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**INVESTIGATION OF THE PROBIOTIC POTENTIAL OF
LACTOBACILLUS GASSERI ATCC 33323 STRAIN**

MASTER OF SCIENCE

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BOLU, TEMMUZ - 2023

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ABSTRACT

INVESTIGATION OF THE PROBIOTIC POTENTIAL OF *LACTOBACILLUS GASSERI* ATCC 33323 STRAIN

MSC THESIS

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BOLU ABANT IZZET BAYSAL UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF BIOLOGY

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(xiii + 74)

Lactic acid bacteria (LAB) as a prime part of the human gut microbiota, have gained increasing interest as a research subject for characterization and the potential to be used as a probiotic candidate to promote human and animal health. The introduction of beneficial bacterial species into the gastrointestinal tract (GIT) may be a very appealing option to restore microbial equilibrium and prevent diseases given that the use of antibiotics among other forms of treatment, may change the composition and have an effect on the GIT microbiota. The aim of this study was to investigate and characterize the probiotic potential of the *L. gasseri* ATCC33323 strain. Probiotic features of the strain were screened using several tests, which included antimicrobial activity, acidifying activity, cell surface properties testing, tolerance to pH, bile salts, and phenol, antibiotic susceptibility, and anti-microbial activity tests including PCR and cloning. Results showed that *L. gasseri* ATCC33323 had an antagonistic effect against three pathogenic bacteria, *S. aureus*, *E. coli*, and *L. monocytogenes* RSKK. This strain also showed a high percentage of auto-aggregation with the highest value being 97.026% at the 5h mark, and high co-aggregation with all five pathogenic bacteria. *L. gasseri* ATCC33323 showed low acidifying activity, tolerance to varying pH points including pH 2, tolerance to bile with a growth rate of 50.6% in 0.3% concentration at the 4h mark, and resistance to differing phenol concentrations (0.1%, 0.2%, 0.3%, 0.4%) with 8.515log for 0.4% concentration at 24h mark, and it was susceptible to all four antibiotics which are ampicillin, kanamycin, erythromycin, and neomycin. In-silico analysis for bacteriocin screening results indicated the presence of a small protein obtained using specially designed primers, but further studies are required to identify bacteriocins in this strain. This study demonstrated that the *L. gasseri* ATCC33323 strain fulfilled the basic desired properties as a probiotic candidate and therefore has considerable potential to be used in the pharmaceutical and food industry.

KEYWORDS: Probiotic, Lactic acid bacteria, *L. gasseri* ATCC33323, antimicrobial activity

ÖZET

***LACTOBACILLUS GASSERİ* ATCC 33323 SUŞUNUN PROBİYOTİK
POTANSİYELİNİN ARAŞTIRILMASI
YÜKSEK LİSANS TEZİ
MARWAH MAHMOOD ABDULRAZZAQ AL-JANABI
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ
BİYOLOJİ ANABİLİM DALI**

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İnsan bağırsağı mikrobiyotasının önemli bir parçası olan laktik asit bakterileri (LAB), karakterizasyon için bir araştırma konusu olarak artan bir ilgi ve insan ve hayvan sağlığını geliştirmek için probiyotik bir aday olarak kullanılma potansiyeli kazanmıştır. Yararlı bakteri türlerinin gastrointestinal sisteme (GIT) sokulması, diğer tedavi biçimleri arasında antibiyotik kullanımının bileşimi değiştirebileceği ve GIT mikrobiyotası üzerinde bir etkiye sahip olabileceği göz önüne alındığında, mikrobiyal dengeyi yeniden sağlamak ve hastalıkları önlemek için çok çekici bir seçenek olabilir. Bu çalışmanın amacı, *L. gasseri* ATCC33323 suşunun probiyotik potansiyelini araştırmak ve karakterize etmektir. Suşun probiyotik özellikleri, antimikrobiyal aktivite, asitleştirme aktivitesi, hücre yüzey özellikleri testi, pH, safra tuzları ve fenole tolerans, antibiyotik duyarlılığı ve PCR ve klonlama dahil anti-mikrobiyal aktivite testleri dahil olmak üzere çeşitli testler kullanılarak tarandı. Sonuçlar, *L. gasseri* ATCC33323'ün üç patojen bakteriye, *S. aureus*, *E. coli* ve *L. monocytogenes* RSKK'ya karşı antagonistik bir etkiye sahip olduğunu gösterdi. Bu suş ayrıca, en yüksek değer 5 saat işaretiinde %97,026 olmak üzere yüksek bir oto-agregasyon yüzdesi ve beş patojenik bakterinin tümü ile yüksek ko-agregasyon gösterdi. *L. gasseri* ATCC33323, düşük asitleştirme aktivitesi, pH 2 dahil değişen pH noktalarına tolerans, 4 saatlik işaretiinde %0,3 konsantrasyonda %50,6'lık bir büyüme oranıyla safraya tolerans ve 24 saat işaretiinde %0,4 konsantrasyon için 8,515log ile farklı fenol konsantrasyonlarına (%0,1, %0,2, %0,3, %0,4) direnç gösterdi. Ve ampisilin, kanamisin, eritromisin ve neomisin olan dört antibiyotiğe de duyarlıydı. Bakteriyosin tarama sonuçları için in-silico analiz, özel olarak tasarlanmış primerler kullanılarak elde edilen küçük bir proteinin varlığını göstermiştir, ancak bu suştaki bakteriyosinleri tanımlamak için daha ileri çalışmalar gerekmektedir. Bu çalışma, *L. gasseri* ATCC33323 suşunun bir probiyotik adayı olarak istenen temel özellikleri karşıladığını ve bu nedenle farmasötik ve gıda endüstrisinde kullanım için önemli bir potansiyele sahip olduğunu göstermiştir.

ANAHTAR KELİMELE: Probiyotik, Laktik asit bakterileri, *L. gasseri* ATCC33323, antimikrobiyal aktivite

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LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	: American-type culture collection
LAB	: Lactic acid bacteria
GI	: Gastrointestinal
WHO	: World Health Organization
FAO	: Food and Agriculture Organization
GIT	: Gastrointestinal tract
CFU	: Colony forming unit
β	: Beta
AAD	: Antibiotic associated diarrhea
CDAD	: <i>Clostridium difficile</i> -associated diarrhea
IBS	: Irritable bowel syndrome
SCFAs	: Short-chain fatty acids
HPV	: Human papillomavirus
DNA	: Deoxyribonucleic acid
BSH	: Bile salt hydrolase
H₂O₂	: Hydrogen peroxide
ACE	: Angiotensin-converting enzyme
TLR	: Toll-like receptors
IL8	: Interleukin 8
GC	: Guanine-Cytosine
rRNA	: Ribosomal ribonucleic acid
RAPD	: Randomly amplified polymorphic DNA
ARDRA	: Amplified ribosomal DNA restriction analysis
CO₂	: Carbon dioxide
GRASS	: Generally Recognized As Safe
nM	: Nanomolar
EMP	: Embden-Meyerhof-Parnas pathway
LABGC	: Lactic Acid Bacteria Genomics Consortium
rDNA	: Recombinant Deoxyribonucleic acid
Da	: Dalton
KDa	: Kilodalton

C	: Celsius
PCR	: Polymerase chain reaction
Amp^r	: Ampicillin resistant
M	: Molar
μl	: microliter
OD	: Optical density
rpm	: Rotation per minute
CFS	: Cell free supernatant
mM	: Millimolar
PBS	: Phosphate buffer saline
μg	: Microgram
GCA	: Glycocholic acid
GDCA	: Glycodeoxycholic acid
GCDCA	: Glycochenodeoxycholic acid
TCA	: Taurocholic acid
TDCA	: Taurodeoxycholic acid
TCDCa	: Taurochenodeoxycholic acid
SD	: Standard deviation
CLSI	: Clinical and Laboratory Standards Institute
UV	: Ultraviolet
EtBr	: Ethidium Bromide
Bp	: Base-pair
μM	: Micromolar
PEG	: Polyethylene glycol
IPTG	: Isopropyl β-D-1-thiogalactopyranoside

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1. INTRODUCTION

1.1. The history and definition of probiotics

Probiotics are live microorganisms that are regarded as advantageous for the health of the host, and they are usually referred to as beneficial bacteria or friendly bacteria (1). The use of probiotics dates back to a time before microorganisms were understood. Without being aware of it, the early food producers used bacteria and yeasts to ferment milk into fermented dairy products. Many medical and widespread sources have suggested that the first use of probiotics in the history of humanity dates back to earlier than 2000 BC when man first discovered how to preserve milk for longer periods. Images of fermented milk products were found in Egyptian hieroglyphs dating back to 3500 BC, as well as evidence from wall paintings from 2500 BC suggesting the Sumerians had a habit of inoculating milk to induce fermentation. Collected evidence using modern, sophisticated genetic archeological techniques showed that the usage of probiotics may be dated back to 10,000 years ago (2–4). Researchers found in the 1800s that consuming a certain amount of fermented milk products had an influence on health, but they were unable to explain these health influences. Louis Pasteur acknowledged that yeast and bacteria were responsible for the fermentation process but did not associate these germs with any health advantages (2).

The concept of probiotics was first introduced when Eli Metchnikoff, the 1908 Nobel Prize recipient, claimed that Bulgarian peasants' long-life spans were caused by their ingestion of fermented milk products (2,5,6). Then Werner Kollath, a German scientist, coined the term "probiotic" in 1953 to describe "active substances that are essential for a healthy development of life." which comes from the Latin pro and the Greek βίος that literally means "for life." (7). When the scientists Lilly and Stillwell used this concept in 1965, they used this term in a different context to refer to compounds generated by one organism that helps in the growth and development of another (2,6,8–10). In the years after that, the term "probiotic" was employed to refer to various things based on how they functioned and how they influenced people's health. Parker refined the definition in 1974, bringing it as close to our present usage as possible describing probiotics as "organisms and substances which contribute to intestinal microbial balance" (10). The later work of Roy Fuller in 1989 changed Parker's connotation by

deleting the reference to "substances," which is largely responsible for the popularity of the new idea since the word can describe something that is dead or even have antibiotics. As a result, a probiotic was defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Soon after that, in the year 1992 Havenaar and Huis, In't Veld expanded the term to encompass the microflora of habitats other than the intestinal, including the upper respiratory tract or the urogenital tract defining probiotics as "a mono- or mixed culture of live microorganisms which applied to animals or man, affects beneficially the host by improving the properties of the indigenous microflora" (8,10). In the years that followed many researchers studied probiotics and attempted to define them, this list includes Guarner & Schaafsma in 1998 who specified the definition to include the dosage as a requirement describing probiotics as "living microorganisms which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition". Naidu et al. (1999) definition expanded on the effect of probiotic mechanism on immunomodulation and the enhancement of nutritional status describing probiotics as "a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as enhancing nutritional and microbial balance in the intestinal tract" (10,11). The most frequently used definition was developed by World Health Organization (WHO) and the Food and Agriculture Organization (FAO) which was reported in 2001 defined probiotics as "Live microorganisms which when administered in adequate amounts confer benefits on the host" (10). In 2013, the World Gastroenterology Organization showed that probiotics' effectiveness is dose- and strain-dependent. Then in 2014, The International Scientific Society for Probiotics and Prebiotics published a consensus statement on the three primary probiotic categories including probiotics without health claims, which are generally regarded as safe and do not need to provide proof of efficacy; probiotics with health claims which are used as food and dietary supplements, and Probiotic medication that satisfies regulatory requirements for pharmaceuticals (2).

1.2. Types of probiotics

Probiotics are commonly known as microorganisms that have positive effects on both people and animals. These microbes assist in maintaining gut microbial

balance and good health. An enormous number of genera and species are regarded as probiotics. Probiotics are made up of a variety of organisms, including bacteria, yeast, and mold but the major part of probiotic microorganisms are members of the heterogeneous group of lactic acid bacteria which includes *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Lactococcus*, *Streptococcus*, and *Leuconostoc*. the most common species used in probiotic production are *Lactobacillus* and *Bifidobacterium* and they have been increasingly introduced to various food products and therapeutic applications. Apart from a few *Streptococci* and *Enterococci*, it is widely agreed that LAB is rarely harmful to both humans and animals (12,13). However, fungi, and mold species are also thought of as probiotics such as *Saccharomyces boulardii*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Candida pintolopesii*, *Aspergillus oryzae*, *Saccharomyces boulardii* (14). Probiotics from many different genres are listed below in Table 1.1 (12).

Table 1.1. Microorganisms used as probiotics (12).

<i>Lactobacillus</i> species	<i>Bifidobacterium</i> species	Other species
<i>L. acidophilus</i>	<i>B. animalis</i>	<i>Enterococcus faecalis</i>
<i>L. rhamnosus</i>	<i>B. bifidum</i>	<i>Enterococcus faecium</i>
<i>L. reuteri</i>	<i>B. breve</i>	<i>Lactococcus lactis</i> subsp. <i>Lactis</i>
<i>L. casei</i>	<i>B. longum</i>	<i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>
<i>L. gasseri</i>	<i>B. infantis</i>	<i>Propionibacterium freudenreichii</i>
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>B. lactis</i>	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>
<i>L. plantarum</i>	<i>B. adolascensis</i>	<i>Leuconostoc mesenteroides</i>
<i>L. crispatus</i>	<i>B. laterosporus</i>	<i>Saccharomyces boulardii</i>
<i>L. salivarius</i>		<i>B. coagulant</i>
<i>L. johnsonii</i>		<i>B. subtilis</i>
<i>L. lactis</i>		
<i>L. paracasei</i>		
<i>L. fermentum</i>		

1.3. The human gut microbiota

The body of a human being contains significantly more microbial cells than human cells. At birth, microbes start to colonize the exposed surfaces of the human body, such as the gut, skin, mouth, and vagina. The gastrointestinal tract is one of the greatest interfaces between the host, and external environment (250–400 m²), and more than 60 tons of food and many environmental microbes go through the gastrointestinal tract of humans causing it to be constantly populated by a variety of species like bacteria, viruses, archaea, and fungus making up the gut microbiota. Nevertheless, bacteria are the predominant microbial species in the human intestinal tract. The intricate interaction between the human host and the microbiota is mutually beneficial and has an important effect on the health of humans (15,16). The term microflora has been wrongly used interchangeably with microbiota for many years to refer to the different microorganisms that live throughout the entire GIT in a harmonious symbiotic relationship with the host. Since the term microflora refers to plants, and bacteria share little resemblance with them, scientists studying microorganisms transitioned to using the term intestinal microbiota to refer to the collection of microbes in the human gut.

Up to 10¹⁴ different genera, species, and strains of bacteria make up the intestinal microbiota, which outnumbers the somatic cells and germ cells by a factor of 1 to 10 and makes up 1 to 3% of the body mass. The microbiome of the gut can encode more than three million genes that create thousands of metabolites, whereas the human genome has approximately 23000 genes. When microorganisms have a mutually beneficial relationship with the host it preferably colonizes the GIT. The composition of the gut microorganism can be categorized into two groups, residual or essential group which is thought to be the core species that resides in the gut throughout adulthood and can reestablish itself. The other type is transient bacteria, which includes nonpathogenic and potentially pathogenic bacteria sourced from the environment and can stay in the human gut for hours, days, or even weeks but they do not establish a long-term residence. In addition to that, the existence of native commensal bacteria within the gut supports the notion that microorganisms have a significant role within the host. Commensal bacteria that do not have a negative effect on the host coevolve and have a crucial effect on many physiological processes which includes improvement of the intestinal epithelial barrier by preventing the colonization of

pathogenic bacteria, development of the immune system, and synthesizing micronutrients from food sources. However commensal microorganisms can become pathobionts causing a pathological effect. As a result, the host-microbial-environmental interactions can modify the microbiota and impact the host's health by controlling the commensal microorganism distribution (17).

1.3.1. The composition of the gut microbiota

The GIT is a complicated system that starts in the oral cavity and terminates at the anus. Several physiological conditions influence the different microbial populations in the upper and lower GIT like pH, bile concentration, and transit duration. More than 1000 taxa compose the oral cavity microbiota with six main phyla composing 96 percent including *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Spirochaetes*. Some of the genera found in the saliva include *Streptococcus*, *Prevotella*, *Pseudomonas*, and *Actinomyces*. Similar to the oral cavity, the genus *Streptococcus* and the phylum *Firmicutes* make up the majority of the bacteria in the human esophagus. Different community types were dominant in a healthy host which includes *Streptococcus*, *Prevotella*, *Veillonella* or *Haemophilus*, and *Rothia*. The upper GIT contains bile, pancreatic secretions, and gastric acid, which prevent most bacteria from growing in a concentration greater than 10^3 CFU per milliliter. The first digestive organ is the stomach, and it is believed that its hostile environment due to low pH and digestive enzymes acts as a defense against pathogens. In spite of the low pH, the genera *Bacillales incertae sedis*, *Streptococcaceae*, *Enterobacteriaceae*, *Leptotrichiaceae*, and *Pseudomonadaceae* are often seen in the stomach (18). The density and diversity of bacteria increase along the intestinal tract, reaching 10^4 to 10^7 in the jejunum and ileum and coming closer to 10^{11} to 10^{12} cell/g in the lumen of the colon. There is also heterogeneity of bacteria, showing lower ratios of anaerobes to aerobes at the epithelial surfaces compared to the lumen of the intestine and feces (17). Up to 90% of the population of the intestinal microbiota is made up of the phyla *Firmicutes* and *Bacteroidetes*. The jejunum and ileum common communities include *Streptococci*, *Lactobacilli*, *Enterococcus* group, and the *Bacteroides* group. The colon of a healthy human contains the phyla *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, *Proteobacteria*, and *Actinobacteria*. There aren't many distinctions between the ascending and descending colon with

Clostridiaceae, *Lachnospiraceae*, and *Bacteroidaceae* families dominating the microbiota structure. The big diversity of bacteria especially in the colon provides significant advantages to the host which include breaking down substrates that the host is unable to digest, supplying nutrients to the intestinal tract cells, activating, and helping the immune system to tolerate the beneficial bacteria, and preventing pathogenic bacteria from colonizing (18,19).

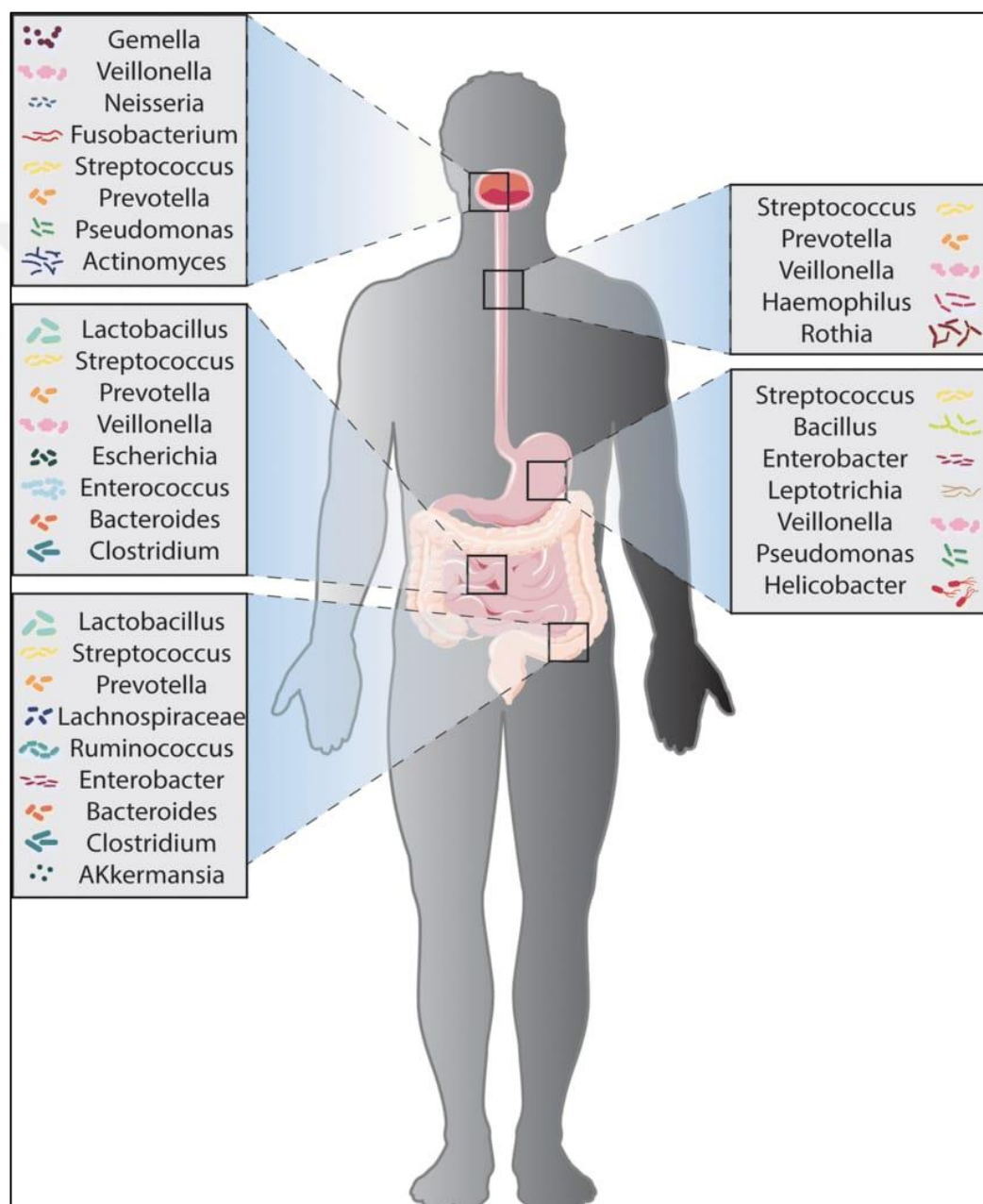


Figure 1.1. The predominant genera of the microbiome in the oral cavity, the esophagus, stomach, small intestine, and colon (retrieved from <https://link.springer.com/article/10.1007/s10620-020-06118-4/figures/1>).

1.3.2. Development of the gut microbiota

The infant's gut microbiota development is believed to begin after delivery, although the colonization in the gut of the infant may start in the lower uterus. Several factors affect the early formation of the gut microbiota like the style of birth- cesarean delivery vs. vaginal delivery- the type of nutrition- breast milk vs. formula milk- the use of antibiotics, as well as the timing of introduction of solid food. A stable gut microbiota is formed by two major shifts in the infancy period. Following birth, Enterobacteriaceae and *Staphylococcus* temporarily dominate the gut microbiota of the baby. The first shift happens shortly after birth, during nursing, and as a result, *Bifidobacterium* and some lactic acid bacteria dominate the gut microbiota. The *Bifidobacterium*-dominated microbiota stage continues up until the beginning of consumption of solid food. The second stage entails the creation of an adult-type complex microbiota that happens through the weaning stage with the introduction of solid meals and continued breast milk feeding dominated by the phyla *Bacteroidetes* and *Firmicutes* and the genera *Prevotella*, *Ruminococcus*, *Clostridium*, and *Veillonella*. These changes persist until the age of three, after which the human has a stable gut microbiota kept in a well-balanced host-microbiota symbiotic state called "Enterotypes". Infancy is a critical window for the development of the microbiota and has a significant impact on the host immune system's early development and function, along with the host's subsequent health, and disruptions to this process may result in immunological disorders such as food allergies, atopic dermatitis, and asthma (20).

1.4. Health benefits of probiotics

It is now widely accepted that the native microbial communities are location- and host-specific, have an extremely complex composition, and have advantageous features for the host (9). The claim that probiotics have beneficial effects, including improving gut health, enhancing immune function, lowering blood cholesterol, and prevention of cancer, is supported by increasing scientific evidence. These health properties are influenced by a variety of mechanisms and are strain specific. While some of the health advantages are well known, others need further research to be confirmed. In reality, there is proof to support the usage of probiotics in the management of health issues like acute diarrheal disorder, antibiotic-associated diarrhea prevention, immunomodulatory effects,

irritable bowel disease management, and the enhancement of lactose metabolism (21). Probiotics may also be more rationally seen as preventative than remedial medicines. In the next subsection, some of the health impacts will be briefly discussed (13).

1.4.1. Lactose intolerance

Beta-galactosidase deficiency is the cause of lactose intolerance and it prevents the hydrolysis of lactose into the monosaccharides galactose and glucose and it is genetically determined. Many people, particularly those of Asian and African heritage, are deficient in the intestinal mucosal enzyme β -galactosidase or experience decreased lactase activity as a result of intestinal infection. In those with a low level of this enzyme, the disaccharide lactose can result in significant intestinal discomfort characterized by bloating, gas, diarrhea, and abdominal pain. As a result of this ailment consumption of calcium-rich dairy products is restricted when they are vitally needed owing to bone loss in the elderly since this disease tends to get worse with age. Lactobacilli generate lactase during fermentation, which hydrolyzes lactose in dairy products into glucose and galactose. Lactase is continually produced throughout fermentation. High quantities of β -galactosidase are produced by *Streptococcus salivarius* subsp. *Thermophiles* and *L. delbrueckii* subsp. *Bulgaricus*. Due to the bile salt sensitivity of both species, they release a significant amount of β -galactosidase in the gut. Compared to the other two species *L. acidophilus* is bile resistant and contains less β -galactosidase, but it develops in the gastrointestinal tract and may generate β -galactosidase over an extended duration of time. Those with low-galactosidase activity are better able to absorb lactose from yogurt and milk that includes the probiotic *L. acidophilus*. Probiotics are claimed to have the ability to improve lactose metabolism though this effect appears to depend on the concentration and kind of strain more than others. Probiotic supplementation is being considered as an alternative therapeutic agent since many people have responded well to the treatment (21–23).

1.4.2. Antibiotic-associated diarrhea

One of the most commonly prescribed drugs in the world is probiotics. Diarrhea is a typical side effect for them, because antibiotics alter the variety and population of bacteria inhabiting the gut, upsetting the ecology of the microbiota

of the intestine. The intestinal microbiota balance can have a long-lasting effect on the patient's susceptibility to infection and other disorders, even after the overall bacterial numbers have been restored to normal. Antibiotic-associated diarrhea (AAD) has been documented in individuals receiving antibiotics in 5%-39% of cases depending on the antibiotic being used, the patient's health, and their exposure to infections with the upper range being individuals over the age of 65. Instead of happening during treatment, AAD can happen up to 2-3 weeks after antibiotic medication is stopped. Antibiotics of any kind can result in AAD but a higher risk is specifically linked to antibiotics that have a broad spectrum and act on anaerobes in particular clindamycin, and cephalosporin and they carry a higher risk than narrow spectrum ones. The signs and symptoms can range from moderate, self-limiting diarrhea to severe diarrhea, the latter being more common in infections caused by *Clostridium difficile*. Probiotics are administered to patients with digestive issues with the aim that they may help restore a healthy balance to the gut microbiota. It is well-recognized that antibiotics can disturb the delicate balance of the gut microbiota, allowing harmful organisms like *C. difficile* to colonize causing *Clostridium difficile*-associated diarrhea (CDAD). Several hypothesized mechanisms exist for how probiotics improve intestinal health. Probiotics are thought to briefly colonize the gut, competing with the normal bacteria for resources and epithelial adherence while also releasing bactericidal acids and peptides. The *Lactobacillus* genus contains several species that produce acid, reduce the pH of the surrounding environment, and release exotoxins that prevent the pathogenic bacteria from sticking to the intestinal epithelium. *Lactobacillus* and *Saccharomyces*, work through a variety of methods to enhance both the innate and adaptive immune systems (24–26).

1.4.3. Irritable bowel syndrome

Irritable bowel syndrome (IBS) is among the most typical digestive issues, and it has a complex etiology. IBS affects 12%-20% of people globally, 18%-23% of people in China, and 5%-11% in the civilizations of Asia and North America, the majority of IBS patients are young between 20-39 years old with the frequency for women being 2-3 times higher than men (27,28). Major symptoms of IBS include gut pain, altered stool, bloating, straining, and abdominal discomfort brought by alterations in bowel movements (diarrhea, constipation, or mix). The

pathophysiology of IBS involves genetic makeup, environmental circumstances, a family history of inflammatory bowel disease, and psychological components, such as stress. IBS severity is influenced by several variables, such as decreased bowel permeability, visceral hypersensitivity linked to gut-brain connections, and persistence of immune reactivity after intestinal microbial modification. Unfortunately, it is uncertain yet what specifically causes IBS (28). Probiotics are known to have a number of positive effects on the GIT even though their precise biological functions in humans are still only partially understood. It can restore the digestive tract motility and visceral sensitivity, as well as strengthen the intestinal mucosal barrier. Also, some *Lactobacilli* strains have shown some influence on intestinal pain by the modulation of μ -opioid and cannabinoid receptors expression in the epithelial cells (27). Probiotic microorganisms may also exert their effect by reducing the development of pathological bacteria, preventing their invasion of the host, and producing or secreting chemicals such as short-chain fatty acids (SCFAs) and neurotransmitters in addition to bacteriocin. Some probiotic strains can lower intestinal cytokine release and enhance the barrier function in intestinal inflammation, and probiotic administration has been linked to the reduction in IBS symptoms in patients and improved cytokine profile (29).

1.4.4. Cancer

Several lines of evidence showed that modifications in the gut microbiota have been associated with many prominent illnesses, including cancer. As a result, restoring the balance by employing probiotics for treatment and prevention has been deemed beneficial. The anti-cancer properties of probiotic microorganisms have gained scientific interest. The exact underlying mechanisms are complex and still under research. Probiotic anti-cancer actions include immune system control, modification in carcinogen metabolism, and inhibition of the growth of microbiota involved in the creation of mutagens and carcinogens. It has also been demonstrated that they are involved in the maintenance of cancer stem cells, metastasis, as well as cell cycle regulation (30). Other suggested mechanisms include restricting the development of microorganisms with enzymes that may transform pro-carcinogens into carcinogens, altering intestinal pH to affect bile solubility and bacterial activity, and modifying colonic transit time for more

effective elimination of fecal mutagens (10). Probiotics have demonstrated an anti-proliferative or pro-apoptotic effect in human cancer cells, with colon cells and stomach cells being the most researched. Many *Lactobacilli* strains exhibited antagonistic activity against *Helicobacter pylori*, which is a source of stomach cancer. It was also found that giving a probiotic beverage every day for six months helped in the clearance of human papillomavirus (HPV) and cervical cancer precursors in the women involved in the trials (31). In vitro, studies demonstrated the cell-type-specific modulatory effects on the signaling pathways linked to cancer. Experiments with fermented milk showed the suppression of the growth of MCF-7 breast cancer but not the healthy mammary epithelial cells, suggesting that the bioactive components cause reactions that are only recognized in tumor cells (30). Orally taken lactic acid bacteria have been proven to lessen DNA damage brought on by chemical carcinogens in rat stomach and colonic mucosa. Healthy humans who consumed *Lactobacilli* have been shown to lessen the mutagenicity of their urine and feces when exposed to carcinogens in cooked meat(10). The findings of multiple clinical trials showed the usefulness of probiotics in preventing, treating, and lowering the advancement of various forms of cancer including breast, liver, colorectal, bladder, and colon cancer in patients (32).

Table 1.2 lists some of the health advantages of probiotics and their alleged processes, though still under investigation, most of these advantages are attributed to several species of microorganisms (33).

Table 1.2. Probiotic health benefits and their speculated mechanisms (33)

Health benefit	Proposed mechanism
Modulation of the immune system	Improvements in nonspecific and antigen-specific immunity toward infections and cancers, adjuvant effects in antigen-specific immune responses, regulation of Th1/Th2 cells, creation of anti-inflammatory cytokines, and reduction in toxin N-metabolites release.
Allergy	Prevention of the translocation of antigen into the circulation, prevents the excessive immunologic responses to the elevated quantities of gut antigen stimulation.
Blood lipids/heart disease	Bacterial cell assimilating cholesterol, a change in BSH enzyme activity, and antioxidant effect.
Urogenital infections	Adherence to cells in the urinary and vaginal tract, competitive exclusion, and generation of inhibitors (H ₂ O ₂ , bio-surfactants).
Infection caused by <i>Helicobacter pylori</i>	Competitive colonization, reduction in the amount of <i>H. pylori</i> in the stomach, and prevention of growth and attachment to mucosal cells.
Resistance to enteric pathogens	Antagonism activity, the adjuvant impact that boosts antibody formation, systemic immunological effect, colonization resistance, resisting access of enteric pathogens (bacteriocin/defensing, pH, antimicrobial peptides, lactic acid generation, and toxic oxygen metabolites).
Antihypertensive effect	Bacterial peptide's action on milk protein produces antihypertensive tripeptides, and cell wall components function as ACE inhibitors.
NEC (necrotic inflammation of the distal small intestine)	reduction in TLRs and the signaling molecules and rise in the negative regulations, decrease in the IL-8 response.
Small bowel bacterial overgrowth	Lactobacilli influence the activity of overgrown flora, decreased toxic metabolite synthesis, restore the antibacterial characteristics of the community of small bowel microbes to normal.

1.5. Lactic acid bacteria

Food preservation has long been achieved using lactic acid bacteria (LABs). The significance of LABs in wine spoilage was first identified by Louis Pasteur when he was studying lactic acid fermentation in 1857. Following ten years of this

discovery, Louis Pasteur's student Joseph Lister isolated pure lactic acid bacteria from milk. "*Bacterium lactis*" was then given as the name of the bacteria (34). Lactic acid bacteria or *Lactobacillales* are an order of gram-positive organisms. They can generally be defined as non-spore-forming, rod- or cocci-shaped, and often non-motile bacteria. The rods are irregular, can be found alone, in pairs, or in irregular short chains, and sometimes forming clusters. Because lactic acid is the prominent byproduct of various carbohydrate sources used by these bacteria during the fermentation process, they are known as lactic acid bacteria (34–36). They lack catalase and oxidase enzymes, cytochromes, and the ability to produce porphyrins. Most LABs are aero-tolerant anaerobes. Although they can develop in the presence of oxygen, they do not need it for growth (34). They are acid tolerant and have a low GC content. Under settings of fermentation, when organic acids like lactic acid are formed, lactic acid bacteria may live and compete with other bacteria because they can resist acidity (36). Lactic acid bacteria are a collection of microorganisms that share similar physiological and metabolic traits (37). The 16S rRNA gene can be used for DNA-based molecular identification to distinguish between closely related bacterial species of LAB. Randomly amplified polymorphic DNA (RAPD) and amplified ribosomal DNA restriction analysis (ARDRA) were utilized successfully to distinguish LAB (38).

There are more than 110 species of *Lactobacillus* found among LAB members and they can be categorized into three main categories, obligate homo or fermentative lactobacilli, which turn hexoses into lactic acid; facultative hetero-fermentative lactobacilli, which turn hexoses into lactic acid solely or a combination of lactic acid with acetic acid, ethanol, and formic acid under glucose limitation; and obligate hetero-fermentative lactobacilli, which converts hexose to lactic acid, acetic acid, ethanol, and CO₂ and ferment pentose (36). LAB is a part of the phylum *Firmicutes*, the class *Bacilli*, and the order *Lactobacillales*. LAB contains 17 genera which include *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Lactosphaera*, *Enterococcus*, *Leuconostoc*, *Aerococcus*, *Alloiococcus*, *Dolosigranulum*, *Globicatella*, *Carnobacterium*, *Mlissococcus*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Weissella*, and *Vagococcus*. LAB are "Generally Recognized as Safe" (GRAS) with the exception of a small number of species like *Streptococcus pneumoniae* and *Streptococcus pyogenes* (34,39).

LABs are prevalent in a wide range of settings that are rich in nutrients, particularly when the carbohydrates and protein levels are high. They are native to environments that are associated with food such as plants (vegetables, fruits, and cereals), wine, meat, and milk settings. They are naturally connected to mucosal surfaces like the GI system. Additionally, they are essential in the creation of cocoa, wine, silage, and sourdough (34,36).

Many LABs operate as probiotics, and one of the numerous ways that LAB improves the host's health is by producing bacteriocin. In the gut, these substances prevent the growth of harmful Gram-positive bacteria like *Staphylococcus aureus* and *Listeria monocytogenes*. In the presence of other stress conditions like low pH or osmotic shock, the bacteriocin activity has also been shown against Gram-negative bacteria including *Salmonella* and *E. coli* (40). Other antimicrobial substances created by LAB include reuterin, hydrogen peroxide, carbon dioxide, and acetaldehyde. These microorganisms prevent the development of pathogenic and degrading microorganisms keeping the food nutrient-rich and extending its shelf life (36).

1.5.1. *Lactobacillus*

Among the genera found in LAB, *Lactobacillus* is the biggest by far. It includes species with a wide range of biochemical, morphological, and physiological characteristics, making it quite diverse. The variation in the G+C percentage of the DNA of the species included in the genus reflects their heterogeneity. Their range of 32-53% is twice as wide as what is often considered acceptable in a genus (41). There are numerous varieties of Lactobacilli that can be found in nature, they are called Döderlein's bacillus and can be classified as a genus of facultatively anaerobic or microaerophilic Gram-positive rod-shaped bacteria with standard dimensions of 0.5-1.5x10nM, catalase-negative, and aciduric or acidophilic and quite stable in salt. They are strictly fermentative, fermenting a variety of carbohydrates. Their development depends on complicated dietary needs such as amino acids, vitamins, minerals, carbohydrates, fatty acid esters, and salts (41–43). Their DNA concentration of G+C is typically around 50%, certain lactic acid bacteria are grouped under the *Actinomycetes* branch because of their larger percentage of G+C contents, whilst other LABs including *Lactobacillus* are classified under the branch of *Clostridium*.

They can be found in a variety of environments such as the mucosal membrane of both humans and animals (mouth, gut, and vagina). Many of these species are prevalent in the decomposing plant matter, with the surroundings becoming acidic as a result of the creation of lactic acid preventing the growth of some other bacteria. Lactobacilli are significant industrial bacteria with a long history of usage in cheese production, yogurt, and other types of fermented food(41,43).

Orla Jensen's early description of LAB in 1919 was to pinpoint the bacteria that would be helpful in the dairy business, especially the Danish "dairy cheese". Orla-Jensen recognized ten species, and gradually the number rose to 44 species in the 9th edition of Bergey's manual. The emergence of new taxonomic techniques and the ongoing studies on the ecosystem enabled more accurate identification of strains and are one of the reasons why the number of species is still rising. The most recent categorization of Lactobacilli by Kandler and Weiss (1986) depends on biochemical-physiological parameters and disregards the traditional Orla-Jensen criteria like growth temperature and morphology. However, the description of novel species often does not include the investigation of the byproducts from the pentose fermentation. So, both definitions must be utilized while being aware of their limitations.

With the carbon source being glucose, Lactobacilli can be hetero-fermentative or homo-fermentative. While hetero-fermentative creates lactic acid, carbon dioxide, ethanol, or acetic acid. Homo-fermentative strains can produce more than 85% lactic acid. Acetate production may increase in the presence of oxygen or other oxidants at the expense of lactate or ethanol. Three metabolic groups have been created from a total of 56 species of Lactobacilli including group A of obligatory homo-fermentative Lactobacilli where the hexoses are fermented by the EMP pathway into lactic acid. Group B is facultative hetero-fermentative Lactobacilli where hexoses are fermented by the EMP pathway, with the organisms possessing both aldolase and phosphoketolase enabling fermentation of alsopentoses and gluconate. Group C is obligatory hetero-fermentative Lactobacilli with the phosphogluconate pathway fermenting the hexoses, and pentose may be fermented in this pathway (41).

1.5.2. *Lactobacillus gasseri*

Lactobacillus gasseri is one of the most common species of the *Lactobacillus* genus which is classified as GRAS (generally recognized as safe). It is an autochthonous human microorganism that settles in healthy people's gastrointestinal system, vagina, and oral cavity and displays niche-related traits involved in colonizing the human mucosa which can contribute to or enhance probiotic activity. It is homo-fermentative, acid-resistant, bile tolerant, and considered group B acidophilus complex bacteria and can be distinguished from group A by the lack of significant surface layer (S-layer) proteins (44–47). *L. gasseri* has a long history of being safely consumed by humans and has health benefits well supported by studies, leading to the adoption of well-characterized strains of probiotics. *L. gasseri* has a significant level of intra-species variation, unlike the closely related and well-conserved *L. acidophilus* species (46).

Due to the great degree of physical similarity, *L. gasseri* was typically categorized as *Lactobacillus acidophilus* and could not be separated by conventional taxonomic features, like carbohydrate consumption and the lactic acid isomer generated. *L. gasseri*'s categorization as *L. acidophilus* did not change till 1980. The *L. acidophilus* group was first identified as "*Bacillus acidophilus*" by Moro in 1900 after isolating it from newborn feces. Later, the term *Bacillus acidophilus* was added to the list of *Lactobacillus* species. In 1980, then Francis Gasser's work on hybridization patterns of DNA/DNA allowed the *L. gasseri* strain to be separated from *L. acidophilus*. In 2000, Kullen et al. used a quick and accurate DNA sequence-based identification method to identify *L. gasseri* and other *L. acidophilus* complex bacteria via amplifying the 16S rRNA gene (47,48).

L. gasseri possesses a number of probiotic qualities. Clinical studies revealed the ability of *L. gasseri* to maintain gut and vaginal homeostasis, alleviate allergic diseases, have anti-inflammatory properties, activate macrophages, reduce *Helicobacter pylori* infection, and inhibit some virus infections by various mechanisms, including the production of lactic acid, bacteriocin, and hydrogen peroxide. Oxaloacetate may be broken down by *L. gasseri* in the digestive system, which also helps in keeping serum and renal homeostasis (45,49,50).

Lactobacillus gasseri ATCC 33323 was the first strain to have its whole genome sequenced completely, and it has been demonstrated to play an important role in the defense and protection of the human gastrointestinal system, furthermore demonstrating the absence of transmissible antibiotic resistance genes or undesirable genes. Nevertheless, it can't be presumed that all the strains of *L. gasseri* are free of transportable antibiotic-resistance genes therefore each potential probiotic strain must have its genome sequenced (44,50). *L. gasseri* ATCC 33323 genome was sequenced by the collaboration of the Department of Energy-Joint Genome Institute and the Lactic Acid Bacteria Genomics Consortium (LABGC). The project of LABGC which was concluded by Makarova et al., compared the *L. gasseri* sequence to other bacteria including several *Lactobacillus* species like *Lactobacillus brevis* and *Streptococcus thermophilus*. Results revealed by comparative in-silico analysis at the species level in the *Lactobacillus* group showed lots of parallels between *L. johnsonii* NCC533 and *L. gasseri* ATCC 33323 at the DNA and protein levels. The human-derived strain *Lactobacillus gasseri* ATCC 33323 is a typical resident of both children and human microbiota. The bacteria are homo-fermentative, rod-shaped with rounded ends that range in size from 0.6 to 0.8 by 3 to 5 μm with an optimal growth range of 35 to 38°C. *L. gasseri* has been identified as one of the *Lactobacillus* species endemic to the human GI system of newborns and adults, depending on dietary preferences and geographic location (48).

Table 1.3. The scientific classification of the *L. gasseri* ATCC33323 strain retrieved from https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=962654#null

Classification of <i>Lactobacillus gasseri</i> ATCC33323	
Kingdom	Bacteria
Subkingdom	Posibacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	<i>Lactobacillus</i>
Species	<i>Lactobacillus gasseri</i>
subspecies	ATCC 33323

1.6. Bacteriocin

Living organisms produce a range of antimicrobial compounds as a part of a defensive system or an immune response, improving their capacity to compete for space and resources. Living organisms show their microbial antagonism by producing antimicrobial substances like fatty acids, ethanol, organic acid, antibiotics, lytic enzymes, and bacteriocin (51). Given their potential as a natural food preservative and as therapeutic agents, focus on bacteriocin research, particularly from LAB, has increased significantly in recent years (52).

Klaenhammer's definition of bacteriocin is currently accepted by the scientific literature and research community, which states that "bacteriocins are a heterogeneous group of ribosomally synthesized, extracellularly released bioactive peptides or proteins displaying antimicrobial activity against other bacteria" (53).

Historically, the first information to be published regarding to bacteriocin was in 1925, when scientists discovered that a biologically active chemical generated by *E. coli* V demonstrated an antagonistic action against another strain of the same species (*E. coli* F). Then the first indication of antibiosis in lactic acid bacteria was made by Rogers in 1928, after that significant attempts were

undertaken to describe the antibiotic compound. In 1944, Oxford isolated an inhibitory chemical which they termed diplococcin from *Streptococcus cremoris* culture. After that, the inhibitory substance generated by *S. lactis* was concentrated by Mattric and Hirsh (1944). This chemical which inhibited several organisms was identified as nisin, which was produced from group N Streptococci. In 1976, these chemical compounds were classified as bacteriocins by Tagg et al. and described as proteinaceous substances with inhibitory activity against species closely related to the producer of the bacteriocin and can be produced by both Gram-negative and Gram-positive bacteria (53,54).

Bacteriocins are substances produced by bacteria including LAB, with a bactericidal or bacteriostatic effect and a physiologically active moiety. They are ribosomally synthesized, protease-sensitive, heat-stable peptides that could or could not undergo post-translational modifications. The vast majority of bacteriocins are tiny, cationic compounds that have a restricted to broad inhibitory effect against related or unrelated species. They vary in length, biochemical characteristics, genetic origin, molecular weight, cellular response, and immune system interactions. Bacteriocins exhibit different mechanisms of action, including cytoplasmic membrane permeability induction by pore creation, inhibition of cell wall production, and disruption of metabolic processes pathways (36,51). To avoid the bacteriocin damage to the bacteria that produce them, a lipoprotein is produced by LAB which binds to the bacteriocin outside the cells. As a result, the cells undergo a reversible self-clustering to lessen their exposure to bacteriocin. LAB can also protect itself by producing an inactive precursor of bacteriocin to avoid the self-toxicity caused by them (40). Bacteriocins are particularly appealing for a variety of applications because of several advantageous characteristics. LAB bacteriocins are known for their activity over a wide range of pH values without losing their antibacterial agency and they are resilient to high-temperature stress without degrading. Bacteriocins' potential usage is enhanced by the fact that they are tasteless, odorless, and colorless, especially in the food industry. LAB-produced bacteriocin is considered safe for consumption since it's harmless to eukaryotic cells (40,52). Studies focusing on bacteriocins from *Lactobacilli* are due to two basic factors, the starting cultures that produce bacteriocins may lead to a more dependable fermentation process by

limiting the spoilage bacteria. The other factor is the potential of the genetic determinants of bacteriocin production and bacteriocin resistance as genetic markers in rDNA technology for the usage in the development of food additives or bacterial supplements. There are two primary categories for antimicrobial compounds made by lactic acid bacteria, which are low molecular mass chemical compounds having a molecular mass of less than 1000 Da and high molecular mass chemical compounds that possess a molecular mass of more than 1000 Da including bacteriocins (36).

Despite the widespread and long history of bacteriocin usage, no reports of the emergence of resistant bacteria have been made. One explanation might be that bacteriocins work quickly and create holes in the targeted bacteria membrane, even with a very small dose. Also, they are readily broken down by proteolytic enzymes since they are proteinaceous. As a result, they are short-lived both in the environment and in the human body, which reduces the chances of interaction with the target bacteria. Bacteriocins offer several advantages over traditional antibiotics, but being a primary metabolite is the most important characteristic, which increases their capacity to be genetically modified through bioengineering to boost their activity and selectivity against particular bacteria (51,52).

1.6.1. Classification of bacteriocin

LAB bacteriocins have been categorized into four groups based on amino acid composition, post-translational modifications, and genetic traits. Class I bacteriocin is called lantibiotics. They are membrane-active peptides that undergo post-translational modifications; they are small peptides with less than 5 kDa and contain unique amino acids such as dehydroalanine, dehydrobutyrine, lanthionine, and methyllanthionine. These amino acids combine to produce a ring-rigid shape giving lantibiotics their distinctive structural characteristics and allowing lantibiotics to be divided into two subgroups, class Ia, which includes nisin has a spiral structure and a positive charge and contains residues of 21 to 38 amino acids. Additionally, they have a hydrophobic group and tight stiffness. Class Ib is spherical, neutrally, or negatively charged. Typically, has less than 19 amino acids (55,56). Class II bacteriocins contain the majority of naturally occurring bacteriocins and are often known as non-lantibiotics. They have a low molecular weight of less than 10 kDa, are positively charged, heat stable, and formed from

non-modified amino acids. Their amino acids are usually inactive and get activated by post-translational cleavage of the N-terminal (36,52,55). Class II contains four subgroups, class IIa is a tiny S-S bridge peptide that contains a Listeria resistance activity with the sequence YGNGVXC at the N- terminus. Class IIb is two peptide oligomers. Class IIc is cyclic and is covalently joined at the N- and C-termini (52,55,57). Class IId is non-pediococin that has not undergone modification after translation (55). Class III of bacteriocins is the macromolecular thermo-labile peptides with a molecular weight higher than 30 kDa. These bacteriocins can be inactive after 30 minutes of exposure to high heat (100°C). *Lactobacillus* is the main producer of this class, Helveticins J and V and lactacin B are examples of this class (55,56). Hydrophobic, heat-stable bacteriocins make up class IV. They are complex bacteriocins with lipid or carbohydrate moieties (34,36,55,56). The prior categorization of Klaenhammer which classifies bacteriocin into four classes was suggested to undergo a revision by Cotter et al. (2005), dividing bacteriocin into two main groups which are lantibiotics and non-lanthionine bacteriocins. The third group added was classified as bacteriolysins since the bacteriocins are lytic enzymes in this group (34,52).

1.6.2. Mode of action of bacteriocins

Bacteriocins can operate in a bactericidal effect which can include or exclude cell lysis or bacteriostatic effect which inhibits the growth of the cell. The majority of bacteriocins act against sensitive bacteria in a bactericidal manner. Bacteriocins have several unique methods of action. The majority of LAB bacteriocin works to kill the bacteria by affecting the processes connected to the cell envelope. Peptidoglycan production is inhibited by a number of lantibiotics; nisin inhibits cell wall development by forming a complex with lipid II, an intermediate in the biosynthesis machinery found in the bacterial cell envelope. This process causes the integration of more peptides to create a hole in the bacteria membrane. Nisin and other bacteriocins employ lipid II as a docking molecule that alters the membrane potential leading to cell death. A number of ideas have been put forth to clarify the precise method with which antimicrobial peptides kill bacteria. The 'barrel-stave' method showed a group of peptides create transmembrane channels or pores with the pore size increasing constantly as more peptide monomers were added causing cell death from the leakage of intracellular

components through the pores. Other bacteriocins destroy their target cell by inhibiting protein synthesis (36,58).

1.7. Selection criteria

There are specific conditions that must be met and a variety of qualities that should be considered when employing innovative strains as a potential probiotic candidate for them to have a beneficial impact. According to Ibnou-Zekri et al. (2003), the benefits of probiotics vary depending on the strain. Therefore, even within the same species, a positive benefit attributed to one strain may not always be predicted from another and no candidate should be able to meet all probiotic quality requirements. One of the crucial things to take into consideration is the original source of the strain since microorganisms from the gut microbiota have a greater chance of survival in their natural habitats and weather difficult digestive circumstances. One of the main selection factors for probiotics is the capacity of bacteria to colonize the GIT. Here are some requirements that a probiotic candidate should be able to meet (13,59).

1.7.1. Acid and bile resistance

In the GIT, probiotic bacteria must be immune to the enzymes found in the oral cavity. The voyage will then continue in the stomach. At this point, strains ought to be able to survive passage in order to reach the intestinal tract. The production of stomach acid acts as the body's main defense system against the majority of ingested bacteria. These abrasive conditions of stomach (pH 1.5-3.0) and upper intestine require the strains to be durable. The capability of probiotic organisms to endure a pH value as low as 1.5 and to endure high concentrations of bile in the gut after being produced in the liver from cholesterol are the most crucial selection criterion. Probiotic organisms must enter the lower digestive system and survive there in order to demonstrate their sufficiency. Only a few strains can survive under such extreme conditions. As was pointed out by Lankaputhra and Shah (1995) in their research, it is not true to say that all probiotic bacteria can tolerate acid and bile (10,41,60).

1.7.2. Antimicrobial activity

One of the most crucial probiotic screening factors is antimicrobial activity. The main target for the antimicrobial action is the enteric undesirables and the gut

pathogens. LAB produce several substances which attributes to their antimicrobial activity which include organic acids, diacetyl, and hydrogen peroxides, in addition to low molecular weight antimicrobial compounds and bacteriocin. They also create digestive enzymes that enhance digestion and increase the stress tolerance of the host. In addition to the antibacterial effect, probiotics have antiviral and antifungal effects. The most extensively studied products however are likely bacteriocins and proteinaceous compounds with inhibitory action against related species. There are several of them but till now only nisin, which is made by *Lactobacillus lactis* subsp. *Lactis* has been authorized for use in goods and products meant for human consumption (10,60,61).

1.7.3. Bile salt hydrolase activity (BSH)

Potential probiotic microorganisms, especially LAB, may generate a bile salt hydrolase, which can hydrolyze conjugated bile salts, enhancing cholesterol absorption by the formation of micelles from cholesterol and conjugated bile salt. This trait has developed as a defense mechanism against the intestinal-conjugated bile acids' antibacterial effect. Probiotic microorganisms have a few mechanisms for cholesterol removal which include cholesterol assimilation into the cell membrane of bacteria, short-chain fatty acids production, and de-conjugation of bile salts by the BSH enzyme. In the colon, bacteria usually employ primary bile acids as substrates to bio-transform them into secondary bile acids, in addition, it catalyzes the de-conjugation of bile salts coupled with glycine and taurine by the BSH enzyme. In persons with hypercholesterolemia, the unconjugated bile acids are ultimately eliminated in the feces decreasing the blood cholesterol (2,62).

1.7.4. Cell surface properties

A key factor in the selection process for probiotic strains is microbial adherence to biological surfaces. This phenomenon controls the probiotics' survival and colonization within the lumen of the gut, pathogens exclusion, and interaction of bacteria with the host cells for their therapeutic effect. The fragile non-specific contact between biological surfaces and the specific interactions between the legend-receptor serve as the main mediators for the microbial interactions. For this, the cell surface characteristics of a potential probiotic strain are taken into consideration as a key contributor to the evaluation of the beneficial

effects on the host. Adhesion and aggregation interactions between surface macromolecules have been regarded as key indicators for assessing the colonization capacity of probiotics and are measured using the metrics auto-aggregation, hydrophobicity, and co-aggregation (63).

1.7.4.1. Auto-aggregation

Cellular aggregation refers to the ability of comparable cell types to gather. Numerous bacteria can self-bind to one another in an unspecific way which is called auto-agglutination or auto-aggregation and it is the initial stage in the development of biofilm, along with surface colonization. Cell surface proteins and a small number of soluble proteins are thought to mediate the auto-aggregation process of probiotics (2,63,64).

1.7.4.2. Co-aggregation

The intercellular adhesion between different strains from various species is known as co-aggregation, and it is associated with the ability to prevent enteric pathogens from adhering to host cell surfaces. The direct aggregation of probiotics with the pathogenic bacteria forms a protective barrier that inhibits the adherence of pathogens to the mucosa (2,65).

1.7.5. Antibiotic resistance

A crucial factor in the evaluation of potential probiotics is the study of antibiotic resistance of prospective probiotic microorganisms and their antimicrobial susceptibility profile. It is desirable that putative probiotic bacteria exhibit sensitivity to antibiotic components. Probiotic microbial strains shouldn't act as a repository for antibiotic resistance genes that could subsequently spread later to gut pathogens. Probiotic bacteria have the ability to carry mobile and intrinsic genetic components that enable resistance to different drugs. Probiotics do not have a safety risk when the resistance to antibiotics is intrinsic or acquired from mutations involving chromosomes. The safety concern for probiotics has been focused on the acquired genes that can be transferred by transposases, conjugative plasmids, and prophage/bacteriophage elements (2,66,67).

2. THESIS AIM

The use of probiotics and their antimicrobial metabolites as a substitute antimicrobial strategy for infections' treatment and prevention has gained increasing interest in light of present concerns regarding the widespread and reckless application of antibiotics for disease treatment and livestock growth promotion as well as the emergence of antibiotic-resistant pathogens. Antibacterial metabolites and specifically bacteriocin of lactic acid bacteria have inhibitory or bactericidal effects on different bacteria as well as possessing the potential for usage as a natural preservative to control spoilage and pathogenic bacteria in food. So, desirable qualities of a possible probiotic strain in the food and pharmaceutical sectors include the antibacterial action against infections and bacteriocin production as an efficient substitute for various conventional antibiotics. Given their importance on human health and their therapeutic effect within the gut, novel probiotics and prebiotics are constantly in demand for the modulation of gut microbiota to improve the welfare of the host.

This study seeks to add value to the scientific efforts to progress the benefits of *Lactobacillus* bacteria to present a probiotic with excellent probiotic characteristics. The aim of this work is to conduct an in-vitro characterization and screening of *L. gasseri* ATCC33323 in respect to probiotic selection criteria.

The objectives of this thesis include an in-vitro investigation of *L. gasseri* ATCC33323 for probiotic characteristics using variable tests like pH and bile tolerance, cell surface properties (auto-aggregation, co-aggregation), antimicrobial activity, antibiotic susceptibility, acid production test, and phenol tolerance. Screening for bacteriocin-coding genes is performed using multiple primers by polymerase chain reaction (PCR) assay, cloning of the PCR results using a cloning vector, and applying 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 work were obtained from the stock culture of the BAIBU/Biology Department. The bacterial and plasmid stock cultures were stored at -80°C with 30% glycerol. The bacterial strains, plasmid DNA, and their growth conditions were listed in Table 3.1. The *Lactobacillus gasseri* ATCC33323 (American-type culture collection 33323) strain used in this work is a commercial strain.

Table 3.1. The bacterial strains and plasmid

Bacteria		Strains	Growth media	Growth conditions	
				Temp	Time
<i>Lactobacillus gasseri</i>		ATCC33323	MRS	37°C	24-48h
<i>Staphylococcus aureus</i>		Food-borne	NA/PCA	37°C	24h
<i>Escherichia coli</i>		Food-borne	NA/PCA	37°C	24h
<i>Salmonella typhimurium</i>		ATCC13311	NA/PCA	37°C	24h
<i>Listeria monocytogenes</i>		RSKK	NA/PCA	37°C	24h
<i>Yersinia enterocolitica</i>		ATCC23715	NA/PCA	30°C	24h
Bacteria	Genotype	Strain	Growth media	Temp	Time
<i>E. coli</i>	rec A end A 1 gyr A986 thi 1hsdr17supE44 rel A1 lac	XL1-Blue ¹	LB	37°C	16-18h
Plasmid	Genotype/Phenotype	Strain	Growth media	Temp	Time
pBluescript ²	SKII+/Amp ^r	XL1	LB	37°C	16-18h

1. XL1-Blue was obtained from Stratagene, USA.
2. pBluescript was obtained from Thermo Scientific (Fermantas, Europe).

3.1.2 Chemicals and Enzymes

The chemicals and enzymes used in this study and their sources are listed in Appendix A,

3.1.3 Growth media and buffers

The growth media used in this study included de Man, Rogosa, and Sharpe (MRS), Nutrient Agar (NA), Plate Count Agar (PCA), and Luria Bertani (LB) media, their preparation is listed in Appendix B. Also, the buffers, the solution, and their preparation are listed in Appendix C.

3.2 Method

3.2.1 Characterization of *L. gasseri* ATCC33323

3.2.1.1 Acid tolerance

The most crucial factors for probiotic bacteria to perform beneficial actions are their survival and viability. When probiotics move through the GIT they are exposed to a series of stressors that affect their viability. Therefore, probiotics are selected according to the resistance to different pH conditions. The acid tolerance of *Lactobacillus gasseri* ATCC33323 was assessed based on the method described by Li et al., and El-Sayed et al., with modifications (65,68). To assess the acid tolerance with different pH points, the MRS broth was adjusted to pH values of pH 2, pH 3, pH 4, pH 5, and pH 6 using 1M HCl and 1M NaOH with one tube containing normal MRS broth as control. *L. gasseri* ATCC33323 was grown anaerobically on an MRS plate for 24h, at 37°C. Then a colony from the streaked plate was inoculated in 5ml MRS broth for 16-18h, at 37°C in anaerobic conditions. 100µl of the overnight grown culture (2%) was added to 5 ml of MRS broth with different pH values and incubated at 37°C for 6 hours. The measurements were made at hours 2, 4, and 6 using a spectrophotometer at OD₆₀₀. The bacteria were re-suspended in normal MRS broth (2%) at the 6h mark for 2 hours to observe the growth rate. The obtained results were calculated as Log₁₀ (cfu/ml).

3.2.1.2 Bile tolerance

To assess the ability of LAB as efficient probiotics, it is typically considered necessary to test their ability to tolerate the effects of bile. Since the concentration of bile in the intestine is estimated to be 0.3% (w/v), and the time for passage through the intestine is thought to be 4 h, this test was conducted with bile concentrations of 0.1% and 0.3%, with an incubation period of 5h (10, 60). The bile tolerance test for *L. gasseri* ATCC33323 was conducted according to the method used by Li et al., with modifications (65). The bacteria were grown in 4ml MRS broth at 37°C for 16-18h, in anaerobic conditions. The MRS broth was supplemented with 0.1% and 0.3% of Bile bovine powder (Oxgall, Merck) and 100µl of the overnight culture (2%) was inoculated into 5ml of MRS containing the bile salts, and normal MRS as a control and incubated for 5h at 37°C. The measurements were made each hour using a spectrophotometer AT OD₆₀₀. Data were calculated as Log₁₀ (cfu/ml), growth rate of the bacteria was calculated as follows:

$$\% \text{ of growth} = (\text{Growth in bile salt medium} / \text{Growth in control medium}) \times 100$$

3.2.1.3 Antimicrobial activity against pathogens

The antimicrobial activity of *Lactobacillus gasseri* ATCC33323 was assessed using the Kirby-baur method in a nutrient agar medium (69,70). The bacteria were grown anaerobically for 24 hours in 4ml MRS broth at 37°C, the pellet was then collected by centrifugation at 6000 rpm for 5 minutes, and then the cell-free supernatant (CFS) was filtered with a 0.22µm Millex syringe filter. The pellet was lysed with 200µl of lysis buffer (Lysozyme (30mg/ml), Tris-HCl (1mM), EDTA (50mM), Triton 100X 1.2%) and incubated at 37°C for 45 minutes, then the mixture was centrifuged for 5min at 13000 rpm. The supernatant and the pellet were both used to test the antimicrobial activity. The antipathogenic activity of *L. gasseri* ATCC33323 was tested against five pathogenic bacteria, which are *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* ATCC13311, *Listeria monocytogenes* RSKK, and *Yersinia enterocolitica* ATCC23715. The pathogenic bacteria were grown on a nutrient agar (NA) plate and incubated for 24h at 37°C for four of the pathogenic bacteria, and at 30°C for *Yersinia enterocolitica*. Then the pathogenic bacteria were inoculated into 5ml nutrient broth (Neogen, UK) and spread on nutrient agar plates using a cotton swap, after

adjusting the concentration to 0.5-0.7 OD₅₇₀, blank disks (Oxoid, UK) were impregnated with the CFS and the lysed pellet of *L. gasseri* ATCC33323, then they were placed on the plate with each pathogen. The plates were then incubated for 24h, four of the plates were incubated at 37°C, except *Yersinia enterocolitica*, which was incubated at 30°C. The inhibition zones were measured using a millimetric ruler.

3.2.1.4 Phenol tolerance

Testing for phenol tolerance can provide further information on the survival ability of *Lactobacillus* bacteria in the GIT since the phenol can possess a bacteriostatic impact on some strains of *Lactobacillus*. The phenol tolerance capacity of *L. gasseri* ATCC33323 was examined according to the method described by Reuben and colleagues (71). 5ml of MRS broths with four different concentrations of phenol (0.1%, 0.2%, 0.3%, and 0.4%) were inoculated with 100µl of an overnight grown *L. gasseri* ATCC33323 culture and incubated for 24h at 37°C. The measurements were taken at times 0h and 24h using a spectrophotometer at OD₆₀₀. The obtained measurements were calculated as Log₁₀ (cfu/ml).

3.2.1.5 Acidifying activity

The acidifying activity was tested using pH measurement (72). *L. gasseri* ATCC33323 was grown in MRS broth in anaerobic conditions for 24h at 37°C, then the bacterial culture was inoculated in skimmed milk at a level of 50 µl/5ml. The acidity was determined using a pH meter after 8, 10, 24, and 48 hours of incubation at 37°C.

3.2.1.6 Cell surface properties

3.2.1.6.1 Cell auto-aggregation

Auto-aggregation of *L. gasseri* ATCC33323 was tested using the method described by Collado and colleagues with some modifications (65,73). *L. gasseri* ATCC33323 was grown anaerobically overnight at 37°C in MRS broth. Followed by centrifugation at 4000 rpm, 4°C, for 15 minutes and washed 2 times with phosphate-buffer saline (PBS) with a pH of 7.4 then suspended in PBS buffer and gently vortexed. 100µl of *L. gasseri* ATCC33323 culture was added to 3.9ml of PBS buffer and the tubes were incubated at 37°C, measurements were taken from

the upper layer of the solution using a spectrophotometer (OD₆₀₀) at the beginning of each hour of the incubation (1, 2, 3, 4, 5h). After every measurement, 100µl from the upper layer of the bacterial solution was transferred into a fresh 3.9ml of PBS buffer. The auto-aggregation was measured using the following formula:

$$\text{Auto - aggregation\%} = \left[1 - \frac{A_t}{A_0} \right] \times 100$$

A_t: represents the absorbance at times 1, 2, 3, 4, and 5 hours.

A₀ represents absorbance at time 0.

3.2.1.6.2 Cell co-aggregation

The co-aggregation was tested using the method described by Li et al., with differences (65). *L. gasseri* ATCC33323 bacterial broth culture was prepared as mentioned in section 3.2.1.5.1. The five pathogenic bacteria, *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* (ATCC13311), *Listeria monocytogenes* RSKK, and *Yersinia enterocolitica* (ATCC23715, were grown for 16-18h in a nutrient broth at 37°C, with the exception of *Yersinia enterocolitica*, that was grown at 30°C. 100µl of *L. gasseri* ATCC33323 and 100µl of pathogenic bacteria were added to 3.8ml PBS buffer, then the tubes were incubated at room temperature. The absorbance of the samples was measured using the upper layer of the solution at times 0, 2, 4, 6, and 24 hours using a spectrophotometer (OD₆₀₀), then after every measurement, 100µl from the upper layer of bacterial solution is transferred to a 3.9ml of fresh PBS buffer. The formula used for calculation is:

$$\text{Co - aggregation\%} = \left[\frac{(A_{pro} + A_{pat}) - A_{mix}}{(A_{pro} + A_{pat})} \right] \times 100$$

A_{pro} in this formula represents the absorbance of probiotic bacteria at time 0. A_{pat} represents the absorbance of pathogenic bacteria at time 0. A_{mix} represents the absorbance of the mixture for both *Lactobacillus* bacteria and pathogenic bacteria at times 0, 2, 4, 6, and 24 hours.

3.2.1.7 Antibiotic susceptibility

The antibiotic resistance of *Lactobacillus gasseri* ATCC33323 was tested using the disk diffusion method (65). The bacteria were grown anaerobically overnight in nutrient broth at 37°C, then the concentration was adjusted to 0.5-0.8 using a spectrophotometer at OD₅₇₀, and the bacteria were streaked on a nutrient agar plate using cotton swap. The disks of antibiotics (Oxoid, UK) were laid on

the plates, each of which including ampicillin (10µg), erythromycin (15µg), kanamycin (30µg), and neomycin (30µg). The plates were incubated for 24h at 37°C, and the inhibition zones' diameters were measured using a millimeter ruler and described according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) as susceptible (S), intermediate (I), and resistant (R).

3.2.1.8 BSH activity

The bile salt hydrolase (BSH) enzyme which is typically found in probiotic bacteria allows the decrease in cholesterol levels and helps in bacterial colonization by the catalysis of the hydrolysis of conjugated bile salts. In a previous study in BAIBU labs (74), the BSH enzyme of *L. gasseri* ATCC33323 was expressed, and its activity was tested using Ninhydrin assay. The activity and the specificity of the enzyme were tested with Ninhydrin assay by using six bile salts 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

The characterization tests results were represented as means with standard deviation (SD), and all the tests were performed in triplicates to determine the means±SD. Probiotic characterization tests' results were assessed using a one-way ANOVA, Turkey's HSD test to evaluate the statistical significance of data ($p < 0.05$).

3.2.2 Screening for bacteriocin coding gene

3.2.2.1 Bacterial growth and DNA isolation of *L. gasseri* ATCC33323

L. gasseri ATCC33323 pellet was obtained by inoculating one colony from a previously streaked MRS plate into 4ml MRS broth and incubation in anaerobic conditions at 37°C and after 24h, 2% from the first broth was inoculated into a 1.5ml MRS broth then incubated in the same conditions for 18h, the broth culture was centrifuged for 5min at 6000 rpm. The supernatant was then removed, and the pellet was then used for DNA isolation.

To isolate the genomic DNA, the *L. gasseri* ATCC33323 pellet was washed using MRS broth, and bacterial cells (2×10^9 cells/ml) were harvested using

centrifugation for 10min, at 5000 rpm. The isolation of the DNA was accomplished following the protocol of the GeneJET™ genomic DNA purification kit (Thermo Scientific).

3.2.2.2 DNA amplification by PCR

The screening for bacteriocin genes in *L. gasseri* ATCC33323 was conducted using three different primers supplied by Oligomer (Table 3.2) (75,76). The PCR reaction conditions were optimized for each of the primers as shown in Table 3.3 with a final volume of 50µl, containing dNTP mix (5mM), MgCl₂ (25mM), template DNA of *L. gasseri* ATCC33323, the forward and reverse for each of the primers (25µM), with the enzyme *i*-pfu™ DNA polymerase (INTRON biotechnology) used in this reaction which has a proofreading activity and its supplemented buffer (10X). The PCR results were then visualized using agarose gel electrophoresis (0.8%) for 1h at 75 volts, and then treated with Ethidium Bromide (EtBr) for visualization using an ultraviolet (UV) light transilluminator.

Table 3.2. Primers for bacteriocin-gene screening using PCR(75,76)

Bacteriocin name	Primers	Nucleotide sequence	primer length	Tm
Gassericin A	Gas A-F	5'-CACTCGTAATGGATTTCTTA-3'	F-20bp	F-53°C
	Gas A-R	5'-GCCTTTTTTGAATACTCTATTTC-3'	R-22bp	R-53°C
Gassericin T	Gas T-F	5'-TGGATTTAAATTGCCTGAAAC-3'	F-21bp	F-57°C
	Gas T-R	5'-CATTCCCCCACTTGTTTCC-3'	R-19bp	R-52°C
Gassericin A	Gas A2-F	5'-GAACAGGTGCACTAATCGGT-3'	F-20bp	F-57°C
	Gas A2-R	5'-CAGCTAAGTTAGAAGGGGCT-3'	R-20bp	R-57°C

Table 3.3. PCR reactants

Reactants	Volume
UpH ₂ O	32μl
LB. GASS F primer (25μM)	2μl
LB. GASS R primer (25μM)	2μl
dNTP mix (5mM)	2μl
MgCl ₂ (25mM)	4μl
Template DNA (5ng/μl)	2μl
Pfu DNA Polymerase buffer (10X)	5μl
Pfu DNA Polymerase enzyme (2.5U/μl)	1μl
Total volume	50μl

Table 3.4. PCR conditions optimized for each of the primers.

Primers	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Gas-A	95°C, 10 min	94°C, 1 min	48°C, 1 min	72°C, 3 min	72°C, 10 min
Gas-T	95°C, 5 min	95°C, 30 sec.	48°C, 40 sec.	72°C, 2.5 min	72°C, 5 min
Gas-A2	95°C, 5 min	95°C, 30 sec.	52°C, 40 sec.	72°C, 3 min	72°C, 5 min
Number of cycles				30×	

3.2.2.3 PCR product purification

The PCR products were obtained using electrophoresis by loading the product into 1% w/v agarose gel (Sigma, USA) for 1.20h at 75 volts using TAE buffer (1X). Then the products were purified individually following the GeneJET gel extraction kit (Thermo Scientific) protocol.

3.2.2.4 Plasmid isolation of pBluescript vector

pBluescript was chosen as a cloning vector since it contains an extensive polylinker with restriction enzyme sites and a *lacZ* gene which act as a reporter gene. A plate of LB with Ampicillin was streaked using pBluescript vector stock and incubated for 14-16h at 37°C. A liquid culture of LB+amp was started using one colony from the streaked plate and incubated for 16h, at 37°C, at 175 rpm.

After the overnight incubation, the pellet was collected using centrifugation at 4000 rpm, 4min, 25°C. Then the collected pellets were washed with 1ml LB and centrifuged at 6000 rpm, for 3min. The pBluescript plasmid was isolated from the pellets by following the protocol from GeneJET Plasmid Miniprep Kit (Thermo Scientific).

3.2.2.5 pBluescript digestion with *Sma*I restriction enzyme

*Sma*I fast digest restriction enzyme (Thermo Scientific) was chosen to digest the pBluescript vector to obtain a blunt end for the linear vector since the PCR product has a blunt end. The digestion reaction components have been shown in Table 3.5. The incubation of the reaction in the PCR machine was for 23min at 37°C. Then the obtained linear pBluescript vector was purified according to the GeneJET PCR gel extraction Kit (Thermo Scientific) protocol.

Table 3.5. Reaction components for the digestion of pBluescript vector using *Sma*I enzyme.

Reactants	Volume
UpH ₂ O	21µl
PBluescript DNA plasmid	30µl
Fast digest green buffer (10X)	6µl
<i>Sma</i> I enzyme (10U/µl)	3µl
Total volume	60µl

3.2.2.6 Insertion of *L. gasseri* ATCC33323 (*gasA*) gene into pBluescript vector

L. gasseri ATCC33323 gene was ligated into pBluescript vector DNA using T4 DNA ligase enzyme (Fermentas) and its accompanying buffer with an incubation period of 1h at 22°C (Table 3.6). Polyethylene glycol (PEG) (Thermo Scientific, EU) was added to improve the efficiency of the ligation reaction. The new construct was given the name pMMA. The exact amounts of the insert DNA and vector DNA were calculated according to the following formula:

$$\text{Insert mass (ng)} = \text{Ratio} \times \left[\frac{\text{Insert length (kb)}}{\text{Vector length (kb)}} \right] \times \text{vector mass (ng)}$$

Table 3.6. Ligation reaction conditions for *L. gasseri* ATCC33323 and pBluescript

Reactants	Volume
UpH ₂ O	9µl
pBluescript vector	1µl
<i>L. gasseri</i> ATCC33323 DNA	5µl
PEG (50%w/v)	2µl
T4 Ligase buffer (10X)	2µl
T4 DNA Ligase enzyme (5U/µl)	1µl
Total volume	20µl

3.2.2.7 Preparation of XL1-Blue

E. coli XL1-Blue competent cells were prepared according to the calcium chloride method as follows. XL1-Blue competent cells were streaked on an LB plate and then incubated at 37°C, overnight. In the next step, inoculation of a single colony in a 3ml liquid LB media was done and incubated at 37°C for 14-16h, at 175rpm. A second culture of 50ml LB medium was made using 500µl from the first culture (1%) and incubated at 37°C at 125rpm for 2-2.5h till the desired OD is obtained at 600nm wavelength between 0.04-0.06, using the sample at a ratio of 1 to 10. The sample was divided into two 20ml and centrifuged at 4000rpm, 4°C, 5min. The obtained pellets were re-suspended in 10ml CaCl₂ (100mM), then it was put on ice for 30min, and centrifuged at 4000rpm, 4°C, for 10min. The pellet was then dissolved in 1 ml of CaCl₂ (10% glycerol). The competent cells were divided into Eppendorf tubes, each containing 100µl. storage is at -80°C.

3.2.2.8 Transformation of pMMA into *E. coli* XL1-Blue

pMMA was transformed into XL1-Blue using the heat shock procedure. 10µl of pBluescript ligation product was transferred into an Eppendorf tube containing 100µl of competent cell and treated with heat shock, 45min incubation on ice, then 5min at 37°C, followed by incubation 5min on ice. After transferring it into a glass tube containing 1ml LB broth, it was incubated for 1h, 37°C, 175rpm. The cells were then collected by centrifugation at 6000rpm for 3min. The re-suspension of the pellet was made in 100µl of LB. The sample was then

streaked on an LB plate containing Ampicillin (25mg/ml), IPTG (Isopropyl β -D-1-thiogalactopyranoside), X-gal (5bromo 4 chloro 3indolyl β D galactopyranoside), using spread plate technique. The plate was incubated at 37°C for 14-16h. The next day, 4 white colonies were selected randomly from the plate and inoculated into a 5ml LB+Amp medium and incubated for 14-16h, 175rpm, at 37°C. After the pellet was collected plasmid DNA isolation was performed using GeneJET Plasmid Miniprep Kit (Thermo Scientific, EU). Then the recombinant DNA was digested using *EcoRI* restriction enzyme and then visualized using electrophoresis for confirming the presence of the inserted gene.

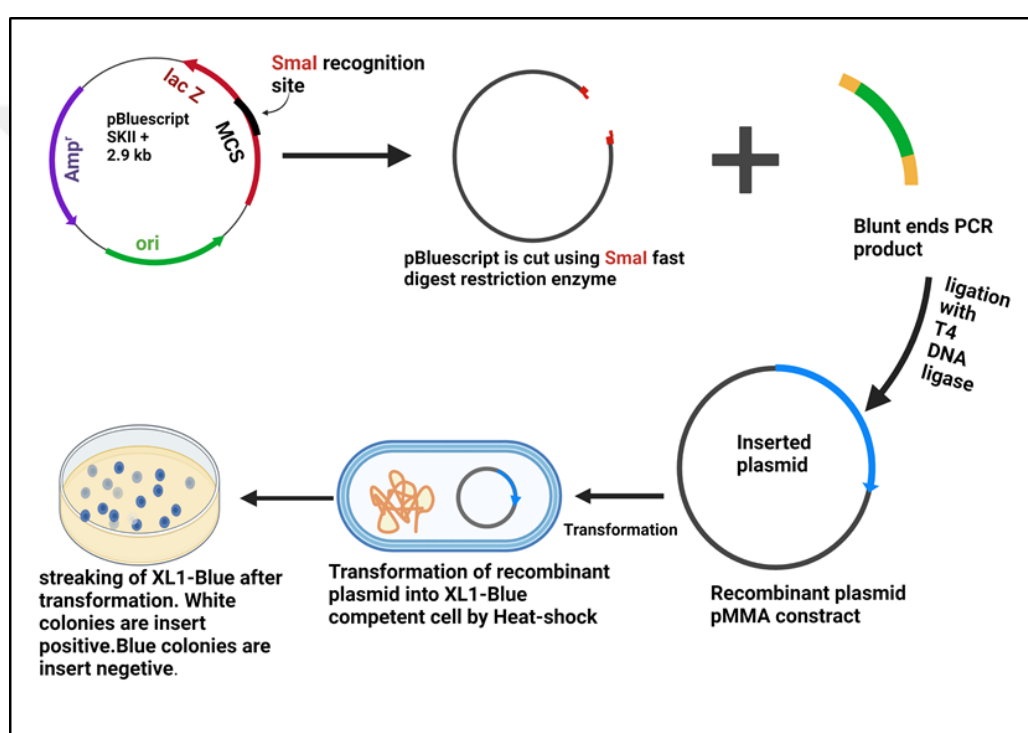


Figure 3.1. Preparation of pMMA construct by cloning process: cutting of pBluescript vector using SmaI restriction enzyme, insertion of PCR product into the plasmid, the transformation of pMMA construct into XL1-Blue competent cell.

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'-GTAAACGACGGCCAGT-3') and M13/pUC Reverse (5'-CAGGAAACAGCTATGAC-3').

3.2.2.10 In-silico analysis

The obtained nucleotide sequence from MacroGen Inc. was verified by the BioEdit program and translated into its amino acid sequence using the ExPASy translate tool (<https://web.expasy.org/translate/>). The amino acid sequence was then aligned against the ones present in the selected target database using BLAST (Basic Local Alignment Search Tool),

(https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_1932051478) in NCBI (National Center for Biotechnology Information), (<https://www.ncbi.nlm.nih.gov/>), and MEGA5 software (Molecular Evolutionary Genetic Analysis).



4. RESULTS

4.1 Characterization of *L. gasseri* ATCC33323

4.1.1 Acid tolerance

The acid resistance of *L. gasseri* ATCC33323 was tested at different pH points. As shown in Table 4.1, *L. gasseri* ATCC 33323 strain showed an increase in the log values at all different pH points with some fluctuations, pH 2 and pH 4 log values had a decrease of 0.14 and 0.13 respectively at the 6th hour mark, and pH 5 had a fluctuation at the 2nd hour. According to statistical analysis, in comparison to time 0h, *L. gasseri* ATCC 33323 showed significant growth ($p<0.05$) at the 6th hour of incubation for all pH values with the exception of pH 2 and pH 3. The bacteria also had significant values at the 2nd and 4th hours mark for pH 4 and 5 and pH 2 and 5 respectively. The viability of the bacteria was controlled by inoculation into a control MRS medium with a normal pH value for two hours and *L. gasseri* ATCC33323 showed growth in all tubes at the 8-hour mark. The results indicated resistance of *L. gasseri* ATCC33323 to different pH values.

Table 4.1. Acid tolerance results of *L. gasseri* ATCC 33323 to five different pH values

	Time (h)	0:00	2:00	4:00	6:00	8:00
Log ₁₀ (cfu/ml)	Control	8.321±0.260	8.397±0.494	8.574±0.428	8.943± 0.065*	–
	pH2	7.502±0.522	8.254±0.168	8.574±0.287*	8.436± 0.416	9.227
	pH3	7.988±0.357	8.306±0.389	8.128±0.531	8.184± 0.487	9.286
	pH4	7.495±0.255	8.151±0.195*	8.089±0.432	8.028± 0.198*	9.314
	pH5	7.763±0.365	9.020±0.110*	8.558±0.334*	8.668± 0.383*	9.199
	pH6	7.336±0.378	8.024±1.052	8.336±0.720	8.920± 0.118*	9.208

Data are expressed as mean± SD (n=3), with the exception of the data at time 8:00 having one repeat as a control. * $p<0.05$. The significance is expressed in comparison to time 0:00.

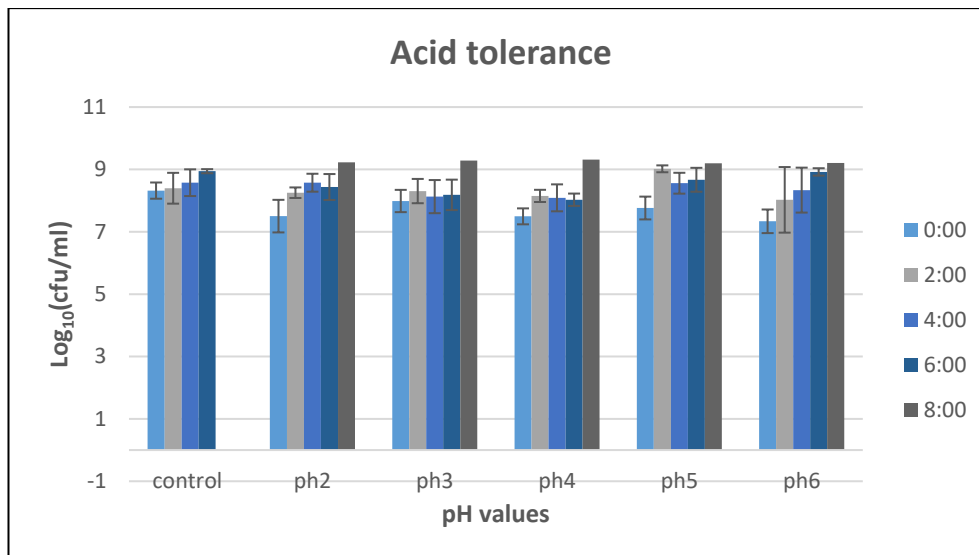


Figure 4.1. pH tolerance of *Lactobacillus gasseri* ATCC 33323.

4.1.2 Bile tolerance

The bile in the intestine is an essential element that influences LAB cell viability since bile is toxic for cells and it can disrupt the cell membrane structure (1). *L. gasseri* ATCC33323 tolerance to bile was tested against Oxgall with concentrations 0.1% and 0.3% as a replacement for human bile salts due to their resemblance to them. According to the obtained data shown in Table 4.2, *L. gasseri* ATCC33323 was able to grow in bile concentration 0.1% with an increase in the log value by 0.25 and a growth rate of 68.9%. The log value of the bacteria in 0.3% increased by 0.05 at the 4th hour of incubation but decreased by 0.08 at the 5th hour mark with a growth rate of 50.6%. According to the statistical analysis, the bacteria showed a significant increase ($p < 0.05$) at the 5th hour for 0.1%, and no significant value for 0.3%. These results suggest the ability of this bacteria to tolerate these bile concentrations at different times.

Table 4.2. Bile tolerance of *L. gasseri* ATCC33323 to concentrations 0.1% and 0.3%

Log ₁₀ (cfu/ml)	Bile %	Time (h)					
		0:00	1:00	2:00	3:00	4:00	5:00
	Control 0%	8.614±0.052	8.670±0.029*	8.832±0.083**	8.881±0.041**	8.997±0.016**	9.038±0.046**
	0.1%	8.655±0.101	8.646±0.054	8.653±0.102	8.773±0.024	8.820±0.148	8.909±0.112*
	0.3%	8.645±0.130	8.695±0.023	8.699±0.014	8.723±0.043	8.695±0.096	8.616±0.054

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

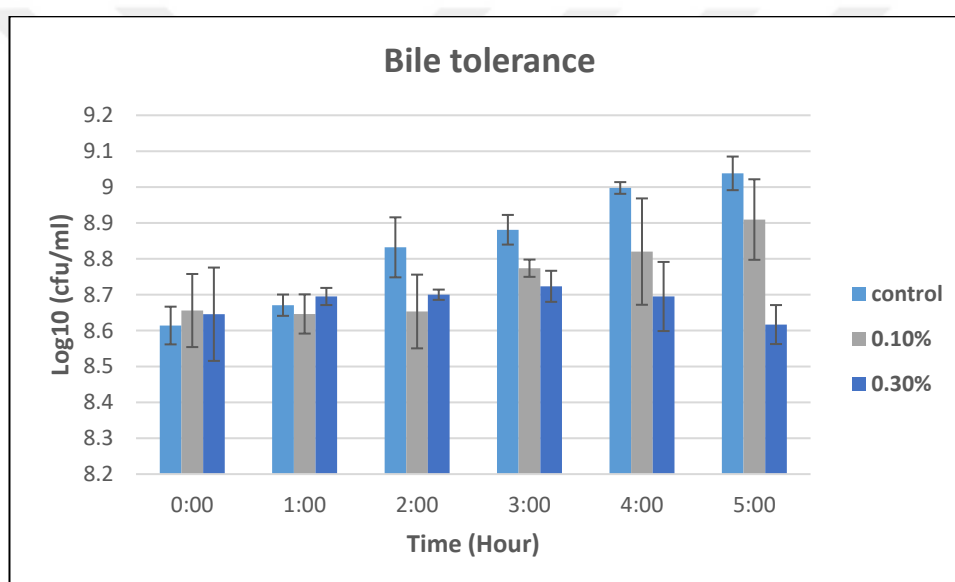


Figure 4.2. Bile tolerance of *L. gasseri* ATCC33323 to different bile concentrations.

4.1.3 Antimicrobial activity against pathogens

One of the main probiotic properties of LAB is their antagonistic effect against pathogenic bacteria. In this work, the antimicrobial activity of *Lactobacillus gasseri* ATCC33323 was tested against five pathogenic bacteria including *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* (ATCC13311), *Listeria monocytogenes* RSKK, and *Yersinia enterocolitica* (ATCC23715). Both cell-free supernatant (CFS) and cell lysate were used in the Kirby-baur method to test the activity of *Lactobacillus gasseri* on nutrient agar plates. Our results showed that *L. gasseri* ATCC33323 cell lysate exhibited an inhibitory zone for both *Staphylococcus aureus* and *E. coli* with 14 mm and 15 mm zones

respectively. The CFS of *L. gasseri* ATCC33323 had an antagonistic activity against *E. coli* and *Listeria monocytogenes* RSKK with an inhibitory zone of 8 mm and 15 mm respectively.

Table 4.3. Diameter of inhibition zones for the antimicrobial activity of *L. gasseri* ATCC33323

Pathogens	<i>L. gasseri</i> ATCC33323 cell lysate	<i>L. gasseri</i> ATCC33323 CFS
<i>Staphylococcus aureus</i>	14mm	--
<i>E. coli</i>	15mm	8mm
<i>Salmonella typhimurium</i> (ATCC13311)	--	--
<i>Listeria monocytogenes</i> RSKK	--	15mm
<i>Yersinia enterocolitica</i> (ATCC23715)	--	--

4.1.4 Phenol tolerance

It is anticipated for probiotics to be tolerant to phenol. The results of this experiment showed differences in the resistance of *L. gasseri* ATCC33323 at different phenol concentration points with declining values as the concentration of phenol increases as shown in Table 4.4. In comparison to the values at the 0h mark, the *L. gasseri* ATCC33323 values at the 24h mark showed significant growth at all phenol concentrations ($p < 0.01$), except 0.4%, where the bacterial growth showed no significant value. The strain survived at 0.4% phenol concentration at the 24 h mark with an increase in the log value by 0.51. The highest growth of bacteria was at 0.1% concentration with an increase in the log value by 1.57 at the 24 h mark. With bacterial growth at 0% phenol was used as a control group for this test.

Table 4.4. Tolerance of *L. gasseri* ATCC33323 to phenol

Phenol concentration	Time (hour)	0%	0.1%	0.2%	0.3%	0.4%
Log ₁₀ (cfu/ml)	0:00	7.970±0.138	7.873±0.652	7.873±0.069	7.917±0.139	8.005±0.451
	24:00	9.582±0.050**	9.452±0.054**	9.311±0.041**	9.054±0.072**	8.515±0.320

Data are expressed as mean± SD (n=3). **p<0.01. The significance is expressed in comparison to time 0:00

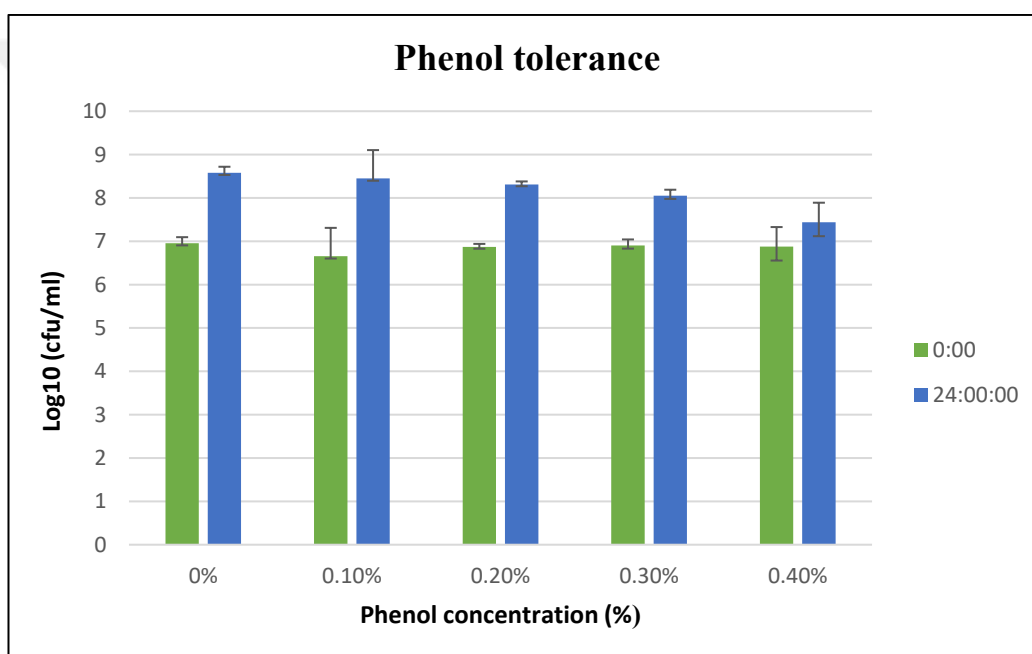


Figure 4.3. Tolerance of *L. gasseri* ATCC33323 to phenol concentrations 0% (control), 0.1%, 0.2%, 0.3%, and 0.4%.

4.1.5 Acidifying activity

The acidifying capability of *L. gasseri* ATCC33323 was determined using a pH meter. The pH measurements of skimmed milk inoculated with bacteria showed that *L. gasseri* ATCC33323 made a significant value change (p<0.05) in the acidity degree with a decrease in the pH value by 1.006 degrees from 6.3333 to 5.3266 in the duration of 48h in comparison to time 0h.

Table 4.5. The pH values of acidifying activity of *L. gasseri* ATCC33323

Time (hour)	0:00	8:00	10:00	24:00	48:00
pH value	6.33±0.100	5.92±0.072*	5.87±0.117**	5.56±0.165**	5.32±0.126**

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

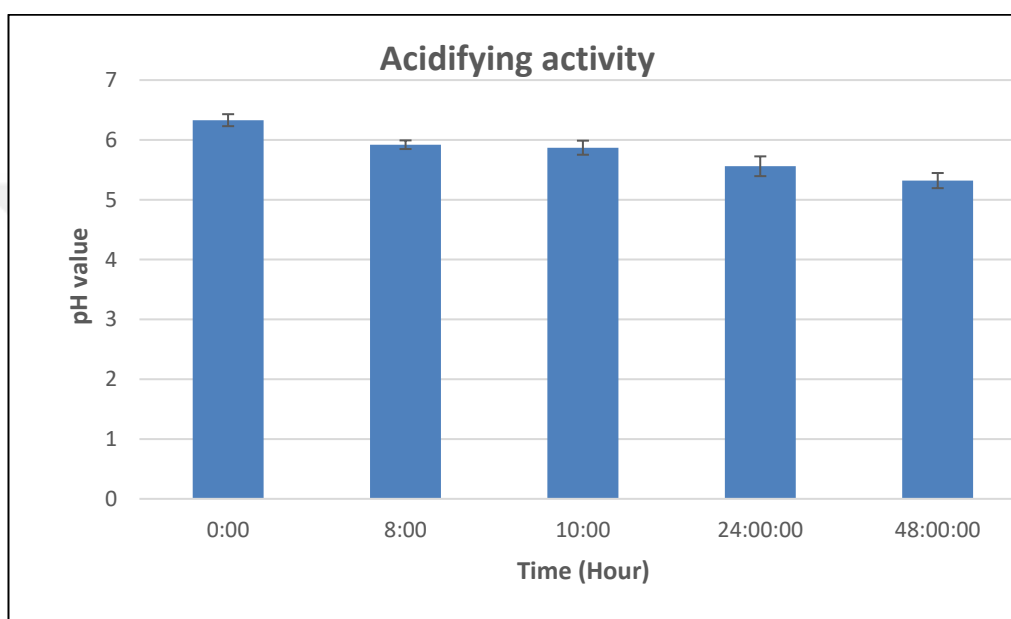


Figure 4.4. The acidifying activity for *L. gasseri* ATCC33323

4.1.6 Cell auto-aggregation

Auto-aggregation is the capacity of bacteria strains to aggregate in a non-specific manner with one another, which is thought to be a need for colonization in order for probiotic bacteria to have positive effects (2). Auto-aggregation of *L. gasseri* ATCC33323 was evaluated in terms of sedimentation rate, measured using a spectrophotometer (OD₆₀₀), then calculated using the auto-aggregation% formula. As shown in Table 4.6, *L. gasseri* ATCC33323 exhibited a high ability to self-aggregate, with the sedimentation rate exceeding the 90% mark after the 2nd hour of incubation, the highest value is 97.02% with a small fluctuation at the 5th hour of incubation. The auto-aggregation percentage showed significant changes (p<0.01) throughout the 5h duration regarding the first hour.

Table 4.6. The auto-aggregation% of *L. gasseri* ATCC33323 in a duration of 5h

Auto-aggregation (%)	1:00	2:00	3:00	4:00	5:00
	17.156±9.078	95.637±7.053*	92.745±8.188*	97.026±4.650*	92.647±0.871*

Data are expressed as mean± SD (n=3). **p<0.01. Significance is expressed in regard to the time 1:00.

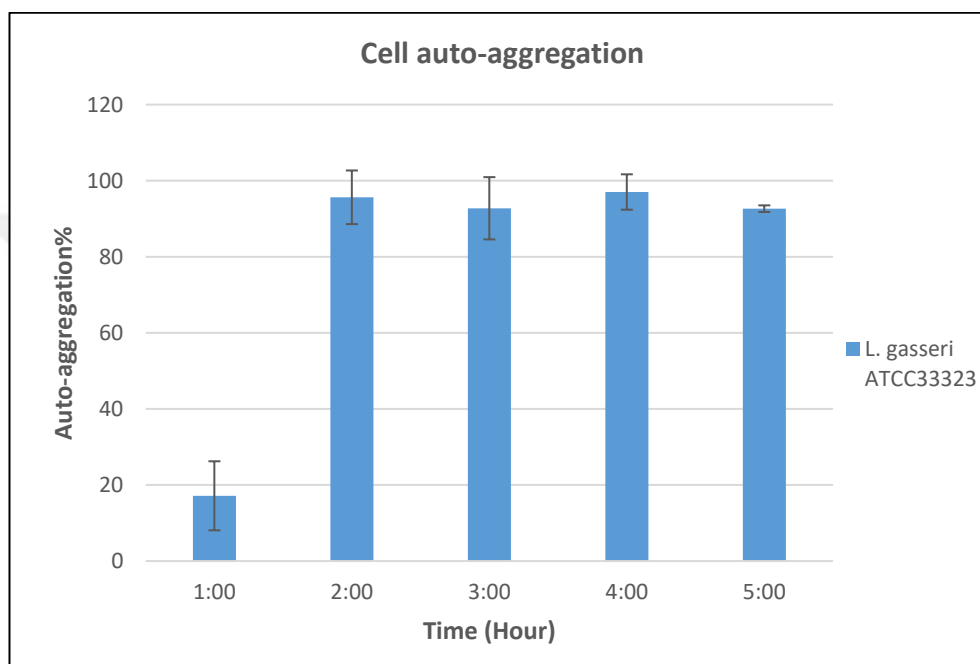


Figure 4.5. Auto-aggregation% of *L. gasseri* ATCC33323

4.1.7 Cell co-aggregation

The creation of bacterial aggregates between various bacterial species is known as co-aggregation. Co-aggregation with a possible pathogenic microorganism enables the probiotic strain to prevent pathogens' colonization in the gut and create antimicrobial substances in close range to them, which may prevent the development of pathogenic strains in GIT. Table 4.7 displays the results of the co-aggregation of *L. gasseri* ATCC33323 with five pathogenic bacteria namely *L. monocytogenes* RSKK, *Y. enterocolitica* ATCC23715, *S. typhimurium* ATCC13311, *E. coli*, and *S. aureus* individually. The results indicated an increase in the co-aggregation% with the pathogenic bacteria during the incubation time with small fluctuations. *L. gasseri* ATCC33323 showed significant co-aggregation% (p<0.01) against *E. coli* at the 6 and 24h marks and

against *L. monocytogenes* at the 24h mark and showed lower co-aggregation% ($p<0.05$) with *E. coli*, *Y. enterocolitica*, and *L. monocytogenes* at an incubation time of 4h, 24h, and 6h respectively. Also, *S. aureus* showed one significant value ($p<0.05$) at the 6h mark obtained by one-way ANOVA analysis. The co-aggregation% ($p<0.05$) against *S. typhimurium* increased gradually with time and showed a small fluctuation at the 24h mark, with a significant value at an incubation time of 4-24h.

Table 4.7. Co-aggregation% of *L. gasseri* ATCC33323 with five pathogenic bacteria in duration of 24h

	Pathogenic bacteria				
Time	<i>L. monocytogenes</i>	<i>Y. enterocolitica</i>	<i>S. typhimurium</i>	<i>E. coli</i>	<i>S. aureus</i>
0:00	75.693±5.827	50.866±19.260	63.942±1.783	72.853±8.799	49.503±22.412
2:00	81.553±9.198	66.020±25.254	77.997±9.465	86.258±5.571	56.916±23.052
4:00	81.679±2.383	85.969±13.468	90.240±8.080*	89.259±3.253*	98.269±7.010
6:00	92.198±6.927*	75.433±9.630	95.802±11.221*	96.072±4.187**	87.637±7.209*
24:00	97.701±3.981**	88.501±6.7059*	92.063±13.746*	97.924±1.828**	95.225±21.292

Data are expressed as mean± SD (n=3). ** $p<0.01$, * $p<0.05$. Significance expressed in regard to time 0:00

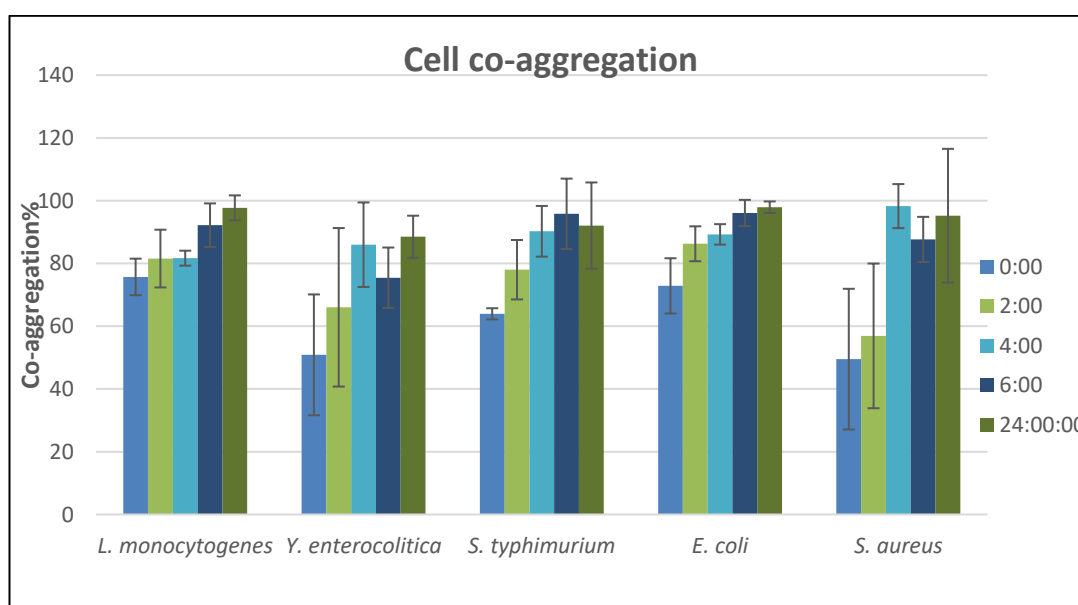


Figure 4.6. Co-aggregation% of *L. gasseri* ATCC33323 with five pathogenic bacteria.

4.1.8 Antibiotic susceptibility

The antibiotic susceptibility of probiotics is considered an important safety aspect for choosing potential probiotic bacteria. The results obtained from the disk diffusion method against four antibiotics were categorized using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) as follows, R resistant with a zone of ≤ 14 mm, I intermediate with a zone of 15-19 mm, S susceptible with a zone of ≥ 20 mm. According to the results shown in Table 4.8, *L. gasseri* ATCC33323 was susceptible (S) to all four antibiotics used, ampicillin (10 μ g), erythromycin (15 μ g), kanamycin (30 μ g), and neomycin (30 μ g) with zones of inhibition ranging from 20mm to 38mm in diameter.

Table 4.8. Antibiotic resistance of *L. gasseri* ATCC33323 against 4 antibiotics

Antibiotic	Zone diameter (mm)	Susceptibility
Ampicillin (10 μ g)	30	S
Erythromycin (15 μ g)	38	S
Kanamycin (30 μ g)	25	S
Neomycin (30 μ g)	20	S

S: susceptible, I: intermediate, R: resistant. Data Interpreted according to CLSI guidelines.

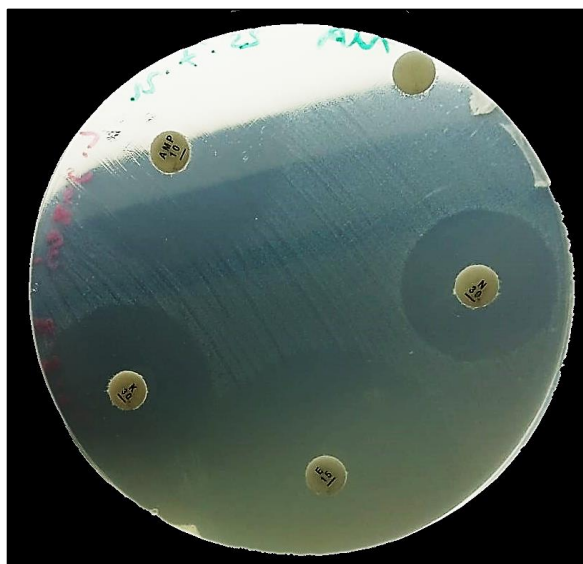


Figure 4.7. Antibiotic susceptibility test of *L. gasseri* ATCC33323 against four antibiotics, Ampicillin (AMP), Erythromycin (E), Kanamycin (K), and Neomycin (N). A blank disk was used as a negative control.

4.1.9 BSH activity

Bile salt hydrolysis is a desirable characteristic in probiotics since it has a role in the elimination of cholesterol from the body. Many *bsh* genes have been identified and characterized from *Lactobacillus* species due to the substantial correlation between BSH and harmful or beneficial health effects. The BSH activity of *L. gasseri* ATCC33323 has been examined in a previous study conducted by Ba and Öztürk (74). To determine the substrate specificity, the BSH activity was tested using the ninhydrin assay against six bile salts which are GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA. The results showed that the highest hydrolysis activity was against GCDCA with 100% activity and the activity against the remaining bile glyco-conjugated was 97% and 30% for GCA and GCDCA respectively. On the other hand, there was a low preference for tauro-conjugated substrates with a percentage of 12% and 4% for TCA and TDCA respectively, furthermore, BSH had no preference for TCDCA salt.

4.2 Screening for bacteriocin gene

4.2.1 PCR amplification of bacteriocin and PCR product purification

For the screening of bacteriocin genes, *L. gasseri* ATCC33323 genomic DNA was isolated, then a PCR amplification reaction was performed using three species-specific designed primers. The obtained result for Gas A, which was visualized using agarose gel electrophoresis and a UV transilluminator, was approximately 700bp in size (Figure 4.8a). Then the product was purified using 1% agarose gel electrophoresis to remove unwanted non-specific PCR products (Figure 4.8b).

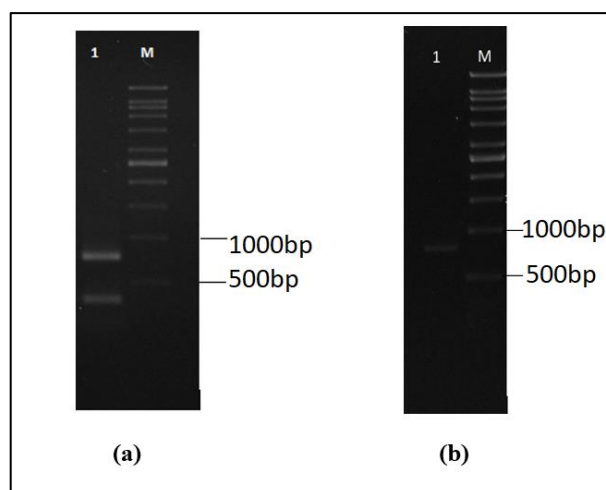


Figure 4.8. The PCR amplification product of *L. gasseri* ATCC33323 and purification of the PCR product. M: VC 1kb DNA ladder. (a), Line 1: Gas A PCR product. (b), line 1: Gas A purified PCR product.

4.2.2. Preparation pMMA construct

4.2.2.1. Preparation of pBluescript cloning vector

For the preparation of the cloning vector, the pBluescript vector was isolated from the *E. coli* XL1 strain and digested with *Sma*I fast digest restriction enzyme to obtain a blunt-ended linear vector, then it was loaded and purified from 1% agarose gel. The results were visualized using agarose gel electrophoresis and UV transilluminator.

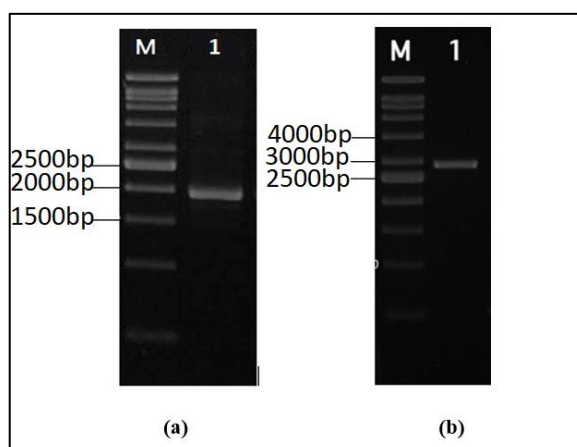


Figure 4.9. Preparation of pBluescript cloning vector. M: VC 1kb DNA ladder. (a), line 1: isolated circular pBluescript cloning vector. (b), line 1: linear pBluescript vector digested with *Sma*I.

4.2.2.2. Transformation of pMMA into *E. coli* XL1-Blue competent cells

The purified recombinant DNA product of ligation, pMMA, was transformed into XL1-Blue competent cell treated with CaCl_2 . The insert-containing colonies were selected according to the color of the colony. The white colonies were insert-positive and the blue colonies were insert-negative. A few white colonies were selected and their DNA was isolated to be visualized by agarose gel electrophoresis and UV transilluminator to assert the presence of the recombinant DNA. To assert the presence of the insert inside the plasmid, the recombinant plasmid DNA was digested with *EcoRI* and visualized.

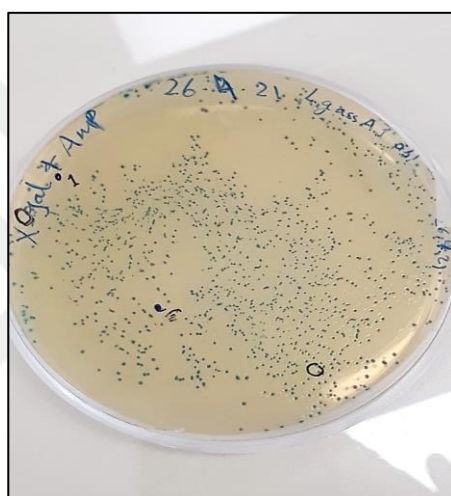


Figure 4.10. Transformation result grown on LB plate supplemented with ampicillin, IPTG, and X-gal, white colonies contain recombinant DNA, and blue colonies lack recombinant DNA.

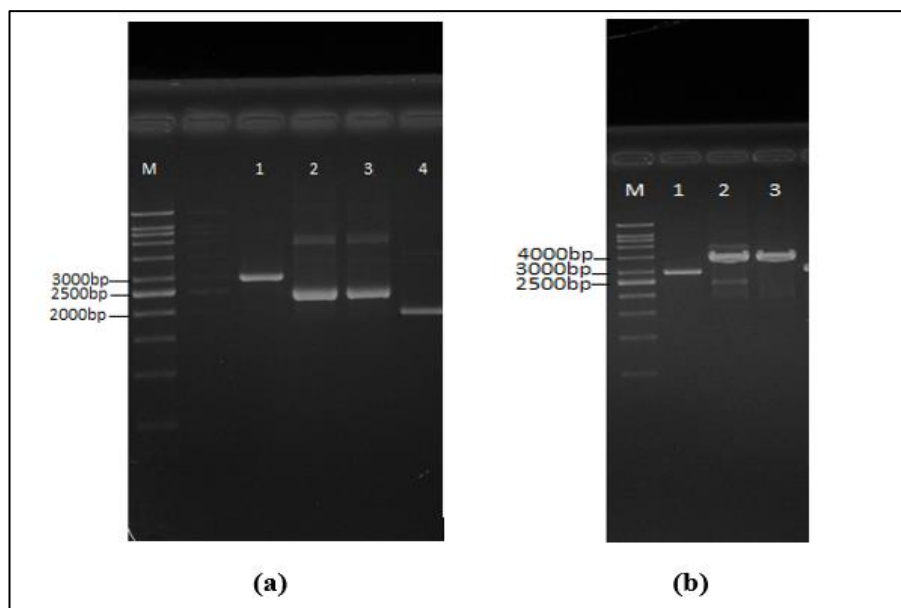


Figure 4.11. Transformation of pMMA into XL1-Blue competent cells. M: VC 1Kb DNA ladder. (a), line 1: pBluescript cloning vector DNA digested with *Sma*, lines 2 and 3: uncut pMMA DNA from two different colonies, line 4: uncut pBluescript cloning vector DNA. (b), line 1: pBluescript cloning vector DNA digested with *Sma* I, lines 2 and 3: pMMA digested with *EcoR* I from different colonies.

4.2.3. DNA sequence analysis for pMMA

The pMMA construct was 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 was verified by the BioEdit program to be 750bp in length (Figure 4.12). The sequence was then translated into its respective amino acid sequence using the SIB (Swiss Institute of Bioinformatics) ExpASy tool (Figure 4.13). The obtained sequence for pMMA construct coded for several amino acid sequences. Then the amino acid sequences were aligned with closely related proteins in the database of NCBI using the BLAST tool and MEGAX software (Figure 4.14).

```
GGTGRGCATTACCTAAAGAAGTAACGTCGAAATGACMACAAGAAAGGTACTGAGTAACGTTGATTAGTTT
GTTTAGTCGGGCTTGACTCTTTATTCTTCGTATTATTTAATGACCTATTATATGGAAATTTGGTTCGTAA
GATTCACGGTAATTCTATATCCGACATCGTACCCGCAATCCCTTGTATATGAATTAGAAGTTTGTCTTCC
TTACATGGTCAGTCCTTAGAATAGTAAAAAGAAGTCTTAAAGGTACAAAAGTAGTGATTAAGGGAATGGTA
CTGCATGTCTAAGGTAACATTTCTTTCGTTGAAGACCAAAATGAGGGGTCACTTAATTCAGGTAGTACTAC
AATAGTGCTGTTGGTTAATGCAAGAAATTGAACGTTCAAGGCAATAATTAATTTAGAAGACGTAAAGGATT
GGTATGTAGTCAGGGCACTCTGTTTGGACTTTAGACGTTGTTAAGACTTTTTCCACCAACCGAAACAAAGAA
GTTTCGTAAGGAGCTTGTTTAAACATGGTTTAAAGACCTTGTGGGTTTCATGGTCAGAAACTGACTTAAACG
AGTCCTCATTGTGGATTTTAAACCCCTGAACTTTTATTCCTGTAATGAGGACCTAGTAGTCCGCCTTATCA
ATTACCCAGTTAAGGACTATTTAGAACGTCGTAAGACTAGTATCAACCCAGTAGTACGGGATCTTATAGTT
TAAAGTTTTATAATAGCACTTATCTCATAAGTTTTTCCG
```

Figure 4.12. The nucleotide sequence of pMMA construct obtained from MacroGen is 750bp in length; the red letters represent an unread nucleotide.

5'3' Frame 1
GXHYLKK-RRNDXKKGTE-R-FSLFSRA-LFILRII--PIIWKFGSLRFTVILYPTSYPQSLVYE
LEVCSLHGQSLE--KEVLKVQK--LREWYCM**SKVTFLSLKTKMRGH**LIQVV**LQ**-CCW**LMQEIER**
SRQ-LI-KT-RIG**M**-SGHSVWTLDVVKTFSP**TET**KKFVRS**LF**KTWFKTLWVSWSETDLNESSLWI
LKP-TFIPV**MRT**--SALSITQLRTI-NVVR**LV**STQ-YGIL-FKVL--HLSHKFFP

5'3' Frame 2
VXIT-RSNVEM**XT**RKVLSNV**DLVCLVGLDSLFFVLFNDLLYG**NLVR-DSR-FYIRHRTNPLY**MN**
-KFVLPY**MVSP**-NSKKKS-RYKSSD-GNGTACLR-HFFR-RPK-GVT-FR-YYNSAVG-CKKLN**V**
QGN**N**-FRRRKGLVCSQGT**LFGL**-TLLRLFHQPKQRSS-GAC**LKHGLR**PCGFHGQ**KL**T-TSPHCGF
-NPELLFL--GPSSPPYQLPS-GLFRTS-D-YQPSS**TGSYS**LKFYNSTYLISFF

5'3' Frame 3
XALPKEVTSK-XQERY-VTLI-FV-SGLTLYSSYYL**MTYYMEIW**FVKIHGNSISDIVPAIP**CI**-I
RSLFFLTWSVLRI**VK**RS**LK**GT**KV**VIK**G****MVLHV**-GNISFVEDQNEGSLNSG**STTIVLLVN**ARN-TF
KAIINLEDVKDWYVVRALCLDFRRC-DFF**TNR**NKEVRKELV-N**MV**-DLVGF**MVRN**-LKRVLIVDF
KTLNFYSCNEDLVVRLINYPVKDYLERRKTSINPVVRDLIV-SFI**IALIS**-VFS

3'5' Frame 1
RKKL**MR**-VLL-NFKL-DPVLLG-Y-SYDV**LN**SP-LGN--GGLLGPHYRNKSSGF-NPQ-GLV-VS
F-P-NPQGLKPCFKQAPYELL**CFGW**-KSLNNV-SPNRVP-LHTNPLRL**LN**-LLP-TFNFLH-PTA
LL-YYLN-VTPHFG**LQ**RKKCYLRHAVPPF-SLLLYL-DF**LLF**-GLT**M**-GRTNF-FIYKGLRVRC
RI-NYRES-RTKFPY**NR**SLNNTKNKESSPTKQTKSTLLSTFLXVISTLL**L**-V**MX**T

3'5' Frame 2
GKNL-DKCYKTLNYKIPYYWVDTS**LT**TF-IVLNWVIDKADY-VLITGIK**VQ**GFKIH**NEDS**FKSV
SDHETHKVLNHVLNKLLTNFFVSVGEKVL**TSKVQTE**CPDYIPILYV**F**-INYCLERSIS**CINQ**QH
YCSTT-**IK**-PLILVFNERNV**TLD****MQYHSLNH**Y**FCTFKTSFYYSKD**-PCKEEQTSNSYTRDCGYDV
GYRITVNLNEPNFHIIGH-IIRRIKSQARLNKLNQRYSV**PFLXSF**RRYFFR-CX

3'5' Frame 3
EKTYEISAI**IKL**-TIRSRTTGLILVLR**RSK**-SLTG-LIRRTTRSSLQ**E**-KFRVLKST**MR**TRLSQ**F**
LTMK**P**TR**S**-**T**MF-TSSLRTSLFRLV**KKS**-QRLKSKQSAL**TTYQ**SFTSSKLI**IALNVQ**FLALT**N**ST
IVVLPELSDPSFWSSTKE**MLP**-TCSTIPLITTFVPLRL**LFTIL**RTDHVRKNKLLIHIQGIAG**TMS**
DIELP-ILTNQ**ISI**--VIK-YEE-RVKPD-TN-INV**TQYL**SCXHFDV**TS**LG**N**XH

Figure 4.13. The amino acid sequence for pMMA construct obtained using ExPASy tool, four amino acid sequences were analyzed from different reading frames.

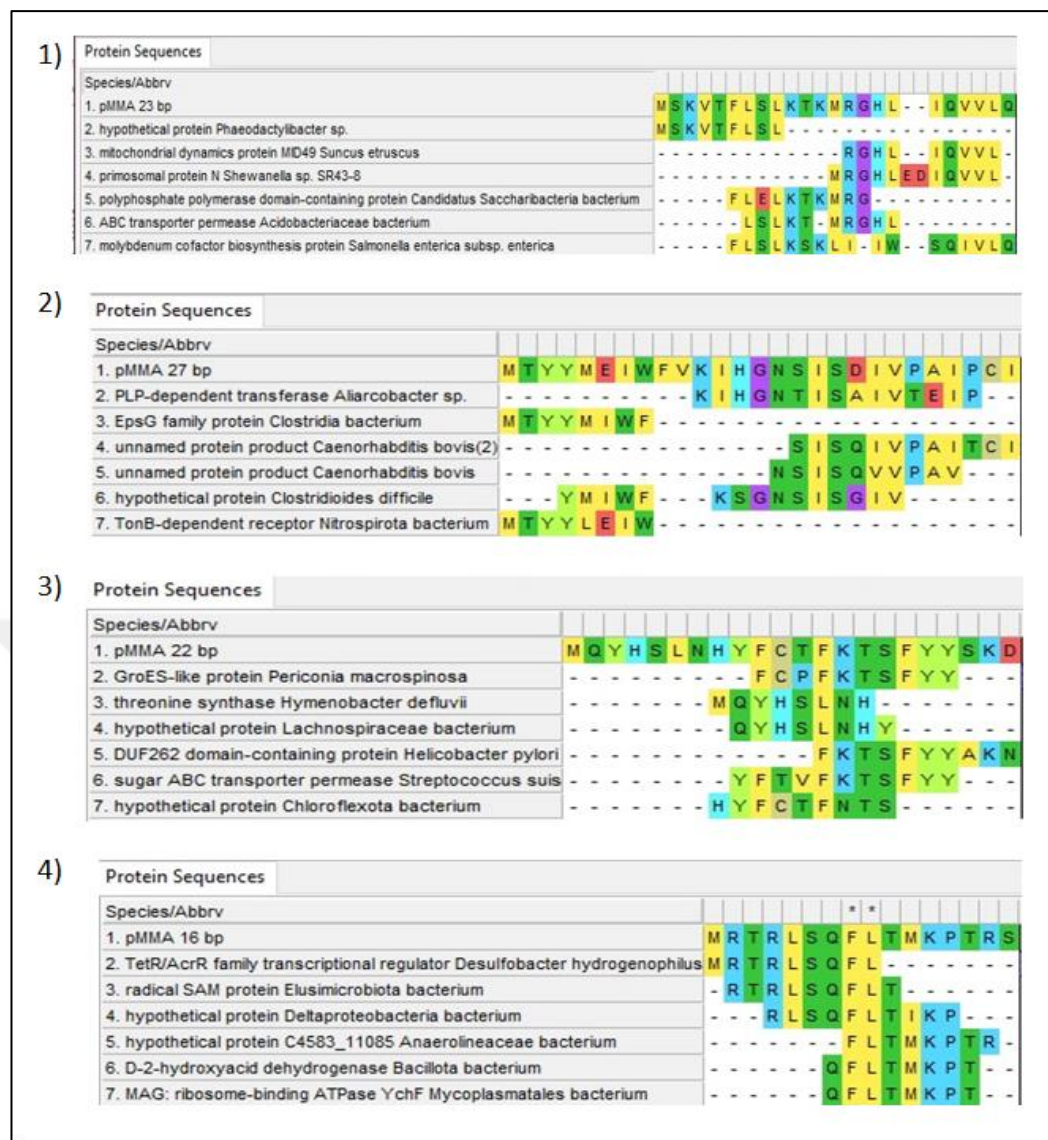


Figure 4.14. Multiple sequence alignment of pMMA construct amino acid sequences obtained using MEGAX software, the amino acid sequences were each aligned separately with six different proteins obtained from the BLAST tool, 1) the first sequence is 23 amino acids in length and encoded with the stop codon tag, 2) the second sequence is 27 amino acids in length and encoded with the stop codon tga, 3) third sequence is 22 amino acids in length and encoded with the stop codon tga, 4) fourth sequence is 16 amino acids in length and encoded with the stop codon taa, the dashes represent gaps in the sequence produced during alignment. Asterisk (*) represents the fully conserved sequences.

5. DISCUSSION

Lactic acid bacteria as a prime part of the human gut microbiota, have gained increasing interest as a research subject for characterization and the potential to be used as a probiotic candidate to promote human health and help in disease prevention. One of the major elements of the human gastrointestinal microbiota is the genus *Lactobacillus*, which is made up of a diverse range of species that are essential to maintaining gut health. These species include the probiotic *Lactobacillus gasseri*, which has a variety of characteristics. These bacteria demonstrated their ability to create significant bacteriocins, excite macrophages, and survive in the GI tract. As a result, this probiotic has been widely applied in a huge variety of industries (74). In order for a bacterial strain to exert health-promoting and beneficial probiotic effects it is essential for the bacteria to survive in an inhospitable habitat such as the human GIT. Another critical criterion is to establish the safety of these bacteria for host consumption. This underlies the significance of characterizing and assessing LAB microorganisms (77). In-vitro evaluation is a key technique for the selection of probiotics since in-vivo experimentation to assess potential probiotic candidates is costly and time-consuming (71). Earlier studies have shown the various effects of LAB, however, because each strain has different characteristics and effects upon application it is essential for the characterization to be strain-specific (78). Therefore, investigating, and characterizing *L. gasseri* ATCC33323 strain probiotic properties are of vital importance.

The first requirement for a probiotic candidate is the ability to reach the targeted region in the GIT. Probiotics must tolerate low pH values to be able to transit through the stomach and express their activity in the small intestine. Therefore, experiments are conducted to assess the degree of acid tolerance in the *L. gasseri* strain (60,61,65). As shown in Figure 4.1, *L. gasseri* ATCC33323 showed growth in all different pH values including pH 2 with small fluctuations at different hours during the 6h incubation period with significant values at different time points, and then the strain showed an increase in growth after being inoculated in normal MRS broth as a control group, compared to the growth in the different five pH values, indicating the ability of *L. gasseri* ATCC33323 to

tolerate the low pH values of the stomach and upper intestine for a few hours. Statistical analysis showed that the bacteria had the most significant results ($p < 0.05$) at pH 5, it also showed significant growth at the 4th hour mark for pH 2, and there was significant growth at pH 4 at times 2h and 6h as well as at the 6th hour for pH 6. According to Erkkilä et al., (79), lactic acid bacteria log values showed a little decline at the 4 h marks at pH 3 which is in parallel with *L. gasseri* results that showed a small decline of log 0.07 at that pH value. Hassanzadazar et al., (80) found that *Lactobacillus* isolates were not resistant to pH 2 whereas *L. gasseri* ATCC33323 showed stable growth in that pH with a little decline of the log value by 0.14 at the 6 h mark showing resistance. These differences can be due to this characteristic being strain specific.

Tolerance to bile salts is considered vital for the colonization of probiotic bacteria in the host's intestine since bile salts are poisonous to the bacteria, disrupting their cellular homeostasis leading to cell death. Bile salt concentration in the intestine is not constant, but the average bile concentration is thought to be 0.3%, with a retention time of four hours for the food in the intestine (1). So this test was conducted with media supplemented with 0.1% (w/v) and 0.3% (w/v) of Oxgall as a replacement for bile due to its resemblance, and the incubation time was 5h. In this study, *L. gasseri* ATCC33323 showed an increase in the log values for 0.1% throughout incubation with very little fluctuation and at 0.3%, the log values increased till the 3rd hour of incubation by 0.08 with a growth rate of 69.5% then a decrease by 0.11 at the 5th hour mark with a growth rate of 38.04%. A growth rate of 68.9% for 0.1% concentrations at the 4th hour mark indicates the ability of *L. gasseri* ATCC33323 to withstand the bile concentration at 0.1% but is affected by 0.3% concentration after the 3rd hour mark with a decrease in the growth rate to 50.6% at the 4th hour. Statistical analysis showed a significant value ($p < 0.05$) at the 5th hour for 0.1% and no significant value at the 0.3% concentration. Li et al., (65) reported that *L. salivarius* and *L. mucosae* had a survival rate of 88.11% and 61.2% in 0.3% concentration of bile after 4h of incubation, in contrast to *L. gasseri* ATCC33323 that had a less survival rate at the 4th hour mark.

One of the major properties of all probiotic candidates is antimicrobial activity. The antimicrobial effects are credited to their metabolites like hydrogen

peroxide, bacteriocin, alcohol, and organic acids (61). *L. gasseri* ATCC33323 was tested against five pathogenic bacteria, two gram-positive bacteria (*Listeria monocytogenes*, and *Staphylococcus aureus*), and three gram-negative pathogens (*E. coli*, *Yersinia enterocolitica*, and *Salmonella typhimurium*) and inhibition zones were categorized according to Sirichokchatchawan et al., (81). *L. gasseri* ATCC33323 showed different degrees of antagonistic activity against the five pathogens. The CFS showed activity against *E. coli* with a small inhibition zone indicating weak antagonistic activity, and *Listeria monocytogenes* with a big-size inhibition zone indicating a strong activity, suggesting the secretion of the antagonistic substances outside of the cell. The cell lysate of the *Lactobacillus* strain also showed antagonistic activity against two strains which are *E. coli* and *Staphylococcus aureus* both with a big-size inhibition zone showing strong activity. The obtained results, even though they demonstrated the antimicrobial activity of the tested strain, did not point out the exact cause of this activity, whether it was a bacteriocin or another antimicrobial compound in the bacteria. In a study conducted by Idoui (82), the CFS of *L. gasseri* showed antagonistic activity against *E. coli* similar to our findings. It also showed activity against *Salmonella* spp. differing from our results. Rastogi et al., (83) also reported that *L. monocytogenes* and *S. aureus* showed resistance to *L. gasseri* CFS in contrast to results obtained in this study. Similar studies conducted on Lactic acid bacteria by Bin Masalam et al., (38), and Jose et al., (84) indicated the activity of LAB strains CFS against *E. coli* and *L. monocytogenes* respectively.

Resistance to phenol is a crucial criterion for potential probiotics and it gives the bacteria a better possibility to survive and persist in the GIT (85). According to the obtained results in Table 4.4, *L. gasseri* ATCC33323 demonstrated an increase in the log₁₀ values in the duration of 24h, indicating resistance and the ability to proliferate in the presence of different phenol concentrations. The one-way ANOVA statistical analysis indicated that the increase in the log₁₀ values of the tested strain was significant ($p < 0.01$) for phenol concentrations 0.1%, 0.2%, and 0.3% in comparison to time 0h, on the other hand, the result for 0.4% phenol concentration showed growth but with no significant value compared to time 0h. These results indicated the ability of *L. gasseri* ATCC33323 to endure toxic metabolites like phenol. A previous study conducted

by Reube et al., (71) indicated poor tolerance of LAB strains to 0.4% phenol concentration in contrast to our result which indicates better resistance. Shehata et al., (85) reported varying degrees of phenol tolerance for LAB strains.

One of the main characteristics of LAB is its ability to create an acidic environment which can help in killing pathogenic bacteria and has a positive effect on the fermentation process and food flavor. LAB can be divided roughly into three categories according to Nieto-Arribas et al., 1) those that decrease the pH value by more than 2 degrees have a high acidifying capacity, 2) those that reduce the pH value between 1.5-2 are considered to have medium acidifying activity, 3) the strains that can only reduce the pH value by 1.5 degrees are considered to have low acidifying activity, in a duration of 24h (86). According to this categorization, *L. gasseri* ATCC33323 had low acidifying activity, decreasing the pH value of skimmed milk by only 0.77 degrees in the duration of 24h. The results of the test (Table 4.5) demonstrated a significant change ($p<0.05$) in the pH value during the incubation period of 48h in comparison to time 0h for each of the measured points. According to Barzegar et al., (86) results, LAB strains showed moderate acidifying activity in contrast to our results.

Another beneficial effect of probiotics is their ability to generate sufficient mass through aggregation making this a desirable property. Auto-aggregation helps microorganisms that are from the same species to bind and make self-forming groups, the formation of biofilms, and adhesion and colonization in the intestine (65,71,87). In this study, Auto-aggregation results of *L. gasseri* ATCC33323 showed values between 17.15% and 92.64% during the duration of 5 hours (Table 4.6). The auto-aggregation % had an incline with the increase of incubation time with a fluctuation from 97.02% at the 4th hour to 92.64% at the 5th, and all the obtained results were found to be significant ($p<0.01$) indicating a strong aggregation ability of *L. gasseri* ATCC33323. These results may be attributed to the complex relations between bacterial surface molecules. El-Sayed et al., (68) findings showed medium auto-aggregation ability for some LAB strains at both the 5 h and 24 h marks, unlike *L. gasseri* ATCC33323 results.

Co-aggregation enables probiotics to form an effective barrier for preventing pathogen adhesion in the intestine; furthermore, it gives a merit over

non-co-aggregating organisms since they are more easily removed from the intestine. The co-aggregation ability of *L. gasseri* ATCC33323 with the five pathogenic bacteria varied with each of the pathogens with results that are strain specific. The statistical analysis showed that *L. gasseri* ATCC33323 had significant results ($p<0.05$) with the five pathogenic bacteria at different time points during the incubation period. Reuben et al., (71) results showed a high co-aggregation value of LAB and *L. monocytogenes* similar to the results of *L. gasseri* ATCC33323 at the 2 h mark. The co-aggregation ability results of *L. plantarum* from Nallala et al., (88) showed a 62.2% co-aggregation with *E. coli* while *L. gasseri* ATCC33323 had an 86.2% rate at the 2 h incubation mark, and it showed a low co-aggregation ability with *L. monocytogenes* (35.5%) compared to *L. gasseri* ATCC33323.

The assessment of probiotic bacterial susceptibility to antibiotics is a major criterion for the evaluation of probiotics. This is considered a controversial subject since probiotics are thought to have the ability to serve as reservoirs for antibiotic-resistance genes that could be transferred to intestinal pathogens (71). In the literature, *Lactobacillus* resistance to antibiotics is considered intrinsic and encoded on the chromosome so it is considered nontransferable (67). Three types of antibiotics were used in this test, those that inhibit protein syntheses like erythromycin and neomycin, the ones that inhibit cell wall resistance like ampicillin, and the ones that target cellular RNA like kanamycin(61). *L. gasseri* ATCC33323 showed susceptibility to all four antibiotics. Similar to the data acquired from this test, studies conducted by Mazlumi et al., (61) showed the susceptibility of LAB isolates to erythromycin and ampicillin, and similarly Reuben et al., (71) and Rodrigues da Cunha et al. (89) results showed LAB isolates' susceptibility to ampicillin. Bin Masalam et al., (61) studies reported the sensitivity of LAB isolates to neomycin which agrees with the obtained results from this study. On the other hand, Somashekaraiah et al., (90) reported resistance of LAB isolates to kanamycin and fluctuation of sensitivity to ampicillin erythromycin.

In general, *L. gasseri* is one of the most prevalent and often found species in the GI tract of humans and is often linked to the synthesis of bacteriocin. The combined effect of some of the bacteriocins in the *L. gasseri* strain like gassericin

A, and gassericin T have recently been shown to have the potential for application as a food preservative. the strain that produces the bacteriocin may gain a competitive edge, increasing its dominance over other species in a given biological niche. the generation of bacteriocin by strains with human intestinal origin has only been the subject of a small number of research to date. In this study, screening for bacteriocin gene was performed and the PCR product was identified by sequencing, and in-silico analysis was conducted on the obtained strain, BLAST analysis, and multiple sequence alignment revealed that the detected protein sequences of *L. gasseri* ATCC33323 did not coincide with any bacteriocins in the database of NCBI, the resultant proteins aligned with different proteins from multiple species including hypothetical proteins. These results may be due to fault in the sequencing of the *L. gasseri* ATCC33323 gene or the attachment of the specially designed primers in a different site on the strain. *L. gasseri* ATCC33323 have positive phenotypic probiotic characteristics but the gene coding the desired bacteriocin was not detected with these specific primers, these results may be due to the existence of multiple bacteriocin genes in *Lactobacillus* species.

6. CONCLUSIONS AND RECOMMENDATIONS

Characterization of *L. gasseri* ATCC33323 for probiotic properties was the goal of this work. To evaluate these probiotic properties several tests were conducted. According to the results, the *L. gasseri* ATCC33323 strain was found to demonstrate desirable in-vitro properties as a probiotic candidate which include antimicrobial activity against several pathogens, good cell surface properties such as the ability to self-aggregate and to aggregate with pathogens, survivability in stress conditions including acidic environment, bile, and different phenol concentrations, and showing susceptibility to antibiotics; which is considered a safety assessment; as well as possessing BSH activity. These results indicate that this *Lactobacillus* strain can be a suitable probiotic candidate for human consumption and application in the food industry, however, this is considered preliminary testing, and further detailed work on this strain's effect in-vitro and in-vivo is required. Future studies on *L. gasseri* ATCC33323 should be performed to use this strain reliably. It is beneficial to perform clinical studies regarding human health and test the viability of this strain in products. Regarding the limitations of this study, although protein-coding genes were detected using PCR, a more detailed molecular investigation for identifying the antimicrobial agents can be performed on this strain. In-vivo testing was not performed in this study which is a required step to validate the probiotic potential claim for this strain to be used in different applications including the pharmaceutical industry.

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8. APPENDICES

Appendix A

CHEMICALS AND ENZYMES

Chemical	Supplier
Agar	Merck
Agarose	Sigma, USA
Ampicillin	Sigma
Antibiotic disks	OXOID, UK
Calcium chloride (minimum 93%, granular anhydrous)	Sigma
EDTA (Ethylenediaminetetraacetic acid)	Sigma
EtBr (Ethidium Bromide)	Sigma
Ethanol	Merck
Glasial acetic acid	Carlo Erba
Glucose	Merck
Glycerol	Sigma
Hydrochloric acid (HCl)	Merck
IPTG (isopropyl-beta-D-thiogalactopyranoside)	Thermo Scientific
Magnesium chloride (MgCl ₂)	Thermo Scientific
MRS broth	Merck, Germany
Nutrient agar	OXOID, UK
Nutrient broth	NEOGEN, UK
Phenol	Merck
Potassium chloride (KCl)	Thermo Scientific
Potassium phosphate Monobasic	Merck
Sodium Chloride (NaCl)	Merck
Sodium Phosphate monobasic	BDLab
Sodium phosphate Dibasic	Merck

Sodium Hydroxide (NaOH)	Merck
Triton X-100%	Sigma
Trypton	Merck
Trizma base	Sigma
Trizma Hydrochloride	Sigma
Yeast extract	Merck

Enzymes and chemicals	Supplier
Dntp Mix	Fermentas
<i>EcoRI</i> Fast Digest	Thermo Scientific
i-pfu™ DNA polymerase	INTRON biotechnology
Lysozyme	Fluka
Polyethylene glycol (PEG)	Thermo Scientific, EU
<i>SmaI</i> enzyme	Thermo Scientific
T4 DNA Ligase	Fermentas
VC 1kb DNA ladder	Vivantis
6X loading dye	Fermentas

Appendix B

GROWTH MEDIA

- **MRS (de Man, Rogosa, and Sharpe) liquid media**

For the preparation of MRS broth, 52.2g of MRS broth powder is added to 1 liter distilled water, and then autoclaved for sterilization at 121°C for 15min.

- **MRS plate**

For the preparation of the MRS plate, the agar percentage to be used is 1.5% of the amount of liquid media.

For 1 liter of liquid MRS media, 15g of agar-agar (Merck) powder is added and then sterilized at 121°C for 15min.

- **Nutrient broth**

8g of medium is added in 1liter distilled water, mixed thoroughly, and autoclaved at 121°C for 15min.

- **Nutrient agar (NA)**

28g of the media is suspended in 1 liter of distilled water, the mixture is heated to dissolve completely, then the media is autoclaved at 121°C for 15min.

- **Plate count agar (PCA) media**

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

These contents are dissolved in 900ml of distilled water and mixed thoroughly at 25°C, then the pH is adjusted to 7.0±0.2 using 5M NaOH. The volume of the media is then completed to 1liter and sterilized by autoclave at 121°C for 15min.

- **LB (Luria Bertani) liquid media**

Contents	For 1 liter
Tryptone	10g
Yeast extract	5g
NaCl	10g

The components are dissolved in 900ml of distilled water and mixed thoroughly, and then the pH is set at a value of 7.5 using 5M NaOH. The volume of the media is completed to 1 liter and then autoclaved at 121°C for 15min.

- **LB plate**

For the preparation of the LB plate, the agar should be used at a percentage of 1.5% of the amount of liquid media. 15g of agar-agar is added to 1 liter of LB liquid media and autoclaved at 121°C for 15min.

- **Ampicillin**

Contents	Amount
Ampicillin Sodium Salt	0.25g
UpH ₂ O	10ml

The ampicillin powder is dissolved completely in UpH₂O, then it was sterilized using a 0.22µm filter. Storage at -20°C.

- **LB plate+ ampicillin**

To supplement ampicillin to the LB plate, 4ml of ampicillin (25mg/ml) is added to 1 liter of sterilized LB medium.

Appendix C

BUFFERS AND SOLUTIONS

For Electrophoresis

- **10X TAE buffer**

Contents	For 1 liter
Trizma base (Sigma)	48.5g
Glacial acetic acid	11.42g
EDTA (0.5M, pH 8.0)	20ml

These components are dissolved in UpH₂O and mixed thoroughly, then the volume is completed to 1 liter. The buffer is stored at room temperature.

For the preparation of TAE buffer (1X), 100ml of TAE buffer (10X) is added to 900ml of UpH₂O.

For competent cell preparation

- **CaCl₂ (100mM)**

To prepare 50ml of CaCl₂ solution, 0.735g of powder is added to 50ml of UpH₂O and the pH adjusted to 7.0, then autoclaved for sterilization for 15 min at 121°C.

- **CaCl₂ (100mM) with 10% glycerol**

1ml of glycerol is added to 9ml of CaCl₂ and sterilized using autoclave for 15min at 121°C.

For cell lysis

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

30mg of lysozyme is dissolved in 1ml of UpH₂O. Storage at -20°C.

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

For the preparation of Tris-HCl solution, 12.2g of Trizma base is dissolved in 80ml UpH₂O and the pH is set at a value of 8.0. then the volume of the solution is completed to 100ml with UpH₂O and then autoclaved for 15min at 121°C.

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

For the preparation of 0.5M of EDTA solution, 2.9224g EDTA is dissolved in 15ml UpH₂O, and the pH is adjusted to a value of 8.0. then the volume of the solution is completed to 20ml and autoclaved for 15min at 121°C.

- **Lysis buffer**

Contents	For 1ml
Lysozyme (30mg/ml)	666.6µl
Tris-HCl (1mM)	20µl
EDTA (50mM)	12µl
Triton 100% (1.2%)	12µl
UpH ₂ O	261.4µl

These components are mixed thoroughly using a pipette. Storage at -20°C.

- **PBS (phosphate buffer saline) buffer (1X)**

Contents	For 1 liter
Sodium chloride (0.137M)	8g
Potassium chloride (0.0027M)	0.2g
Sodium phosphate Dibasic (0.01M)	1.44g
Potassium phosphate monobasic (0.0018M)	0.245g

The components are dissolved one after the other in a beaker then the buffer is filtered using a 0.22µm filter. The pH is adjusted to 7.4, then autoclaved for 15min at 121°C.

- **Phenol (1%)**

For the preparation of 1% phenol (94.11g/mol), the phenol is heated to melt then 1.1ml of phenol solution is added to 100ml UpH₂O. Stored at room temperature.

- **IPTG (1M)**

To prepare 1M IPTG, 1.1915mg is dissolved in 5ml UpH₂O then filtered using 0.22µm filter. Storage at -20°C.

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

For the preparation of X-gal (20mg/ml), 0.2g of the powder is added in 10ml DMF solution and vortexed to dissolve. The X-gal is stored in the dark at -20°C.