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**MOLECULAR AND BIOCHEMICAL CHARACTERIZATION
OF HUMAN ORIGINATED *LACTOBACILLUS REUTERI***

MASTER OF SCIENCE

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ABSTRACT

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF HUMAN ORIGINATED *LACTOBACILLUS REUTERI*

MSC THESIS

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BOLU ABANTIZZET BAYSAL UNIVERSITY

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DEPARTMENT OF BIOLOGY

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According to clinical research intestinal probiotics are live microorganisms that provide health benefits when ingested in sufficient quantities and their main functions were related to modulating and maintaining the health of the intestinal tract. Probiotics have several novel applications because of their special ability to produce bioactive molecules including vitamins, bacteriocins, 3-indoleacrylic acid, Indole-3-aldehyde, short-chain fatty acids, exopolysaccharides, peptides, and proteins. These substances are essential in giving the host a variety of health advantages. Although *Lactobacillus* (*L.*) species are commonly used as probiotics, only limited data are available for the probiotic properties of human originated *Lactobacillus reuteri*. This study aims to characterize human originated *L. reuteri* for its probiotic potential according to the international guidelines for the evaluation of probiotics. In the present study, the probiotic potential of *L. reuteri* isolated from healthy humans was evaluated. The probiotic properties of *L. reuteri* were tested for aspects of antimicrobial activity, antibiotic susceptibility, adhesion and aggregation ability, bile salt tolerance, low pH resistance and antimicrobial peptide production capacity. While clear zones of inhibition test results indicated that the cell free supernatant and cell lysate of *L. reuteri* had antagonistic activity against three pathogenic bacteria, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Salmonella typhimurium*, the antibiotic test results showed that *L. reuteri* was sensitive for Erythromycin, but resistance for Neomycin and Kanamycin. On the other hand, *L. reuteri* showed a strong ability to adhere to pathogenic bacteria as well as self-binding because it has a high sedimentation rate. Furthermore, *L. reuteri* showed a high survival rate under acidic and bile salt containing environment that means gastrointestinal tract conditions. While in-silico analysis for BSH screening results revealed the existence of a protein acquired using specifically designed primers, further research is needed to identify BSH in this strain. Obtained results show that such properties of the human originated *L. reuteri* may be used as probiotics in the healthcare sector and various food products.

KEYWORDS: Probiotics, *Lactobacillus reuteri*, human health, PCR

ÖZET

İNSAN KAYNAKLI *LACTOBACILLUS REUTERI*'NİN MOLEKÜLER VE BİYOKİMYASAL KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

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Klinik araştırmalara göre bağırsak probiyotikleri, yeterli miktarda tüketildiğinde sağlığa fayda sağlayan canlı mikroorganizmalardır ve temel işlevleri bağırsak kanalının sağlığını düzenlemek ve korumakla ilgilidir. Probiyotiklerin, vitaminler, bakteriyosinler, 3-indoleakrilik asit, indol-3-aldehit, kısa zincirli yağ asitleri, ekzopolisakkaritler, peptitler ve proteinler dahil olmak üzere biyoaktif moleküller üretme konusundaki özel fonksiyonlarından dolayı birçok yeni uygulaması bulunmaktadır. Bu maddeler, konakçıya çeşitli sağlık avantajları sağlamak için gereklidir. *Lactobacillus* (L.) türleri yaygın olarak probiyotik olarak kullanılsa da insan kökenli *Lactobacillus reuteri*'nin probiyotik özelliklerine ilişkin sınırlı veri mevcuttur. Bu çalışma, insan kökenli *L. reuteri*'nin probiyotik potansiyelinin, probiyotiklerin değerlendirilmesine yönelik uluslararası kılavuzlara göre karakterize edilmesi amaçlanmıştır. Bu çalışmada sağlıklı insanlardan izole edilen *L. reuteri*'nin probiyotik potansiyeli değerlendirilmiştir. *L. reuteri*'nin probiyotik özellikleri, antimikrobiyal aktivite, antibiyotik duyarlılığı, yapışma ve toplanma yeteneği, safra tuzu toleransı ve düşük pH direnci ve antimikrobiyal peptit üretim kapasitesi test edilmiştir. İnhibisyon testi sonuçları, *L. reuteri*'nin hücre içermeyen süpernatantının ve hücre lizatının üç patojenik bakteri olan *Staphylococcus aureus*, *Yersinia enterocolitica* ve *Salmonella typhimurium*'a karşı antagonistik aktiviteye sahip olduğunu gösterirken, antibiyotik test sonuçları *L. reuteri*'nin: Eritromisin' e duyarlı olduğu fakat Neomisin ve Kanamisine dirençli olduğunu göstermiştir. Öte yandan *L. reuteri*, yüksek sedimentasyon hızına sahip olması nedeniyle kendi kendine bağlanmanın yanı sıra patojen bakterilere de güçlü bir şekilde bağlanma yeteneği olduğunu göstermiştir. Ayrıca *L. reuteri*, asidik ve safra tuzu içeren bir ortamda, yani gastrointestinal sistem koşullarında yüksek bir hayatta kalma oranı göstermiştir. BSH tarama sonuçlarına yönelik in silico analiz, özel olarak tasarlanmış primerler kullanılarak elde edilen bir proteinin varlığını ortaya çıkarmış olsa da bu suşta STH'yı tanımlamak için daha fazla araştırmaya ihtiyaç vardır. Elde edilen sonuçlar insan kökenli *L. reuteri*'nin bu özelliklerinin sağlık sektöründe ve çeşitli ürünlerde probiyotik olarak kullanılabileceğini göstermektedir.

ANAHTAR KELİMELELER: Probiyotikler, *Lactobacillus reuteri*, insan sağlığı, PCR

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LIST OF ABBREVIATIONS AND SYMBOLS

LAB	: Lactic acid bacteria
WHO	: World health organization
FAO	: Food and agriculture organization
BSH	: Bile salt hydrolase
PCR	: Polymerase chain reaction
bp	: Base pair
rpm	: Rotation per minute
IPTG	: Isopropyl β -D-1-thiogalactopyranoside
OD	: Optical density
PBS	: phosphate buffer saline
EtBr	: Ethidium Bromide
μl	: Mikroliter
ml	: Milliliter
SD	: Standard deviation
CLSI	: Clinical and Laboratory Standards Institute
CFU	: Colony forming unit
IBD	: Inflammatory bowel disease
VEGFR	: Vascular endothelial growth factor
CRC	: Colorectal cancer
ROS	: Reactive oxygen species
EFSA	: European food safety authority
qPCR	: Quantitative polymerase chain reaction
PBS	: Phosphate-buffered saline
GIT	: Gastrointestinal tract
UV	: Ultraviolet

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1. INTRODUCTION

1.1 Probiotics importance

The word "probiotic" refers to bacterial relationships and signifies "for life" in modern usage. with positive impacts on the health of humans and animals " (1). In the early 1990s, Metchnikoff (2) Probiotics are defined in scientific terms as altering the diversity of plant/microbial in the human body and replacing harmful bacteria with healthy ones. But still, a breakthrough came with the work of Henry Tissier who found that the microbial concentration of a specific bacterial species was significantly lower in stool samples from children with infected diarrhea compared to youngsters in good health (3). His recommendations were the first of their type for restoring a healthy intestinal flora and giving patients with diarrhea (infantile diarrhea) live organisms (bifidobacteria) orally. What a probiotic is today comes from Havenar & Huisint veld (4) and describes a living monoculture or mixed culture of bacteria. that, when given to people or animals, enhances the properties of the local flora and benefits the host. After preliminary hiccups, probiotic Study has made significant progress over the previous 20 years, and notable progress has been achieved in the identification and description of certain probiotic microorganisms that provide significant advantages for health when consumed. The colonic material's moist weight is composed primarily of bacterial cells, which outnumber tissue cells by a factor of 10 that make up the anatomy of a person. Normally, there are 10³ distinct species in the stomach. of bacteria, and the colon's overall microbial population is roughly 10¹¹–10¹² Cfu/g (5). When neonates are initially exposed to an unsterile environment, bacteria start to colonies their intestines. From then on, it changes throughout time and changes throughout life, relying on the intricate and dynamic interactions between antibiotic usage, host lifestyle, food, and genetics. Notable changes in age-specific the essential gut microflora's makeup includes a drop in the ratio of Bacteroidetes to Firmicutes and a detectable decline in Bifidobacteria in individuals older than 60, Approximately the same time as The beginning of immune system to deteriorate (6). In general, nevertheless, the essential gut microflora's makeup is thought to be mostly steady all through adulthood. Helpful usage of the gut microflora, Additionally mentioned as a "colonization resistance", "barrier effect", is a crucial system employed by native (autochthonous) of gut bacteria to preserve its existence and provide niche

protection against newly microorganisms ingested that includes pathogens (7, 8). Consequently, it might be hypothesized that alterations the microbiota in the gut would lead to an increase in the relative number of (Beneficial bacteria), which may have an impact on metabolism, digestion, immune system, and gut-brain connection (9). Any diversity changes can lead to various disorders and diseases against which traditional medications have very low effectiveness, mostly as a result of the rise in pathogenic microorganisms that are tolerant to and resistant to antibiotics (10). Attempts to overcome this critical problem via improving the method for delivering drugs to targeted locations utilizing nonencapsulated multiplex additives were reported as a possible remedy (11). Even so such approaches seem to be costly and Uncomfortable for general use. Therefore, a simple, inexpensive, susceptible, and means of intrinsic for improving host health is of critical importance in the modern day. Within this framework, probiotics often function as an addition to the human microbiota, offering defense against a range of intestinal infections. Probiotics also yield encouraging outcomes, such as improved gut barrier function and, in addition, their special capacity to enhance colonization and compete with pathogenic microorganisms for adherence in the gut (12). Probiotics activate particular genes from localized host cells, which in turn stimulate, alter, and regulate the host immune response. As a component of the gut, they even control the release of hormones from the gastrointestinal tract and govern brain activity through bidirectional transmission of neural signals-axis of the brain (13). Through the activation of intestinal angiogenesis via the vascular endothelial growth factor receptor (VEGFR), probiotics have a significant impact on the regulation of both chronic/ acute inflammatory in the mucous lining the intestine brought on by the Inflammatory Bowel Disease (IBD) advancement (14, 15). Probiotics are beneficial in the fight against overweight and obesity and have physiological roles that regulate the microbiota and improve the health of the host environment (16). Fortunately, probiotics might have significant potential for clinical and nutritional applications. Considerable research is still needed to use probiotics for human health, nutrition, and the regulation of various abnormalities.

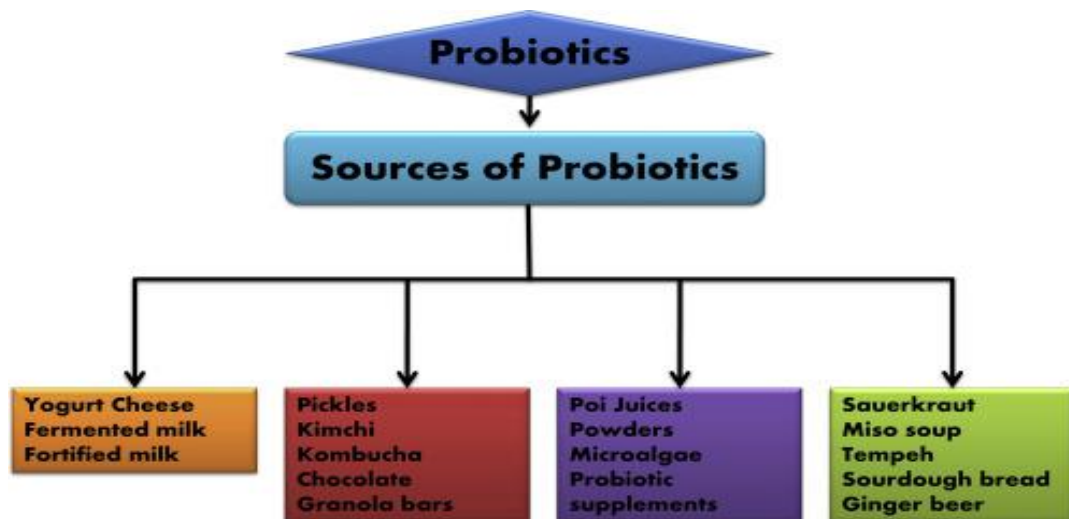


Figure 0.1 Different sources of probiotics retrieved from ([1-s2.0-S1021949818300309-gr1.jpg \(498×292\) \(els-cdn.com\)](#))

1.1.1 Antipathogenic activity of probiotics

The antipathogenic effect is considered among the most useful impacts of probiotics is that, unlike traditional medications, it prevents disruption or alteration of the composition of the complex population of the microbiota of the intestine. The antipathogenic effect of probiotics or probiotic mixtures has been extensively studied (17). They looked at how probiotics affected the *Serovar typhimurium*'s continued existence and *Clostridium difficile*, and *Salmonella enterica* within a vitro model. They hypothesized that Probiotics prevent infections by producing short chain -fatty acids (SCFAs), which include butyric, propionic, and acetic and propionic acids. The SCFAs contribute to the maintenance of a suitable pH within the lumen of the colon, which is essential for the manifestation of many enzymes produced by bacteria and for the metabolism of xenobiotics and the gut carcinogens (18). Islam (11) also pointed out that many probiotics produce variety of antipathogenic compounds such as hydrogen peroxide (H_2O_2), peptides, ethanol, organic acids, diacetyl, and acetaldehydes. Among these compounds, Target cells' membrane permeability is increased by peptides and bacteriocins in particular, which causes the membrane potential to depolarize and eventually results in cell death (19). Likewise, these bacterial groups' production of H_2O_2 causes sulfhydryl group oxidation, which denatures multiple enzymes and peroxidizes membrane lipids. This increases the pathogenic microorganism's membrane permeability,

which ultimately results in the death of the cell (20). Several of these substances could function by decreasing the pH. through organic acids such as lactic and acetic acid (18). Furthermore, to produce antipathogenic bioactive compounds they directly influence pathogens. probiotics also stimulate host antipathogenic defense mechanisms by stimulating or activating the production of defensins, which are cationic antimicrobial peptides that inhibit the entry of pathogens in various cell types including intestinal epithelial cell and paneth cells found in the small intestine's crypts (21).

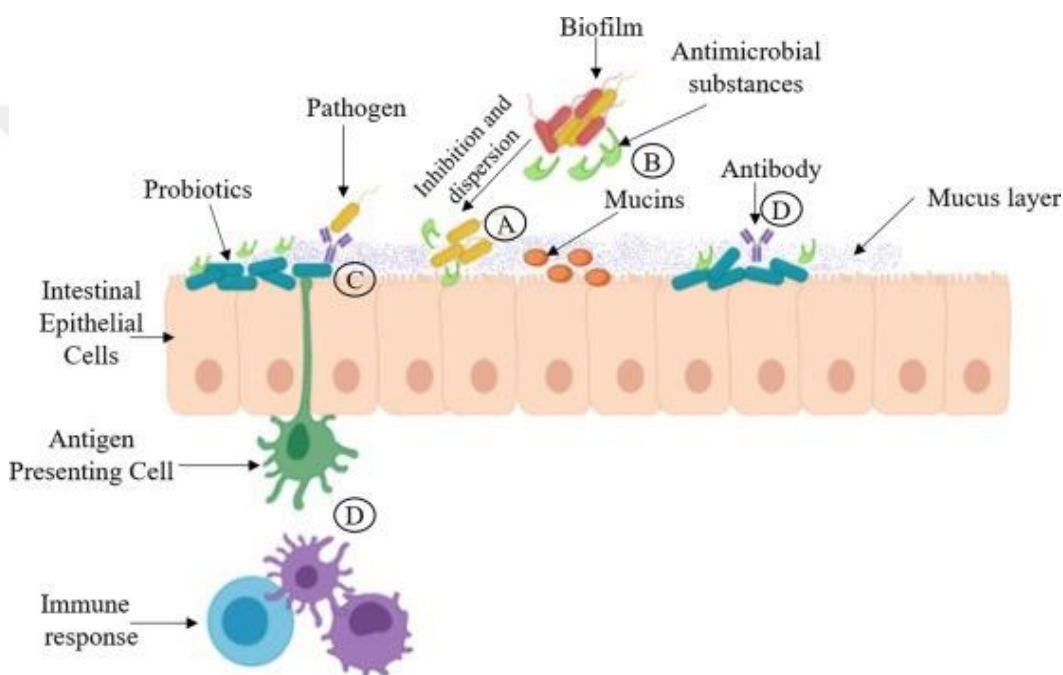


Figure 0.2 Mechanisms of probiotics action. (a- Competitive exclusion for pathogenic microorganism (b- production of the antimicrobial Substances (c) adhesion increased towards the mucosa of intestinal and enhancement for the epithelial barrier (d- immune system stimulation the retrieved from (https://www.researchgate.net/figure/Mechanisms-of-action-of-probiotics-A-Competitive-exclusion-of-pathogenic_fig1_342923972).

1.1.2 Anti-obesity activity of probiotics

Unusual or over accumulation of fat (obesity), which has a direct impact on health is proportional to higher energy ability, more sedentariness and better regulation of body temperature, all of which lead to an unbalance between the intake of energy and consumption (16). It's been demonstrated that transferring the intestinal microbiota of obese mice to germ free animals may mimic the obesity

phenotype, enhance dietary energy extraction, and promote lipogenesis. Probiotics provide physiological actions that benefit the microbiota by modulating the environment of host. In most situations, weight loss is supported by thermogenic/lipolytic reactions that stimulate The system of sympathetic nerves (22). Probiotic strains such as *L. gasseri* BNR17 had been shown to inhibit the proliferation of adipocyte tissue, this is the principal source of adiponectin and leptin, therefore restricting leptin secretion (23). It has been shown that other probiotic microbes, including *L. acidophilus*, *Bifidobacterium longum*, and *L. casei*, exhibit hypocholesterolemia benefits (22).

1.1.3 Anti-cancer activity of probiotics

According to the World Health Organization (WHO) cancer fact sheet (24), cancer is a terrible disease that affects people all over the world, and by 2012, there were about 14 million new cases and 8.2 million cancer-related deaths. More than 70% of the worldwide cancer mortality is on the continents of Asia, Africa, and America (25). In the last decade, intensive cancer research including molecular pathology, proteomics, and genomics has improved cancer knowledge and public awareness. At the same time, many new drugs have been discovered using nano- and biotechnology (nanocapsules) with fascinating luminous properties, but tolerance to their exposure and side effects remains a major limitation. Natural sources that have anticarcinogenic activity, such as probiotics, have come to the fore In the past few years (26). They have attracted the interest of clinical nutritionists and the scientists and industry representatives who want to work together to combat the illness and create a medication that works With low or no adverse effects (27, 28).

In vitro investigations have demonstrated that the probiotic bacteria *L. fermentum* NCIMB-5221 , -8829 are highly effective at inhibiting colon cancer cells while boosting the proliferation of normal epithelial colon cells by producing SCFAs ferulic acid, that capacity was also tested in conjunction with other probiotics, including *L. acidophilus*-ATCC 314 , and *L. rhamnosus*- ATCC 51303 both of them previously defined as possessing tumorigenic activity (29). Two other strains of probiotic, *L. acidophilus* LA102 and *L. casei* LC232, were similarly found to have pronounced cytotoxic activity, exhibiting antiproliferative activity against two colorectal cancer cell lines which are in vitro Caco-2 and HRT-18 (30).

Although probiotics might play an important role in neutralizing cancer, the research is to some extent limited to in vitro testing only. Therefore, Probiotics' ability to prevent cancer must be demonstrated in vivo models and tested in the animal and the clinical trials.

1.1.4 Colorectal cancer (CRC)

The third most frequent intestinal cancer worldwide is CRC (31). Reactive oxygen species (ROS) have been linked to mucosal inflammation-induced intestinal tissue damage, according to studies (32). In IL-10 deficient mice models showed that *L. gasseri* causes excessive superoxide dismutase activity and greatly lessens inflammatory damage, indicating its ability to decrease inflammation and restore gut health. Consequently, anti-oxidant medications including *Lactobacillus* may avert CRC caused by IBD (33). for a model mouse of azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colitis-associated CRC, it was suggested that the probiotic *L. gasseri* 505 and *Cudrania tricuspidata* leaf extract work together synergistically to inhibit the abnormal proliferation and the intestinal tract structural impairment. The development of CRC has been associated with both inflammation and aberrant tumor growth. However, it has been shown that *Lactobacillus* can lessen the expression of mRNA of proinflammatory substances including (TNF, IFN, IL-1, and IL-6), hence reducing inflammation and the risk of cancer. Furthermore, *Lactobacillus* downregulates anti apoptotic markers like Bcl-2 and Bcl-xL while upregulating apoptosis-related markers including p53, p21, and Bax, enhancing apoptosis and having effects of anti-tumor (34) in CRC mouse model produced by AOM//DSS, discovered that *L. acidophilus* lysate mixed with anti ctla-4 antibody suppresses T-cells immunity and has anti-tumor effects. Escamilla and others (2012) showed that *L. casei* and *L. rhamnosus* GG (LGG) inhibit matrix metalloproteinase 9 and zona occludens-1 to prevent metastasis, suggesting that early modification of the microecology of the digestive system may be a viable strategy to enhance the outcome of CRC patients. It has also been discussed elsewhere that *Lactobacillus* may increase an anticancer response and lessen the side effects of chemotherapy (35). Proof from a clinical investigation (n=150) revealed that taking *Lactobacillus* GG supplements is safe and may lessen how often people experience diarrhea and pain in their stomach area (36). In a postoperative clinical trials of CRC patients (n=52), Consuming a supplemented

probiotic containing six live microorganisms including different strains of *Lactobacillus* and *Bifidobacterium* at a 30 1010 CFU concentration was safe and demonstrated to lower the levels of some pro-inflammatory cytokines (exclusive of IFN-), but it did not significantly improve the severity of diarrhea (37). The advantages of the health of *Lactobacillus* in CRC are currently not sufficiently supported by the available research. Furthermore, it is challenging to draw inferences from this research or offer more firm advice on the best course of therapy due to the heterogeneity in the types and dosages of probiotics utilized.

1.1.5 Industrial requirements and technological properties.

Probiotic preparations are becoming considered medical or pharmaceutical merchandise whilst components related to fitness, which include the prognosis of a disorder, prevention, or remedy, are claimed (38). Thus, EFSA has rejected all fitness statements made regarding probiotics, classifying them a food dietary supplements or nutritional dietary supplements (39, 40). In this regard, a selected probiotic must not have unfavorable results on the product's taste or aroma or the growth of the acidity over shelf existence (41-43).

Additionally, the probiotic should live to tell the tale of meal-processing stressors, along with fermentation, freeze drying, harvesting, and versions in temperature and pH, oxidative, and osmotic stress throughout the garage. Finally, from a protection perspective, to prevent pathogenicity or production loss, it is imperative that probiotic cells maintain their genetic stability.

For the probiotic efficacy, populations about 10^6 to 10^8 CFU/g due to the consumption point are required (44). The ratio population could be affected by the bacteriophages, by which infect the probiotic cells, consequently causing cell lysis (45, 46). Selection of bacteriophage resistant probiotics strains could be executed via traditional strategies, which include plaque assays as well as acidification monitoring or through more sophisticated equipment, consisting of qPCR, biosensors, and go with the flow cytometry (45, 47). Furthermore, Products may show changes all through how long they last, along with post acidification, consequently resulting inside the lack of viability of probiotics. The oxygen presence at some stage in some processing and storage also might affect the viability of cells (48). The viable cells controlling may be completed via the

microbiological techniques, together with plating into a traditional cultivation medium or in qPCR the use of dye (Propidium monoazide) (49, 50).

1.1.6 Probiotics as a weapon in the fight against COVID-19

Probiotics increase the immune machine, decorate characteristics of the mucosal barrier, and to inhibit bacterial adherence and the invasion potential in the epithelium of the intestine by using being in a right away antagonism with a pathogenic microorganism (51). The intestine-lung axis contributes to the pathogenicity of the infections caused by bacteria and viruses, just like the microbiota of the intestine boosts the alveolar macrophage pastime, thus having a protecting position in host protection in opposition to pneumonia (52). Along these lines the current medical proof connects the intestine, lung, and brain as a whole with verbal exchange mediated via complicated neural and immunologic inflammatory and the neuroendocrine networks; so referred to as the gut/brain/lung axis (53). There are warning signs in animals additionally human beings that intestinal microbiota offers microorganisms to the lungs. Since the abundance of *Bacteroides* sp. was located within the lungs following sepsis (54). More than ever, following sepsis neurologic and cognitive results are found (55, 56). Without a doubt, the microbiome importance of the gut is said (57). A gut microbiome's makeup might be utilized as a predictive tool for ailment improvement and contamination severity (58-60). Pattern reputation receptors (PRRs) are of foremost importance in developing the response of innate immune (61-63). Probiotics alter the cells of innate immune through interactions among cell wall additives or the metabolites with a host PRRs (sixty-two, sixty-three). Yet probiotic microorganisms are activated. Dendritic Cells (DCs), and macrophages by boosting the adaptive immune response (B mobile differentiation and T cells homing, Th17 cellular stimulation) (64, 65). The expressions of PRRs are inflamed inside the lung cells throughout infection methods. In the following context macrophages and neutrophils and monocytes are reacting by rising degrees of Pathogen-Associated Molecular styles (PAMPs) and Danger-Associated Molecular Patterns DAMPs (66). The effects of probiotics as a possible therapy strategy for COVID-19 are suggested by all the aforementioned factors should probiotics still be administered with caution, especially to patients who are seriously unwell (67). Finally, a few of

them having high levels of enzymatic activity and expressing a CYP enzymes, particularly P450, which may affect the absorption and metabolism of medicines.

In conclusion, for a probiotic to be designated as effective in the prevention or field treatment of COVID-19, a better knowledge of the underlying mechanisms of action of probiotics, definition of the biochemical profile of the mysterious SARS protein E, and clinical studies are required.

1.1.7 Concluding remarks and prospects

Traditionally probiotics had been decided on primarily according to phenotypes strain-resistance that assures their survival via GIT and next patient within the gut; The principal criteria used within most research are those suggested by the WHO, which include host-associated stress resistance and epithelium adhesion potential, and antimicrobial hobby. Using those characteristics, a wide range of microbial species which claimed to be probiotics is currently becoming remoted from unconventional sources and humans. However biological residences answerable for the fitness-promoting outcomes, consisting of anti-carcinogens, antidepressants, antioxidants, and LDL cholesterol-lowering homes, have been in the historical past for much research. Furthermore, safety features for antimicrobial resistance, harmful metabolites manufacturing, and virulence determinants must be addressed in the vivo usage of a prior of recent probiotics, which might be regularly unnoticed.

Recent related technology advances into high-throughput strategies-i.e., genomics, transcriptomics, proteomics, and metabolomics facilitated the improvement of a predictive model for an accurate choice of probiotics. At first vast genome sequencing packages have discovered an exquisite diversification of gene-content within one species of bacteria (68). Strains of Probiotic include genes relevant to acids and bile salts stresses adhesion capability, and antimicrobial substances secretion that were not observed in domesticated, business LAB lines (69, 70). The existence of those genes might be utilized as stable medical evidence in probiotics searching that own the requirement of genetic arsenal for adapting to the intestine milieu, specifically with the rising price reduction of excessive throughput of sequencing analyses. Thus, proteome, transcriptome, and the metabolome profiling may be utilized to evaluates the expressions of those genes beneath different host-related condition for example, proteins to address the poisonous effects of intestine

environment are differently expressed in probiotics traces. Some of the defense mechanisms located in probiotics consist of protein overproduction worried in the energy metabolism and translation and trendy strain responses, lipids metabolism and exopolysaccharide synthesis and reactions of oxidation-discount (71). The transcriptome and even proteome additionally offer a distinct framework for becoming aware of posttranslational adjustments that have an effect on protein functionality and that aren't detected with the aid of different omics strategies (72, 73). Safety evaluation for probiotics could significantly be improved using mechanistic omics equipment's. Safety-related genes, along with antibiotic resistance and harmful production of metabolites, had been stated in certain probiotic lines (74, 75). Even so, in standard, there are not any toxin or hemolysis-encoding genes and safety associated genes which are hardly ever transferable. Furthermore, genomics has revealed best a theoretical hazard level due to the fact gene expression is environmental situations dependent. Thus, genomic statistics should be shown by means of studies in vitro and human medical trials for choice of a correct probiotic. The assays also steps mentioned in this evaluation are extraordinarily useful aids for forthcoming starter lifestyle choice and validation studies (76).

1.1.8 Role of probiotics in human intestinal health

The gut of human is largely inoculated during birth. Microbials variety develops as well as feeding and nutritional patterns mature, and by age from 3–5 years the microbiota resembles that within the grownup (77). Because of versions in pH and substrate concentration and Eh (redox potential and activity of electrons) and time of transit and microbial numbers range among unique anatomical areas of the intestine (78). The belly harbors fewer microorganisms rather than small and big intestines (79). Studies employing metagenomic techniques have shown the complicated interplay Between the two human resident gut bacteria and mammalian metabolisms (78). Through the procedure of fermentation and anaerobic gut microorganism metabolize substrates forming give up merchandise inclusive of natural gases and acids (80). The principal precursors for fermentation which are proteins, dietary carbohydrates and lipids, additionally indigenous secretions which includes mucin; this anaerobic metabolisms contributes undoubtedly towards host every day energy necessities and gut1 homeostasis (81). The humans host should

ideally live in equilibrium with its complicated intestine microbiota in the kingdom that promotes physiological resilience (78). However, dysbiosis can end the outcome of challenges which include medicines, infections and aging, lifestyle, surgical procedures, and bad nutrition (82). In humans, quite a number of acute and continual issues could be an effect of perturbation for microbial communities of the gut (83). On chronic foundation, inflammatory bowel diseases (IBD), weight problems, and irritable bowel syndrome (IBS) have all been connected to the intestinal bacteria and their interest (84, 85). This raises the prospect of microbiota influencing to lessen disorder risk, reinforce homeostasis, and in a few cases improve healing reputation. Diet is a foremost driving force of gut fermentation and Consequently can substantially influence the capability of the native microbiota (78).

1.2 Organism used as probiotics

Numerous bacteria colonize the pores, skin, vagina, oral hollow space, GIT and top animals' respiratory tract and incorporate a surprisingly complex micro-ecosystem of organisms that are in near concord between each other and the host; These resident microorganisms perform useful roles inside the body structure host through digestions and nutrients assimilation, stimulations and maturation of the host tissue, law of host immune response and prevention of pathogenic colonization (86). Among those microbes *Lactobacillus* traces are essential participants of animal microbiomes and humans and have gained huge-unfold interest due to their various outcomes on the health of the host; Because of those properties and lactobacilli or lactic acid microorganism are most of the maximum widely probiotics used, LAB ferment Carbohydrate's to provide lactic acids as the main cease product (87).

The genus *Lactobacillus* which taxonomically related to the phylum Firmicutes, magnificence Bacilli order *Lactobacillales*, and family *Lactobacillaceae*, consists of extra than 170 species of facultative, anaerobic, catalase-poor, Gram-fantastic, non-spore-forming rods (88). For centuries, lactobacilli were used in the manufacturing of fermented meals derived from each animal (milk and meat) and plants (greens and cereal). Besides conventional makes use of in food fermentation, gathering genomic facts keeps increasing our knowledge of the metabolic capacities of lactobacilli. The quantities of lactobacilli determined inside the human GIT

range, depending on the age of the host and area in the intestine and so on. In person feces, lactobacilli bill for simplest 0.01 to 0.06% (10^5 to 10^8 CFU/g) of all bacterial species. The essential indigenous *Lactobacillus* species are *L. gasseri*, *L. reuteri*, *L. crispatus*, *L. salivarius*, and *L. ruminis*. As compared with the grownup microbiota, the infant fecal microbiota is greater enormously unstable and variable, ranging from one hundred and five CFU/g within the feces of neonates to 10^6 – 10^8 CFU/g inside the feces of toddlers and elderly > 1 month. The commonplace isolates in infant feces are *L. plantarum*, *L. salivarius*, *L. rhamnosus*, *L. paracasei*, *L. fermentum*, *L. gasseri*, *L. delbrueckii*, and *L. reuteri* (89). In comparison with the GIT, *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. Vaginalis*, and *L. iners* arise greater frequently in the vagina (90). Moreover, the wholesome lady urogenital tract consists of a massive and solid proportion of *Lactobacillus* spp. that guards against pathogenic infections (91). In this study, we summarize what is currently known about the roles that lactobacilli play in both human and animal health.

Table 0.1 Current microorganisms used as probiotics. This is an adaptation of (Marc o et al., 2006, Lesbros-Pantoflickova et al., 2007; Reddy & Narendara, 2010; Sikorska and Smoragiewicz, 2013, Ouwehand et al., 2016, Markowiak and Śliżewska, 2017 a, Markowiak and Śliżewska, 2017b, Wong et al., 2019).

<i>Lactobacillus</i>	<i>Bifidobacterium</i>	Other bacteria
<i>L. acidophilus</i>	<i>B. bifidum</i>	<i>Saccharomyces boulardii</i>
<i>L. amylovorus</i>	<i>B. infantis</i>	<i>Propionibacterium freudenreichii</i>
<i>L. casei</i>	<i>B. lactis</i>	<i>Enterococcus faecalis</i>
<i>L. crispatus</i>	<i>B. adolescentis</i>	<i>Enterococcus faecium</i>
<i>L. plantarum</i>	<i>B. animalis subsp. lactis</i>	<i>Lactococcus lactis</i>
<i>L. casei Shirota</i>	<i>B. longum R0175</i>	<i>Leuconstoc mesenteroides</i>
<i>L. paracasei</i>	<i>B. breve</i>	<i>Pediococcus acidilacticii.</i>
<i>L. rhamnosus</i>		<i>Sporolactobacillus inulinus</i>
<i>L. reuteri</i>		<i>Streptococcus thermophilus</i>
<i>L. johnssonii</i>		<i>Escherichia coli</i>
<i>L. helveticus</i>		<i>Saccharomyces cerevisiaevarboulardi</i>
<i>L. fermentum</i>		<i>Bacillus coagulans</i>

1.2.1 Probiotic selection criteria

Probiotics are live microorganisms (yeasts or bacteria) that provide a variety of health benefits to humans when consumed in the right amounts. Bifidobacterium and lactic acid bacteria (LAB) species, such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Enterococcus*, are among the recognized probiotic microorganisms with a long history of safe usage (92-94). These microbial communities are resistant to the harsh environments found in the human body, such as low pH, pancreatic juice, and salivary enzymes. colonize intestinal epithelial cells and carry out biological activities to maintain the host environment's health by controlling bacteria (95, 96).

Even though a variety of LAB functions have been used in probiotics fermented foods sold commercially around the world, the biofunctional products market is always in need of new products and product diversification. An increasing number of scientific investigations have used novel selection strains with distinct and unique functional characteristics for this reason. More specialized strains of LAB and other microbial groupings (including *Bacillus* and yeast) are always being discovered. As a safe isolation source of microorganisms for product development, these novel germs are typically isolated from humans. Currently, however, new isolation sources are being used, like waste, fruits, grains, dairy products (97-99). A multi-step, comprehensive strategy is necessary for the identification and assessment of novel probiotic candidates because of the broad range of technology applications and goal functions associated with probiotics (100). The safety and effectiveness of probiotic bacteria were confirmed by international regulations before 2002. Therefore, safety and efficacy criteria for probiotic was created by the Food / Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) in their publication of the "Guidelines for Evaluation of Probiotics in Food (101). Many parameters for choosing probiotics are recommended in this guideline. comprising antibacterial activity, epithelial adhesion ability, safety assessment, and resist to adverse condition imposed by human body. New methods for choosing probiotics have been developed because of mounting data showing the connection between microbes and elements essential to human health and wellbeing. The biological qualities of probiotics, such as their ability to lower cholesterol, fight obesity, treat diabetes, treat depression, and prevent cancer, are

among them. Safety considerations include virulence, toxicity, and the possibility of resistance spreading for antibiotic. This review we offer a thorough theoretical framework to help researchers choose criteria and procedures when selecting probiotic bacteria (102).

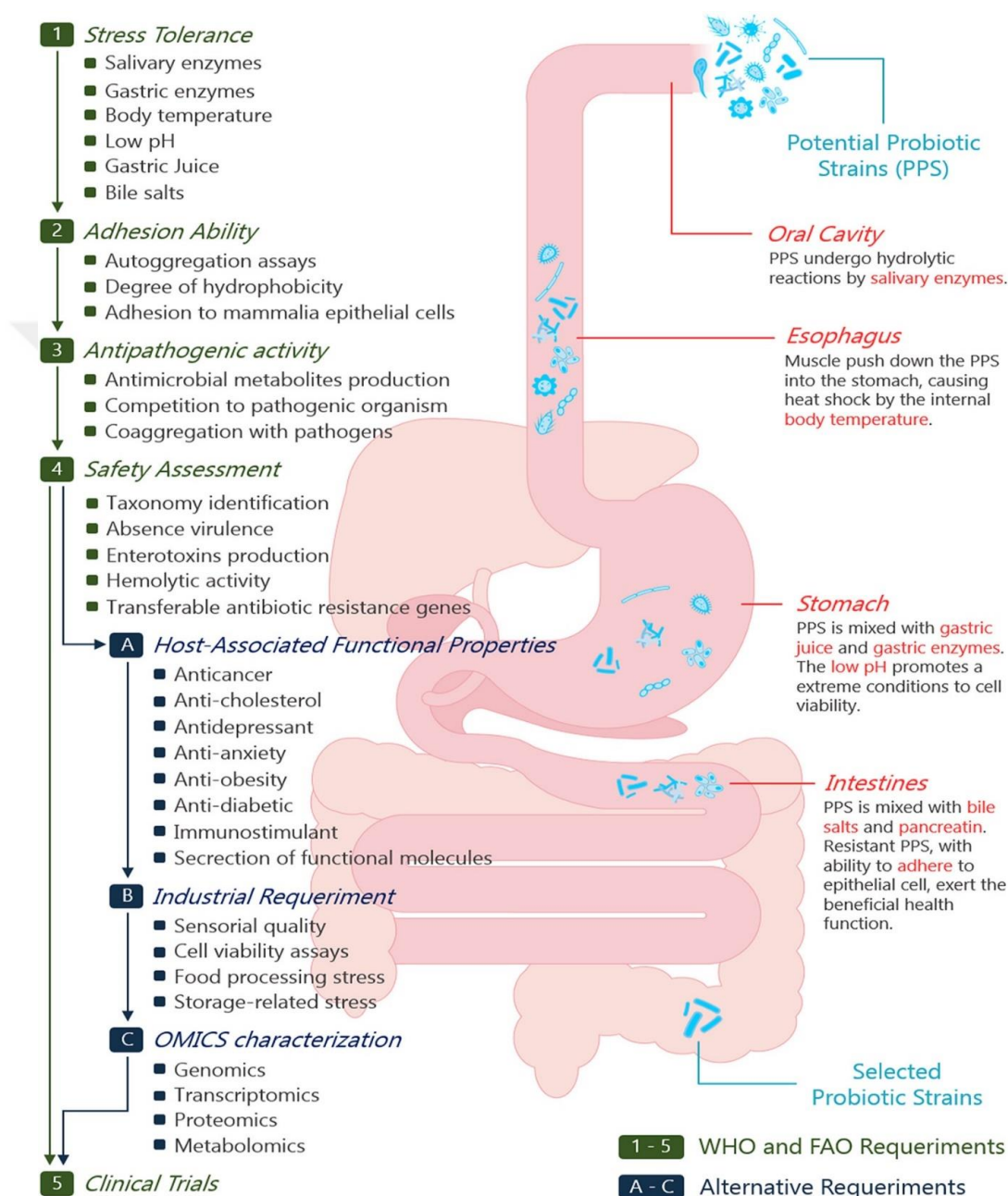


Figure 0. 3 A Screening approaches used for characterization of probiotics strains according to the WHO/FAO requirements (102).

1.2.2 Stress- tolerance ability

Firstly, the candidate lines are assessed to address the stress condition that the human frame imposes at the time of management, the probiotic must be proven against the enzymes gift within the oral cavity, like lysozyme and amylase. Although gram-high-quality microorganisms are commonly touchy to lysozyme some LABs are extra resistant than other gram-superb bacteria and are capable of being a part of the microbiome resident of the oral hollow space of people (103, 104). After ingestion, probiotic cells interact with antimicrobial factors within the belly (low pH and gastric juice and pepsin) and intestines (pancreatin and bile salts), as well as slight warmth surprise due to the inner body temperature. The probiotic ought to have acids and bile tolerance or the exclusion mechanisms to continue to exist in the intestine. Moreover, the accumulation of intracellular loss is one of the excellent-known responses related to the thermo-tolerance of probiotics microorganisms (105). Gastrointestinal resistance varies consistent with species-as an instance, *Lactobacillus* are extensively resistant whereas Bifidobacteria are exceptionally sensitive to lower pH and exhibit lower or no survival fees at pH 2 and pH three (106). For this reason, tolerance to inhibitory conditions usually eliminates an extensive range of isolated microorganisms. From 29 *Lactobacillus* strains that (107) evaluated, the simplest six may want to continue to exist 1 hour at the pH of 1, and 8 strains can't survive with pepsin answer at the pH of 2. A similar result became also suggested in (108) in which from 100 bacterial lines, such as *Streptococcus* and *Lactobacillus* and *Bifidobacterium*, most effective 51 could continue to exist at pH 2. In precis, pH starting from pH 2 to pH 5 and bile salts concentration from zero to three are taken into consideration important for the choice of probiotic microorganisms (109, 110). Such a resistance homes can be examined by means of strain cultivating hobby at a one-of-a-kind pH with the existence of enzymes, including pepsin, lysozyme and amylase, NaCl, phenol, porcine gastric juice, Ovgall, pancreatic, and taurodeoxycholic acid. Resistance to those compounds is determined by the colony remember or absorbance at distinct time intervals (111-113).

1.2.3 Adhesion ability

The following action is to determine whether the capacity probiotic applicants could colonize epithelial cells of the GIT. Microbial adherence on epithelial cells is a complex touch procedure regarding two membranes (i.e., microbial and human cells) that rely upon the chemicals and physiochemical composition of the probiotics pressure cell surface. This behavior relies upon the equilibrium of electrostatics and the Van der Waals interactions at the target surfaces; however, investigations have shown that the components of bacterial extracellular and the encompassing compositions can impact adhesion (114-116). Adhesion of the microbes to the epithelial cells is related to each the auto aggregation ability and hydrophobic houses of the cellular surface; Microbial cellular auto aggregation ability guarantees that the probiotics reach an excessive mobile density within the intestine contributing to the adhesion mechanism, even as cellular floor hydrophobicity permits an advanced interplay between microbes and the human epithelial cells, Auto aggregation could be done via a simple procedure whereas the absorbance of the strain suspension using phosphate-buffered saline is evaluated at various intervals time (117-119).

1.2.4 Safety assessment

There is a long history of safe usage of lactobacilli in food and dairy products (120) Lactobacilli is naturally occurring in both plants and animals, and they are associated with human flora (121). Many cultures throughout the world have long employed lactic acid bacteria in fermented milks to cure intestinal disorders, particularly in young patients (122). In rare cases, LAB can infect humans and cause bacteremia or endocarditis, especially in immunocompromised hosts (123). The group of organisms categorized as "generally regarded as safe" includes lactobacilli (124). Regarding the use of probiotics in people, a few potential negative effects have been brought up (125). These possible hazards include the possibility of transmigration and the possibility of adverse effects, including metabolic and physiological ones, on gastrointestinal physiology and function from probiotic colonization (126-128). Adverse immunologic effects, both localized and generalized, may also occur (126). Furthermore, commensal or probiotic bacteria may transfer their antibiotic resistance to other bacteria or possible pathogens within the gastrointestinal system (127, 129).

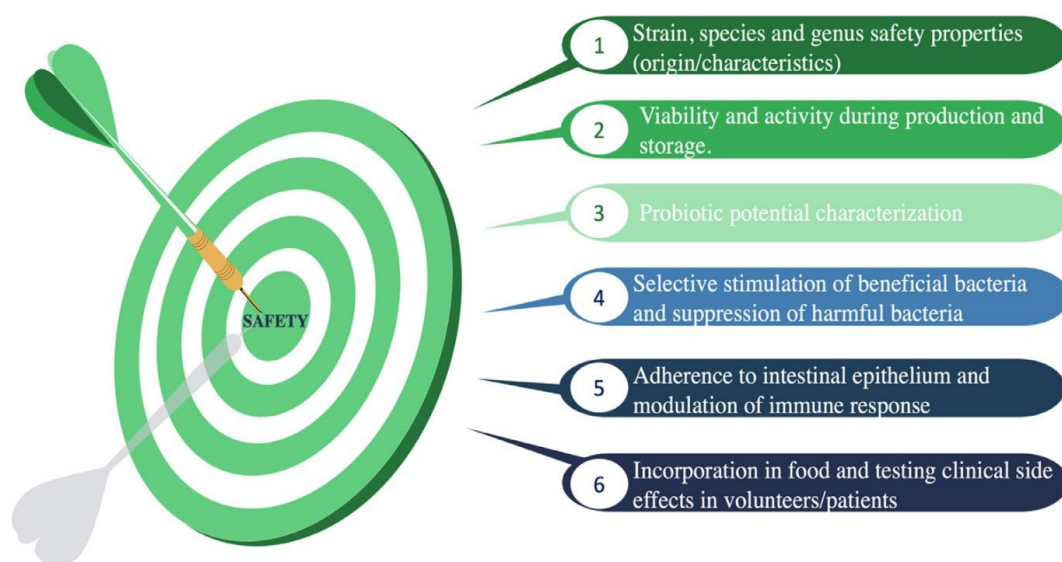


Figure 0.4 General procedures for the safety evaluations of probiotic for application in foods and beverages derived from (https://ars.els-cdn.com/content/image/1-s2.0-S2666833521000034-gr2_lrg.jpg).

1.3 The genus of *Lactobacillus*

Among the many and phylogenetically varied orders of bacteria that produce lactic acid are the *Lactobacillales*, which comprises the genera *Facklamia*, *Granulicatella*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* in addition to the type of genus *Lactobacillus*. They use fermentative methods to use carbohydrates, and one of their main byproducts is lactic acid (130). *Lactobacillus* species are a Gram-positive facultative anaerobic and catalase-negative which are non-spore-forming rods that frequently grow more readily in microaerophilic environments. They can appear as short, fat rods, long, in chains, slender rods, or in palisades, depending upon the Gramme stain morphology. Their grey colonies can range in size from small to medium, and when they grow on blood agar, they typically show alpha hemolysis. Other media, such as Man, Rogosa, and Sharpe (MRS) agar, are also used by lactobacilli to thrive. On these media, the bacteria develop as white, typically mucoid colonies. The molecular method (16S rRNA genes) is used to identify *Lactobacillus* species because phenotypic identification is typically incorrect. Some *Lactobacillus* species, such as *Atopobium* (*A. minutum* and *A. rimae*), *Eggerthia* (*E. catenaformis*), *Olsenella* (*O. uli*), and *Weissella* (*W. confusus*), were incorrectly placed to the genus and have now been reallocated to new or other genera, according to more recent taxonomic procedures. There are around 170 species and 17 subspecies of *Lactobacillus* that have been reported with validity

and are well-established in the nomenclature. Though they can occasionally be opportunistic pathogens, they are native to the human GIT and vagina. Within the digestive system of human's Numerous lactobacilli, such as *L. fermentum*, *L. plantarum*, *L. casei*, and *L. rhamnosus*, have been isolated from the gut and represent a range of ecological niches. From the stomach mucosa, *L. antri*, *L. gastricus*, *L. kalixensis*, *L. reuteri*, and *L. ultunensis* have been isolated (131). Common vaginal isolates include *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. vaginalis*, *L. gasseri*, and *L. iners* (132). The oral and digestive tracts of both humans and animals naturally contain *Lactobacillus acidophilus* (133).

1.3.1 Lactic acid bacteria (LAB)

A new strategy for preventing bacterial and fungal diseases of the digestive tract in humans has been developed because of the development of multidrug-resistant pathogens that have been created by the traditional use of antibiotics. This strategy makes use of safe and natural antimicrobial biomolecules, such as peptides and proteins synthesized in ribosomes and low molecular weight compounds, which are extracted from LABs (134, 135). A class of gram-positive bacteria known as facultative aerobic bacteria (or cocci) that do not produce spores and are thought to be non-pathogenic are known as LABs. These bacteria can be either faculty or anaerobic, able to use carbohydrates as fuel and produce lactic acid as the main byproduct of fermentation (136, 137). Typically, they can be found in feces, decomposing plants and animal matter, a variety of raw materials used to make fermented foods like milk, meat, and wheat, and even in the symbiotic relationship between humans and herbivorous animals in their digestive tracts (138-140). According to reports, LABs have been employed for food and alcoholic fermentation for 6000 years Probiotic LAB strains have been shown to produce a variety of anti-microbial compounds, such as lactic acid, acetic acid, propionic acid, alcohol, diacetyl, and Bacteriocin, which exhibit excellent anti-bacterial, anti-fungal, and anti-viral properties. LAB produces antimicrobial compounds that interact with harmful pathogens' cell membranes, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Helicobacter pylori*, *Candida albicans*, and *Listeria monocytogens*. This interaction inhibits the pathogens' growth by lowering the pH intracellularly and denaturing the

membrane's proteins, which causes the membrane permeability to malfunction (137, 141-145).

Because of this ability, LAB is frequently used as a starting culture in food fermentation operations. It prolongs the shelf life of food by preventing the growth of other hazardous pathogens and contributes to the preservation of food flavor (134, 146, 147).

1.3.2 History of LAB

LABs have been utilized for more than 4,000 years in fermentation procedures to increase the shelf life of various foods. The LABs were initially identified by Carl Wilhelm Scheele when he first isolated lactic acid from sour milk. In 1857, Pasteur discovered that bacteria that cause milk to spoil may be heat-killed by boiling the milk. He also established that the bacteria were responsible for lactic acid generation. The first firm to commercially create lactic acid using fermentation techniques was M/s Clinton Processing Company (USA) in 1881. Around the turn of the 20th century, unknown microbial mixtures were used in Denmark to create the first commercial starter cultures from raw milk. In the 1940s and 1950s, the concept of employing pure single-strain starter cultures, made up of LAB, first emerged and was made commercially available. Researchers noticed that LAB were being used extensively after the 1980s in biomedicine, food preservation, food processing, fermentation, and animal husbandry (148-150).

1.3.3 Classification of LAB acid bacteria

Additionally, LAB has been divided into several genera and species based on the formation of acids through the fermentation of sugars and their development at temperatures (151, 152). The traditional method of classifying LABs relied on physiological and biochemical traits, but more recently, molecular characterization has emerged as a key tool for LAB identification and classification. Random amplified polymorphic DNA profiling, 16S rRNA gene sequencing, PCR-based fingerprinting, soluble protein patterns, and species separation by multiplex PCR test by employing unique recA-derived primers are all examples of molecular characterization (153, 154).

1.3.4 Homofermentative and heterofermentative

Based on their capability to ferment carbohydrates, lactic acid microorganisms are labeled as either homofermentative or heterofermentative organisms (155). *Lactococcus* and *Streptococcus* are homofermentative LAB that produce molecules of lactate from an unmarried glucose molecule, whereas *Leuconostoc*, *Wiessella*, and some lactobacilli are heterofermentative lactic acid microorganisms that produce lactate, carbon dioxide, and ethanol from a single glucose molecule (155).

1.3.5 Taxonomical classification

In the most current taxonomic classification, the LAB come below the Firmicutes Phylum; class Bacilli and order *Lactobacillus* which are created from numerous genera; however, the foremost genera consist of *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Weissella*, *Streptococcus*, *Enterococcus*, *Melissococcus*, *Leuconostoc*, *Lactosphaera*, *Oenococcus*, *Carnobacterium*, *Vagococcus* and, *Tetragenococcus*. Other genera consist of *Aerococcus*, *Microbacterium*, *Propionibacterium*, and *Bifidobacterium* (154, 156, 157). However, the largest genus in this group is *Lactobacillus* and it includes greater than eighty identified species such as *Acidophilus*, *Lactobacillus*, *Lactococcus*, *Lactis cremoris*, *L. plantarum*, *Bifidobacterium bifidum*, *L. caseia*, *Lactobacillus rhamnosus*, and *L. bulgaricus*, toddler *Bifidobacterium*, LAB, *Bifidobacterium adolescentis*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Enterococcus faecalis*, and *Enterococcus faecium* (158).

1.3.6 *Lactobacillus reuteri*

According to WHO, the probiotics are a "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Although probiotics have long been thought to provide health advantages, interest in them has grown dramatically in recent years (159). This could be partly attributed to the rise of antibiotic resistance, especially when treating gastrointestinal disorders, and the growing public demand for natural health promoters. *Lactobacillus* species, *Bifidobacterium* species, *Saccharomyces boulardii* and *Propionibacterium* species, *Streptococcus* species, *Bacillus* species and *Enterococcus* species, and

certain strains of *Escherichia coli* are among the probiotic microorganisms that have been demonstrated to have advantageous qualities (160).

L. reuteri, one species of *Lactobacillus*, has several positive benefits on host health, including the prevention and/or treatment of a variety of illnesses. 1962 saw the first isolation of *L. reuteri*. It has been described as a heterofermentative species that inhabits the gastrointestinal tracts of both humans and animals and develops in environments with low oxygen levels (161). Its probiotic qualities may stem from the fact that it typically colonizes the GI tract. This organism has been demonstrated to secrete antimicrobial intermediates, can tolerate a broad range of pH conditions, and uses several strategies to effectively suppress pathogenic microbes (162, 163). According to research, *L. reuteri* is one of the GI tract's genuinely native bacteria (164). It naturally settles on a variety of animals, such as rats, chickens, and pigs. Indeed, via long-term evolution, it has become more diverse into lineages that are adapted to their hosts (165, 166). Most often, this bacterium is discovered in the host's proximal digestive tract (167). Numerous research investigations have evaluated the organism's safety in adults and children, newborns, and also in HIV-infected communities (168). The findings demonstrated that 2.9×10^9 colony-forming units (cfu)/day were still safe, effective, and well tolerated in humans. Numerous publications listing the advantages of *L. reuteri* as a probiotic have also been written. These advantages include promoting health, lowering infections and improving the feed tolerance, boosting the absorption of minerals, nutrients and vitamins, modulating the host responses immune, and enhancing gut mucosal integrity (169).

Table 0.2 The scientific classification of the *L. reuteri* (strain retrieved from (ITIS - Report: *Lactobacillus reuteri*)).

Classification of <i>Lactobacillus reuteri</i>	
Kingdom	<i>Bacteria</i>
Subkingdom	<i>Posibacteria</i>
Phylum	<i>Firmicutes</i>
Class	<i>Bacilli</i>
Order	<i>Lactobacillales</i>
Family	<i>Lactobacillaceae</i>
Genus	<i>Lactobacillus</i>
Species	<i>Lactobacillus reuteri</i>

1.4 *L. reuteri*'s probiotic characteristics

Potential probiotics need to meet a few requirements to prevail, such as the capacity to undergo lower pH and surrounding rich in enzymes, sticking to the epitheliums to interact with the host probiotics, competing with pathogenic microbes, and maximum crucially, protection (170). All these conditions are glad through *L. reuteri*. Here, extra probiotics qualities. of *L. reuteri* that guide its range of beneficial effects on host health and infection prevention and/or ameliorations are highlighted (171).

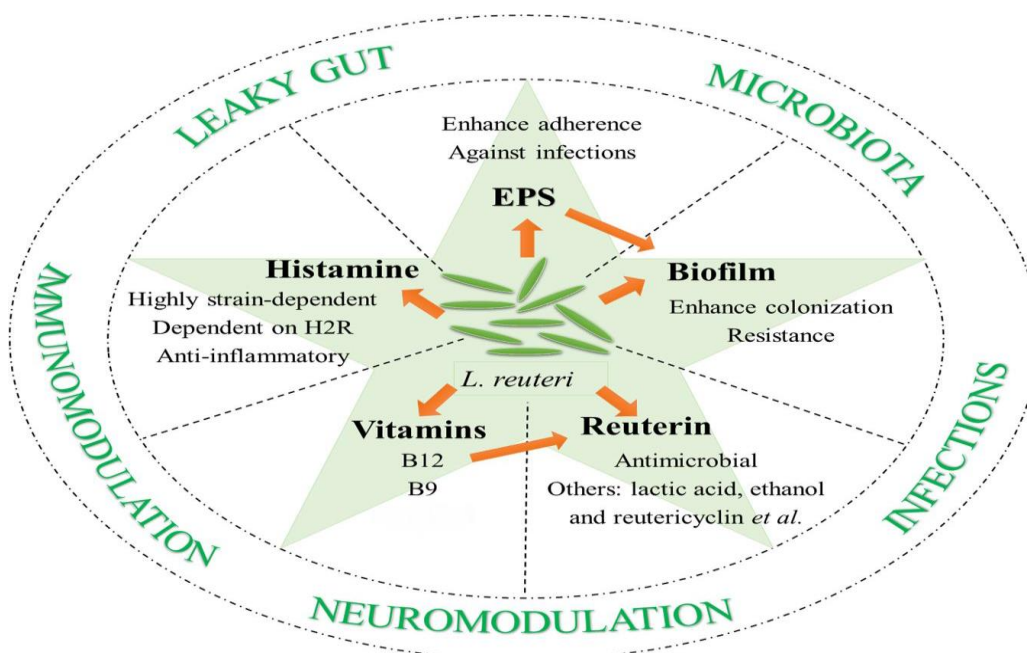


Figure 0.5 Probiotic properties of *L. reuteri* retrieved from (https://www.frontiersin.org/files/Articles/315828/fmicb-09-00757-HTML/image_m/fmicb-09-00757-g001.jpg).

1.4.1 *L. reuteri* gut colonization

Some areas of the GI system, which were designed for digestion and absorption, have turned out to be difficult for microbial colonization. Instances of this include the low pH conditions in the upper small intestine brought on by the stomach acids and the bile salts. So, surviving in such settings is the first step in colonizing the GI tract. Several *L. reuteri* strains are immune to bile salts and low pH (172, 173). This resistance is thought to depend at least in part on the formation of biofilms (174). In addition to mucin and intestinal epithelia, several strains of *L. reuteri* may also cling to gut epithelial cells in a variety of vertebrate hosts (175, 176). The binding of the bacterial surface molecules to the mucus layer is one potential route for adhesion. As so-called adhesions, mucus-binding proteins (MUBs) and MUB-like proteins, which are expressed by clusters of orthologous protein-coding genes peculiar to the Lactobacilli genus, operate as adhesion mediators (177-179). Significant correlations exist between the variety in cell-surface MUB abundance and substantial diversity of the MUBs found across *L. reuteri* strains (180) and their capacity to bind mucus. It is possible to understand how MUBs affected *L. reuteri*'s adherence by considering their strain-specific roles in identifying mucus components and/or their capacity to promote aggregation. Several major surface proteins are among the factors that mediate the adhesion to the surface (181, 182).

Studies have been conducted on the control of *L. reuteri* biofilm secretions and its relationship with the adhesion of bacteria to host GI epithelium because *L. reuteri* which has colonized GI tract of the host can produce biofilms. The role of GtfA and Inu in the biofilm production of *L. reuteri* TMW1.106 was discovered by Water, J. et al. using an in vitro biofilm experiment (184). The origin of the strains' hosts appears to have an impact on how well *L. reuteri* biofilms develop in vivo. Nine distinct *L. reuteri* strains were isolated from the human and mouse, rat, and chicken, and pig hosts were fed to germ-free mice in one research, and the biofilms were assessed after two days. Even though the luminal populations among the various strains were comparable, it is interesting to note that only the rodent strains were capable of forming biofilms and adhering to the epithelium forestomach (167). The SecA2-SecY2 system was a unique transport channel found only in the rodent and porcine strains, according to another investigation by the same scientists (182). They contrasted extracellular and cells wall-associated protein between wild-type

strain and secA2 mutant using a mouse strain of *L. reuteri* 100-23. The secA2 mutant was deficient in only one surface protein, *L. reuteri* 70902. The lack of *L. reuteri* 70902 results in virtually entirely abolished biofilm development, according to in vivo colonization tests. This clearly implies that *L. reuteri* 70902 and the SecA2-SecY2 system play important roles in controlling the formation of *L. reuteri* 100-23 biofilms in germ-free mice (167). Another team looked at the function of the two-component systems bfrKRT and cemAKR in the development of *L. reuteri* 100-23 in vitro biofilms (186). They discovered that operon genes might be deleted to increase biofilm development and adhesion. The role of the bfrKRT and cemAKR in the production of in vivo biofilms has yet to be fully understood. *L. reuteri* 100-23 was also used to study how exopolysaccharide (EPS) aids in colonization. A mutation in the fructosyl transferase (ftf) gene resulted in the elimination of EPS synthesis (187). The colonization of the ftf mutant in the forestomach and cecum following administration to *Lactobacillus*-free mice was significantly reduced in comparison to the wild-type strain. This suggests that strain 100-23's capacity to colonize the gut may be improved via EPS synthesis. Interestingly, it has been shown that *L. reuteri* RC-14 may ingest mature *E. coli* biofilm and integrate into it (188). Recently, *L. reuteri* was administered as a biofilm on a microsphere, and it was discovered that this administration encouraged *L. reuteri*'s adhesion to the intestinal epithelium and improved its probiotic properties (189, 190).

1.4.2 Reuterin

L. reuteri strains of humans and poultry ancestry may generate and excrete the majority of reuterin, a well-known antibacterial chemical (191). Numerous types of 3-hydroxypropionaldehyde (3-HPA) can be discovered in reuterin (192). *L. reuteri* is well regarded for its ability to metabolize glycerol to create 3-HPA through a coenzyme B12 required mechanism (193, 194). Furthermore, it is known that certain other bacterial species generate 3-HPA (195). The capacity of *L. reuteri* to manufacture and secrete 3-HPA in a way that goes beyond what is necessary for bioenergetics makes it special (196). Additionally, 3-HPA appears to spontaneously transform into the cytotoxic electrophile acrolein, which is necessary for reuterin's antimicrobial action (197). Reuterin can suppress a variety of microorganisms, mostly Gram-negative bacteria (198). Unsurprisingly, most *Lactobacillus* species

are reuterin resistant, with *L. reuteri* strains exerting the highest resistance reuterin not only has antibacterial properties but also can conjugate heterocyclic amines, which appear to be depend on the production of the acrolein (199). This indicates acrolein is a crucial substance for reuterin's function. In addition to reuterin, numerous additional antimicrobial compounds produced by some *L. reuteri* strains have been identified, including lactic acid, acetic acid, ethanol, and reutericyclin (200-202). It's been demonstrated that *L. reuteri* is effective for a range of gastrointestinal bacterial illnesses with the manufacture of those compounds. *Clostridium difficile*, *Salmonella*, *E. coli*, and *Helicobacter pylori* are among these illnesses (203-206). The use of *L. reuteri* to treat *H. pylori* is one of the most known examples of how effective it is as a probiotic against infections. A key contributor to peptic ulcers and chronic gastritis, *H. pylori* infection also increases the chance of developing stomach cancer (207-209). Numerous research has investigated the effectiveness of *L. reuteri* against *H. pylori* (Table 1.3). It has been hypothesized that *L. reuteri* inhibits *H. pylori*'s ability to bind to glycolipid receptors by competing with it (210). The competition reduces the *H. pylori* bacterial burden and the associated symptoms (211, 212). *L. reuteri* has the capacity to entirely remove *H. pylori* from the gut, according to certain research (213). A crucial point is that *L. reuteri* is helpful in the treatment of *H. pylori* since supplementation eliminates the virus without generating the typical side effects connected with antibiotic therapy (214).

Table 0.3 Clinical efficacious of *L. reuteri* against *H. pylori* retrieved from (https://www.frontiersin.org/files/Articles/315828/fmicb-09-00757-HTML/image_m/fmicb-09-00757-t001.jpg)

Strain	Treatment	Subjects	Result	Citation
DSM 17648	14 days	Adults	Decrease in pathogen load in the stomach	Holz et al., 2015
DSM 17938	20 days	Patients	93% successful eradication of the pathogen with inhibitor-tetracycline-metronidazole – <i>L. reuteri</i> therapy	Dore et al., 2016
ATCC 55730	10 days	Infected children	Improvement of GI symptoms	Lionetti et al., 2006
–	7 days	Patients	No improvement of the standard triple therapy	Scaccianoce et al., 2008
ATCC 55730	4 weeks	Patients	Significant decrease of pathogen load and improvement of dyspeptic symptoms	Francavilla et al., 2008
SD2112	4 weeks	Patients	Decrease of pathogen density and suppression of urease activity	Imase et al., 2007
DSMZ 17648	14 days	Patients	Decrease in pathogen load	Mehling and Busjahn, 2013
DSM 17938, ATCC PTA 6475	During therapy	Patients	Reduction of antibiotic-associated side effects in eradication therapy	Francavilla et al., 2014
DSM 17938	8 weeks	Patients	Decrease of urease activity in pantoprazole therapy	Dore et al., 2014

The effectiveness of *L. reuteri* against viruses and/or fungus has been the subject of much investigation. There is proof that *L. reuteri* protects against coxsackieviruses, rotaviruses, pneumoviruses, and papillomaviruses (215). According to some theories, *L. reuteri* reduces viral infection via controlling the microbiota and secreting compounds with antiviral properties (216). Additionally, some research indicates that *L. reuteri* may also possess antifungal capabilities, whereby it competes with, halts the growth of, and ultimately eliminates several species of *Candida* (217).

1.4.3 Histamine

Several lines of *L. reuteri* may rework the dietary amino acid L-histidine into the biogenic amine histamine (218). *L. reuteri* 6475, a human commensal bacterium, served as the histamine research pressure in *L. reuteri*. According to J. Versalovic's crew, histamine from *L. reuteri* 6475 inhibited the era of tumor necrosis aspect (TNF) by means of activated human monocytes (219). Histamine H2 receptor activation protein kinase A and extended intracellular cAMP ranges, and inhibition MEK/ERK signaling have been All need for this inhibition. Full *hdc*

chromosomal histidine decarboxylase gene cluster, which is composed of *hdcA*-*hdcB*, -and *hdcP*, which controls synthesis of the histamine and the consequent in vitro of TNF-suppressive movement (219, 220). The equal team of researchers located that oral treatment of *hdc*⁺ *L. reuteri* may additionally efficiently lessen intestinal irritation in the mouse colitis version generated through trinitrobenzene sulfonic acid (TNBS) (218). Furthermore, animals given TNBS also observed a similar reduction of colitis after being injected intraperitoneally of *L. reuteri* 6475 lifestyle supernatants. These findings surely endorse that *L. reuteri* metabolites, along with histamine, are involved within the regulation of intestinal immunity (221). Further studies discovered that the manufacturing of the *hdc* gene clusters in *L. reuteri* ri6475 required the presence of a gene referred to as *rsiR* (222). Reduced TNF inhibition in vitro and reduced anti-inflammatory efficacy *in vivo* have been the consequences of the *rsi R* gene being inactivated. Additionally, a gene called *folC2* appears to be worried in controlling each the in vivo anti-colitis actions and the in vitro TNF suppression(221). The *hdc* gene cluster turned into suppressed and histamine synthesis decreased because of the *folC2* gene's inactivation. Notably, *L. reuteri*'s potential to supply histamine may be very stress-structured, and maximum of the studies have been on traces with human foundation (223).

1.4.4 Vitamins

Thirteen essential vitamins are necessary for human health because the human body cannot generate them (224). Like numerous other *Lactobacillus* species, some strains of *L. reuteri* possess the ability to synthesize a range of vitamins, including vitamin B9 (folate) and B12 (cobalamin). Since glycerol needs to be converted to 3-HPA, coenzyme B12 is necessary for the synthesis of reuterin (225), With more research, its demonstrated that at least four different strains of *L. reuteri* generate B12 (226). The strains *L. reuteri* CRL1098 and JCM1112 have been the subject of most studies (227, 228). When B12-deficient pregnant female mice were administered *L. reuteri* CRL1098 together with is a diet lacking in vitamin, it was shown that the mice and their offspring had diseases (229). This provides strong evidence in favor of using *L. reuteri* to treat B12 deficiency. In addition to producing vitamin B12, several strains of *L. reuteri*, including *L. reuteri* 6475 and *L. reuteri* JCM1112, can also create folate (225).

1.4.5 Exopolysaccharide (EPS)

The EPS produced by the organism is vital for developing of biofilms and *L. reuteri* adherence to epithelial surfaces (230). Furthermore, EPS made by *L. reuteri* may inhibit *E. coli*'s in vitro adhesion to pig epithelial cells (231). More importantly, the proinflammatory cytokine (IL-1 and IL-6) that are produced when an *E. coli* infection occurs are also inhibited by EPS-mediated suppression of adhesion. More in vivo experiments conducted on pigs yielded similar results. Proving that EPS made from *L. reuteri* decreased piglet diarrhea caused by bacterial infection by preventing *E. coli* from adhering (232). Furthermore, when EPS of *L. reuteri* origin is present, enterotoxigenic *E. coli* have been shown to not adhere to porcine erythrocytes (233). It has also been demonstrated that *L. reuteri* 100-23 EPS from rodents stimulates Foxp3⁺ regulatory T (Treg) cells in the spleen (187).

2. AIM OF THE STUDY

In view of current worries concerning the widespread and careless use of antibiotics for disease treatment, the promotion of livestock growth, and the emergence of Pathogens resistant to antibiotics, the use of probiotics and their antimicrobial metabolites as an alternative antimicrobial strategy for infections' treatment and prevention has gained increasing interest. Probiotics, or LAB, are essential microorganisms. that are part of the gut microbiota of humans and other animals. To achieve this goal, *L. reuteri* was put through multiple tests to look at its probiotic qualities, such as its resistance to acid, antipathogenic activity, ability to produce acid, and characteristics of its cell surface, such as co- and auto-aggregation and antibiotic susceptibility.

Using numerous primers, a polymerase chain reaction (PCR) assay, cloning of the PCR amplicons, using a cloning vector, and in-silico analysis are the methods used to screen for BSH-coding genes.

This work aims to contribute to the scientific community's efforts to advance the advantages of *Lactobacillus* bacteria and offer a probiotic with superior probiotic qualities.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains and growth conditions

The BAIBU/Biology Department stock culture provided the plasmid DNA and all the bacterial strains utilized in this study. The stock cultures of bacteria and plasmids were kept in 30% glycerol at -80°C. Table 3.1 and Table 3.2 included a list of the plasmid DNA, bacterial strains, and growing conditions. This study employed the human-originated *L. reuteri* strain.

Table 3.1 Bacterial strains and plasmid

Bacteria	Genotype	Strain	Growth media	Temp	Time
<i>E. coli</i>	rec A end A 1 gyr A986 thi 1hsdr17supE44 rel A1 lac	XL1-Blue1	LB	37°C	16-18h
Plasmid	Genotype/Phenotype	Strain	Growth media	Temp	Time
pBluescript	SKII+/Ampr	XL1	LB	37°C	16-18h

XL1-Blue was obtained from Stratagene, USA.

pBluescript was obtained from Thermo Scientific (Fermantas, Europe).

Table 3.2 Bacterial strains and plasmid

Bacteria	strain	Growth media	Growth conditions	
			Temp	Time
<i>Lactobacillus reuteri</i>		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>	ATCC1311	NA/PCA	37°C	24h
<i>Listeria monocytogenes</i>	RSKK	NA/PCA	37°C	24h
<i>Yersinia enterocolitica</i>	ATCC23715	NA/PCA	37°C	24h

3.1.2 Chemicals and Enzymes

Appendix A contains a list of the compounds and enzymes utilized in this Study along with their suppliers.

3.1.3 Growth media and Buffers

Appendix B lists the growth medium preparations utilized in this Study, which comprised de MRS, Nutrient Agar (NA), Plate Count Agar (PCA), and Luria Bertani (LB) media. Appendix C also contains a list of the buffers, the solution, and how they were prepared.

3.2 Method

3.2.1 Characterization of *L. reuteri*

3.2.1.1 Acid tolerance

The survival and viability of probiotic bacteria are the most crucial conditions for them to carry out beneficial functions. Probiotics encounter several stresses that have an impact on their viability as they pass through the GIT. Probiotics are therefore chosen based on their ability to withstand various pH levels. *L. reuteri* acid tolerance was evaluated using a modified version of the methodology outlined by Li et al. and El-Sayed et al (234). Using 1M HCl and 1M NaOH, the MRS broth was changed to pH values of pH 2, pH 3, pH 4, pH 5, and pH 6 to evaluate the acid tolerance with different pH points. The control tube had normal MRS broth. Anaerobic growth of *L. reuteri* was conducted for 24 hours at 37°C on an MRS plate. Next, during 16–18 h at 37°C under anaerobic conditions, a colony from the streaked plate was injected in 5 ml of MRS broth.

5 ml of MRS broth with varying pH values was mixed with 100µl of the overnight developed culture (2%) and incubated for 6 hours at 37°C. The spectrophotometer was used to measure OD₆₀₀ during hours 2, 4, and 6. To track the growth rate, the bacteria were re-suspended in regular MRS broth (2%) at the 6-hour point and left for two hours. Log¹⁰ (cfu/ml) was used to determine the findings that were obtained.

3.2.1.2 Antimicrobial activity against pathogens

The Kirby-Bauer technique was utilized to evaluate the antibacterial activity of *L. reuteri* in a nutritional agar medium (235). The bacteria were cultured in 4 milliliters of MRS broth at 37°C without oxygen for a whole day. The pellet was then separated by centrifuging the mixture for 5 minutes at 6000 rpm, and the resulting cell-free supernatant (CFS) was filtered through a 0.22µm Millex syringe filter. After incubating at 37°C for 45 minutes, the pellet was lysed using 200µl of lysis buffer (Lysozyme (30 mg/ml), Tris-HCl (1 mM), EDTA (50 mM), and Triton

100X 1.2%). The mixture was then centrifuged for 5 minutes at 13000 rpm. The antibacterial activity was evaluated using both the pellet and the supernatant.

Five pathogenic bacteria, namely *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* ATCC13311, *Listeria monocytogenes* RSKK, and *Yersinia enterocolitica* ATCC23715, were used to assess *L. reuteri* antipathogenic activities. Four of the pathogenic bacteria were cultivated on nutritional agar (NA) plates and incubated for 24 hours at 37°C, whereas *Yersinia enterocolitica* was incubated for 30°C. Next, using a cotton swap, the pathogenic bacteria were dispersed on nutritional agar plates after being inoculated into 5 milliliters of nutrient broth (Neogen, UK). The CFS and the lysed pellet of *L. reuteri* were impregnated into blank discs (Oxoid, UK) after the concentration was adjusted to 0.5-0.7 OD₅₇₀. The discs were then put on the plate containing each pathogen. Subsequently, the plates were incubated for a whole day at four different temperatures: 37°C for four of the plates and 30°C for *Y. enterocolitica*. Using a millimetric ruler, the inhibition zones were measured.

3.2.1.3 Acidifying activity

Using a pH measurement, the acidifying action was examined (236). After being cultured in MRS broth under anaerobic conditions for 24 hours at 37°C, *L. reuteri* was injected at a rate of 50 µl/5ml into skim milk. A pH meter was used to measure the acidity following 8, 10, 24, and 48 h of incubation at 37°C.

3.2.1.4 Cell surface properties

3.2.1.4.1 Cell auto-aggregation

With minor adjustments, the procedure outlined by Collado, and colleagues (234) was used to evaluate the auto-aggregation of *L. reuteri*. In MRS broth, *L. reuteri* was cultivated anaerobically for the whole night at 37°C. centrifuged for 15 minutes at 4000 rpm and 4°C, after which the sample was rinsed twice with phosphate-buffer saline (PBS) at a pH of 7.4. The sample was then suspended in PBS buffer and gently vortexed. After mixing 3.9 ml of PBS buffer with 100µl of *L. reuteri* culture, the tubes were incubated at 37°C. A spectrophotometer (OD₆₀₀) was used to measure the top layer of the solution at the start of each incubation hour (1, 2, 3, 4, 5h). Following each measurement, a new 3.9 ml PBS buffer was filled

with 100µl of the bacterial solution's top layer. The following formula was used to calculate the auto-aggregation:

$$\text{Auto-aggregation\%}=[1-\text{At}/\text{A0}] \times 100$$

At: stands for the absorbance at times 1, 2, 3, 4, and 5 hours.

A0 stands for absorbance at time 0.

3.2.1.4.2 Cell co-aggregation

With a few modifications, the co-aggregation was examined utilizing the Li et al. approach (234). As stated in section 3.2.1.4.1, a broth culture of *L. reuteri* bacterium was created. *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* (ATCC13311), *Listeria monocytogenes* RSKK, and *Yersinia enterocolitica* (ATCC23715) were the five pathogenic bacteria that were cultured for 16–18 hours at 37°C in a nutrient broth, except for *Yersinia enterocolitica*, which was grown at 30°C. After adding 100µl of pathogenic bacteria and 100µl of *L. reuteri* to 3.8ml of PBS buffer, the tubes were incubated at room temperature. Using a spectrophotometer (OD₆₀₀), the absorbance of the samples was measured using the top layer of the solution at times 0, 2, 4, 6, and 24 hours. Following each measurement, 100µl of the bacterial solution was transferred to 3.9 ml of fresh PBS buffer. The computation formula that is employed is:

$$\text{Co-aggregation\%}=[((\text{Apro}+\text{Apat})-\text{Amix})/(\text{Apro}+\text{Apat})] \times 100$$

In this formula, Apro stands for the probiotic bacteria's absorbance at time zero. The absorbance of pathogenic bacteria at time 0 is shown by Apat. Amix is a representation of the mixture's absorbance for pathogenic and *Lactobacillus* bacteria at periods 0, 2, 4, 6, and 24 hours.

3.2.1.5 Antibiotic susceptibility

Using the disc diffusion technique, *L. reuteri* resistance to antibiotics was assessed (234). The bacteria were cultured anaerobically in nutritional broth for a whole night at 37°C. After that, the concentration was adjusted with a spectrophotometer to 0.5-0.8 at OD₅₇₀, and the bacteria were streaked by cotton swab on a nutrient agar plate. The antibiotic discs (Oxoid, UK) containing ampicillin (10µg), erythromycin (15µg), kanamycin (30µg), and neomycin (30µg)

were placed on 31 plates. The plates were incubated for twenty-four hours at 37°C. The widths of the inhibitory zones were measured using a millimeter ruler and classified as susceptible (S), intermediate (I), and resistant (R) based on the recommendations provided by the Clinical and Laboratory Standards Institute (CLSI).

3.2.1.6 Statistical analysis

The results of the characterization tests were expressed as means with standard deviation (SD), and the means SD were calculated by running each test three times. Turkey's HSD test and a one-way ANOVA were used to analyze the probiotic characterization test findings to evaluate the data's statistical significance ($p < 0.05$).

3.2.2 Bile salt hydrolyses (BSH) coding gene screening.

One colony from a previously streaked MRS plate was inoculated into 4 ml of MRS broth, and the mixture was then incubated in anaerobic conditions at 37°C for 24 hours. Additionally, 2% of the first broth was inoculated into 1.5 ml of MRS broth, which was then incubated in the same conditions for 18 hours. Finally, the broth culture was centrifuged for 5 minutes at 6000 rpm. This process produced the *L. reuteri* pellet. After removing the supernatant, DNA was isolated using the pellet. Centrifugation was used for 10 minutes at 5000 rpm to harvest bacterial cells (2×10^9 cells/ml) and wash the *L. reuteri* pellet in MRS broth to separate the genomic DNA. The DNA was isolated by using the GeneJET™ genomic DNA purification kit (Thermo Scientific) methodology.

3.2.2.1 DNA amplification by PCR

Two distinct primers provided by Oligomer were used to test for BSH genes in *L. reuteri* (Table 3.3). Table 3.4 and Table 3.5 illustrate the optimized PCR reaction settings for each primer, which included a final volume of 50 µl, dNTP mix (5 mM), MgCl₂ (25 mM), *L. reuteri* template DNA, the forward and reverse for each primer (25 µM), with the proofreading-active enzyme i-pfu™ DNA polymerase (INTRON biotechnology) and its enhanced buffer (10X) utilized in this procedure. The PCR results were then processed with ethidium bromide (EtBr) for visualization using an ultraviolet (UV) light transilluminator, and the results were visualized using agarose gel electrophoresis (0.8%) for 1 hour at 75 volts.

Table 3.3 Primers that are used to amplify the target BSH gene.

Primers	Nucleotide sequence	Primer length	Tm
REU BSH F1	ATGTGCACATCAGTAAT	F-17bp	54°C
REU BSH R1	TTAATTTTGGTTGGTTAATTATTTG	R-24bp	53°C
REU BSH F2	ATGGGAATGTGCAC	F-14bp	54°C
REU BSH R2	TTAATTTTGGTTGGTTAATAATTTG	R-24bp	53°C

Table 3.4 Reactants for PCR

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

Table 3.5 The ideal PCR conditions for every primer

Primers	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Reu BSH 1	95°C,5 min	95°C,0:30 sec	45°C,0:40 sec	72°C,2 min	72°C,5 min
Reu BSH 2	95°C,5 min	95°C,0:30 sec	45°C,0:40 sec	72°C,2 min	72°C,5 min

Number of cycles: 30

3.2.2.2 Purification of PCR products

The PCR products were placed onto a 1% of agarose gel (Sigma, USA) and then separated by electrophoresis using 1× TAE buffer at 75 volts for 1.15 hours to exclude undesired non-specific PCR products. Subsequently, each PCR result was isolated from agarose gel by following the directions provided by Thermo Scientific's GeneJET gel extraction kit.

3.2.2.3 Isolation of pBluescript plasmid

After streaking stock of pBluescript cloning vector into an LB plate and adding 30µg/ml ampicillin, the plate was cultured for eighteen hours at 37°C. One colony from the streaked plate was used to initiate a liquid culture of LB+amp, which was then incubated at 37°, 16 hours, and 175 rpm. Following a 16–18-hour incubation period, the pellet was extracted by centrifuging the tubes for 4 minutes at 4000 rpm and 25°C. Following a 1 ml LB wash, the pellets are centrifuged for 3 minutes at 6000 rpm. Then, using the GeneJET plasmid Miniprep-Kit (Thermo Scientific) instructions, the DNA plasmid was extracted from the pellets.

3.2.2.4 pBluescript digestion using the restriction enzyme *SmaI*.

Since the PCR result has a blunt end, the circular pBluescript vector were digested using *SmaI* fast digest enzyme (Thermo Scientific, EU) to produce a linear vector with a blunt end. Table 3.6 displays the components of the digesting reaction. The PCR machine was incubated at 37° C for 23 minutes. Next, in accordance with the instructions, the linear pBluescript vector was extracted from 1% of agarose gel by using a GeneJET gel extraction kit from Thermo Scientific.

Table 3.6 pBluescript digestion reaction condition

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

Reaction was incubated at 37° C for 23 min.

3.2.2.5 Linear pBluescript vector insertion of *L. reuteri* genes

Using Fermentas' T4 DNA ligase enzyme and its corresponding buffer, the *L. reuteri* gene was ligated into the pBluescript vector DNA after a one-hour incubation time at 22°C (Table 3.7). Thermo Scientific, EU's polyethylene glycol (PEG) was added to increase the ligation reaction's efficiency. The following formula was used to determine the precise amounts of vector and insert DNA:

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

Table 3.7 *L. reuteri* and pBluescript ligation reaction conditions

Reactants	Volume
UpH ₂ O	6 µl
pBluescript vector	3 µl
<i>L. reuteri</i> DNA	6 µl
T4 Ligase Buffer (10X)	2 µl
T4 DNA Ligase enzyme (5U/µl)	1 µl
PEG (50%w/v)	2 µl
Total volume	20 µl

3.2.2.6 Preparation of XL1-Blue

The calcium chloride technique was used to create *E. coli* XL1-Blue competent cells. After streaking XL1-Blue competent cells onto an LB plate, they were cultured for an entire night at 37°C. The second stage involved inoculating a single colony into a 3 ml liquid LB medium, which was then cultured for 14–16 hours- at 175rpm at 37°C. Using 500µl from the initial culture (1%) and a ratio of 1 to 10, a second culture of 50ml LB media was created. It was then incubated at 37°C and 125 rpm for 2 to 2.5 hours, or until the desired OD at 600nm wavelength between 0.04-0.06 was reached. The sample was divided into two 20 ml parts, and it was centrifuged at 4000rpm and 4°C for five minutes. The produced pellets were left on ice for half an hour, and then they were re-suspended in 10 milliliters of 100 millimolar CaCl₂. They were then centrifuged for 10 minutes at 4000 rpm at 4°C. Next, one milliliter of 10% glycerol CaCl₂ was used to dissolve the pellet. After being divided, the competent cells were put into 100µl Eppendorf tubes and kept at -80°C for storage.

3.2.2.7 Transformation of *L. reuteri* into *E. coli* XL1-Blue

The heat shock protocol was used to convert *L. reuteri* into XL1-Blue. 10µl of the pBluescript ligation product was put into an Eppendorf tube with 100µl of competent cell, and it was heated to 45°C for 45 minutes, then it was incubated on ice for another five minutes. It was then placed in a glass tube with 1 milliliter of LB broth and incubated for 1 hour at 37°C at 175 rpm. Centrifugation was then used to gather the cells, for 3 minutes at 6000 rpm. The pellet was again suspended in

100µl of LB. The sample was then distributed using the spread plate technique on an LB plate with IPTG (Isopropyl β-D-1-thiogalactopyranoside), X-gal (5bromo 4 chloro 3indolyl β D galactopyranoside), and ampicillin (25 mg/ml). The plate was incubated for 14–16 hours at 37°C. The next day, four white colonies were chosen at random from the plate (Figure 3.1), inoculated into a 5 ml LB+Amp media, and then incubated at 37°C for 14–16 hours at 175 rpm. Using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, EU), plasmid DNA isolation was carried out following pellet collection. To verify that the inserted gene was present, the recombinant DNA was then digested using the *EcoRI* restriction enzyme and then visualized using electrophoresis.

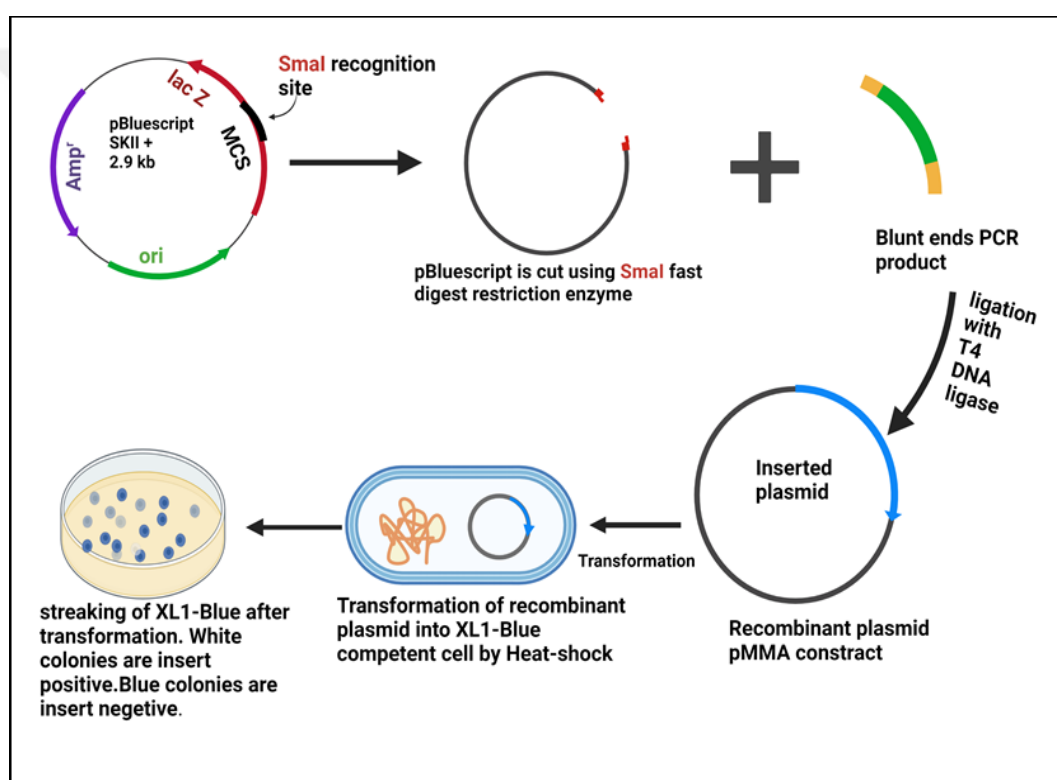


Figure 3.1 Preparation of *L. reuteri* construct by cloning process: cutting of pBluescript vector using *SmaI* restriction enzyme, insertion of PCR product into the plasmid, the transformation of *L. reuteri* construct into XL1-Blue competent cell.

3.2.2.8 DNA sequencing

Macrogen Inc. (Seoul, Korea) provided the recombinant DNA sequence, which was produced using universal primers M13/pUC Foreword (5'-ATGGGAATGTGCAC-3') and M13/pUCReverse (5'-TTAATTTTGTGGTT-AATAATTTG 3').

3.2.2.9 In-silico analysis

Using the BioEdit program, the nucleotide sequence result was confirmed after being obtained. Analysis in silico was carried out. Using the ExPASy translation tool (<https://web.expasy.org/translate/>), the sequence of nucleotides was initially converted into an amino acid sequence. Next, the amino acid sequence was aligned against those in a chosen data base using the Molecular Evolutionary Genetic Analysis (T-coffee) software and the BLAST tool in the National Centre for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).



4. RESULTS

4.1 Characterization of *L. reuteri* strain

4.1.1 Acid tolerance

Probiotics must have the ability to endure at different pH levels as one of their fundamental requirements. At various pH values, *L. reuteri*'s acid resistance was examined. As demonstrated in Table 4.1 and Figure 4.1, At all pH levels, the *L. reuteri* strain demonstrated an increase in OD values with occasional variations. pH3 and pH4 from 0 (h) to 6 (h), there is a notable shift in growth and reduction in all hours relative to pH2, pH5 and pH6.

Based on statistical analysis, in comparison from 0 (h) to 6 (h) for each pH2, pH3, pH4, pH5 and pH6 *L. reuteri* didn't showed any significance growth ($p < 0.05$).

Table 4.1 Acid tolerance results of *L. reuteri* strain to five different pH values.

Time (h)	0:00	2:00	4:00	6:00
Control	0.476±0.688	0.473±0.582	0.486±0.647	0.773±0.464
pH2	0.6±0.943	0.305±0.313	0.125±0.078	0.176±0.348
pH3	0.373±0.456	0.176±0.187	0.082±0.121	0.226±0.269
pH4	0.456±0.678	0.133±0.096	0.142±0.162	0.222±0.191
pH5	0.61±0.850	0.422±0.484	0.385±0.468	0.400±0.434
pH6	0.924±1.304	0.637±0.744	0.474±0.775	0.772±0.546

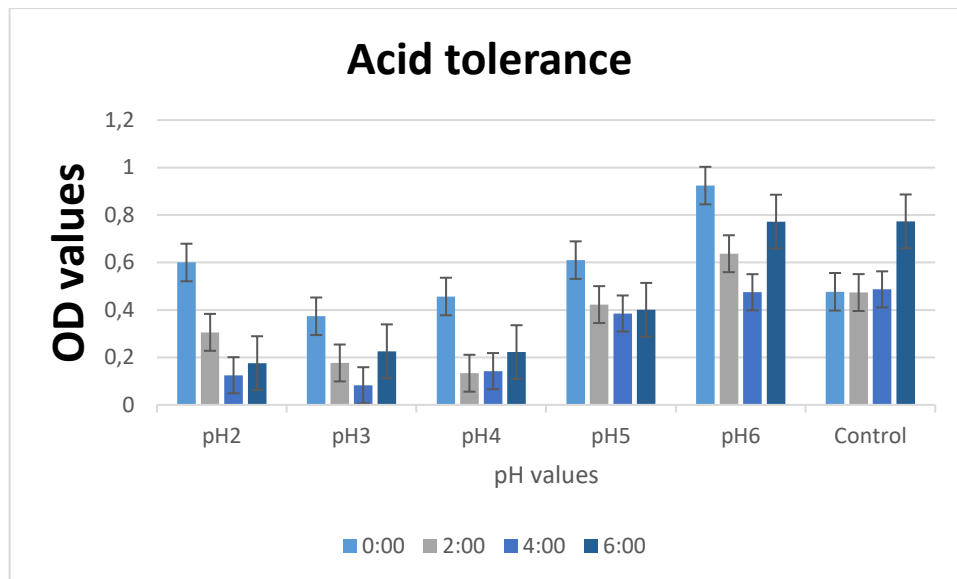


Figure 4.1 *L. reuteri*'s tolerance to pH.

4.1.2 Antimicrobial activity against pathogens

The antagonistic action of LAB against pathogenic bacteria is one of its primary probiotic qualities. The antibacterial activity of *L. reuteri* was examined in this study against five pathogenic bacteria: *E. coli*, *S. typhimurium* (ATCC13311), *Y. enterocolitica* (ATCC23715), *S. aureus*, and *L. monocytogenes* RSKK. Using the Kirby-Bauer method, *L. reuteri* activity was assessed on nutrient agar plates using both cell-free supernatant (CFS) and cell lysate materials. The *L. reuteri* cell lysate demonstrated an inhibitory zone for, *Staphylococcus aureus* with 18mm, with an inhibitory zone of 10 mm, 6 mm, 8 mm, and 12 mm, the CFS of *L. reuteri* exhibited antagonistic activity against *S. typhimurium*, *Y. enterocolitica*, *S. aureus*, and *E. coli* (Table 4.2).

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

Pathogens	<i>L. reuteri</i> cell lysate	<i>L. reuteri</i> CFS
<i>Staphylococcus aureus</i>	18 mm	6 mm
<i>Escherichia coli</i>	---	10 mm
<i>Salmonella typhimurium</i> (ATCC13311)	---	8 mm
<i>Listeria monocytogenes</i> RSKK	----	-----
<i>Yersinia enterocolitica</i> (ATCC23715)	----	12 mm

4.1.3 Acidifying activity

A pH meter was used to assess *L. reuteri*'s acidifying capacity. The results of the pH tests on skim milk infused with microorganisms revealed that the acidity degree was significantly changed ($p<0.05$) by *L. reuteri* when the pH value dropped by 2.5 degrees in 48 h as opposed to the initial 0 h (Table 4.3 and Figure 4.2).

Table 4.4 The pH values of acidifying activity of *L. reuteri*

Time (hour)	0:00	8:00	10:00	24:00	48:00
pH (value)	6.333±0.100	5.846±0.245	5.643±0.317 *	4.733±0.339 **	3.833±0.214 **

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

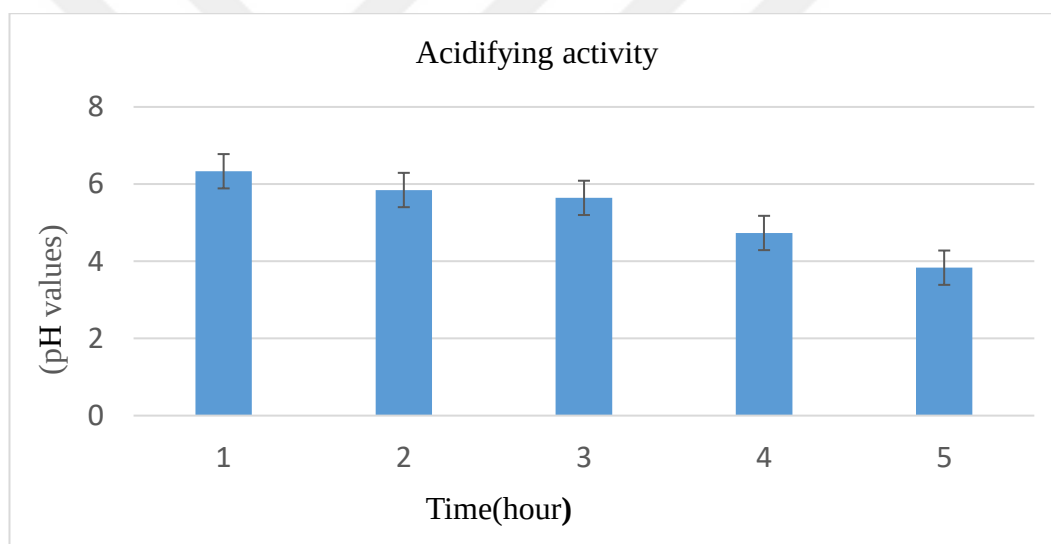


Figure 4.2 The acidifying activity for *L. reuteri*

4.1.4 Antibiotic susceptibility

A crucial safety consideration when selecting possible probiotic bacteria is the probiotics' sensitivity to antibiotics. Following the recommendations provided by the Clinical and Laboratory Standards Institute (CLSI), the disc diffusion method findings against four antibiotics were categorized (Figure 4.3). Susceptible has a zone of ≥ 20 mm, I intermediate has a zone of 15–19 mm, and R resistant has a zone of ≤ 14 mm (Table 4.4).

Table 4.5 Antibiotic resistance of *L. reuteri* against 4 antibiotics

Antibiotic	Zone diameter (mm)	Susceptibility
Ampicillin (10µg)	50mm	S
Erythromycin (15µg)	35mm	S
Kanamycin (30µg)	-----	-----
Neomycin (30µg)	10mm	R

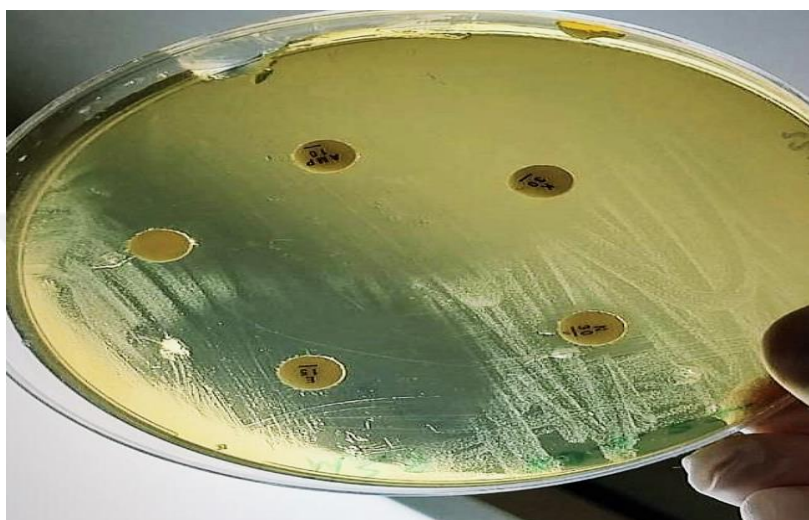


Figure 4.3 Antibiotic resistance test results of *L. reuteri*. Amp: Ampicillin, K: Kanamycin, N: Neomycin, E: Erythromycin. Blank disc used as negative control.

4.1.5 Cell auto-aggregation

Numerous bacteria possess the ability to attach themselves with each other. Additionally, to host cells the extracellular matrix of the host tissues or the inorganic surfaces. This self-binding, also known as auto-aggregation or auto agglutination, is one of the initial-processes in the creation of biofilm, along with surface colonization (237). *L. reuteri* auto-aggregation was assessed in terms of sedimentation rate, which was determined using the auto-aggregation% formula and an OD₆₀₀ spectrophotometer. Table 4.5 illustrates the significant degree of self-aggregation that *L. reuteri* exhibited, with the sedimentation rate above 90% after the second hour of incubation. 98.38% is the maximum value, with a minor change from the second to the fifth hour of incubation. Regarding the first hour, the auto-aggregation % changed significantly ($p<0.01$) in the second, third, fourth, and fifth hours (Figure 4.4).

Table 4.6 The percentage of *L. reuteri* auto-aggregation over a 5-hour period

Auto-aggregation (%)	1:00	2:00	3:00	4:00	5:00
	18.262±21.14	95.542±2.79 **	98.380±1.98 **	93.974±7.77 **	96.671±2.525 **

The data is shown as mean ± SD for n = 3. Significance indicated with express to time 1:00, **p<0.01.

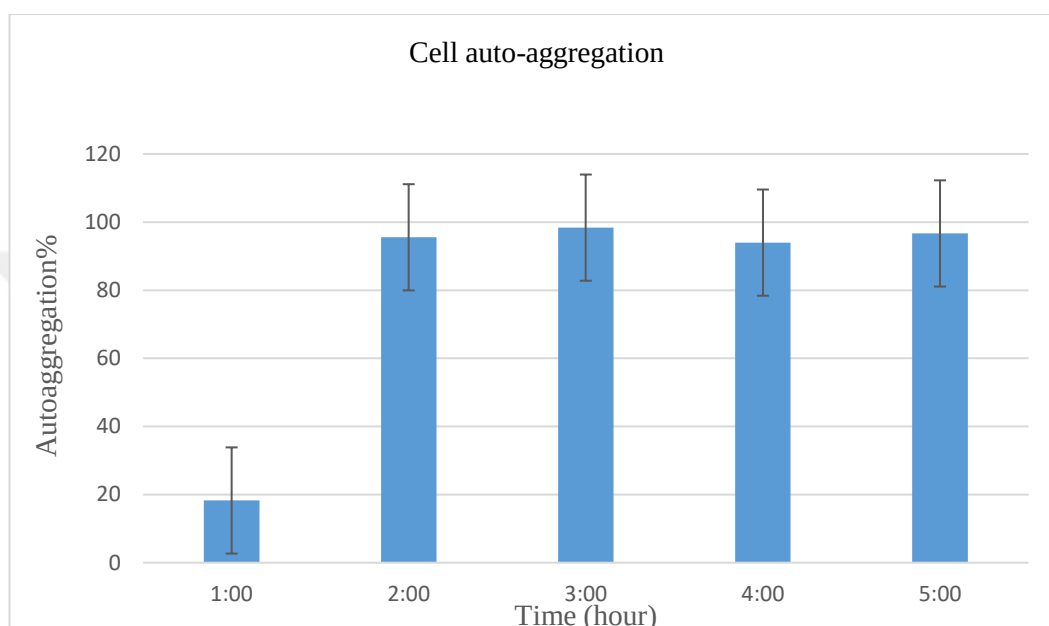


Figure 4.4 Auto-aggregation% of *L. reuteri*

4.1.6 Cell co-aggregation

Co-aggregation is the formation of bacterial aggregates between different species of bacteria. The probiotic strain can inhibit pathogen colonization in the gut and produce antimicrobial compounds near a potentially harmful microbe through co-aggregation. This may impede the growth of pathogenic strains in the gastrointestinal tract. Table 4.6 shows the outcomes of *L. reuteri* co-aggregating with five pathogenic bacteria, each of which is represented by an individual: *L. monocytogenes* RSKK, *Y. enterocolitica* ATCC23715, *S. typhimurium* ATCC13311, *E. coli*, and *S. aureus*. The findings showed that, with only minor variations, the co-aggregation percentage with the pathogenic bacteria increased during the incubation period. Significant co-aggregation percentage (p<0.05) was demonstrated by *L. reuteri* against *L. monocytogenes* at 4, 6, and 24 hours, as well as against *Y. enterocolitica* at 6 and 24 hours, and *S. typhimurium* at 4, 6, and 24

hours (Figure 4.5). Simultaneously, for all incubation durations, there is no significant value ($p < 0.05$) for *E. coli* and *S. aureus*.

Table 4.7 Co-aggregation percentage of *L. reuteri* throughout a 24-hour period with five pathogenic bacteria

	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	61.656±7.472	55.061±23.498	52.629±17.007	74.087±3.105	40.356±50.389
2:00	82.595±20.763	90±26.128	73.240±14.594	79.682±27.144	70.321±42.541
4:00	95.989±0.984*	86.641±6.87	93.166±6.331*	96.468±1.143	90.427±8.353
6:00	97.616±11.758*	95.580±10.191*	94.092±17.455*	92.460±8.947	91.367±9.764
24:00	98.303±6.708*	97.950±6.927*	90.555±15.485*	99.087±6.181	105.45±15.659

The data is shown as mean ± SD for n = 3. Significance indicated with express to time 0:00, ** $p < 0.01$, * $p < 0.05$.

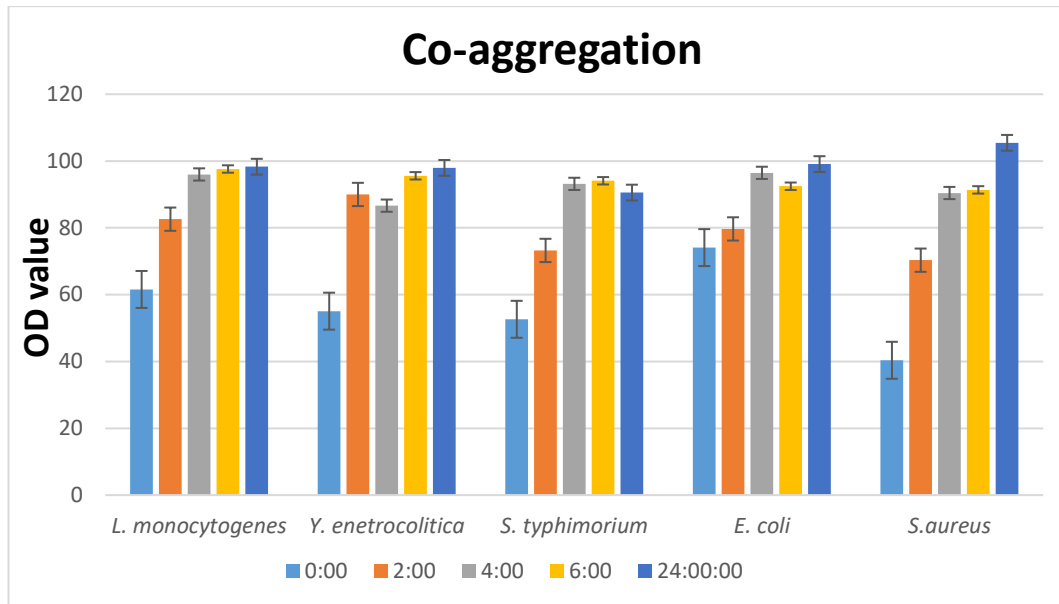


Figure 4.5 *L. reuteri* co-aggregation percentage with five pathogenic bacteria.

4.2 Screening for *bsh* gene

4.2.1 PCR amplification of BSH and PCR product purification

After isolating the genomic DNA of *L. reuteri*, two specially created primers were used in a PCR amplification reaction to test the *bsh* genes. *L. reuteri* produced a result that was roughly 750 bp in size when visualized using agarose gel electrophoresis and a UV transilluminator (Figure 4.6. a). After that, undesired non-specific PCR products were eliminated from the product using 1% agarose gel electrophoresis (Figure 4.6. b).

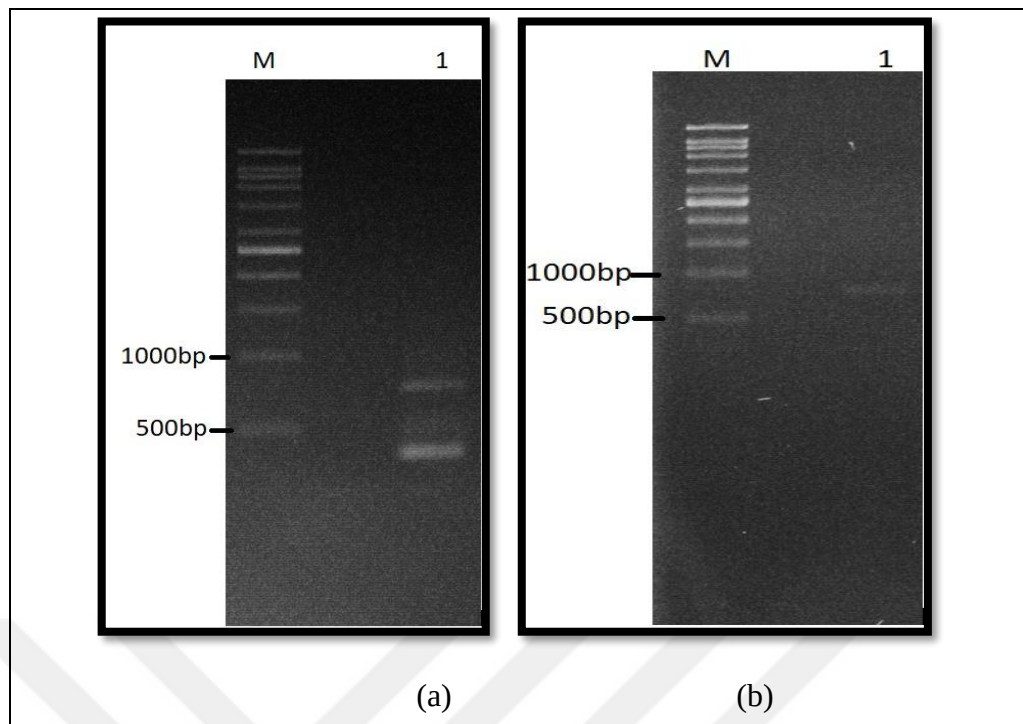


Figure 4.6 The purified *L. reuteri* PCR product and its amplification result. M: 1kb DNA ladder from VC. (A), Line 1: PCR product of *L. reuteri*. (B), line 1: Purified PCR result of *L. reuteri*.

4.2.2 Preparation of *L. reuteri* construct

4.2.2.1 Preparation of pBluescript cloning vector

The pBluescript vector was extracted from the *E. coli* XL1 strain and digested with the *Sma*I fast digest restriction enzyme to produce a blunt-ended linear vector, which was subsequently loaded and purified from 1% agarose gel to prepare the cloning vector. A UV transilluminator and agarose gel electrophoresis were used to visualize the results (Figure 4.7).

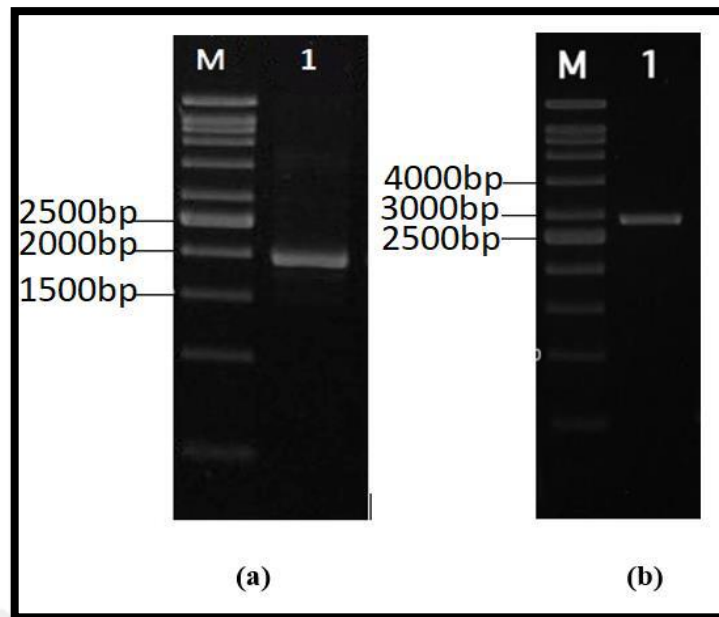


Figure 4.7 Preparation of pBluescript cloning vector. M: VC 1kb DNA ladder. (a), first line: isolated circular pBluescript cloning vector. (b), first line: linear pBluescript vector digested with *Sma*I.

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

After being treated with CaCl_2 , the ligation's pure recombinant DNA product, *L. reuteri*, was converted into an XL1-Blue capable cell. The colonies that contained inserts were chosen based on their color. In contrast to the blue colonies, which were insert-negative, the white colonies were insert-positive (Figure 4.8). A few white colonies were chosen, and their DNA was extracted so that the presence of the recombinant DNA could be confirmed using an agarose gel electrophoresis and a UV transilluminator. After the recombinant plasmid DNA was digested with *Eco*RITM and visualized, the insert's presence within the plasmid was confirmed (Figure 9).

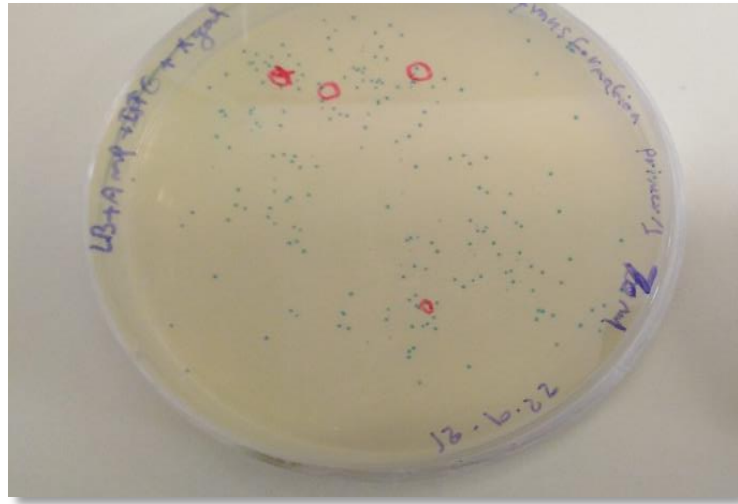


Figure 4.8 Transformation resulted in white colonies with recombinant DNA and blue colonies without, when cultivated on LB plates treated with ampicillin, IPTG, and X-gal.

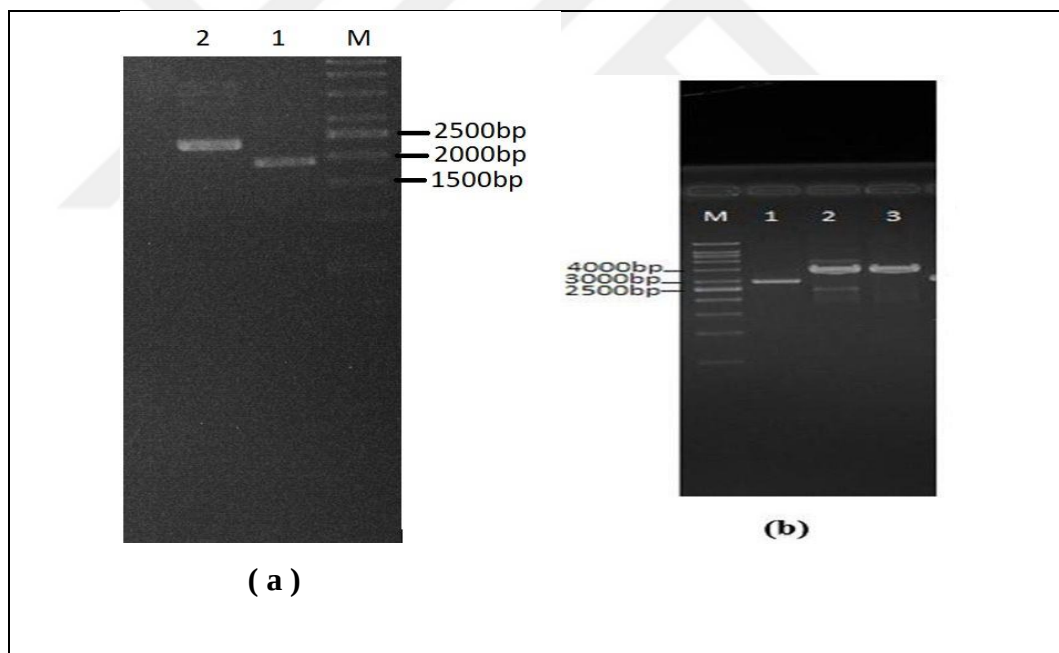


Figure 4.9 Transformation of *L. reuteri* into XL1-Blue competent cells. M: VC 1Kb DNA ladder (a), line 1: pBluescript cloning vector DNA digested with *SmaI*, lines 2 uncut *L. reuteri* DNA from two different colonies (b), line 1: pBluescript cloning vector DNA digested with *SmaI*, lines 2 and 3: *L. reuteri* DNA digested with *EcoRI* from different colonies.

4.2.3 DNA sequence analysis for *L. reuteri*

Using the universal primers which are M13/pUC Foreword (5'-ATGGGAATGTGCAC-3') and M13/pUCReverse(5'-TTAATTTTGTGG-TTAATAATTTG 3'), the *L. reuteri* construct was sequenced by Macrogen (Seoul, Korea). The BioEdit program confirmed that the nucleotide sequence was 748 bp long (Figure 4.10).

The Swiss Institute of Bioinformatics (SIB) ExPASy program was then used to translate the nucleotide sequence into its corresponding amino acid sequence (Figure 4.11). The protein was aligned with seven distinct proteins from the NCBI database using the BLAST algorithm (Figure 4.12).



```

TTCAAGNTCCTATAGGGCGAATTGGGTACCGGGCCCCCCTCGAGGTCGACGGTA
TCGATAAGCTTGATATCGAATTCCTGCAGCCCATGGGAATGTGCACCAGTCAATG
CTAGTACAACACTTTTAGTATAATAACCTTTACTAATTAGATCAATATTGAATTT
ATTTAAGCACCATGATATTGCACTAAGCTGTAAATATCAACATCAGGCAACTTTT
CTCTACCCTTTTAAATCAGGCAATTCAACGTAAAATGCAGCACCTACAAGCTTACC
ACCAAGATTTTCAATTAATTCCTTAGAAGCTCTTAAAGTACCTGCAGTAGCCATT
AAATCGTCACAGAGAACAACCTCTTTGGCCAGGCTTAATTGCATCTTTATGCATTT
CAAGGCTATTTGAACCGTATTCCAAGTCATATGAAGCACGTTCAACTTCTCTTGG
CAACTTATGTGGCTTTCTTGCTGGAACAAAACCTAAGCCTAACTCAGTTGCAACA
GGACAGCCTACAATAAAGCCACGTGCTTCAGGACCTACGATTACATCTGCCTTTC
TACTCTTTGCATATTTTGCCAATTCATGAGTAGCAGCACGATACAATTCACCATC|
TTGCAAAATAGGTGTAATATCGCGAAAGACAATCCCTTATTAGGAAAGTCCTTA
ACACTTGCAATATATTTACTAAAATCAATCGCCATAAGAAGAAAAACCTCCATAA
TTCTGTGCGTTTGTATTCAAACACTTTTGACTTGTATATTTTACCAAAGATTTGT
TATAAAAGCACTGAAATCTTGATATTTATCTATCTTTTTTACCAAATTATTAACCA
ACAAAATTAAAGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC
CAGCTTTTGTTCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCA
TAGCTGTTTCTGGGGGAAATTGTTATCCGCTCACAATTCACACAACATACGAGC
CGGAAGCATAAATGGTAAAGCCTGGGGTGCCCAAGGAGGGACCTAACTCCATTAA
TTGGGTGGGCTNNNCTGCCCTTTTCATCAGGGGAAACCGTCTGGCCCATGGATTA
AGGA

```

Figure 4.10 The *L. reuteri* construct, acquired from MacroGen, has a 748 bp nucleotide sequence; the yellow letters stand for our target primers.

```

MEVFLLM AIDFSKYIASVKDFPNKGIVFRDITPILQDGELYRAATHELAKYAK
SRKADVIVGPEARGFIVGCPVATELGLGFVPARKPHKLPREVERASYDLEYGS
NSLEMHKDAIKPGQRVVLCDDL MATAGTLRASKELIENLGGKLVGAIFYVELP
DLKGREKL PDVDIYSLVQYHGA-

```

Figure 4.11 Using the ExPASy technique, the amino acid sequence for the *L. reuteri* construct was determined.

```

ADX70041.1      MEVFLMLAIDFSKYIASVKDFPNKGIVFRDITPILQDGELYRAAT
AHI12086.1      MEVFLMLAIDFSKYIASVKDFPNKGIVFRDITPILQDGELYRIAT
KRL64040.1      MEVQVNMTVDFKKYIASVQDFPNKGIVFRDITPILQDGEIFRAAT
WP_089148115.1  -----MAVDFSKYIASVQDFPNKGIVFRDITPILQDGELYHAAT
WP_103661781.1  -----MAIDFSKHIAVQDFPNKGIVFRDITPILQDGELFRAAT
WP_215485483.1  -----MAIDFKEHIAVQDFPNKGIIIFRDITPILQDGKLYRAAT
WP_269256582.1  -----MAIDFKEHIAVQDFPNKGIIIFRDITPILQDGKLYRAAT
seq_15786      MEVFLMLAIDFSKYIASVKDFPNKGIVFRDITPILQDGELYRAAT
cons           *: : ** . : : ***** : ***** : ***** : : : **

ADX70041.1      HELAEYAKSRKADVIVGPEARGFIVGCPVATELGIGFVPARKPHK
AHI12086.1      HELAEYAKSRKADVIVGPEARGFIVGCPVATELGIGFVPARKPHK
KRL64040.1      HELATYAKKRGADVIVGPEARGFIVGCPVATELGIGFVPARKPHK
WP_089148115.1  HELAEYAKSKKADVIVGPEARGFIVGCPVATELGIGFVPARKPHK
WP_103661781.1  HELAEYAKSRNADVIVGPEARGFIVGCPVAAELGIGFVPARKPHK
WP_215485483.1  HELAEYAKSRGAEVIVGPEARGFIVGCPVATELGIGFVPARKPHK
WP_269256582.1  HELAEYAKSRGAEVIVGPEARGFIVGCPVATELGIGFVPARKPHK
seq_15786      HELAKYAKSRKADVIVGPEARGFIVGCPVATELGIGFVPARKPHK
cons           ****  *** . : * : ***** : *** : *****

ADX70041.1      LPREVERASYDLEYGSNSLEMHKDAIKPGQRVVLCDDLMTAGTL
AHI12086.1      LPREVERASYDLEYGSNSLEMHKDAIKPGQRVVLCDDLMTAGTL
KRL64040.1      LPRDVERASYDLEYGSNSLEMHKDAIKPGQKVVICDDLMTAGTL
WP_089148115.1  LPREVERASYDLEYGSNSLEMHKDAIKPGQRVVLCDDLMTAGTL
WP_103661781.1  LPREVERASYDLEYGSNSLEMHMDAIKPGQRVVVCDDLMTAGTL
WP_215485483.1  LPREVESASYDLEYGSNVLEMHKDAIKPGQKVVICDDLMTAGTL
WP_269256582.1  LPREVESASYDLEYGSNVLEMHKDAIKPGQKVVICDDLMTAGTL
seq_15786      LPREVERASYDLEYGSNSLEMHKDAIKPGQRVVLCDDLMTAGTL
cons           *** : ** ***** ***** : ** : *** : *****

ADX70041.1      RASKELIENLGGKLVGAIFYIELPDLKGREKLPDVDIYSLVQYHG
AHI12086.1      RASKELIENLGGKLVGAIFYIELPDLKGREKLPDVDIYSLVQYHG
KRL64040.1      HATIELINRLGGEVVGAIFYVELPDLNGREKLPDIDIFSLVQYHG
WP_089148115.1  RASKELIENLGGKLVGAIFYIELPDLHGREKLPDVDIYSLVKYHG
WP_103661781.1  RASKELIENLGGKLVGAIFYVELPDLKGREKLPDVDIYSLVKYHG
WP_215485483.1  HATKELIENLGGGEVVGAIFYIELTDLKGREQFPDLDIYSLVKYHG
WP_269256582.1  HATKELIENLGGGEVVGAIFYIELTDLKGREQFPDLDIYSLVKYYG
seq_15786      RASKELIENLGGKLVGAIFYVELPDLKGREKLPDVDIYSLVQYHG
cons           : * : *** . *** : ***** : ** . ** : *** : : ** : ** : *** : * :

```

Figure 4.12. The *L. reuteri* builds protein multiple sequence alignment by T-COFFEE (Version_11.00). The ExPASy tool was utilized to acquire protein. Using the BLAST algorithm, the protein was aligned with seven distinct proteins from the NCBI database.

5. DISCUSSION

Diet and health are closely related, as seen by better lifestyle choices and self-care practices. Beyond basic nutrition, food supplements can assist in improving human health. Thus, a tiny portion of functional food includes probiotics which have received a lot of notice (238). There are three times as many bacteria in the human body as there are human cells, and GIT is home to a massive bacterial ecosystem (239). Dietary supplements containing live bacterial strains that can swiftly colonize the (GIT) and produce a variety of bioactive substances that have a positive impact on the host's physiology are known as probiotics (240). These are mostly made up of LAB (241). The most researched genera of probiotics are *Lactobacillus* (242). *L. reuteri*, one species of *Lactobacillus*, has several positive benefits on host health, including the prevention and/or treatment of a variety of illnesses. It has been described as a heterofermentative species that inhabits the (GIT) of both humans and animals and develops in environments with low oxygen levels (161). For a bacterial strain to have positive and health-promoting effects, it must be able to thrive in an unpleasant environment, like the human (GIT). Determining if these bacteria are safe for the host to eat is another crucial requirement. This emphasizes how important it is to describe and evaluate LAB bacteria (243).

Due to the high cost and duration of in-vivo testing required to evaluate potential probiotic candidates, in-vitro examination is a crucial method for probiotic selection (244). Previous research has demonstrated the range of effects of LAB; however, as every strain is unique and has distinct effects when applied, strain-specific characterization is crucial (245). It is therefore crucial to investigate and characterize the probiotics qualities of the *L. reuteri* strain.

Bacteria need to survive the GIT to perform their probiotic activity. As soon as the bacteria enter the stomach, their surroundings quickly alter, and they are exposed to stressors such different enzymes and a very acidic pH (246). To ascertain the *L. reuteri* strain's level of acid tolerance, the acid tolerance assay was employed. As shown in Figure 4.1 *L. reuteri* shows growth at all pH values, even pH 2. With oscillations of varying intensity at different time points and after being injected into regular MRS broth as a control group, the strain displayed an increase in growth.

The outcome demonstrated a significant increase in time zero for pH 2, 3, 5, and 6. With a decrease in growth during the remaining hours of incubation but growth remained present until pH 6, with a difference between the zero hour and the sixth hour in comparison with a study conducted for acid resistance of *L. reuteri* showed survival of *L. reuteri* after acid shock.

The survival of *L. reuteri* ATCC 55730 after a sudden shock of acid is shown in the Fig (247). After one hour at pH 2.7, almost 80% of the acid-treated cells were still alive. Roughly 60% of the cells were still alive after two hours (data not shown). Within 30 minutes, the cells in the control group started growing, and within an hour, the cell count had more than doubled.

One of LAB's primary qualities is its capacity to produce an acidic environment, which can aid in the destruction of pathogenic bacteria and improve food flavor and fermentation. Nieto-Arribas et al. classify LAB into three broad categories: 1) strains with a high acidifying capacity are those that lower the pH value by more than two degrees; 2) strains with a medium acidifying activity are those that lower the pH value by 1.5 degrees; and 3) strains with a low acidifying activity are those that can only lower the pH value by 1.5 degrees over the course of a 24-hour period (246) This classification indicated that *L. reuteri* had mild acidifying activity, lowering the pH of skim milk by just 1.6 degrees over the course of a 24-hour period. The test's results (Table 4.5) showed that, as compared to time 0h, the pH value changed significantly ($p < 0.01$) during the 48- and 24-hour incubation periods. and a notable shift in the pH value ($p < 0.05$) from time 0 to time 10 throughout the incubation period. In contrast to our results, which are the same as Barzegar et al.'s (248) results, LAB strains demonstrated mild acidifying activity.

The ability of bacterial strains to aggregate with one another in an indiscriminate way is measured by auto-aggregation, and it is thought that colonization is required for the probiotic bacterium to carry out its advantageous function (249).

After incubation for five hours, *L. reuteri* demonstrated a significant degree of auto-aggregation, reaching over 96% in the current investigation. Except for the

first hour of incubation, all the data were determined to be significant ($p < 0.05$), suggesting that *L. reuteri* has a strong ability to aggregate (Table 4.5).

The strains were classified as (1) high (HAAg,70%AAg), (2) medium (MAAg,20–70%AAg), and (3) low (LAAg,20%AAg) based on their AAg ability (250). Findings showed medium auto-aggregation ability for some LAB strains at both the 5 h and 24 h marks, unlike *L. reuteri*.

Probiotics can effectively build a barrier that hinders enteric pathogens from attaching to intestinal mucosa by co-aggregation. The capacity of probiotics to clump together with pathogens may be essential for food preservation as well as the therapeutic effect they have on the intestinal flora (244). *L. reuteri*'s capacity to co-aggregate with the five pathogenic bacteria differed depending on the pathogen, yielding results that were strain specific. The ability of *L. reuteri* to co-aggregate with the five pathogenic bacteria that were examined varied according to the strain, with values ranging from 98.3% for (*L. monocytogenes*) to 105.4% for *S. aureus*. *L. reuteri* exhibited significant ($p < 0.05$) outcomes with three pathogenic bacteria at various stages of the incubation period, according to the statistical analysis (Table 4.6). According to previous study (244), the coaggregation test findings demonstrated that, following two hours of incubation, the coaggregation percentage of the *L. reuteri* strain was 75.81% for *E. coli*, 71.83 for *L. monocytogenes*, and 66.30 for *S. Typhimurium*. The *L. reuteri* strain demonstrated a greater rate of co-aggregation at the 2-hour incubation period in contrast to our results.

The primary characteristic shared by all probiotic candidates is their antibacterial action, which is attributed to their metabolites, which include organic acids, bacteriocin, alcohol, and hydrogen peroxide (251).

To assess the viability of antimicrobial substances inside the cell and their secretion into the medium, the anti-pathogenic activity of the *L. reuteri* strain was tested in this study against five pathogenic bacteria: two gram-positive pathogens, *S. aureus* and *L. monocytogenes*, and three gram-negative pathogens, *E. coli*, *Y. enterocolitica*, and *S. typhimurium*. The *L. reuteri* cell lysate demonstrated an inhibitory zone for *S. aureus* with 18mm. With an inhibitory zone of 10 mm, 6 mm, 8 mm, and 12 mm, the CFS of *L. reuteri* exhibited antagonistic activity against *S.*

typhimurium, *Y. enterocolitica*, *S. aureus*, and *E. coli*. The results obtained did not pinpoint the precise reason for *L. reuteri*'s antibacterial activity. Comparing the results of this test to a study in the literature (244), indicating that the inhibition activities against all pathogens tested at varying degrees for *L. reuteri* against *E. coli* were 19.5 mm, whereas our result showed 10 mm against *E. coli*. Additionally, the study showed that the inhibition zone range against *S. typhimurium* was approximately 15.5 mm.

However, our results against *S. typhimurium* indicated an about 8mm inhibitory zone range. Additionally, the study demonstrated an inhibition zone against *L. monocytogenes* that ranged about 16 mm; however, our results did not demonstrate any inhibition zone.

All bacteria, including LAB, can exchange genes to increase their chances of surviving in environments that include antibiotics (252). For horizontal gene transfer via mobile genetic elements to occur, there must be intimate contact between LAB and other bacteria, such as in the intestine, on mucosal surfaces, or in food. Accordingly, ingested, and native LAB may add to the pool of antibiotic resistance genes that can spread to pathogens or other commensal bacteria through the food chain or the gastrointestinal tract (253).

The antibiotics utilized in this study fall into three categories (253): (i) β -Lactams, such as ampicillin, which inhibit cell wall production; (ii) protein synthesis inhibitors, such as erythromycin and aminoglycosides like neomycin; and (iii) RNA-inhibiting antibiotics, such as kanamycin, which inhibit cellular RNA.

Our results demonstrated that the *L. reuteri* strain was sensitive to ampicillin. moreover, erythromycin susceptibility which supports the finding that erythromycin and ampicillin were sensitive to *L. reuteri*, as reported by a study conducted in 2008 by Rosenberg et al (244). Additionally, a study by Reuben et al. (244) revealed that *L. reuteri* was susceptible to erythromycin and ampicillin., which is consistent with our findings. Our research revealed that *L. reuteri* is neomycin resistant (Table 4.4). in contrast to study about Antibiotic MIC distribution across different *Lactobacillus* species (254) demonstrated a strong neomycin sensitivity.

One of the most common and often encountered species in the human gastrointestinal tract is *L. reuteri*. Additionally, it is commonly connected to the secretion of BSH. In a specific biological niche, *Lactobacillus* species that produce BSH may have an advantage over other bacteria in competition. The present investigation involved the identification of the *bsh* gene through screening, followed by sequencing of the PCR product. In-silico analysis was then carried out on the obtained strain, and results from BLAST and multiple sequence alignment demonstrated that the protein sequences of *L. reuteri* were not consistent with any BSH in the NCBI database. It matched up with several proteins from various species, such as Adenine phosphoribosyl transferase. Adenine phosphoribosyl transferase and the protein sequences of *L. reuteri* were found to be roughly identical. These outcomes could be the result of the primers attaching to a different location on the strain. Although the *bsh* coding gene was not found, *L. reuteri* exhibits favorable antimicrobial phenotypic traits. It's possible that the multiple *bsh* gene found in *Lactobacillus* species explains the discrepancy in the results.

6. CONCLUSIONS AND RECOMMENDATIONS

In this study, probiotic characteristics of human-originated *L. reuteri* were characterized. These probiotic qualities were assessed by several assays. Based on the findings, *L. reuteri* was shown to exhibit favorable in vitro probiotic traits, such as a high tolerance to stress conditions like acids and different antibiotic susceptibility, antimicrobial activity against pathogenic bacteria, and cell surface characteristics like a high capacity for coaggregation and self-aggregation with pathogens. These results suggest that, with more research, *L. reuteri* may prove to be a viable probiotic option for ingestion by humans and use in food.

As for the limitations of this work, the co-aggregation and auto-aggregation tests used in this work are connected to attachment to cell, therefore the adhesion to epithelial cell test is necessary. Even though PCR was used to identify the genes that code for proteins, a more thorough molecular analysis is required to identify the genes that code for antibiotics and separate their proteins. Further molecular investigation is needed to assess the transferable likelihood of the antibiotic resistance gene because *L. reuteri* exhibited resistance to some antibiotics.

To use this strain consistently, more research on *L. reuteri* should be done in the future. Conducting clinical research on human health and evaluating the viability of this strain in products are beneficial. Regarding the study's limitations, even though PCR was able to identify the protein-coding genes, a more thorough molecular analysis might be carried out on this strain to determine the antimicrobial compounds. This study lacked in-vivo testing, which is necessary to verify the probiotic potential claim made for this strain to apply it in many applications, including the pharmaceutical industry.

7. REFERENCES

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8. APPENDICES

Appendix A

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

Enzymes and other chemicals

Enzymes and the other chemicals	Suppliers
<i>Eco-RI FastDigest</i>	Thermo Scientific
dNTP mix	Fermentas
<i>Sma</i> I	Thermo Scientific
Lysozyme	Fluka
T4 DNA Ligase	Thermo Scientific
VC1 kb DNA- ladder	Vivantis
6xloading -dye	Fermentas

Appendix B

Growth media:

❖ deMan, Rogosa, and Sharpe (MRS) media

• MRS- broth (per liter)

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

• MRS Agar (per liter)

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

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

❖ Nutrient media

• Nutrient broth per liter

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

• Nutrient agar (NA) per liter

28 grams of nutrient agar powder is dissolved in 1000 milliliters of distilled water and the mixture is heated to dissolve completely, and autoclave at 121°C for 15 min.

❖ Plate count agar (PCA) medium

Contents	For 1 liter
Tryptone	5 grams
Yeast -extract	2.5 grams
Glucose	1 gram
Agar	15 grams

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

❖ Luria Bertani (LB) -medium

- **LB broth/ liter**

Components	For 1 liter
Tryptone	10 grams
Yeast extract	5 grams
NaCl	10 grams

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

- **LB plate**

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

- **Ampicillin (25mg/ml)**

0.25 grams of Ampicillin Sodium Salt is dissolved in Distilled water (10 milliliters) and then it was sterilized by the use of 0.22 µm filter. Then the solution is divided into the desired volume and the storage is at -20°C.

- **LB plate with Ampicillin**

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

Appendix C

Solutions and buffers

❖ For electrophoresis

• 10 X TAE buffer

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

These components are dissolved in UpH₂O and mix thoroughly then the volume complete to 1000 ml and the buffer is stored at room temperature.

To prepare **1X TAE buffer** 100mliter of 10X TAE buffer is added to 900 milliliters UpH₂O.

• For competent cell preparation

• CaCl₂ (0.1 M)

0.7351 g of CaCl₂ powder is added to 50 ml UpH₂O and dissolved, pH is adjusted to the value of 7 then sterilized by autoclave for 15 minutes at 121°C Then stored at +4 °C.

• CaCl₂ (0.1 M) with 10% glycerol

9milliter of 0.1M CaCl₂ and 1 ml glycerol are mixed together then sterilized by autoclave at 121°C for 15 minutes and well stored at +4°C.

❖ For cell lysis

• Lysis buffer

Content	Concentration	Amount
Lysozyme	30 mg/ml	666.6 µl
Tris-HCl	1 mM	20 µl
EDTA	50 mM	12 µl
Triton 100%	1.2%	12 µl
UpH ₂ O		261.4 µl
Total volume		1 ml

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

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

30 mg of lysozyme is dissolved in 1ml Up H₂O. Storage at -20 °C.

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

12.2 gram of Trizma base were dissolved into 80milliter of UpH₂O, the pH is set at a value of 8.0 with HCl then top to a total volume of 100ml with UpH₂O and sterilized by autoclave C for 15minutes at121°then Stored at room temperature.

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

2.9224 grams E.D.T.A is added to 10 ml UpH₂O and dissolved then the pH set at 8.0 with 5 M NaOH and top to volume 20 ml with UpH₂O then autoclaved for15 minutesat121°C and then well stored at room temperature.

- **NaOH (5 M)**

10 g NaOH (MW: 40 g/mol) is dissolved in 50 ml UpH₂O and stored at room temperature.

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

Content	Concentration	Amount for 1L
Sodium- Chloride	0.137 M	8.00 grams
Potassium -Chloride	0.0027 M	0.20 grams
Sodium phosphate -dibasic	0.01 M	1.44 grams
Potassium phosphate -monobasic	0.0018 M	0.245 grams

These components are dissolved in 1000 ml UpH₂O one after the other, pH modified at 7.4, then were filtered using 0.22 µm and then sterilized by autoclave for 15 minutes at 121°C and after that stored at room temperature.

- **Phenol 1%**

To prepare 1% phenol solution, the phenol (94.11 g/mol) is heated to melt then 1.1 ml is added to 100 ml UpH₂O and stored at room temperature.

- **1M IPTG**

1.1915 mg IPTG (MW: 238.3 g/mol) is dissolved in 5 ml UpH₂O then filtered by using a 0.22 µm filter and then well stored at -20°C.

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

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