7). *L. plantarum* B14 showed good coaggregation abilities with the selected pathogens which are *L. monocytogenes* RSKK, and *Y. enterocolitica* ATCC23715, *S, typhimurium* ATCC13311, *E. coli*, *S. aureus*, and the results were strain specific and time dependent. *L. plantarum* B14 presented highest significant ($p<0.05$) co-aggregation rate (97.05%) toward *S. typhimurium* after 6 h of incubation, where is the coaggregation with *Y. enterocolitica* showed a significant ($p<0.05$) result with a value of 96.97% after 4 h of incubation. The results of *S. aureus* showed significant values of coaggregation in a duration of 2-24 h in comparison to time 0:00 results. The results showed coaggregation with *L. monocytogenes* and *E. coli* with a rate of 89.86% and 94.07% respectively after 24 h of incubation time with no significant values obtained by one-way ANOVA analysis.

Table 4.7: Co-aggregation percentage of *L. plantarum* B14 against pathogens in a duration of 24 h of incubation

	Pathogenic bacteria				
Time	*L. monocytogenes*	*Y. enterocolitica*	*S. typhimurium*	*E. coli*	*S. aureus*
0:00	67.26±21.03	70.06±8.38	65.36±18.48	75.46±18.48	62.11±9.58
2:00	87.73±17.37	89.70±10.77	85.53±9.70	88.60±9.70	94.04±10.14*
4:00	95.79±1.17	96.79±2.78*	90.98±10.14	93.31±10.14	94.41±6.58*
6:00	90.18±12.87	93.69±14.34	97.05±6.14*	93.67±6.14	90.10±9.29*
24:00	89.68±9.01	94.01±6.96	94.95±7.42	95.32±7.42	94.07±6.27*

Data are expressed as mean ± SD (n=3).

*p< 0.05, the significance is expressed in comparison to time 0:00

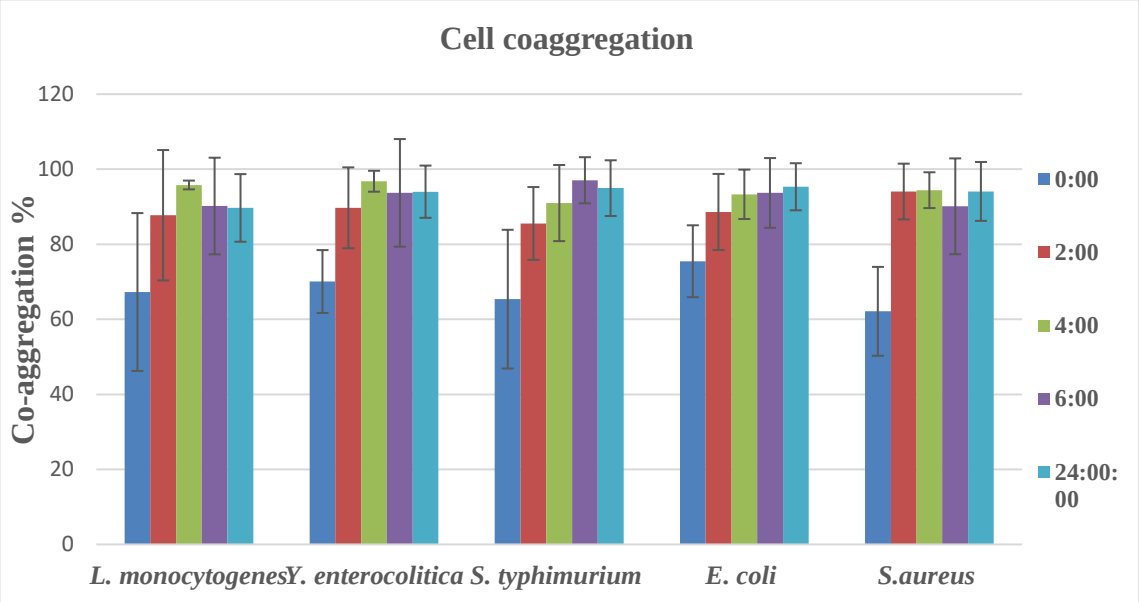

Figure 4.6. The co-aggregation percentage of *L. plantarum* B14 against five pathogenic bacteria in a duration of 24 h of incubation.

4.1.8 Antibiotic susceptibility

Antibiotic susceptibility of potential probiotics is a major safety aspect when selecting bacteria as probiotic because of the possibility of horizontal transfer of bacterial resistance to pathogenic and non-resistant bacteria. The antibiotic susceptibility profile of *L. plantarum* B14 was obtained using disc diffusion method against four commonly used antibiotics: Ampicillin (10µg), Erythromycin (15µg), Kanamycin (30µg) Neomycin (30µg). The results were explained according to the breakpoints recommended by CLSI (2022) guidelines as follows: the inhibition zone less than or equal to 14 mm were considered as resistant (R), and the inhibition zone with more than or equal to 20 mm considered as susceptible (S), and the those having zone of inhibition between 15 and 19 mm considered as intermediate (I). According to the results shown in Table 4.6, *L. plantarum* B14 was resistant to erythromycin and kanamycin with inhibition zone 13 mm and 0 mm respectively, intermediate to neomycin 15 mm, and sensitive to ampicillin with inhibition zone 30 mm.

Table 4.8. Antibiotic resistance test result of *Lactobacillus plantarum* B14.

Antibiotic	Zone size	Sensitivity
Ampicillin (10µg)	30 mm	S
Erythromycin (15µg)	13 mm	R
Kanamycin (30µg)	0	R
Neomycin (30µg)	15 mm	I

(S): susceptible, (R): resistant, (I): intermediate.

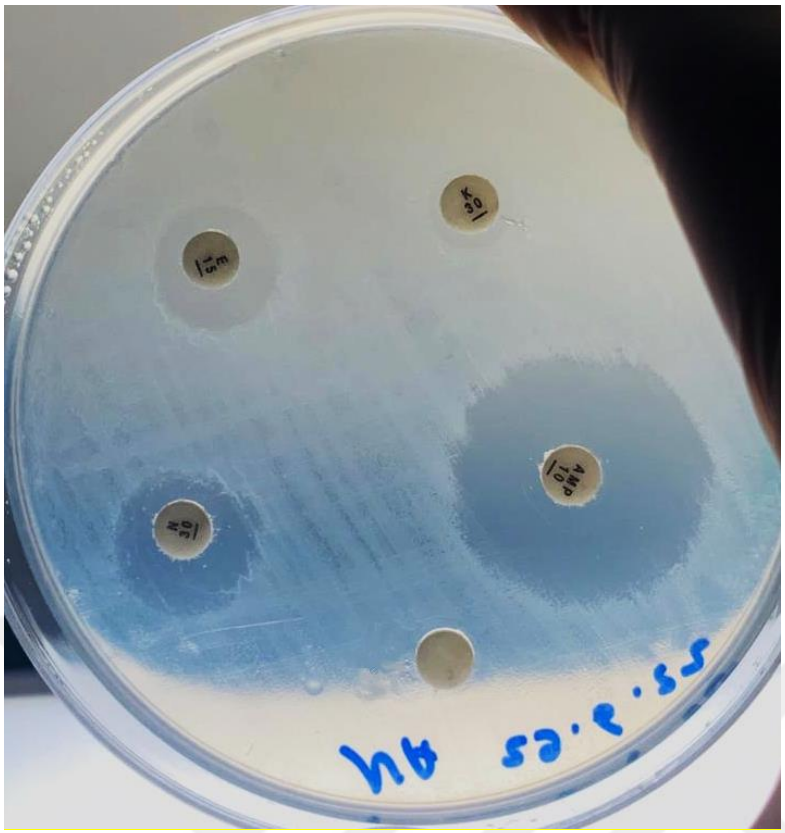

Figure 4.7. Antibiotic resistance test results of *L. plantarum* B14. Amp: Ampicillin, K: Kanamycin, N: Neomycin, E: Erythromycin. Blank disc used as negative control.

4.1.9 Bile Salt Hydrolase (BSH) activity

Probiotic strains can produce an enzyme that hydrolyzes conjugated bile salts and reduces blood cholesterol which is known as bile salt hydrolase (BSH). It has been proposed that probiotic bacteria with BSH activities have a selection advantage in bile salt-rich environments. The BSH activity of *L. plantarum* B14 was tested against different human originated bile salts which are GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA using ninhydrin assay as described previously by Öztürk et al (65). The results showed high BSH enzyme activity against glycoconjugate salts GCA, GDCA, GCDCA with relative activity 90.5%, 100%, 90.4% respectively, and low BSH activity against taurocojugated salts TCA, TDCA, TCDCA, with relative activity 11.2%, 18.4%, 11.7% respectively.

4.2 Screening for bacteriocin gene

4.2.1 PCR amplification of bacteriocin genes and purification of PCR products

After the isolation of the genomic DNA of *Lactobacillus plantarum* B14, screening for bacteriocin gene was done using three species specific designed primers and amplified by PCR reaction. Two results were obtained with approximately 500 bp and 1300 bp from Plan W and Plan S primers respectively. The results were detected with agarose gel electrophoresis then stained with EtBr and visualized under ultraviolet light (UV) (Figure 4.8). Then the PCR products were purified from agarose gel (1%) (Figure 4.9).

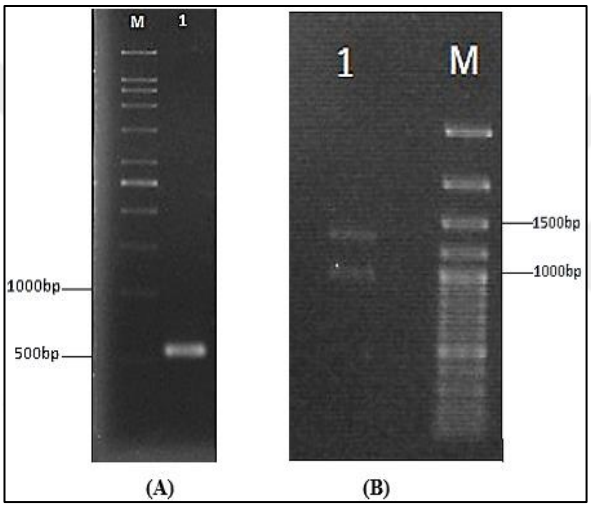

Figure 4.8. PCR amplification products of *L. plantarum* B14. M: VC 1 Kb DNA Ladder. (A): Line 1: Plan W PCR product. (B): Line 1: Plan S PCR product.

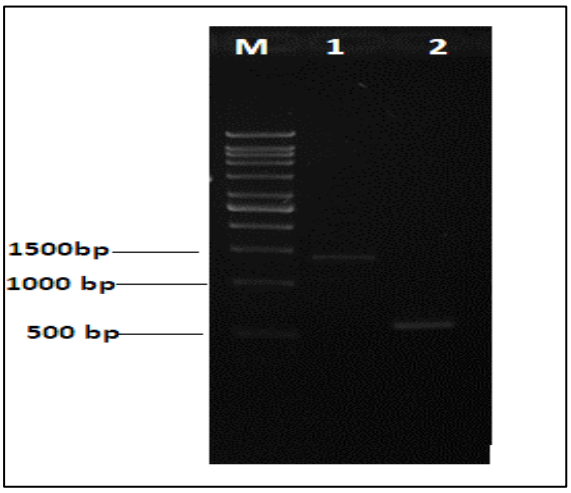

Figure 4.9 Purification of PCR products. M: VC 1 Kb DNA Ladder. Line 1: purified Plan S PCR product. Line 2: purified Plan W PCR product.

4.2.2 Preperation of pLAS and pLAW constructs

4.2.2.1 Preparation of pBluescript cloning vector

For the preparation of pBluescript cloning vector (2966 bp), pBluescript was isolated from *E. coli* XL1 strain and cut with *Sma*I restriction enzyme to obtain blunt-ended linear vector. Then, the digested pBluescript DNAs were purified from agarose gel (1%). The results were visualized using agarose gel electrophoresis and UV transilluminator.

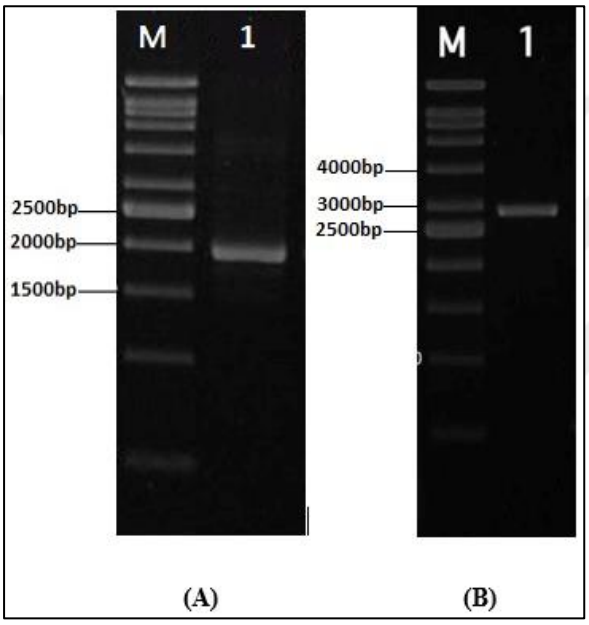

Figure 4.10 Preparation of pBluescript cloning vector DNA. M: VC 1 Kb DNA ladder. (A): Circular pBluescript cloning vector. (B): Linear pBluescript digested with *Sma*I.

4.2.2.2 Transformation of pLAS and pLAW into *E. coli* XL1-Blue competent cell

The purified recombinant DNAs, pLAS and pLAW, were transformed into XL1-Blue competent cell treated with $CaCl_2$. The insert positive colonies were selected according to color. White colonies were insert-positive, and blue colonies were insert negative (Figure 4.11). To confirm the presence of the recombinant DNAs inside the competent cell, plasmid DNAs were isolated and loaded into 0.8% agarose gel and visualized under UV light (Figure 4.12).

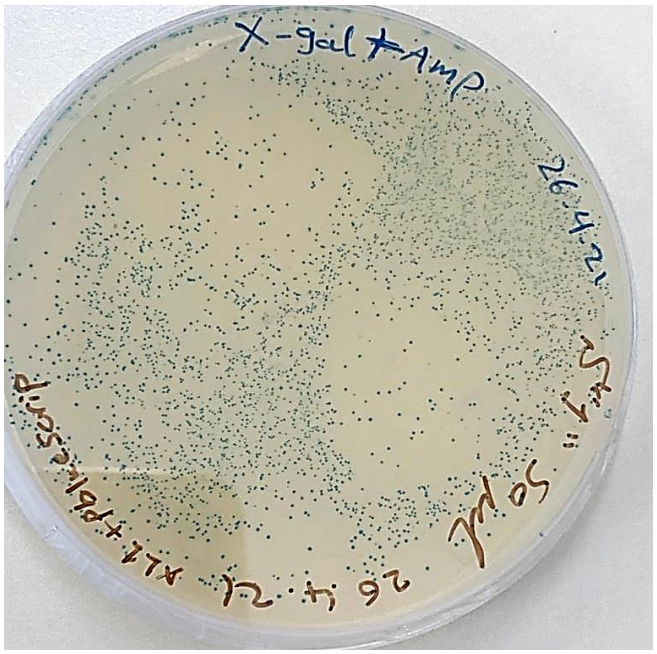

Figure 4.11 Transformation result grown on LB plate supplemented with ampicillin, IPTG, X gal. White colonies contain recombinant DNA, and blue colonies lack recombinant DNA.

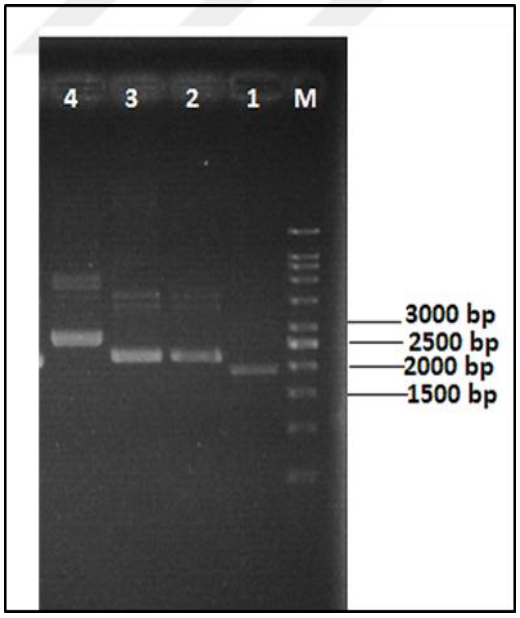

Figure 4.12 Transformation of pLAS and pLAW into XL1-Blue competent cell. M: VC 1 Kb DNA ladder. Line 1: circular pBluescript DNA. Line 2 and 3: uncut pLAW DNAs from first and second white colonies respectively. Line 4: uncut pLAS DNA.

4.2.2.3 DNA sequence analysis for pLAW and pLAS constructs

The pLAW and pLAS constructs were sequenced by Macrogen (Seoul, Korea) using the universal primers M13/pUC Foreword (5´-GTAAAACGACGGCCAGT-3´) and M13/pUC Reverse (5´-CAGGAAACAGCTATGAC-3´). The nucleotide sequence visualized by BioEdit program showed the result to be 552 bp for pLAW and 1327 bp for pLAS construct. Then the sequences were translated into their respective amino acid sequences by ExPASy sequence translation tool. The obtained sequence for pLAW and pLAS construct encode for 30 amino acids with the stop codon TGA and 152 amino acid residues with the stop codon TAA respectively. Then the proteins were aligned with closely related proteins in the database of NCBI by BLAST tool and MEGAX softwere.

TCACACGAAATATTCCAGATGTGGCAGCAACGAGCTGGACGATGATTT
TTTACATGCTGGTTGCCGTATTGTTATATGCCTTTCCGATCTCATTGATC
TCTGGTGAATTTGCCGGAATGTTTCCTCAAAAAGGTGGTCCAGAACTT
TGGGTATCGAATGCTCTAGGAAAAAAATGGGGATTCATTGTCTCCTGG
CTGTTATGGGTCCAAATGTTTCCAGGGATGGTGATGGTTGCTTCAGCA
CTCGCTCCGTTGTTTGGTAATATTATCGATAATGTGCCGCTTGGGCTGA
ACAGCAAATTTACATTAGTTGTTATTTTAGTTGTTTATTGGATCATCAC
TTTTTTGAATTTAACATTCGACATGGCAAAAATCGGAGGAAAAATCGG
TGTTTGGTTAGGTCTATATGTGCCTTTGACGATGTTGCTCTTATTAGGG
CTTGCTGCGTGGATCAAAGTTGGAATCGTACCAACCGGAACATTAGGC
AATTTTTCATGGGACAAAGTAATACCAGATAGTACGACTGCATCCTCA
TTTATTTCTTACGCTTGCC

Figure 4.13 The 552 bp nucleotide sequence of pLAW construct obtained by Macrogen.

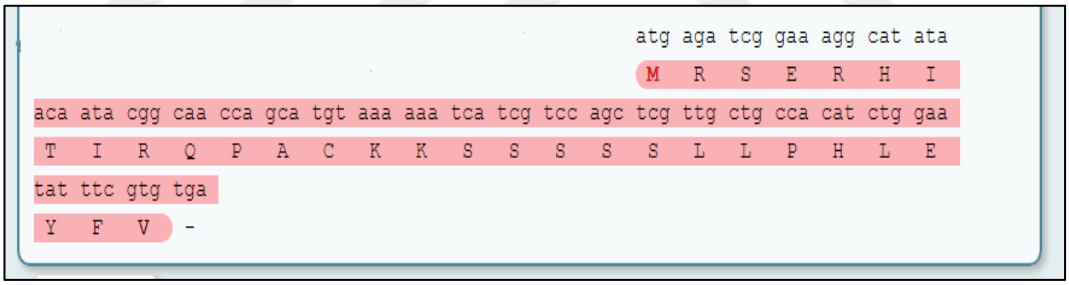

Figure 4.14 The amino acid sequence (30) for pLAW construct obtained using EXPASY tool.

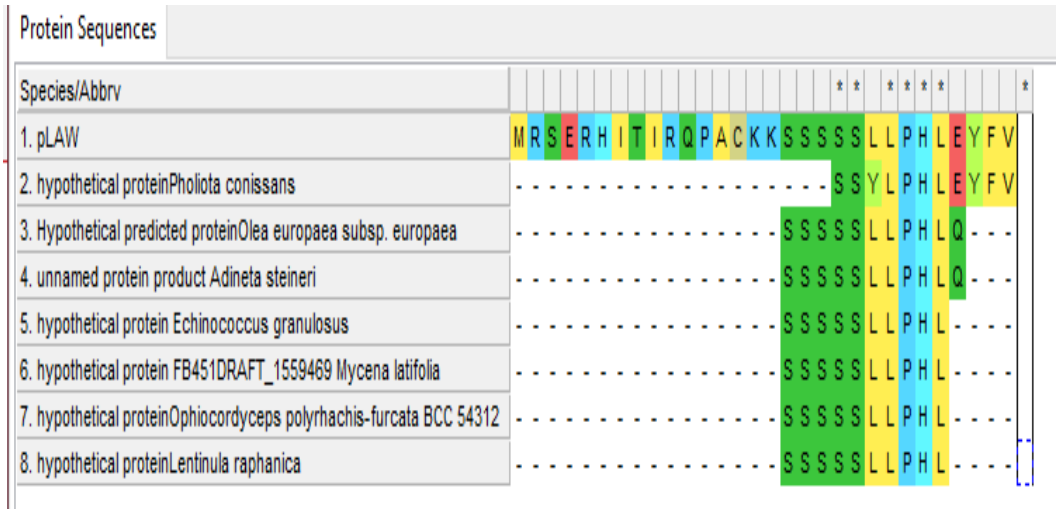

Figure 4.15. The multiple sequence alignment of Plaw construct protein by MEGAX. The protein was obtained using ExPASY tool. The protein was aligned with seven different hypothetical proteins from database of NCBI by BLAST tool. Dashes (-) represent gaps in the sequence introduced during alignment. Asterisk (*) represent fully conserved bases.

TTACCAGCGTAATGCCCCCGTGAGCGGATTAACATTCAACTTTTGTTCATCTCGT
TCAATTGACGTTAATTCATAAGCAAACACAATGGCAAAGTAAATAGATAAAATAC
CCATCGTCATCGTATTGACGATCATGTACAAGTCACTAACTTTGAAAAAAGTTGC
ATTAAAAAATCCTTCTAAAAATGGGAACGGTAATGGCAAAATATTCAAAACCAAA
AACATCGAACCTACGATCGTGAAAGGAATAGAAGCCATCCCCGCTGCCATAACAG
CCCGAACGATTTTAAGTTGCGCAATTTTCCCCATAGGTCCCATCAGGTATTTCTG
CAAAAAATCAAACATTAATTATTCTCCTTTCCTAAATACAACGCTTTTTCATATT
GTTTTATACGGATATACTGCTGATCTTGATTGAGCGAAATTTTCTTTAAACGATA
CTGGATAAATACACTGACAAAAATACCGCTTACAATAAGAATGAAAACAAATAGC
TGTCCTCTATTGTTAATTGCCATAAAAGGATACAAGAGTGTAAATCCTGAGGTGA
ACAACACTGGAATCAATAATATATTGCAGACACCCTGAAACAAAAAATACCGCTT
TGTCCATGGAACATTCGTTTGGTGCTTTCGATAAAGCTTTACTTGTTCTCCTACT
GCAGGCAAAATAGCTAAAAGTAGAACCATTGGTAAACTGCTTCCTAAACTTTTAG
CTGATAGAAGCAGAATCATCCATTGCATGTTCATGAAAAAGAAAAAGGCAGTCAG
ATAGCGTACGAACAGATAACGATTGAAATACATGTTTTTCAAACTAAGTTGCTTT
TTTTCTAATTCGATTTTCCTTTTTACTGTATCCTGCACAATAAATCTTCCTTTCT
ATTTATTTTTTTGATACAAATAATTCATTTCCTGTGCAACTTCCAATAATGTCAT
CGTCGTCATCAGATGATCTTGTGCATGGACCATGATAATTTCCATTTCTATTTTT
GTCCCCCTAGCCAATTCTTTCAAAATTTGAGTTTGTGATTGATGCGCATGAAGAA
TCTCTTCATTTGCTTGTCGCAACTTCTCTTCTACTTCTTCAAACATCTCCTTACG
CATCGAGCGAAACGCTTCATGTATCAGTGTTCGTGCATTTCCGCTATGAAAAATG
ATATCAAAAGACGTCACTTGTAGCGTTTCACTGTTTATTTTTTCAGCCATCTGTT
TTCCTCCTTATTCGATCAGTTTGATAAATTTACTGCTGAAATTCTCAAAAATAGG
TTCAGCTAAGATTTCCTGTTGCACGTTTAGCTGGTCAACTAAACTAACGATTGCA
TCACCAG

Figure 4.16. The 1327 bp nucleiotide sequence from pLAS construct obtained by Macrogen. The underlined sequence refers to the foreword and reverse primers.

Figure 4.17 The amino acid sequence (152) for pLAS construct obtained using EXPASY tool.

Figure 4.18. The multiple sequence alignment of pLAS construct protein by MEGAX. The protein was obtained using ExPASY tool. The protein was aligned with six different hypothetical proteins from database of NCBI by BLAST tool. Dashes (-) represent gaps in the sequence introduced during alignment. Asterisk (*) represent fully conserved bases.

5. DISCUSSION

Lactic acid bacteria (LAB) which are main probiotic microorganisms and essential components of the gut microbiota of humans and other mammals, have significant positive impact on the host's health. Due to their ability to restore the equilibrium of intestinal bacteria, probiotics are utilized to treat intestinal diseases in both humans and animals. Despite extensive study on probiotics, it is still necessary to search for novel stains because probiotic properties are strain-specific and can be dependent on their origin. Therefore, the host origin probiotic bacteria are preferable because they are more adapted to the GIT environment and can proliferate and exert their biological activity more effectively than probiotic bacteria from other sources. Additionally, it can be costly and time-consuming to directly evaluate potential probiotics in vivo, so in vitro evaluation is the major standard for the selection of efficient and desirable strains with optimal probiotic properties. Several research have shown that *L. plantarum* is a useful and important LAB due to its unique probiotic properties. It can tolerate acidic and bile conditions and has antagonistic activity against gut pathogens. Therefore, in the present study, we have investigated *L. plantarum* B14 probiotic characteristics.

Probiotic bacteria are usually administered orally, and they must firstly pass through the stomach before reaching the intestinal tract to colonize. The stomach's acid condition has been reported as the major challenges to the persistant of probiotic bacteria in the host's GIT tract (67). Therefore, the acid tolerance assay was used to determine the degree of acid tolerance of *L. plantarum* B14 strain. This strain has shown high survivability and growth in all five pH points with small fluctuations and slower growth rate in pH 2, pH 3, and pH 5 in comparison to the control group. No significant results were detected because the collected data of the growth rate were relatively close to each other. The increase in bacterial growth after re-suspension in normal MRS indicates that the small decrease in the growth was due to the bacteria being dormant, which means *L. plantarum* B14 can tolerate high acidic conditions for few hours and survive the stomach acidity till it reachs the intestine for colonization.

Tolerance to bile salts is regarded as a necessity for the colonization of probiotic bacteria in the host's small and large intestine, since bile salts are poisonous to the bacteria, affecting the integrity of the cell membrane leading to cell death (6). Bile salt concentration in the intestine is not constant, but the average bile concentration is thought to be 0.3% w/v and the retention time is suggested to be 4 hours (34). So, this test was conducted with media supplemented with 0.1% and 0.3% of oxgall as a replacement for human bile due its resemblance and incubation time 5 hours. The results of this test showed high tolerance of *L. plantarum* B14 to bile salts. It showed continuous rising in the log value in the 0.1% oxgall concentration with 94.4% growth rate after 5 hours of incubation, also with 0.3% oxgall concentration, the bacteria showed continuous increase in the log value with small decline in the 5^{th} hour reaching 74.5% growth rate in comparison to the control group. This bacterial strain showed significant growth ($p<0.05$) and survivability in 0.3% bile concentration after 3^{rd} and 4^{th} hours of incubation. In the light of this finding, the survival rate was higher than 70% which means it survived bile and thus protected its ability to colonize indicating bile tolerant ability.

Antimicrobial activity is a major characteristics of probiotic bacteria are attributed to the secretion of several different metabolic compounds such as organic acids (lactic acid, acetic acid etc), alcohols, hydrogen peroxide, and bacteriocin (61). In this study, the anti- pathogenic activity of *L. plantarum* B14 strain was tested against five pathogenic bacteria, three-gram negative pathogens, *Escherichia coli, Yersinia enterocolitica, Salmonella typhimurium*, and two gram-positive pathogens, *Listeria monocytogenes, staphylococcus aureus*, for both cell lysate and CFS to test the viability of antimicrobial substances inside the cell and the secretion of it into the medium. The cell lysate exhibited antibacterial activity with a big inhibition zone against *S. aureus* and *E. coli*, and CFS exhibited activity against *L. monocytogenes* with a small inhibition zone. The obtained results did not identify the exact cause of antimicrobial activity of *L. plantarum* B14, whether that activity was caused by the presence of bacteriocin or another antimicrobial compounds in the bacteria. The result of this test in agreement with Gheziel et al (70) results which reported antimicrobial activity of several *L.*

plantarum strains against *S. aureus* and *E. coli*, but no result against *L. monocytogenes* in contrast to our result showing positive results agains all three.

Resistance to phenol is an indicator for survival ability for probiotic bacteria under intestinal condition. According to the obtained result in Table 4.4 *L. plantarum* B14 showed resistance to phenol and growth ability with an increase in $\log_{10}$ values in different phenol concentrations after 24 h of incubation. The one-way ANOVA statistical analysis indicated that the growth rate of tested bacteria was significant for 0.1 %, 0.2%, 0.3% phenol concentration in comparison to 0 h, on the other hand, the bacterial growth rate at 0.4% phenol concentration showed no significant change in comparison to 0 h. These results indicate the ability of *L. plantarum* B14 to tolerate toxic metabolites like phenol that are present in the gut. The results obtained from this study are better compared with results obtained by Pinto et al (71), by which different *L. plantarum* strains showed growth inhibition after 24h of incubation in 0.4% phenol.

Acidification is a crucial characteristic in the selection of LAB as starter culture in fermentation process. According to the production of acidic compounds after 24 hours of incubation, isolates are categorized into three categories: (i) those with high acidity capacity can lower the pH by more than 2 degrees, (ii) those with medium acidity capacity can reduce the pH by 1.5-2 degrees, (iii) and those with low acidifying ability to reduce the pH in less than 1.5 units (72) According to these categories, the results in Table 4.5 showed the capacity of *L. plantarum* B14 to decrease the pH value 1.47 units after 24 h of incubation, which can be classified as a low acidifying capacity. According to statistical analysis, the decrease in the pH values was significant ($p<0.01$) in all measured points and reached the 4.6 pH value after more than 24 h showing *L. plantarum* B14 as slow acidifier. In comparison to previous study, *L. plantarum* B14 showed better acidifiying activity than *L. plantarum* strains tested by Herreros et al (73).

Auto-aggregation measures a bacterial strain's ability to aggregate with one another in a nonspecific manner, which is regarded a necessity for colonization in order for the probiotic bacterium to perform its beneficial effect (6). In the present study, *L. plantarum* B14 showed a high autoaggregation ability by reaching 100% after 5 h of incubation. The obtained results during the 5 h duration was all

significant ($p<0.01$) in comparison to the first hour. Our findings are consistent with previous research showing that autoaggregation rate increases with incubation time (7). The autoaggregation ability may be connected to the component of cell surface because this charactertistic had persisted after washing and suspending the cells in PBS buffer.

Co-aggregation enables probiotics to form an effective barrier that helps in preventation of enteric pathogens from adhering to intestinal mucosa. Probiotics' ability to aggregate with pathogens may be crucial for both food preservation and the therapeutic influence they have on the intestinal flora (61). Co-aggregation potential screening could be useful for finding possible probiotic strains that are acceptable for usage in food, human or animal. The coaggregation ability between *L. plantarum* B14 and the 5 pathogenic bacteria tested showed strain specific coaggregation abilities ranging between 89.6% for (*L. monocytogenes*) and 95.3% for (*E. coli*) after 24 h of incubation as shown in Figure 4.6. Co-aggregation test results from a previous study in the literature showed that the coaggregation percentage of *L. reuteri* strain after 2 h of incubation were 75.81%, 66.30%, and 71.83% for *E. coli*, *S. typhimurium*, and *L. monocytogenes* respectively. In comparison to our result, *L. plantarum* B14 stain showed a higher rate co-aggregation at the 2 h of incubation (61).

Antibiotic susceptibility testing for *L. plantarum* B14 strain is performed to test the sensitivity of our strain to a range of antibiotics, which are widely used to treat bacterial infection. It is not recommended to use probiotic strains that are resistant to antibiotics in order to prevent the transmission of resistance genes to intestinal pathogenic bacteria (74). In this study the antibiotics that were used belong to different groups: (i) β-Lactam, cell wall synthesis inhibitors (ampicillin); (ii) protein synthesis inhibitors including macrolide (erythromycin), and aminoglycosides (neomycin), (iii) inhibitor of cellular RNA like aminoglycosides (kanamycin). Our obtained result indicated that *L. plantarum* B14 strain was susceptible to ampicillin which is in agrrement with the earlier findings demonstrating a similar antibiotic susceptibility of LAB (70,75,76), intermediate susceptibility to neomycin and resistant to erythromycin and kanamycin. Also, the same resistance to kanamycin was reported by another study (74). The resistance of *Lactobacillus* genus to aminoglycosides antibiotics,

including streptomycin, kanamycin and gentamicin is thought to be intrinsic since they lack cytochrome-mediated electron transport, which is a key component of drug uptake (76,77). On the other hand, previous studies indicated that *L. plantarum* was susceptible to erythromycin which contrasts with our result (75). Most types of antibiotic resistance are considered to be naturally encoded on the chromosome in lactobacillus strains and are considered to be non-transferable (77).

In the human gastrointestinal system, *Lactobacillus plantarum* is one of the most prevalent and frquantly found species. It is also frequently linked to the synthesis of bacteriocin. Bacteriocin producing Lactobacillus species may have a competitive advantage over other species in a particular ecological niche. In this study, screening for bacteriocin gene was preformed and the PCR product identity was confirmed by sequencing. In-silico analysis was conducted on the obtained result, BLAST analysis and multiple sequence alignment showed that the detected gene by PCR and translated protein sequances did not coincide with any bacteriocin proteins in the database of NCBI. It aligned with different proteins from several species including hypothetical proteins. These results may be due to the atachment of the primers on a different site on the strain. *L. plantarum* B14 has positive antimicrobial phenotypic characteristics but the bacteriocin coding gene was not detected. This difference in results may be due to the existence of multipe bacteriocin gene in *Lactobacillus* species.

6. CONCLUSIONS AND RECOMMENDATIONS

Characterization of human originated *L. plantarum* B14 for probiotic properties was the aim of this study. Several tests were conducted in order to evaluate these probiotic properties. According to the results, *L. plantarum* B14 was found to demonstrate desirable in vitro probiotic characteristics, including high tolerance to stress conditions like acid and bile, and different phenol concentrations, ampicillin susceptibility, antimicrobial activity against pathogenic bacteria, cell surface properties such as high ability to self-aggregate and coaggregation with pathogens. Also, high BSH activity partially reflects a strain's ability to remove cholesterol. These results indicate that *L. plantarum* B14 has promising probiotic features and can be a probiotic candidate for human consumption and food application after further investigation.

Regarding the limitation of this work, the adhesion to epithelial cell test is required since the co-aggregation and autoaggregation assays that were employed in this work are related to attachment to cell. Although protein coding genes were detected using PCR, a more detailed molecular investigation is needed to detect antimicrobial genes and isolate their proteins. Since *L. plantarum* B14 showed resistance to some antibiotics, further work at molecular level is required to test the transferable probability of antibiotic resistance gene.

This work is considered a preliminary testing for probiotic characterization, more in detail testing is recommended. In-vivo studies for this human originated bacterium which showed good in-vitro results will be greatly appreciated to evaluate clinical effects for humans with different health conditions. Further studies are needed to detail the industrial properties of this strain to be applied in diverse fields.

7. REFERENCES

"Vancouver citation system was used in this thesis."

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8. APPENDICES

Appendix A

Chemicals

Chemicals	Suppliers
Agar	Merck
Agarose	Sigma
Ampicillin	Sigma
Antibiotic disks	Oxoid, UK
Calcium chloride [minimum 93.0%, granular anhydrous]	Sigma
EDTA (Ethylenediaminetetraacetic acid)	Sigma
EtBr (Ethidium Bromide)	Sigma
Ethanol	Merck
Glycerol	Sigma
Glucose	Merck
Glacial acetic acid	Carlo ebra
Hydrochloric acid	Merck
IPTG (isopropyl-beta-D-thiogalactopyranoside)	Thermo Scientific
MRS broth	Merck, Germany
Nutrient agar	Oxoid, UK
Nutrient broth	NEOGEN, UK
Potassium chloride (KCl)	Thermo Scientific
Peptone from casein	Merck
Phenol	Merck
Sodium Chloride (NaCl)	Merck
Sodium Hydroxide (NaOH)	Merck
Sodium phosphate dibasic	Merck
Sodium phosphate monobasic	BD lab
Trizma Base	Sigma
Trizma Hydrochloride	Sigma
Triton X-100	Sigma
Tryptone	Merck
Yeast Extract Granulated	Merck

Enzymes and other chemicals

Enzymes and other chemicals	**Suppliers**
*Bam*H	Thermo Scientific
dNTP mix	Fermentas
*Sma*I	Thermo Scientific
*Pst*I	Thermo Scientific
Lysozyme	Fluka
T4 DNA Ligase	Thermo Scientific
VC 1 kb DNA ladder	Vivantis
6x loading dye	Fermentas

Appendix B

Growth media:

❖ **de Man, Rogosa, and Sharpe (MRS) media**

- **MRS broth (per liter)**

To create MRS broth, 52.2 g of MRS broth powder is dissolved in 1000 ml of distilled water, the mixture is then sterilized by autoclaving at 121°C for 15 min.

- **MRS Agar (per liter)**

  the amount of agar needed to make an agar plate is 1.5% of the total amount of the liquid media.

  52.2 g MRS broth powder, 15 g Agar-Agar (Merck) are dissolve in 1000 ml of distilled water and autoclaved at 121°C for 15 min.

❖ **Nutreint media**

- **Nutreint broth per liter**

To prepare nutrient broth, 8 g of nutrient broth powder is thoroughly dissolved in 1000 ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes.

- **Nutreint agar (NA) per liter**

28 g of nutrient agar powder is dissolved in 1000 ml of distilled water and the mixture is heat to dissolve completly, and autoclave at 121°C for 15 min.

❖ **Plate count agar (PCA) media**

Contents	**For 1 liter**
Tryptone	5g
Yeast extract	2.5g
Glucose	1g
Agar	15g

Dissolve these contents in 950 ml of distilled water and mix thoroughly at 25°C, then the pH is set at a value of 7±0.2 with 5M NaOH then fill up to 1000 ml and autoclave at 121°C for 15 min.

❖ **Luria Bertani (LB) media**

- **LB broth per liter**

Components	**For 1 liter**
Tryptone	10g
Yeast extract	5g
NaCl	10g

The components are dissolved in approximately 950 ml distilled water and mix thoroughly then the pH is set at a value of pH 7.5 with 5M NaOH and fill up to 1000 ml and autoclave at 121°C for 15 min.

- **LB plate**

In order to prepear an LB plate, add 15 g (1.5%) Agar-Agar (Merck) and dissolve in 1000 ml of distilled water and autoclaved at 121°C for 15 min.

➢ **Ampicillin (25mg/ml)**

0.25 g of Ampicillin Sodium Salt is dissolved in 10 ml distilled water then it was sterilized by using 0.22 µm filter. Then the soulution is divided into the desired volume and the storage is at -20°C.

- **LB plate with Ampicillin**

Add 400 µl ampicillin to every 100 ml sterilized LB medium (4 ml Ampicillin for 1 liter), and store at +4°C.

Appendix C

Solutions and buffers

- **For electrophoresis**

- **10 X TAE buffer**

Contents	Amount for 1 liter
Tris base (Sigma)	48.50 g
Glacial acetic acid	11.42 g
0.5 M EDTA (pH:8)	20 ML

These components are dissolve in UpH_2O and mix thoroughly then the volume complete to 1000 ml and the buffer is stored at room temperature.

To prepeare **1X TAE buffer** 100 ml of 10X TAE buffer is added to to 900 ml UpH_2O.

- **For competent cell preperation**

- **$CaCl_2$ (0.1 M)**

0.7351 g of $CaCl_2$ powder is added to 50 ml UpH_2O and dissolve, pH is adjusted to the value of 7 then sterilized by autoclave at 121°C for 15 minutes. Storage at +4 °C.

- **$CaCl_2$ (0.1 M) with 10% glycerol**

9 ml of 0.1 M $CaCl_2$ and 1 ml glycerol are mixed togather then sterilized by autoclave at 121°C for 15 minutes and stored at +4 °C.

- **For cell lysis**

- **Lysis buffer**

Content	concentration	Amount
Lysozyme	30 mg/ml	666.6 µl
Tris-HCl	1 mM	20 µl
EDTA	50 mM	12 µl
Triton 100%	1.2%	12 µl
UpH_2O		261.4 µl
Total volume		**1 ml**

These components are mixed thoroughly by pipetting and stored at -20°C

- **Lysozyme (30mg/ml)**

30 mg of lysozyme is dissolved in 1ml UpH_2O. Storage at -20 °C.

- **Tris-HCl (1M) pH 8.0**

12.2 g Trizma base is dissolved in 80ml of UpH_2O, the pH is set at a value of 8.0 with HCl then top to a total volume of 100ml with UpH_2O and sterilized by autoclave at 121°C for 15 minutes, Storage is at room temperature.

- **EDTA (0.5 M) pH 8.0**

2.9224 g EDTA is added to 10 ml UpH_2O and dissolve then the pH set at 8.0 with 5 M NaOH and top to volume 20 ml with UpH_2O then autoclaved at 121°C for 15 minutes and stored at room temperature.

- **NaOH (5 M)**

10 g NaOH (MW: 40 g/mol) is dissolved in 50 ml UpH_2O and stored at room temerature.

❖ **Phosphate Buffer Saline (PBS) 1X**

Content	Concentration	Amount for 1L
Sodium Cloride	0.137 M	8 g
Potassium Chloride	0.0027 M	0.2 g
Sodium phosphate dibasic	0.01 M	1.44 g
Potassium phosphate monobasic	0.0018 M	0.245 g

These components are dissolve in 1000 ml UpH_2O one after the other, pH adjusted at 7.4, then filtered using 0.22 µm and then sterilized by autoclave at 121°C for 15 minutes and stored at room temperature.

- **Phenol 1%**

To prepeare 1% phenol solution, the phenol (94.11 g/mol) is heated to melt then 1.1 ml is add to 100 ml UpH_2O and store at room temperature.

- **1M IPTG**

1.1915 mg IPTG (MW: 238.3 g/mol) is dissolved in 5 ml UpH_2O then filtered using a 0.22 µm filter and stored at -20 °C.

- **X-gal (20mg/ml)**

0.2 g of X-gal powder is added to 10 ml DMF solution and vortex to dissolve, and stored in the dark (light sensitive) at -20 °C.