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Evaluation of probiotic, antibacterial and safety properties of *Lacticaseibacillus rhamnosus* JCM 1136

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ABSTRACT

Probiotics are non-pathogenic and beneficial bacteria that contribute to improving digestive health, preventing various types of cancer, and boosting the body's immune system, among other benefits. The aim of this study was to investigate the probiotic potential and antimicrobial activity of the strain *Lacticaseibacillus rhamnosus* JCM 1136. In this research, the probiotic and safety characteristics of the strain were assessed, including acid resistance at pH 2, 3, and 4, bile resistance (0%, 0.3%, 0.5%, and 0.7% bile salt concentration), hydropHobicity, potential for producing DNase enzyme and biogenic amines, hemolytic activity, antioxidant activity, cholesterol absorption capacity, and resistance to common antibiotics. The antimicrobial effect of the strain against pathogenic bacteria (*Shigella dysenteriae*, *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria innocua*, and *Bacillus cereus*) was evaluated using the agar well diffusion and disk diffusion methods. The results showed that *L. rhamnosus* JCM 1136 exhibited survival capability at different pH levels. The growth of the strain decreased with increasing bile salt concentration. *L. rhamnosus* JCM 1136 showed intermediate to the antibiotic Ampicillin and sensitivity to other antibiotics. The hydropHobicity, antioxidant activity by DPPH and ABTS methods, and cholesterol absorption capacity of the strain were 50.0 ± 50.53%, 50.0 ± 23.44%, 62.0 ± 50.48%, and 43.0 ± 55.41%, respectively. No production of DNase enzyme, biogenic amines, or hemolytic activity was observed in the strain. *L. rhamnosus* JCM 1136 exhibited a greater antimicrobial effect against Gram-positive bacteria. The results indicate that *L. rhamnosus* JCM 1136 possesses desirable probiotic characteristics and can be used in the production of probiotic food products.

1-Introductin

Probiotics are living microorganisms that, when consumed at a sufficient level of CFU/mL 10^7, have beneficial effects on human health [1]. The word "probiotic" means "for life" in the literal sense. Probiotic bacteria, while influencing the gut microbiota, help prevent various infectious diseases related to the digestive system, boost the immune system, lower blood cholesterol, prevent cancer, inhibit the growth of tumors, aid in the absorption of calcium and vitamins B and K, assist in lactose digestion found in dairy products, reduce allergies, and so on [2]. Probiotic microorganisms can be isolated from various sources such as sewage, human and animal digestive, reproductive, and respiratory systems, fermented foods especially dairy products, fermented fruits and vegetables. Probiotic microorganisms separated from fermented foods include *Leuconostoc*, *Veissella*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Bifidobacterium*, and *Pediococcus* [3]. Yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces boulardii* can also be considered as probiotics. The most common probiotic microorganisms are lactic acid bacteria. These bacteria are Generally Recognized as Safe (GRAS) and have the potential to function effectively in the human digestive system [4]. To benefit from the remarkable properties of probiotics, their resistance in human digestive conditions must be evaluated. In other words, probiotics must be able to maintain their function in the human digestive system. Considering a microorganism as a probiotic requires external tests and confirmation by internal tests [1]. Therefore, various tests such as tolerance to acid and bile conditions, bacterial viability in the digestive system, ability to adhere to intestinal epithelial cells, potential cholesterol reduction, ability to hydrolyze bile salts, non-hemolytic properties, antimicrobial activity, and survival capability in the fermentation process are used for their assessment [5]. Lactobacillus are among the Gram-positive, anaerobic, non-spore-forming, catalase-negative, rod-shaped, microaerophilic lactic acid bacteria. Their DNA composition contains less than 50% guanine and cytosine (G + C) [1]. A carbon dioxide level of 5% in the environment promotes their growth, and they exhibit the highest growth at temperatures of 30 to 40 degrees Celsius. Based on previous research, the optimal pH for Lactobacillus growth is considered to be around 5.5 to 8.5, while they can also grow well at pH levels below 5 [6]. Some species of Lactobacillus, aside from their therapeutic properties, have antimicrobial activity and the ability to prevent the colonization of pathogenic bacteria. Lactobacillus contributes to human health and possesses metabolic activity that is widespread throughout the digestive system, forming 1 to 6% of the gut microbiota [2]. *Lacticaseibacillus rhamnosus* has various strains that are effective in enhancing the immune system against various tumors. This bacterium has a genetic relationship with the bacterium *Lactobacillus casei* [7]. The aim of this study was to investigate the probiotic characteristics such as resistance to acid and bile salts, sensitivity to antibiotics, cholesterol reduction, production of DNase enzymes and biogenic amines, strain hydrophobicity potential, hemolytic potential, and antimicrobial activity of the *Lacticaseibacillus rhamnosus* JCM 1136 bacterium against pathogenic disease-causing agents.

2- Materials and Methods

2-1- Isolation and Identification of *L. rhamnosus* JCM 1136 Strain

The strain *L. rhamnosus* JCM 1136 was isolated from local yogurt according to the method of Saboktakin-Rizi et al. (2021) [8]. Samples of local yogurt were collected randomly and transferred to the laboratory under refrigerated conditions. To 5 grams of the samples, 45 mL of 1.0% peptone water was added. The samples were homogenized using a homogenizer (Seaward, made in Germany). After preparation, serial dilutions of 10^{-1} to 10^{-6} were plated on MRS Agar medium. After isolating

the strain from the culture medium, Gram staining and catalase testing were performed. For molecular identification, genomic DNA was isolated using the VI kit and bacteria were cultured overnight in MRS Broth. The universal primers FYM 27 (5'-AGA GTT TGATYMTGG CTC AG-3') and R 1492 (5'-GGT TAC CTT GTT ACG ACT T-3') were used in this study based on the amplified conserved region of the16S rRNA. The results indicated that the isolated strain with catalase-negative and Gram-positive properties, with a 99% similarity, belongs to the *L. rhamnosus* JCM 1136 strain.

2-2- Acid Resistance Test

The acid resistance test was conducted according to the method of Barzegar et al. (2021) [9]. To examine the acid resistance of the target bacterium, *L. rhamnosus* JCM 1136 was inoculated in 5 mL of MRS Broth medium (Merck, made in Germany). Anaerobic conditions were created using an anaerobic jar and then incubated at 37 °C. After 18 to 24 hours, 20 mL of the microbial suspension was added to a sterile falcon tube. To separate bacterial cells from the culture medium, a refrigerated centrifuge (Hermle, made in Germany) at a speed of 6000 g for 10 min at 4 °C was used. The bacterial pellet was washed twice with sterile phosphate buffer solution (Sigma-Aldrich) and the culture medium was completely removed. To separate the liquid layer, the contents inside the Falcon tube were centrifuged. The obtained bacterial pellet was dissolved in sterile phosphate buffer solution at different pH levels of 2 and 3. Inoculation was done with 50 microliters of the prepared microbial suspension in 4 microtubes containing 450 microliters of sterile acidic phosphate buffer with pH values of 2 and 3. After 0, 1, 2, and 3 hours of anaerobic incubation at 37 °C, sequential dilutions of sterile phosphate buffer were prepared up to 10^{-9}. After surface plating of the prepared dilutions, plates were incubated anaerobically at 37 °C for 24 hours on MRS Agar medium. After 24 hours, the formed colonies on the culture medium surface were counted using a colony counter and the survival percentage of *L. rhamnosus* JCM 1136 bacteria was compared and evaluated with the control sample.

2-3- Bile Resistance Test

This test was performed according to the method of Vasiee et al. (2018) [10]. In this method, to activate the bacterium *L. rhamnosus* JCM 1136 in MRS Broth culture medium, it was incubated at 37 degrees Celsius for 24 hours. 100 microliters of the suspension was inoculated on MRS Agar culture media containing 0%, 3.0%, 5.0%, and 7.0% bile salts. The culture media were incubated anaerobically in an anaerobic jar and then in a 37°C incubator for 48 hours. After the completion of the incubation, the results were visually observed.

2-4- Antibiotic Sensitivity Test

The evaluation of the sensitivity of *L. rhamnosus* JCM 1136 to antibiotics was carried out according to the method of Tabatabaei Yazdi et al. (2016) [11]. The sensitivity of the probiotic bacterium to common therapeutic antibiotics such as Ampicillin, Clindamycin, Nitrofurantoin, Chloramphenicol, Ciprofloxacin, Penicillin, Tetracycline, and Erythromycin was assessed. After 48 hours, a solid culture of *L. rhamnosus* strain and a standard 0.5 McFarland suspension were inoculated on the surface of MRS Agar medium. Then antibiotic disks were placed on the culture medium using sterile forceps. Plates were placed in an anaerobic jar and incubated at 37 °C. After 48 hours, the diameter of the growth inhibition zone around the antibiotic disks was evaluated and reported as resistant, intermediate, or sensitive.

2-5- Cholesterol Removal Capability Test

This test was conducted according to the method of Vasiee et al. (2020) [12]. After inoculation of the *L. rhamnosus* JCM 1136

bacterium in MRS Broth culture medium with cholesterol stock solution (Sigma-Aldrich) and 0.3% bile salt at 37 °C for 24 hours, the cultured medium was centrifuged at 6000 rpm for 8 minutes at 4 °C. 0.5 mL of the supernatant samples, 3.0 mL of 95% ethanol (Merck, Germany), and 2 mL of KOH (Merck, Germany) were added. The mixture was heated in a water bath at 60 degrees Celsius for 10 minutes. 5 mL of hexane (Merck, Germany) and 3 mL of distilled water were added. The mixture was kept at room temperature for 15 minutes for phase separation. 2 mL of hexane was transferred to a microtube and evaporated at 60 °C. 2 mL of O-Phthalaldehyde reagent (Sigma-Aldrich) was incubated for 10 minutes. After incubation, 1 mL of concentrated sulfuric acid was added and kept at room temperature for 10 to 15 minutes. The absorbance of the inoculated and non-inoculated samples was read at a wavelength of 570 nm. A standard curve of different cholesterol concentrations (0, 91.3, 81.7, 63.15, 25.31, 5.62, 125, 250, and 500 µg/mL) was plotted. The cholesterol absorption was calculated using the following equation (1):

Percentage of cholesterol absorption = (C-T)/C × 100 Equation (1)

Where C is the cholesterol concentration (µg/mL) in the non-inoculated culture medium and T is the cholesterol concentration (µg/mL) in the inoculated culture medium.

2-6- Cell surface hydrophobicity

The evaluation of the hydrophobicity level of strain *L. rhamnosus* JCM 1136 in adhering to non-polar solvents was performed according to the method of Brzegar et al (2021) [9]. The strain was centrifuged at 6000 g for 15 minutes. After two washes with sterile phosphate buffer, in order to reach an optical density of 0.6 to 0.7 at 600 nanometers (OD0), the strains were suspended in buffer. 3 mL of suspended strain in sterile phosphate buffer were added to 1 mL of n-hexadecane (Merck, Germany) and kept at room temperature for 15 minutes. The test tube containing the strain was vortexed for 3 minutes and then kept at room temperature for 30 minutes. Finally, the absorption of the aqueous phase (OD) was measured using the Equation (2) to determine the percentage of hydrophobicity.

Equation (2): Hydrophobicity= $\left[\frac{OD_0-OD}{OD_0}\right]\times 100$

2-7- Hemolytic activity test

This test was conducted according to the method of Saboktakin-Rizi et al. (2021) [8]. The hemolytic activity of strain *L. rhamnosus* JCM 1136 was examined after linear cultivation on agar medium enriched with 7% sheep blood volume-volume. The inoculated culture medium was incubated at 37 °C for 24 hours. The color changes were observed and analyzed. The formation of a clear halo, a green halo, or no halo around the colonies indicate β-hemolysis, α-hemolysis, and γ-hemolysis, respectively. *Staphylococcus aureus* and *Escherichia coli* bacteria were used as control samples for β-hemolysis and α-hemolysis in this test.

2-8- DNase enzyme production

This test was carried out according to the method of Vasiee et al. (2020) [12].

2-9- Biogenic amine production (BA) test

This test was performed according to the method of Barzegar et al. (2021) [9]. The ability of strain *L. rhamnosus* JCM 1136 to produce biogenic amines through decarboxylation of amino acids on a culture medium containing amino acid precursors such as L-histidine, monohydrochloride, disodium tyrosine salt, L-ornithine, and L-lysine was assessed. Initially, the lactic acid strain was inoculated in MRS Broth culture medium containing 1.0% of the mentioned amino acid precursors and 0.005% pyridoxal-5-phosphate. The strain was then inoculated again in MRS Broth culture medium

without amino acid precursors and containing 0.6% bromocresol purple (Sigma-Aldrich). After 2 to 5 hours of incubation, the formation of a purplish color in the culture medium was reported for biogenic amine production.

2-10- Antioxidant potential

The assessment of antioxidant activity was performed according to the method by Vasiee et al. (2022) [13]. Following the inoculation of *L. rhamnosus* JCM 1136 in MRS culture medium at 37 °C for 18 hours, incubation was carried out. After washing twice with sterile phosphate buffer, the mixture was centrifuged at 4000 rpm for 5 minutes. A solution of 0.2 millimolar DPPH (Sigma-Aldrich) was added to the sample at a volume-to-volume ratio of 1:2. It was kept at room temperature for 30 minutes. A solution of 0.7 millimolar ABTS (Sigma-Aldrich) with 2.45 millimolar potassium persulfate (Merck) at a 1:1 volume-to-volume ratio was prepared. After adding distilled water, the absorption of the solution was read at 734 nanometers, reaching 0.70 to 0.01. 300 mL of the sample and 600 mL of the ABTS solution were kept at room temperature for 30 minutes. The absorption at 517 nanometers for DPPH and 734 nm for ABTS was measured. Ascorbic acid solution was used as a positive control. The percentage of free radical inhibition by the target strain was calculated using Equation (3):

$$\text{Free Radical Inhibition} = (1 - A_{sample} / A) \times 100 \quad \text{Equation (3)}$$

In this Equation, A_{sample} is the absorption of the tested sample, and A is the absorption of the control sample.

2-11- Antimicrobial Test

To evaluate the antimicrobial activity of the *L. rhamnosus* JCM 1136 strain against common pathogenic microorganisms, Well Diffusion Agar and Disk Diffusion methods were used. The antimicrobial activity test included 3 Gram-negative pathogenic strains such as *Shigella dysenteriae* PTCC1188, *Salmonella typhimurium* PTCC1609, *Escherichia coli* ATCC25922, and 3 Gram-positive pathogenic strains like *Staphylococcus aureus* ATCC25923, *Listeria innocua* ATCC33090, and *Bacillus cereus* ATCC14579.

For the antimicrobial test using the Well Diffusion Agar method according to the Noshad et al. (2021) method, after activating the pathogenic strains, a suspension was prepared based on the half McFarland standard at a concentration of CFU/mL 1.5×10^8 The prepared suspension of pathogens was streaked on MHA culture medium using a spreader [1]. Wells with a diameter of 6mm were created on the inoculated plates using a sterile pipette tip. The activated *L. rhamnosus* JCM 1136 strain in MRS Broth culture medium was centrifuged at 5000 g for 10 minutes at 25 °C. The supernatant was filtered through a syringe filter with a diameter of 0.22 µm to obtain a cell-free supernatant. 100 microliters of the acidic and neutral supernatant from the *L. rhamnosus* JCM 1136 strain were poured into the wells on the MHA culture medium. After anaerobic incubation at 37 °C for 48 hours, the zone of growth inhibition around the well was measured using a ruler and reported in millimeters.

For the evaluation of antimicrobial activity of *L. rhamnosus* JCM 1136 by the diffusion method on agar using disks, as per the method by Alizadeh Behbahani et al. (2022) [6], paper disks with a diameter of 6mm were immersed in the supernatant prepared from the *L. rhamnosus* JCM 1136 strain for 15 minutes. Subsequently, the disks were placed on MHA culture medium inoculated with pathogenic bacteria. After anaerobic incubation at 37 °C for 48 hours, the zone of growth inhibition around the disk was measured using a ruler and reported in millimeters.

3- Results and Discussion

After molecular separation and identification, the viability assessment results of *L. rhamnosus* JCM 1136 at different pH levels are shown in

Figure 1. The results indicate that the mentioned bacterium had the capability to survive at various pH levels, although over time, the viability of the bacteria decreased at different pH levels. The number of *L. rhamnosus* JCM 1136 at pH 2 significantly decreased after a 3-hour incubation period, showing the highest logarithmic reduction compared to other pH levels. On the other hand, the number of bacteria at pH 4 exhibited the least logarithmic reduction, indicating that the bacterium at pH 4 had the highest level of resistance and survival potential.

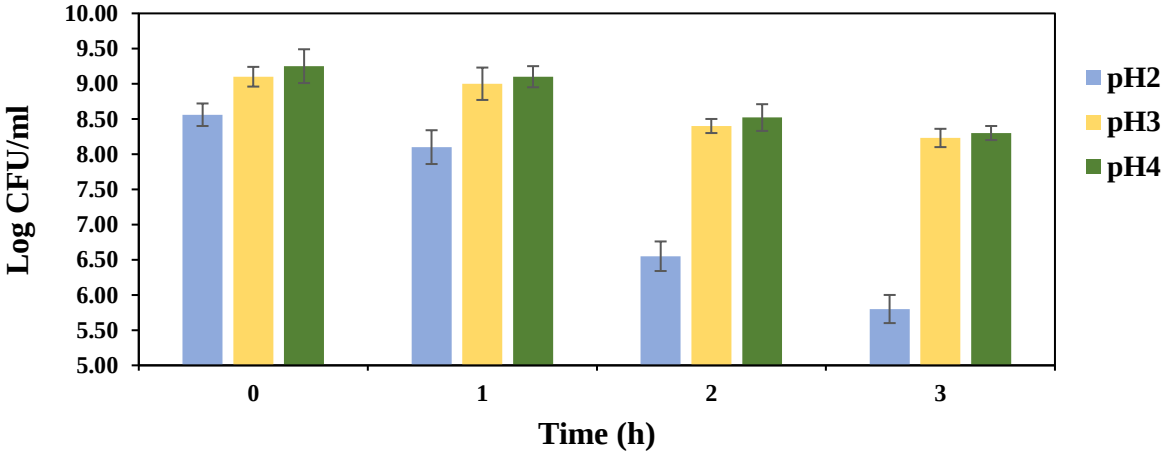

Fig. 1. The capacity of *Lacticaseibacillus rhamnosus* JCM 1136 to endure in an acidic pH environment.

The results of the examination of the resistance of *L. rhamnosus* JCM 1136 bacteria to different concentrations of bile salt are reported in Table 1. The results showed that *L. rhamnosus* JCM 1136 has good resistance to various concentrations of bile salt. In this study, with an increase in the percentage of bile salt, the growth of the tested bacteria gradually decreased. Specifically, at zero percent bile salt concentration (control sample), the highest level of growth was observed, while at 0.7 % bile salt concentration, no growth of the mentioned bacterium was observed.

Table 1. The ability of *Lacticaseibacillus rhamnosus* JCM 1136 to endure varying concentrations of bile salts

	0.3%	0.5%	0.7%	Control
Survivability	Growth	Growth	Not grown	Growth

The results related to the investigation of the sensitivity of *L. rhamnosus* JCM 1136 bacteria to various antibiotics are reported in Table 2. The results of this study showed that *L. rhamnosus* JCM 1136 was moderately sensitive to the antibiotic Ampicillin and sensitive to other antibiotics.

Table 2. Effect of common therapeutic antibiotics on *Lacticaseibacillus rhamnosus* JCM 1136

Antibiotic	JCM 1136 *L. rhamnosus*
Ampicillin	**Intermediate**
Clidamaycin	**Sensitive**
Nitrofurantion	**Sensitive**
ChlorampHenical	**Sensitive**
Ciprofloxacin	**Sensitive**
Penicillin	**Sensitive**
Tetracyclin	**Sensitive**
Erythromaycin	**Sensitive**

In the current study, the result of the test examining the cholesterol absorption capacity by strain *L. rhamnosus* JCM 1136 was 41.55 ± 0.43 %. The result of the cell surface hydrophobicity test by strain was 53.50 ± 0.55 %. Additionally, the strain did not show hemolytic activity, the ability to produce the DNase enzyme, or biogenic amines. *L. rhamnosus* JCM 1136 exhibited antioxidant activity (Figure 2). In this study, the inhibition percentage of free radicals DPPH and ABTS by the strain was reported as 44.23 ± 0.50 % and 48.50 ± 0.62 %, respectively.

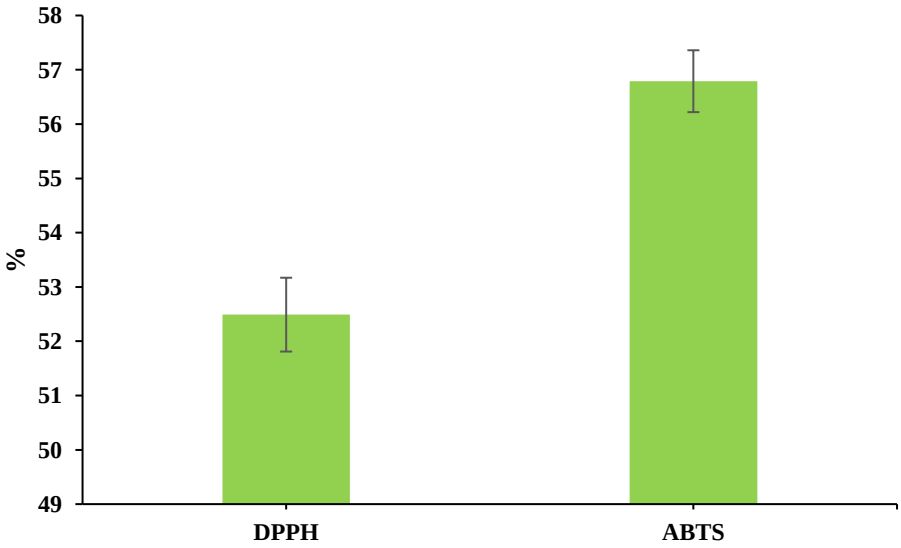

Fig. 2. The antioxidant activity (DPPH & ABTS) of *Lacticaseibacillus rhamnosus* JCM 1136.

The results of evaluating the antimicrobial activity of strain *L. rhamnosus* JCM 1136 against pathogenic bacteria such as *Shigella dysenteriae*, *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria innocua*, and *Bacillus cereus* using the

agar well diffusion and disk diffusion methods are shown in Figures 3 and 4. In this study, the antimicrobial activity of the cell-free supernatant in both acidic and neutral states was investigated against pathogenic pathogens. The results indicate that the strain under study had a significant inhibitory effect on the pathogenic strains. In the agar well diffusion method, the zone of inhibition for *Bacillus cereus*, *Listeria innocua*, and *Staphylococcus aureus* bacteria was 7 mm, 7.30 mm, and 7.50 mm, respectively. While the zone of inhibition due to the antimicrobial activity of the cell-free supernatant in the acidic state for these bacteria was 8.5 mm, 8.30 mm, and 9 mm, respectively. In the neutral state, the strain had no antimicrobial effect on *Escherichia coli*, *Salmonella typhimurium*, and *Shigella dysenteriae*, while the cell-free supernatant in the acidic state had an antimicrobial effect on *Escherichia coli* and *Salmonella typhimurium*. The zone of inhibition due to the antimicrobial activity of the cell-free supernatant in the acidic state of *L. rhamnosus* JCM 1136 strain for *Escherichia coli* and *Salmonella typhimurium* was 6.50 mm and 6.80 mm, respectively (Figure 3).

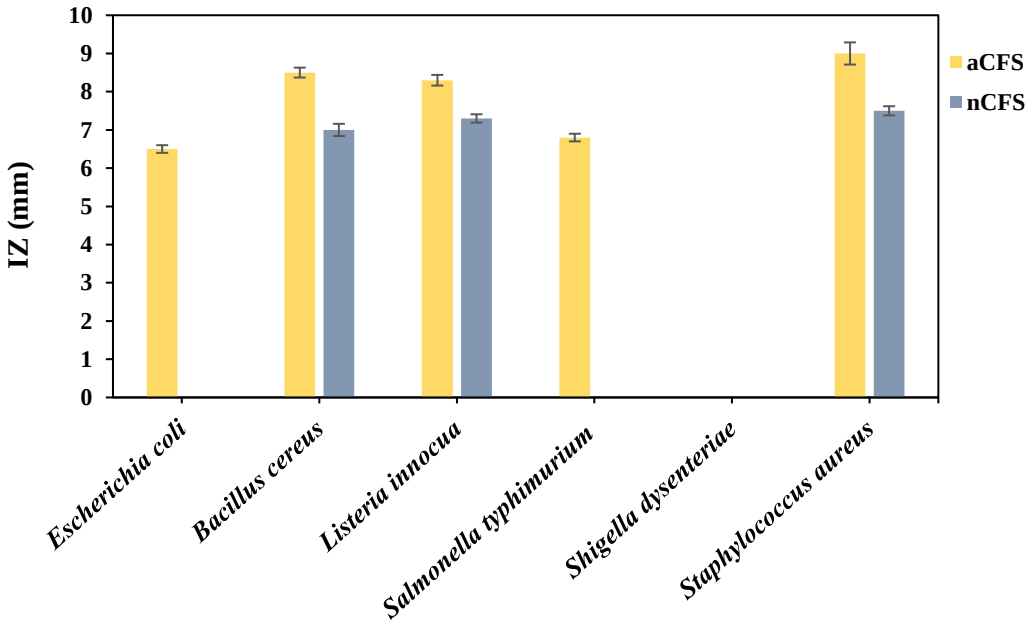

Fig. 3. The antimicrobial potency of *Lacticaseibacillus rhamnosus* JCM 1136 using well diffusion agar. The abbreviations aCFS and nCFS stand for acid cell-free supernatants and neutralized cell-free supernatants, respectively.

The zone of inhibition of the cell-free supernatant without neutral cells using the disk diffusion method on *Bacillus cereus*, *Listeria innocua*, and *Staphylococcus aureus* bacteria was 6.80 mm, 7 mm, and 7.10 mm, respectively. The cell-free supernatant without neutral cells had no antimicrobial effect on *Escherichia coli*, *Salmonella typhimurium*, and *Shigella dysenteriae*. The cell-free supernatant without neutral cells of *L. rhamnosus* JCM 1136 in the acidic state using the disk diffusion method had an antimicrobial effect on *Bacillus cereus*, *Listeria innocua*, *Salmonella typhimurium*, and *Staphylococcus aureus* bacteria. The zone

of inhibition for the mentioned pathogenic bacteria was 7.80 mm, 8 mm, 6.50 mm, and 8.60 mm, respectively. The cell-free supernatant without neutral cells of *L. rhamnosus* JCM 1136 in the acidic state had no antimicrobial effect on *Escherichia coli* and *Shigella dysenteriae* bacteria (Figure 4).

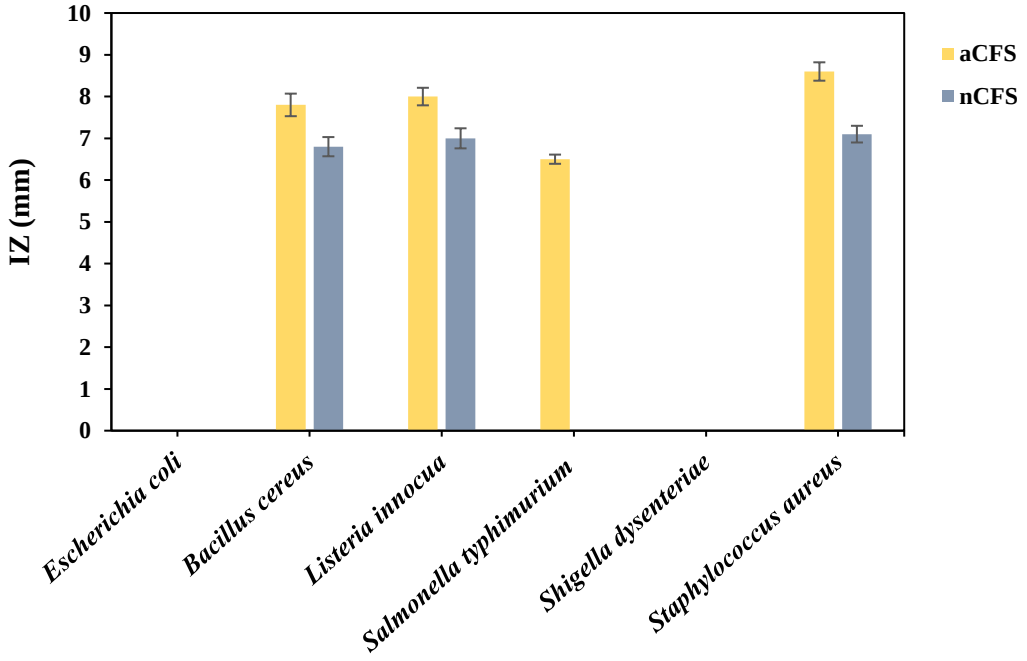

Fig. 4. The antimicrobial potency of *Lacticaseibacillus rhamnosus* JCM 1136 using disk diffusion agar. The abbreviations aCFS and nCFS stand for acid cell-free supernatants and neutralized cell-free supernatants, respectively.

One of the important features of probiotic microorganisms is their survival rate in food products and the human digestive system [12]. This feature is used to select probiotic species in the production of food products. Probiotic microorganisms must be able to tolerate acidity, bile salts, and high osmotic pressure in order to survive in the stomach and small intestine [14]. Control of central metabolic pathways, regulation of proton pumps, changes in cell membrane composition and density, repair of DNA and proteins, as well as neutralization processes are among the various mechanisms involved in regulating acid resistance by probiotic bacteria. Conditions such as acidic pH lead to a decrease in the growth and survival of lactic acid bacteria. In the human stomach, 2 liters of gastric juice with a pH close to 1.5 is secreted daily from the surface cells into the stomach, which can disrupt the biological membrane of bacteria due to their polarity. Therefore, the survival of lactic acid bacteria is due to their resistance to the acidic conditions of the stomach [15]. Resistance to bile salts is an important feature of probiotic microorganisms because bile salts can disrupt the cell walls of microorganisms and ultimately lead to their destruction. Probiotics hydrolyze bile salts through their hydrolyzing enzymes to reduce their undesirable effects. The gallbladder plays a significant role in the specific and nonspecific defense mechanisms of the intestine. According to

previous research, the average concentration of bile salts in the human gastrointestinal tract is 0.3% weight/volume [17].

Sarabi Jamab et al. (2018) investigated the survival rate of *L. rhamnosus* as a key probiotic bacterium in a simulated gastric and intestinal model. They reported that with every 30 minutes passing at different pH levels after incubation, the number of bacteria decreased. After 2 hours in the intestinal environment, the logarithm of live *L. rhamnosus* bacteria reached 0.65 [17]. In another study by Huq et al. (2017), to increase the survival of *L. rhamnosus* bacteria in stomach and intestinal conditions, they encapsulated them with alginate, cellulose nanocapsules, and lecithin capsules [18]. Maleki et al. (2020) used cheese whey isolate, inulin, and crystalline nanocellulose for the microencapsulation of *L. rhamnosus* [19]. Vaseei et al. (2020) reported that the strain *Pediococcus acidilactici* grew well in a 0.3% concentration of bile salts, which was consistent with the results of the present study [12]. Han et al. (2017) examined the probiotic properties of lactic acid bacteria isolated from fermented sausages and reported that some lactic acid bacteria can maintain their growth in 0.3% bile salts and pH levels of 3 and 8 [20]. Lactobacillus bacteria were isolated from dairy products in a study, and their growth was examined at different concentrations of bile salts 0.3%, 0.5%, and 1%. It was reported that the growth of Lactobacillus decreased in the presence of bile salts compared to the control sample, but the highest growth rate was reported at a 0.3% concentration of bile salts [4].

Biswal et al. (2021) reported that L. rhamnosus has the ability to survive at a pH below 2.5 and can grow in a 0.3 % bile salt concentration [21]. Their results were consistent with the findings of the present study. In another study, *L. rhamnosus* was encapsulated with alginate and chitosan. The bacterium was reported to be resistant to pepsin enzyme at pH 1.8 and pancreatin enzyme at pH 6.8, and the number of live bacteria after passing through a simulated gastric system was determined to be an acceptable level of CFU/ml 10^6 [22].

Today, the resistance of various bacteria to antibiotics has become a concern for human societies. Antibiotic resistance may occur due to genetic mechanisms such as gene transfer through plasmids, chromosomal mutations, ineffective transfer of aminoglycosides, and enzymatic modifications, and sometimes it naturally occurs in bacteria [15, 23]. Resistance of lactic acid bacteria to antibiotics may be due to the absence of the antibiotic target site in probiotic bacteria, inactivation of the antibiotic, and other factors. Studies have shown that the transfer of antibiotic-resistant genes in lactic acid bacteria is ongoing. Some lactic acid bacteria are resistant to one or more antibiotics, raising concerns regarding the use of these bacteria in the production of various food products and their consumption by humans. One important mechanism of antibiotic resistance in lactic acid bacteria is the presence of pumps that cause the antibiotic to be expelled from the cell, thereby reducing its concentration inside the cell [23]. The results of the present study indicated that the *L. rhamnosus* bacterium was sensitive to antibiotics such as Clindamycin, Nitrofurantoin, Chloramphenicol, Ciprofloxacin, Penicillin, Tetracycline, and Erythromycin, and semi-sensitive to Ampicillin. In a study by Shamsuddin and Mazharuddin Khan (2019), strains of Lactobacillus isolated from fermented foods such as Bifidobacterium bifidum were examined for sensitivity to twelve antibiotics. The isolated strains were found to be sensitive to antibiotics such as Amikacin, Ampiclox, Ciprofloxacin, Clarithromycin, Cefotaxime, Levofloxacin, Cefuroxime, Cefoperazone,

Gentamicin, Roxithromycin, Cotrimoxazole, and Azithromycin [24]. In another study, the sensitivity of Pediococcus pentosaceus and Lactobacillus paraplantarum strains isolated from various sourdoughs to the antibiotic Ampicillin was reported, while they were resistant to Vancomycin, Streptomycin, Acid nalidixic, and Ciprofloxacin antibiotics. The resistance of lactic acid isolates to Vancomycin was reported to be due to the presence of D-alanine instead of D-lactate or D-serine in the peptidoglycan structure of bacteria [23]. Biswal et al (2021) reported that the CRD4 strain of *L. rhamnosus* is sensitive to antibiotics such as Ciprofloxacin, Chloramphenicol, Amoxicillin, Ampicillin, and Tetracycline [21]. Their results were similar to the findings of the present study.

While the presence of cholesterol is essential for some body tissues, an increase in blood cholesterol levels in humans can lead to heart and vascular diseases, among others. Probiotic bacteria have the ability to absorb cholesterol and eliminate hydroxyl radicals. Therefore, human attention to lactic acid bacteria is increasing. A study reported that the bacterium *Pediococcus acidilactici* IAH-5 showed the highest cholesterol absorption capacity. Generally, the reduction in cholesterol levels by probiotics is related to the strain type, the production of compounds that inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, the activity of bile salt hydrolase, and the cholesterol absorption ability of probiotic strains [12].

Hydrophobicity is a property created by hydrophobic components present in the outer membrane of bacteria. The hydrophobicity of probiotic strains is related to their ability to attach to non-polar sources. This property is known as the hydrophobic effect and plays a significant role in bacterial adhesion to epithelial cells. Compounds such as xylene, n-hexadecane, chloroform, and ethyl acetate can be used to investigate this property. Hydrophobicity is calculated by measuring the water surface before and after adding the solution [1].

In the present study, the hydrophobicity level of the strain L. rhamnosus JCM 1136 was reported to be 53.50%. In a study by Saboktakin-Rizi et al. (2021), the hydrophobicity level of the strain *L. plantarum* TW29-1 was reported to be 53% [8]. The results of these researchers were consistent with the results of the present study. In another study, the hydrophobicity level of the strain *L. rhamnosus* CRD4 was reported to be 70% in the presence of xylene, 70% in the presence of toluene, and 57 % in the presence of chloroform. In a study by Barzegar et al. (2021), the hydrophobicity level of the strain *L. acidophilus* B14 was reported to be 65.9%.

The differences in the hydrophobicity levels of probiotic strains are related to the strain type, structural chemical heterogeneity, cell growth phase, environmental factors, degree of pleomorphism, van der Waals force, Brownian motion, surface charge, and gravitational force. Differences in intracellular proteins, hydrophobic amino acids, proteins and lipids of the cytoplasmic membrane, and polysaccharides also affect the hydrophobicity levels of probiotic strains [1, 9]. The S-layer is a single molecular layer made of identical proteins and glycoproteins. This layer plays an important role in the Brownian motion of probiotic bacteria and their hydrophobic properties. Hydrophobicity in probiotic bacteria used in the food industry, especially in dairy products, is important due to their tendency to bind to milk fat molecules and stabilize emulsions. [1].

In order to evaluate the safety of using probiotic strains, two tests are used to assess hemolytic properties and the production of the DNase enzyme. The DNase enzyme, also known as deoxyribonuclease, causes the hydrolysis of phosphodiester bonds in the DNA structure. In some food products, such as infant formula, arginine exists as an essential amino acid. Therefore, it is crucial when using probiotic bacteria in food products that the mentioned amino acid is not hydrolyzed by the bacteria

[25]. In the current study, no hemolytic activity or production of the DNase enzyme was observed in the *L. rhamnosus* JCM 1136 strain. Hence, this strain is safe for producing probiotic food products. Vasiee et al. (2020) reported that *Pediococcus* strains isolated from Iranian local dairy products did not produce the DNase enzyme and were non-hemolytic. Saboktakin-Rizi et al. (2021) reported that the *L. plantarum* TW29-1 strain isolated from local dairy products also did not produce the DNase enzyme and showed no hemolytic activity [8]. The results of the present study were consistent with the findings of the mentioned researchers.

Lactic acid bacteria, especially Enterococcus and Lactobacillus, have the ability to produce biogenic amines. This feature is considered undesirable in lactic acid bacteria, and lactic acid bacteria lacking this feature are used as probiotic bacteria. In the current study, no potential was observed for producing biogenic amines in the *L. rhamnosus* JCM 1136 strain. The potential for producing biogenic amines can vary among different strains of the same bacterial species, so identifying and determining the characteristics of bacterial strains for use as probiotic strains is important [25]. Biogenic amines are formed by amination and transamination of aldehydes and ketones or by decarboxylation of amino acids. Biogenic amines can be found in food products containing proteins and free amino acids that undergo biochemical and microbial processes such as fermentation. Their origin is usually related to fish products, fermented sausages, cheeses, and other fermented food products. The presence or absence of histamine and tyramine in probiotic food products is evaluated to assess the toxic and allergic properties of probiotics [9]. Barzegar et al. [9] and Fatemizadeh et al. [25] reported that the *Lactobacillus plantarum* M17 and *Lactobacillus acidophilus* B14 strains do not have the potential to produce biogenic amines. The results of the present study were consistent with the findings of the mentioned researchers.

Consumption of antioxidants leads to a reduction in oxidative stress in various tissues of the human body. The antioxidant activity of lactic acid bacteria occurs when they enter the human intestine through the consumption of probiotic food products. These bacteria, upon entering the intestinal tract, contribute to the destruction of various free radicals. Probiotics also have antioxidant activity due to their high levels of 1/3-β-D-glucan and other β-glucans in their cell walls. *Pediococcus acidilactici* IAH-5 has been reported to exhibit higher antioxidant activity compared to *L. rhamnosus*, with the ability to inhibit 58.32% of hydroxyl radicals [12]. Previous research suggests that *Enterococcus durans* can be used in food products as an antioxidant due to its high antioxidant activity and ability to inhibit free radicals like ABTS [26]. Iron ions present in compounds such as EDTA and Bathophenanthroline disulfonic acid (BPS) are effective in preventing oxidation. Lactic acid bacteria, with the presence of iron and copper ions, have the ability to inhibit free radicals. Additionally, compounds such as organic acids, bacteriocins, bacterial exopolysaccharides, manganese, glutathione (GSH), butyrate, folate, and superoxide dismutase enzyme also have the ability to prevent oxidation. Superoxide dismutase is an enzyme that catalyzes the conversion of superoxide to hydrogen peroxide and water [13, 27]. To evaluate the antioxidant activity of strain *L. rhamnosus* JCM 1136, the DPPH and ABTS free radical inhibition tests were used. The strain showed 44.23 ± 0.50 % and 48.50 ± 0.62% inhibition of DPPH and ABTS free radicals, respectively. In a study by Yang and Li (2022), it was reported that the inhibition of DPPH radicals by *L. rhamnosus* was 73.02%. Discrepancies in the mentioned results could be related to the strain type and test conditions [28]. In another study, Kim et al. (2022) reported inhibition rates of 2.55-6.88 % for DPPH radicals and 19.69-86.26% for ABTS radicals by lactic acid bacteria [13].

Researchers have reported that the activity of antimicrobial bacteria of lactic acid against pathogenic disease-causing pathogens is due to competition for attachment sites on the epithelial cells of the intestine and the presence of various acids such as acetic acid, lactic acid, propionic acid, sorbic acid, and benzoic acid, organic compounds such as diacetyl, ethanol, hydrogen peroxide, phenols, ammonia, low molecular weight proteins that are famous for bacteriocins, and inhibitory metabolites similar to bacteriocins (BLIS) [29, 30]. Bacteriocins not only disrupt the synthesis mechanism of lipid II in the cell walls of pathogens but also inhibit the activity of enzymes such as RNA polymerase and tRNA synthetase in them [23]. In the present study, the antimicrobial activity of cell-free supernatant *L. rhamnosus* JCM 1136 against pathogenic disease-causing pathogens using the well diffusion and disk methods was investigated. The results indicated that the diameter of the zone of inhibition of pathogens by the test strain in the well method ranged from 0 to 9 mm. The largest zone of inhibition was related to the antimicrobial activity of cell-free supernatant. The diameter of the zone of inhibition of pathogens compared to cell-free supernatant *L. rhamnosus* JCM 1136 for Gram-positive pathogenic bacteria *Staphylococcus aureus*, *Listeria innocua*, and *Bacillus cereus* was 7.50 ± 0.29 mm, 7.30 ± 27 mm, and 7 ± 0.23 mm, respectively. The diameter of the zone of inhibition of pathogens compared to cell-free supernatant *L. rhamnosus* JCM 1136 for gram-positive pathogenic bacteria Staphylococcus aureus, Bacillus cereus, and Listeria innocua was 9 ± 0.27, 8.50 ± 0.15, and 8.30 ± 19 mm, respectively. The most resistant pathogen to the antimicrobial effect of cell-free supernatant was the gram-negative bacterium *Shigella dysenteriae*, showing no antimicrobial effect. The diameter of the zone of inhibition of cell-free supernatant for gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* was 6.50 ± 0.13 and 6.80 ± 0.12 mm, respectively. The diameter of the zone of inhibition of cell-free supernatant *L. rhamnosus* JCM 1136, which was both acid-free and neutral, using the disk diffusion method, ranged from 0 to 60.8 mm. In this method as well, the highest antimicrobial activity was related to the acid-free cell-free supernatant. The most sensitive pathogens to the cell-free supernatant without acid and neutral using the disk diffusion method were gram-positive bacteria *Staphylococcus aureus*, *Listeria innocua*, and *Bacillus cereus* with zone of inhibition diameters of 7.10 ± 0.25, 7 ± 0.13, and 6.80 ± 18 mm, respectively. The most sensitive strains of pathogens to the cell-free supernatant without acid were gram-positive pathogenic bacteria *Staphylococcus aureus*, *Listeria innocua*, and *Bacillus cereus* with zone of inhibition diameters of 8.60 ± 0.22, 8 ± 0.14, and 7.80 ± 0.11 mm, respectively. The cell-free supernatant without acid and neutral *L. rhamnosus* JCM 1136 did not have any antimicrobial effect on Gram-negative bacteria *Shigella dysenteriae* and *Escherichia coli*. The Gram-negative bacterium *Salmonella typhimurium* was resistant to the cell-free supernatant without neutral, with a zone of inhibition diameter of 6.50 ± 0.10 mm in the acidic state. The results of the agar well and disk diffusion antimicrobial tests indicated that the acid-free cell-free supernatant *L. rhamnosus* JCM 1136 isolated from local yogurt had higher antimicrobial activity compared to the neutral state. One of the important mechanisms of probiotic bacteria in inhibiting the growth of pathogenic bacteria is the production of organic acids. It is obvious that with an increase in acidity, the rate of inhibiting the growth of pathogenic bacteria by them increases [31]. The results of this test were consistent with the results of Vasiee (2020) [12]. These researchers reported that the acid-free supernatant of strain *P. acidilactici* VKU2 had greater antimicrobial effect compared to the neutral supernatant. In a study, the antimicrobial effect of strain *L. rhamnosus* CRL 2244 on the bacterium *Acinetobacter baumanni* was investigated and it was reported that the mentioned probiotic strain led to the destruction of the cell wall of the pathogenic bacterium due to the production

of organic acids such as lactic acid and acetic acid [7].

In the present study, strain *L. rhamnosus* JCM 1136 had a greater antimicrobial effect on Gram-positive pathogenic bacteria. The mechanism of antimicrobial activity of probiotic bacteria against Gram-negative and Gram-positive bacteria is different. Probiotic bacteria destroy Gram-negative pathogenic bacteria through the production of organic acids such as hydroxyl, hydrogen peroxide, carbon dioxide, and fatty acids. Additionally, bacteriocin produced by these bacteria is effective in destroying Gram-positive pathogenic bacteria. In a study, the antimicrobial effect of Lactobacillus bacteria isolated from dairy products in Kerman on pathogenic disease-causing bacteria was investigated, and it was reported that the zone of inhibition diameter ranged from 5.7 to 44.02 millimeters, with the highest antimicrobial effect of the isolated Lactobacillus strains reported against the Gram-positive bacterium *Bacillus cereus*.

4- Conclusion

Today, probiotic bacteria have gained attention as biological preservatives with antimicrobial activity and the potential to improve human health in the production of food products. Based on the results of the present research, the *L. rhamnosus* strain JCM 1136 isolated from local yogurt showed tolerance and survival in acidic pH and various concentrations of bile salts. *L. rhamnosus* JCM 1136 exhibited hydrophobicity, antioxidant activity, cholesterol absorption capability, and desirable antimicrobial potential against various pathogenic bacteria, without producing undesirable metabolites such as DNase enzyme, biogenic amines, and lacking hemolytic activity. The targeted probiotic bacteria showed sensitivity to most common antibiotics and there is no concern about the transfer of antibiotic-resistant genes to pathogenic bacteria by the probiotic bacteria. Therefore, with further external and internal studies such as adhesion to intestinal epithelial cells, bacteriocin production potential, blood sugar reduction, etc., this bacterium can be used in the production of probiotic food products.

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ارزیابی ویژگی‌های پروبیوتیکی، ضدباکتریایی و ایمنی سویه *Lacticaseibacillus rhamnosus* JCM 1136

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چکیده

پروبیوتیک‌ها باکتری‌های غیربیماری‌زا و مفیدی هستند که موجب بهبود سلامت دستگاه گوارش، ممانعت از ابتلا به انواع سرطان، تقویت سیستم ایمنی بدن و ... می‌شوند. هدف از پژوهش حاضر، مطالعه و بررسی پتانسیل پروبیوتیکی و فعالیت ضدمیکروبی سویه *Lacticaseibacillus rhamnosus* JCM 1136 بود. در این پژوهش، ویژگی‌های پروبیوتیکی و ایمنی سویه شامل مقاومت به اسید در PHهای ۲، ۳ و ٤، مقاومت به صفرا (صفر، ۰/۳، ۰/۵ و ۰/۷ درصد غلظت نمک صفراوی)، هیدروفوبیستی، پتانسیل تولید آنزیم DNase و آمین‌های بیوژنیک، میزان فعالیت همولیتیک، میزان فعالیت آنتی‌اکسیدانی، میزان جذب کلسترول، مقدار مقاومت به آنتی‌بیوتیک‌های رایج بررسی شد. اثر ضدمیکروبی سویه علیه پاتوژن‌های بیماری‌زا (*Salmonella typhimurium*، *Shigella dysenteriae*، *Escherichia coli*، *Staphylococcus aureus*، *Listeria innocua* و *Bacillus cereus*) به‌روش انتشار در آگار به‌کمک چاهک و دیسک ارزیابی گردید. نتایج نشان داد سویه *L. rhamnosus* JCM 1136 توانایی زنده‌مانی در PHهای مختلف را داشت. میزان رشد سویه با افزایش غلظت نمک صفراوی کاهش یافت. *L. rhamnosus* JCM 1136 نسبت به آنتی‌بیوتیک Ampicillin نیمه‌حساس و نسبت به سایر آنتی‌بیوتیک‌ها حساس بود. هیدروفوبیستی، فعالیت آنتی‌اکسیدانی به روش های DPPH و ABTS و میزان جذب کلسترول سویه به ترتیب ۰/۵۰ ± ۵۳/۵۰ درصد، ۰/۵۰ ± ٤٤/۲۳، ۰/٦۲ ± ٤۸/۵۰ درصد و ۰/٤۳ ± ٤۱/۵۵ درصد بود. هیچگونه تولید آنزیم DNase، آمین‌های بیوژنیک و فعالیت همولیتیک از سویه مشاهده نشد. *L. rhamnosus* JCM 1136 دارای اثر ضدمیکروبی بیشتری بر باکتری‌های گرم مثبت بود. نتایج نشان داد که *L. rhamnosus* JCM 1136 دارای ویژگی‌های پروبیوتیکی مطلوب می‌باشد و قابلیت کاربرد در تولید محصولات غذایی پروبیوتیکی را نیز دارد.