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Examining the impact of probiotic *Lactiplantibacillus pentosus* 6MMI on inhibiting biofilm formation, adhesion, and virulence gene expression in *Listeria monocytogenes* ATCC 19115

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ABSTRACT

Probiotic bacteria improve human health by secreting pro-microbial substances, balancing intestinal flora, binding to the mucous membrane and epithelium, strengthening the intestinal epithelial barrier, and creating interactions between the gastrointestinal microbiota and the immune system. This study aimed to investigate the probiotic potential, biofilm-related gene expression and anti-biofilm capabilities of *Lactiplantibacillus pentosus* 6MMI. The strain exhibited remarkable resilience to challenging conditions, including acidic environments, gastrointestinal settings, and bile salts. Notably, *Lpb. pentosus* demonstrated significant hydrophobicity (71.89 %), auto-aggregation (42.39 %), co-aggregation (51.28 %), antioxidant activity (ranging from 42.29 % to 64.61 %), and a cholesterol reduction capacity of 50.31 %. Its competitive abilities against *Listeria monocytogenes* were quantified, showing a competition rate of 54.51 %, displacement rate of 48.57 %, and inhibition of adhesion at 27.71 %. Also, *Lpb. pentosus* resulted an adhesion rate of 12.91 % to epithelial cells and showed no DNase or hemolytic activity. The strain exhibited the highest resistance to nalidixic acid, with an inhibition zone measuring 15.20 mm, while it was least resistant to chloramphenicol, which had an inhibition zone of 27.30 mm. Treatment with cell-free supernatant (CFS) from *Lpb. pentosus* significantly reduced biofilm formation by 91.25 % and 24.50 % and diminished mature biofilm formation by 83.82 % and 21.80 % on *L. monocytogenes*. Additionally, the CFS inhibited the transcription of the *plcB*, *hly*, and *prfA* genes in *L. monocytogenes*, suggesting a potential reduction in bacterial virulence through decreased hemolysin release and modulation of phospholipase activity. In the next step of the study, the Gaussian Process Regression (GPR) model accurately predicted bile tolerance and acid parameters with a high R^2 of 0.99 and minimal Mean Absolute Percentage Error (MAPE) values of 0.33 % and 0.21 %, respectively. The residual errors showed a normal distribution, indicating reliable and consistent predictions. Overall, *Lpb. pentosus* 6MMI represents a valuable candidate for further investigation in probiotic development and biofilm management strategies.

1. Introduction

Today, the interest in the consumption of food containing probiotic bacteria is growing among humans. Probiotic bacteria are live microorganisms that provide health benefits to the host when consumed in adequate amounts. The American Food and Drug Administration and the European Food Safety Authority have declared probiotic bacteria as General Recognized As Safe (GRAS) [1]. When consumed continuously at 10^7 colony forming unit (CFU)/mL, these bacteria can have a notable effect on the human body. According to research, probiotics improve human health by secreting pro-microbial substances, balancing intestinal flora, binding to the mucous membrane and epithelium, strengthening the intestinal epithelial barrier, and creating interactions between the gastrointestinal microbiota and the immune system [2–4]. These bacteria can reduce lactose intolerance, alleviate allergies, prevent inflammation and infection, reduce the risk of colon and liver cancer,

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lower blood cholesterol, improve the immune system, treat and prevent diarrhea and constipation, enhance the absorption of minerals and vitamins (B and K), and prevent the growth and proliferation of harmful bacteria [5].

Lactic acid bacteria (LAB) are considered one of the most important groups of probiotic bacteria. LAB are Gram-positive, spherical or rod-shaped, facultative anaerobic, catalase-negative, microaerophilic, and lack the ability to produce spores and indole. LAB produce antimicrobial compounds such as lactic acid, diacetyl, and bacteriocins by consuming available carbohydrates [6–8]. LAB compete with pathogenic microorganisms for nutrients and binding sites, and through the competitive elimination of these pathogens, they contribute to the health of the digestive tract. Additionally, LAB can alter the metabolism of pathogenic microorganisms and prevent their growth and multiplication by stimulating the immune system. Therefore, LAB have been introduced as safe preservatives for food products [2].

One of the bacteria in this family, *Lactiplantibacillus pentosus*, is a member of the *Lactobacillus* genus, which constitutes 1–6 % of the intestinal microbial flora. It has a unique ability to produce probiotic products such as yogurt, cheese, kimchi, and pickles [9]. According to studies, this bacterium improves the taste, texture, and nutritional value of products. While the optimal pH for the growth of this bacterium is between 5.5 and 5.8, it can also grow well at pH 5. Research indicates that the optimal temperature for the growth of *Lpb. pentosus* is between 2 and 53 °C [9]. This bacterium can survive, multiply, and maintain function in the acidic and biliary conditions of the digestive tract, leading to cholesterol reduction, hydrolysis of bile salts, antimicrobial activity, antioxidant effects, and more.

Listeria monocytogenes is one of the important pathogenic factors that cause listeriosis. Consumption of food contaminated with this bacterium, especially red meat, can lead to symptoms such as diarrhea, nausea, muscle pain, fever, and neurological disorders in humans. *L. monocytogenes* can survive and multiply at temperatures ranging from 1 to 45 °C and at pH levels from 4.3 to 9.8 [4]. This bacterium can become resistant to antimicrobial agents by forming bacterial biofilms, which can cause problems by attaching to various surfaces, such as food industry equipment. According to research, LAB exhibit significant antimicrobial effects on the biofilms formed by *L. monocytogenes* and are considered a promising strategy to reduce microbial agents, maintain the quality of food products, prevent spoilage, and create beneficial effects on human health while improving immunity [9,10].

The combination of Artificial Neural Networks (ANN) and Gaussian Process Regression (GPR) models has several promising applications in food science and technology [11]. These models can be used for quality control and prediction, such as forecasting the shelf life of perishable items by analyzing factors like temperature and microbial growth [12]. They also optimize food processing parameters, improving efficiency and product quality in processes like drying and fermentation [13]. Additionally, ANN-GPR models predict sensory attributes like taste and texture based on chemical composition, aiding in the development of new products [14]. They assist in nutritional analysis by estimating the nutritional content of food products, ensuring healthier options and compliance with standards [10]. Furthermore, these models enhance food safety by predicting the presence of contaminants or pathogens, thus preventing foodborne illnesses. Some studies have confirmed the benefits of using ANN in food science and technology [10,11,14–16]. Faradonbeh et al. [13] evaluated the active packaging coating using *Ocimum basilicum* seed mucilage and *Hypericum perforatum* extract and applied a GPR model to predict various laboratory parameters. The modeling results indicated that GPR can accurately predict all output parameters and can be a sustainable solution for meat preservation.

This research focused on exploring the various properties of the *Lpb. pentosus* strain 6MMI, specifically its potential as a probiotic, antimicrobial agent, and antibiofilm producer, along with its cytotoxicity and safety profile. A comprehensive series of tests were performed to assess its tolerance to acid and bile, cholesterol absorption capabilities, and antioxidant activity. Additionally, we examined its anti-adhesive properties through mechanisms such as competition, inhibition, and translocation, as well as its hydrophobic characteristics, self-aggregation ability, and adhesion potential to Caco-2 cells. The study also investigated the strain's anti-adhesion microbial activity using methods like agar disk diffusion, agar well diffusion, modified two-layer techniques, and minimum inhibitory concentration (MIC) assessments. Other evaluations included cytotoxicity assays, biofilm formation and inhibition abilities, production of biogenic amines, DNase activity, absence of hemolytic activity, and resistance to antibiotics.

2. Materials and methods

2.1. Probiotic strain isolation

A portion of 5 g of traditional yogurt samples was thoroughly mixed with 45 mL of 0.1 % peptone water. Following this, serial dilutions were created and plated onto De Man–Rogosa–Sharpe (MRS) agar [17].

2.2. Morphological, physiological and biochemical features

The initial identification of the strain among the pure isolates involved evaluating cell morphology, performing Gram-staining, and conducting a catalase test. Its tolerance to various temperatures (15, 37, and 45 °C), pH levels (2, 4, and 6), and salt concentrations (2 %, 3 %, and 6 %) was assessed. Furthermore, the strain's capacity to ferment a range of carbohydrates was also tested [9,18].

2.3. Molecular identification

Genomic DNA was extracted from the strain after it was cultured overnight in MRS broth. To amplify the 16S rRNA gene, universal primers 27FYM (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were utilized. The processes of PCR amplification, gene sequencing, and homology analysis were conducted following the methods outlined by Saboktakin-Rizi et al. [17]. The isolated strain was identified as *Lpb. pentosus* strain 6MMI, showing a 98 % similarity and registered under ACCESSION ON763298.

2.4. Acid and bile tolerance

The methodology employed in this study was modified from the approach used by Cheruvari and Kammara [19], incorporating some modifications. Initially, the isolated strain was incubated for 24 h in MRS broth, after which it was further cultured at a 10 % concentration in two different growth media. One medium consisted of MRS broth adjusted to pH levels of 2.0, 3.0, and 4.0 to assess acid tolerance, while the other was MRS broth supplemented with 0.1–0.7 % bile salt to evaluate bile tolerance. Throughout the experiment, the cultures were maintained under aerobic conditions with agitation at 200 rpm and a constant temperature of 37 °C. Samples were collected at intervals of 0, 1, 2, and 3 h for analysis. The cultures underwent serial dilutions before being plated on MRS agar, which was then incubated for 24 h at 37 °C. Bacterial growth was quantified by counting the colony-forming units per milliliter (CFU/mL).

2.5. Resistance to simulated gastrointestinal conditions

The cells were concentrated through centrifugation at 6000 rpm for 10 min at room temperature, after which they were re-suspended in sterile MRS broth. A 10 % culture was then added to MRS broth that had been supplemented with simulated gastric fluid containing 0.3 % pepsin at pH 3, as well as intestinal fluid with 0.1 % pancreatin and 0.15 % bovine bile salt at pH 8. These cell suspensions were incubated for 4 h at 37 °C while shaking at 200 rpm. Finally, the viable cell counts were determined and reported as colony-forming units per milliliter (CFU/

Table 1
Primers used in this study [23].

Gene	Primer
flaA	Forward CTGGTATGAGTCGCCTTAG
	Reverse CATTTGCGGTGTTTGGTTTG
inlB	Forward AAGCAMGATTTCATGGGAGAGT
	Reverse TTACCGTTCCATCAACATCATAACTT
sigB	Forward GATGATGGATTTGAACGTGTGAA
	Reverse CGCTCATCTAAAACAGGGAGAAC
agrA	Forward ATGAAGCAAGCGGAAGAAC
	Reverse TACGACCTGTGACAACGATAAA
hly	Forward AACCAGATGTTCTCCCTGTA
	Reverse CACTGTAAGCCATTTCGTCA
prfA	Forward CGGGAAGCTTGGCTCTATTTG
	Reverse GCTAACAGCTGAGCTATGTGC
plcB	Forward CAGGCTACCACTGTGCATATGAA
	Reverse CCATGTCTTCYGTTGCTTGATAATTG

mL) [19].

2.6. Surface hydrophobicity

The strain was subjected to centrifugation at 6000*g* for 15 min. To achieve an optical density (OD) of 0.6–0.7 at 600 nm ($OD_{initial}$), the strain was resuspended in a buffer solution. Subsequently, 3 mL of the resuspended strain was combined with 1 mL of n-hexadecane and incubated at room temperature for 15 min. The test tube containing the mixture was then vortexed for 3 min. After resting at room temperature, the absorption of the aqueous phase was measured (OD_{final}) and the surface hydrophobicity was calculated using equation (1) [7,9]:

$$\text{Surface hydrophobicity } (\%) = \frac{OD_{initial} - OD_{final}}{OD_{initial}} \times 100 \quad (1)$$

2.7. Aggregation

The auto-aggregation and co-aggregation capabilities of *Lpb. pentosus* 6MMl were assessed using modified methods from Alonso García et al. [20]. Cultures of *Lpb. pentosus* 6MMl were grown overnight, then harvested and resuspended in sterile Dulbecco's phosphate-buffered saline (DPBS). The optical density (OD) at 580 nm was measured at both 0 and 2 h of incubation, and the auto-aggregation percentage was calculated from equation (2):

$$Auto-aggregation\ (\%) = \frac{OD_{initial} - OD_{final}}{OD_{initial}} \times 100 \quad (2)$$

To evaluate co-aggregation, overnight cultures of *Lpb. pentosus* 6MMl and pathogenic bacteria were collected, washed, and then resuspended in sterile DPBS until achieving an optical density of OD600 = 1.0. The optical density of the upper suspension was recorded at the initial time point (time = 0) and again after 1 h, following the combination of 3 mL of each suspension in individual tubes, and the co-aggregation percentage was calculated from equation (3):

$$Co-aggregation\ (\%) = \left(1 - \frac{OD_{final}}{OD_{initial}}\right) \times 100 \quad (3)$$

2.8. Adhesion

The adhesion characteristics of the strain were examined using the human intestinal epithelial cell line Caco-2 [21]. Caco-2 cells were grown at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10 % fetal bovine serum and 1 % penicillin-streptomycin, maintained under humidified conditions of 95 % air and 5 % CO_2. For the adhesion assay, the cells were seeded at a density of 5×10^5 cells per mL and allowed to incubate until they formed a confluent monolayer. After washing the cells with PBS, a suspension of the isolates was created through centrifugation and then re-suspended in DMEM free of antibiotics. This suspension was incubated with the Caco-2 cells for 90 min. Subsequently, the cells were washed to eliminate any unbound bacteria, lysed using Triton X-100, and the number of adhered bacteria was determined using the spot plate method on MRS agar, with the results calculated according to a formula (4):

$$Adhesion\ (\%) = \frac{The\ number\ of\ probiotic\ cells\ adhered\ to\ Caco-2\ cells}{The\ total\ number\ of\ inoculated\ probiotic\ cells} \times 100 \quad (4)$$

2.9. Anti-infection

The methods established by Ref. [22], were utilized to assess the anti-infective properties of the strain in preventing the adhesion of *L. monocytogenes* ATCC 19115 to Caco-2 cells, focusing specifically on the mechanisms of competition, inhibition, and displacement.

2.10. Antimicrobial effect

The antimicrobial activity of *Lpb. pentosus* 6MMl was evaluated against various pathogenic strains, including *Shigella dysenteriae*, *Escherichia coli*, *Klebsiella aerogenes*, *Salmonella typhi*, *Bacillus cereus*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *L. monocytogenes*, *Rhizopus stolonifera*, *Aspergillus niger*, and *Botrytis cinerea*. *Lpb. pentosus* 6MMl was cultured in MRS broth at 37 °C for 28 h and then centrifuged to obtain cell-free supernatants (CFSs). A portion of the CFSs was maintained at its original pH, while the other was adjusted to pH 5.5 to reduce the effects of organic acids. Both the acidified (aCFS) and neutralized (nCFS) supernatants were filtered, lyophilized, and reconstituted in sterile distilled water for antimicrobial testing using various methods, including disk diffusion agar, well diffusion agar, modified double layer, and MIC assessments ([9]; Alizadeh Behbahani and Noshad, 2024; [12,23]).

The crystal violet assay was used to evaluate the capacity of CFS to inhibit and degrade biofilms formed by *L. monocytogenes* [9]. Moreover, the expression levels of genes associated with biofilm formation and virulence (Table 1) were assessed using RT-PCR [23].

2.11. Antioxidant effect

The DPPH and ABTS free radical scavenging activity of the strain, along with its effectiveness in inhibiting linoleic acid peroxidation, was assessed using the methodologies outlined by Sreepathi et al. [24] and Shivangi, Devi, Ragul, and Shetty [25].

2.12. Cytotoxicity

The cytotoxic effect of the CFS on Caco-2 cancer cells was assessed using the (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) MTT assay. Initially, 10^5 cells were cultured in a 96-well microplate. Once the cells reached at least 70 % confluence, the culture medium was replaced with fresh DMEM and fetal bovine serum (FBS) (200 μL), and varying concentrations of the CFS (ranging from 0.195 to 200 mg/mL) were added to each well. The samples were then incubated at 37 °C in an environment with 95 % humidity and 5 % CO_2. After 24 h, MTT solution (5 mg/mL, 30 μL) was introduced to each well and further incubated for 3 h. Following this incubation, the medium was removed, and DMSO (200 μL) was added to the wells. Finally, the absorbance was measured using an ELISA microplate reader at a wavelength of 570 nm. The concentration of the CFS (mg/mL) that resulted in a 50 % reduction in cell growth was designated as the IC_{50} [1].

2.13. Cholesterol assimilation

The strain's capability to assimilate cholesterol was assessed in MRS

Table 2
Morphological, physiological and biochemical features of *Lactiplantibacillus pentosus* 6MMI

Characteristics	Results
Gram	Positive
Shape	Rod
Growth at 2 % NaCl	Growth
Growth at 3 % NaCl	Growth
Growth at 6 % NaCl	Growth
Growth at pH 2	Growth
Growth at pH 4	Growth
Growth at pH 6	Growth
Growth at 15 °C	Partial growth
Growth at 37 °C	Growth
Growth at 45 °C	Partial growth
Catalase	Negative
Fermentation of xylose	Growth
Fermentation of arabinose	Growth
Fermentation of glucose	Growth
Fermentation of fructose	Growth
Fermentation of maltose	Growth
Fermentation of lactose	Growth
Fermentation of sucrose	Growth
Fermentation of mannitol	Growth
Fermentation of galactose	Growth
Fermentation of raffinose	Growth

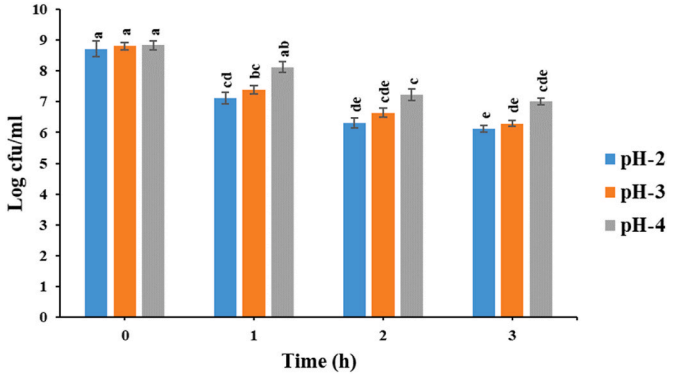

Fig. 1. Survival (log CFU/mL) of *Lactiplantibacillus pentosus* 6MMI in relation to different acidic pH levels. Samples that are labeled with different letters indicate significant differences at $p < 0.05$.

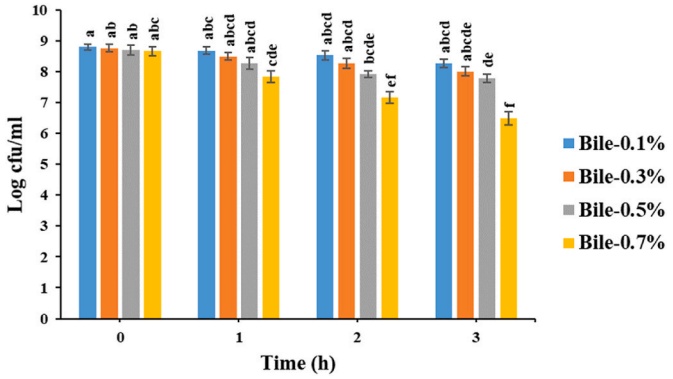

Fig. 2. Survival (log CFU/mL) of *Lactiplantibacillus pentosus* 6MMI in relation to different bile concentrations. Samples that are labeled with different letters indicate significant differences at $p < 0.05$.

broth enriched with cholesterol-polyethylene glycol (100 μg/mL). The inoculum was introduced into the MRS-cholesterol mixture and then incubated anaerobically at 37 °C for a duration of 24 h. Cholesterol was extracted from the MRS broth, and the remaining cholesterol levels were quantified [26,27].

2.14. Safety evaluation

The strain's capability to produce biogenic amines, as well as its DNase and haemolytic activities, was assessed using the methods outlined by Alizadeh Behbahani, Jooyandeh, and Namazi [10]. The sensitivity of *Lpb. pentosus* 6MMl to several antibiotics, including imipenem, chloramphenicol, erythromycin, ampicillin, nitrofurantoin, ciprofloxacin, and nalidixic acid, was tested using a modified method from Zhou et al. [28]. The cells were cultured on MRS agar, antibiotic discs were placed on the medium, and after a 48-h incubation at 37 °C, the diameters of the resulting inhibition zones were measured.

2.15. Statistical analysis

The experiments were carried out using a completely randomized factorial design. Data analysis was performed with Minitab 19 software, using Tukey test ($p < 0.05$). Each measurement was repeated at least three times.

2.16. Gaussian Process Regression (GPR)

GPR is a flexible machine learning technique used for regression tasks [29]. GPR allows to provide not only predictions but also uncertainty estimates for those predictions. The model relies on a covariance function (or kernel) to define the relationship between data points, which helps in capturing the underlying patterns in the data [30]. One of the key advantages of GPR is its ability to handle small datasets effectively and provide smooth predictions. Additionally, GPR is non-parametric, meaning it does not assume a fixed form for the underlying function, making it highly adaptable to various types of data [31]. The GPR model can be mathematically expressed as formula (5) [32]:

$$f(x^*) : N[\mu(x^*), k(x^*, x^*)] \tag{5}$$

Where, $f(x^*)$ is the predicted value at a test point x^*, N denotes a normal distribution, $\mu(x^*)$ is the mean function evaluated at x^*, $k(x^*, x^*)$ is the covariance function evaluated at x^*.

In this study, the Mean Absolute Percentage Error (MAPE) and the coefficient of determination (R^2) were used to evaluate the GPR model, as shown in equation (6) and equation (7), respectively [31]:

$$\mathrm{MAPE} = \frac{1}{n} \times \sum_{j=1}^{n} \left| \frac{d_j - p_i}{d_j} \right| \times 100 \tag{6}$$

$$R^2 = \frac{\sum_{j=1}^{n} \left((d_j - \bar{d}) \times \left(p_j - \bar{p} \right) \right)^2}{\sum_{j=1}^{n} (d_j - \bar{d})^2 \sum_{j=1}^{n} \left(p_j - \bar{p} \right)^2} \tag{7}$$

where d_j and p_j is the components of the actual and desired outputs, also the mean of actual and predicted data was showed by $\bar{d}$ and $\bar{p}$. Finally, n shows the number of all variables.

3. Results and discussion

The bacterium, identified by its rod-like form and positive Gram reaction, thrived in conditions with sodium chloride concentrations between 2 % and 6 % and pH values from 2 to 6. It showed a negative result for catalase and was able to grow at a temperature of 37 °C, with some growth also observed at 15 °C and 45 °C. Furthermore, Table 2 provided data on the fermentation of carbohydrates.

To successfully enhance gut health, probiotic strains must withstand the harsh conditions of the gastrointestinal tract, particularly the acidic

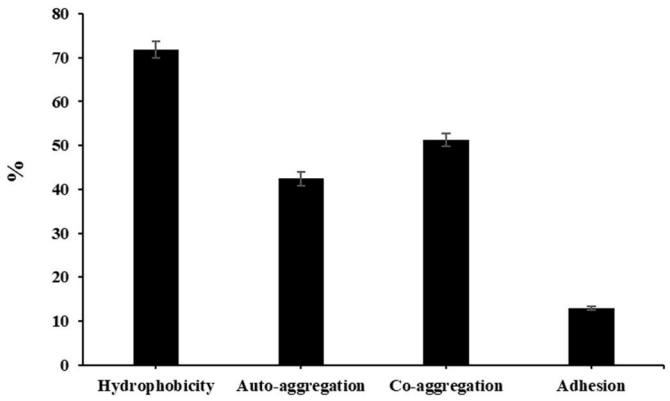

Fig. 3. Cell surface properties of *Lactiplantibacillus pentosus* 6MMI.

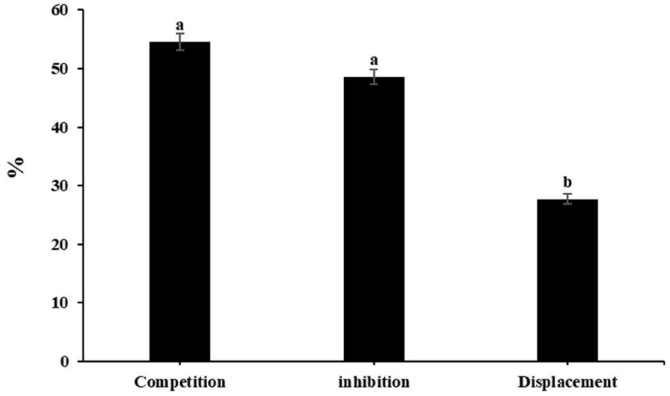

Fig. 4. Anti-adhesion properties of *Lactiplantibacillus pentosus* 6MMI. Significant differences ($p < 0.05$) are denoted by different superscript letters.

environment found in the stomach. Fig. 1 illustrates the pH tolerance of *Lpb. pentosus* 6MMl. It demonstrates that the strain can withstand low pH levels. Although there was a notable decline in viable cell counts with increasing exposure time, the strain maintained viable cell counts of 7.07, 7.28, and 7.79 log CFU/mL at pH levels of 2, 3, and 4, respectively. Additionally, the strain exhibited impressive stability within the gastrointestinal tract (GIT), with a viable cell count of 7.12 log CFU/mL following GIT treatment.

Fig. 2 presents the strain's bile stability, reporting cell counts of 8.56, 8.38, 8.16, and 7.53 log CFU/mL at bile salt concentrations of 0.1 %, 0.3 %, 0.5 %, and 0.7 %, respectively. These findings align with other research that highlights the resilience of *Lpb. pentosus* strains under harsh conditions ([9]; Alizadeh Behbahani and Noshad, 2024). Studies on Gram-positive bacteria, such as LAB, suggest that the F1F0-ATPase contributes to their resistance and survival in acidic environments. Additionally, compounds like polysaccharides offer protection to lactic acid bacteria against stomach acid [2].

The ability of probiotic bacteria to adhere to epithelial cells and subsequently colonize the gastrointestinal tract offers significant advantages, enabling them to effectively compete and thrive within the gut environment. Therefore, this characteristic is crucial to consider when isolating new probiotic strains. Bacterial auto-aggregation refers to the phenomenon where bacteria physically interact and aggregate, settling at the bottom of a static liquid suspension [33]. The capacity of bacteria to form cellular aggregates, whether through auto-aggregation (among bacteria of the same strain) or co-aggregation (among genetically distinct strains), enhances their persistence in the intestinal environment. Additionally, this aggregation can impede the growth of potential pathogenic microorganisms [34,35]. The ability to auto-aggregate is closely linked to adhesion, and for probiotic bacteria to provide their intended benefits, they must be able to develop a sufficiently large biomass through this aggregation process [9]. Fig. 3 illustrates the cell surface characteristics of *Lpb. pentosus* 6MMl. This strain exhibited notable surface hydrophobicity (71.89 %), auto-aggregation (42.39 %), co-aggregation (51.28 %), and adhesion (12.91 %).

It was observed that the self-aggregation rate of *Lpb. pentosus* 68-1 increased over time, peaking at 82.67 % after 24 h of incubation. At the 20-h mark, the self-aggregation rates for *Lpb. pentosus* 68-1 and LGG were recorded at 80.79 % and 29.58 %, respectively. The cell surface hydrophobicity of *Lpb. pentosus* 68-1 was measured at 34.57 %, which is notably lower than that of LGG, which had a hydrophobicity of 53.28 %. Additionally, *Lpb. pentosus* 68-1 demonstrated a higher adhesion rate of 9.79 % to Caco-2 cells compared to LGG's adhesion rate of 4.19 %. This enhanced adhesion has been associated with the presence of adherence-related genes in their genomes, which include those encoding mucus-binding proteins, collagen-binding proteins, fibronectin-binding proteins, moonlighting proteins, and exopolysaccharides [36]. Furthermore, various strains of *Lpb. pentosus* have shown the capability to adhere to epithelial cells, with adherence-related genes also identified in

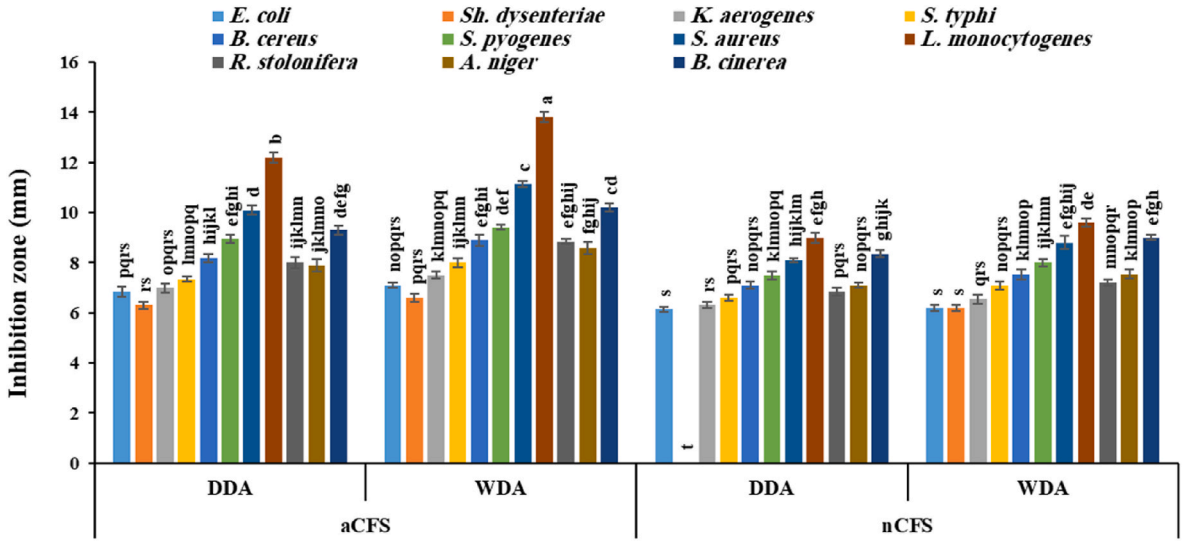

Fig. 5. The antimicrobial effects of cell-free supernatant (CFS) of *Lactiplantibacillus pentosus* 6MMI based on disk diffusion agar (DDA) and well diffusion agar (WDA). Significant differences ($p < 0.05$) are denoted by different superscript letters.

Table 3
The correlation coefficient analysis between all cell-free supernatant (CFS) from *Lactiplantibacillus pentosus* 6MMI on pathogenic microorganisms

Variables	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
X_1	1	0.714[b]	0.972[a]	0.912[a]	0.964[a]	0.971[a]	0.963[a]	0.971[a]	0.971[a]	0.936[a]	0.907[a]
X_2	0.714[b]	1	0.688	0.767[b]	0.729[b]	0.763[b]	0.737[b]	0.774[b]	0.785[b]	0.726[b]	0.770[b]
X_3	0.972[a]	0.788[b]	1	0.979[a]	0.995[a]	0.971[a]	0.980[a]	0.964[a]	0.990[a]	0.989[a]	0.973[a]
X_4	0.912[a]	0.767[b]	0.979[a]	1	0.986[a]	0.947[a]	0.961[a]	0.930[a]	0.967[a]	0.993[a]	0.994[a]
X_5	0.964[a]	0.729[b]	0.995[a]	0.986[a]	1	0.981[a]	0.989[a]	0.974[a]	0.991[a]	0.988[a]	0.980[a]
X_6	0.971[a]	0.763[b]	0.971[a]	0.947[a]	0.981[a]	1	0.991[a]	0.985[a]	0.980[a]	0.947[a]	0.948[a]
X_7	0.963[a]	0.737[b]	0.980[a]	0.961[a]	0.989[a]	0.991[a]	1	0.992[a]	0.992[a]	0.961[a]	0.964[a]
X_8	0.971[a]	0.774[b]	0.964[a]	0.930[a]	0.974[a]	0.985[a]	0.992[a]	1	0.987[a]	0.934[a]	0.938[a]
X_9	0.971[a]	0.785[b]	0.990[a]	0.967[a]	0.991[a]	0.980[a]	0.992[a]	0.987[a]	1	0.973[a]	0.974[a]
X_{10}	0.936[a]	0.726[b]	0.989[a]	0.993[a]	0.988[a]	0.947[a]	0.961[a]	0.934[a]	0.973[a]	1	0.987[a]
X_{11}	0.907[a]	0.770[b]	0.973[a]	0.994[a]	0.980[a]	0.948[a]	0.964[a]	0.938[a]	0.974[a]	0.987[a]	1

X_1: *Escherichia coli*; X_2: *Shigella dysenteriae*; X_3: *Klebsiella aerogenes*; X_4: *Salmonella typhi*; X_5: *Bacillus cereus*; X_6: *Streptococcus pyogenes*; X_7: *Staphylococcus aureus*; X_8: *Listeria monocytogenes*; X_9: *Rhizopus stolonifera*; X_{10}: *Aspergillus niger*; X_{11}: *Botrytis cinerea*.

[a] Correlation is significant at the 0.01 level.

[b] Correlation is significant at the 0.05 level.

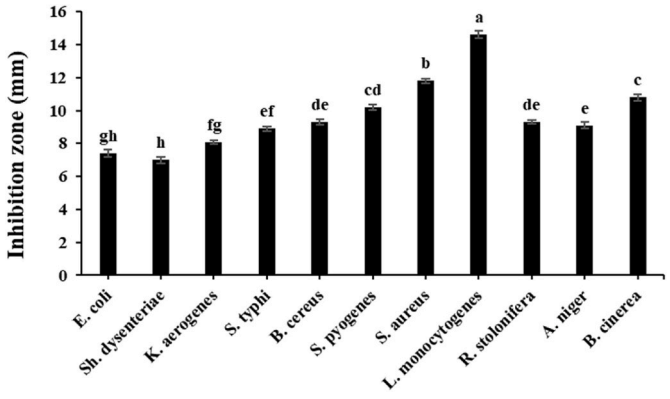

Fig. 6. The antimicrobial effects of cell-free supernatant (CFS) of *Lactiplantibacillus pentosus* 6MMI based on double layer method. Significant differences ($p < 0.05$) are denoted by different superscript letters.

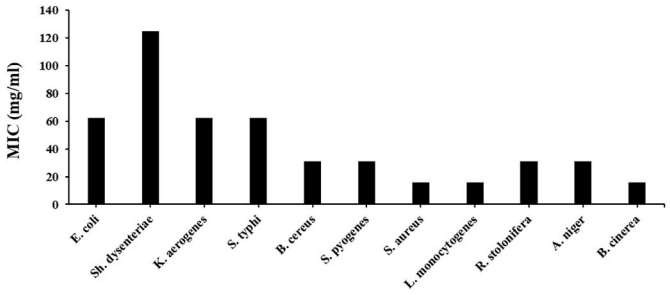

Fig. 7. The antimicrobial effects of cell-free supernatant (CFS) of *Lactiplantibacillus pentosus* 6MMI based on minimum inhibitory concentration (MIC) method.

their genomes [37].

Adhesion is a critical initial step for pathogenic bacteria, enabling them to colonize hosts, proliferate, and release enzymes and toxins that lead to infections. Fig. 4 presents the results of the anti-adhesion activity, demonstrating that the strain achieved anti-infection rates of 54.51 %, 48.57 %, and 27.71 % through competition, inhibition, and displacement methods, respectively. Previous studies on human cell lines indicated that *Lpb. pentosus* enhanced host immunity, inhibited the adhesion of intestinal pathogens such as Salmonella, and contributed to infection prevention [38]. Additionally, research by Jiao et al. [39] revealed that incubating zebrafish with 1×10^6 CFU/mL of *Lpb. pentosus* SF-1 for one week significantly increased the expression of antimicrobial

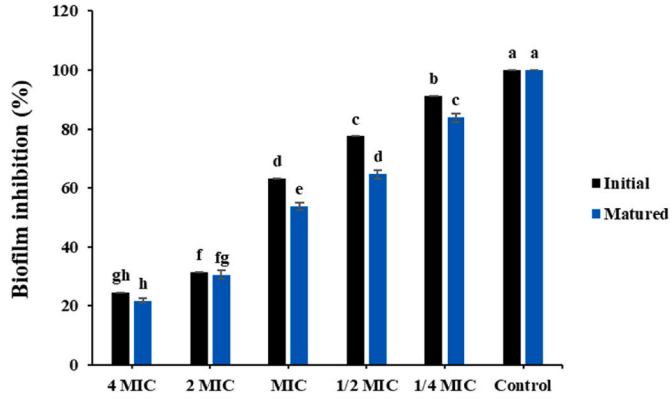

Fig. 8. The antibiofilm activity of the cell-free supernatant (CFS) of *Lactiplantibacillus pentosus* 6MMI was examined against the biofilm formation capacity of *Listeria monocytogenes* at both initial and mature biofilm stages. Significant differences ($p < 0.05$) are denoted by different superscript letters.

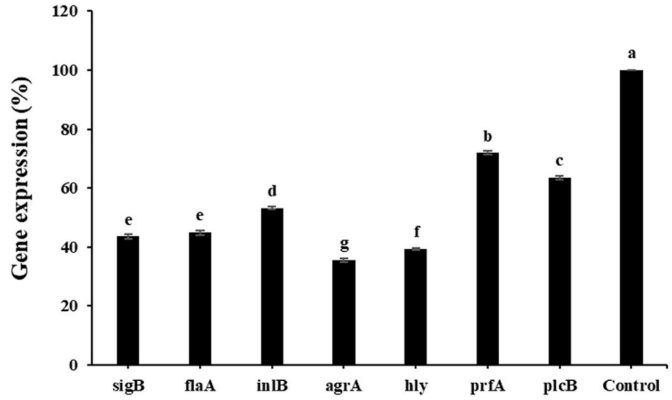

Fig. 9. Gene expression analysis was conducted in *Listeria monocytogenes* treated with a sub-inhibitory concentration (1/2 MIC) of the CFS from *Lactiplantibacillus pentosus* 6MMI. Significant differences ($p < 0.05$) are denoted by different superscript letters.

peptides, particularly *β-defensins* and lysozyme, in their kidneys and intestines. This treatment also notably improved the zebrafish's resistance to infections caused by *Edwardsiella tarda*. Furthermore, *Lpb. pentosus* HC-2, isolated from the intestinal tract of *Acanthogobius hasta*, was effective in colonizing the intestines of white leg shrimp (*Litopenaeus vannamei*), enhancing intestinal health, boosting immune responses, and protecting against pathogenic threats (Y. [40]). The

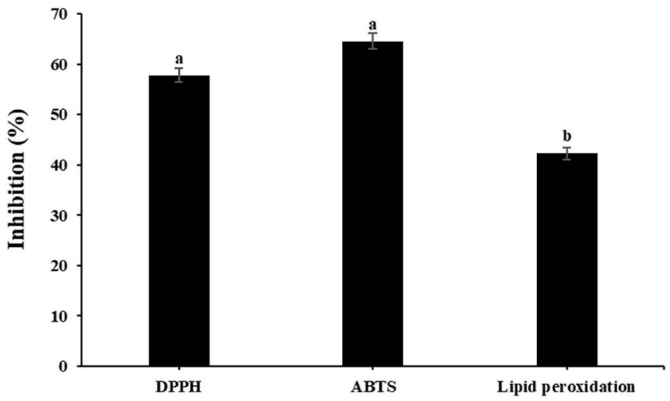

Fig. 10. Antioxidant effect of *Lactiplantibacillus pentosus* 6MMI. Significant differences ($p < 0.05$) are denoted by different superscript letters.

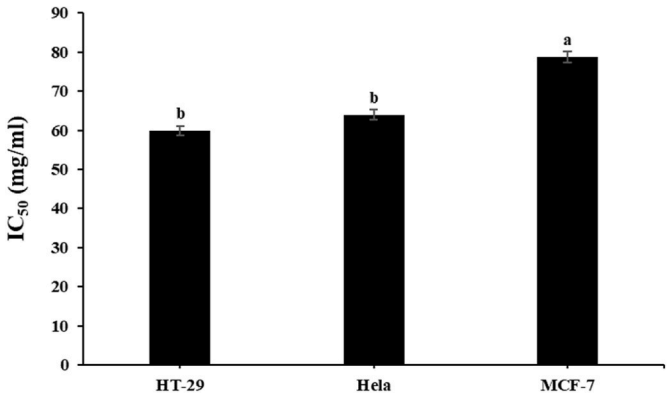

Fig. 11. Cytotoxic effect of *Lactiplantibacillus pentosus* 6MMI. Significant differences ($p < 0.05$) are denoted by different superscript letters.

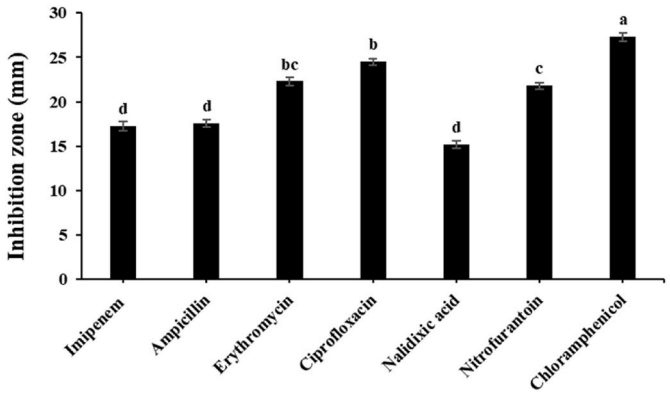

Fig. 12. Antibiotic susceptibility profile of *Lactiplantibacillus pentosus* 6MMI. Significant differences ($p < 0.05$) are denoted by different superscript letters.

anti-infection properties of LAB against pathogens are attributed to their secretion of antimicrobial substances such as organic acids (primarily lactic acid), carbon dioxide, volatile compounds like ethanol and diacetyl, hydrogen peroxide, and bacteriocins. Moreover, probiotic bacteria may produce anti-adhesion compounds, degrade carbohydrate receptors through protein secretion, and generate biosurfactants [22]. Notably, lactate, a secondary metabolite produced by many probiotics, plays a crucial role in resisting infections by inhibiting pathogenic bacterial growth while facilitating the colonization of probiotic strains [41].

Fig. 5 demonstrates the results of antimicrobial activity from disk and well diffusion agar tests. The acidic properties of aCFS yielded a significantly larger inhibition zone of 8.73 mm, in contrast to nCFS, which showed an inhibition zone of 7.13 mm ($p < 0.05$). Among the tested bacteria, *L. monocytogenes* exhibited the greatest sensitivity with an inhibition zone of 11.15 mm, while *Sh. dysenteriae* displayed the lowest sensitivity with a zone of only 4.77 mm ($p < 0.05$).

The correlation coefficient is a vital statistical measure that quantifies the strength and direction of a linear relationship between two variables, ranging from −1 to 1. A coefficient of 1 or -1 indicates a perfect correlation, where all data points lie on a straight line-positive for 1 and negative for −1. In contrast, values between −1 and 1 signify an imperfect correlation, with coefficients closer to these extremes indicating stronger relationships. According to Table 3, the correlation coefficients among all analyzed variables exceeded 0.7, suggesting a strong positive correlation, which is generally considered acceptable in research contexts. This understanding of correlations is crucial for predictive modeling, generating research insights, and informing strategic decision-making across various fields.

This trend was similarly observed with the double layer method (Fig. 6) and the MIC results (Fig. 7). The study also found that CFSs were generally more effective against Gram-positive bacteria than Gram-negative bacteria, potentially due to differences in their cell membrane structures. Furthermore, the well diffusion method produced larger inhibition zones compared to the disk diffusion method (8.36 mm versus 7.51 mm) ($p < 0.05$), likely because of the more direct interaction between CFS and bacteria in the former. In research conducted by Alizadeh Behbahani et al. [10], the *Lpb. pentosus* v390 strain was reported to have a more substantial antimicrobial effect against Gram-positive pathogenic bacteria. *Lpb. pentosus* ESSG2 exhibited notable antibacterial activity against pathogens such as *E. coli, Yersinia enterocolitica, S. aureus, and B. cereus.* Its antifungal properties were also evaluated, revealing the strain's capability to inhibit the growth of pathogenic fungi including *Sclerotium, Pythium, Alternaria, Botrytis, and Fusarium* [42]. Another strain, *Lpb. pentosus* PCZ4, known for its broad-spectrum antibacterial effects, was isolated from traditional fermented kimchi in Sichuan [43]. BAGEL4 analysis identified classes IIa and IIb bacteriocins, including plantaricin S, while two novel antibacterial peptides, Bac1109 and Bac2485, were predicted based on limited open reading frames. In experiments involving refrigerated storage of snakehead fish, the crude extract of PCZ4 significantly reduced the total bacterial count, slowed increases in total volatile basic nitrogen and pH levels, improved the sensory quality of the fish, and extended its shelf life by an additional two days. Additionally, PCZ4 effectively inhibited the growth of *Aeromonas hydrophila* in artificially contaminated snakehead fish [43].

Biofilm represents a highly prevalent microbial community often found in wounds, dental cavities, various food processing environments, and natural ecosystems, frequently leading to serious pathogenic infections. Approximately 80 % of chronic infections are linked to biofilm-related phenomena, which exhibit resistance to a wide array of antibiotics, sanitizers, and chlorine. In non-host settings, pathogenic bacteria can survive on surfaces, with biofilm serving as a key reservoir for infections that may be transmitted via food and water [44]. Treatment with CFS from *Lpb. pentosus* 6MMl has been shown to significantly reduce the biofilm formation of *L. monocytogenes* during the early stages, with reductions measured between 91.25 % and 24.50 % across various MICs (Fig. 8). Furthermore, the presence of *Lpb. pentosus* 6MMl CFS significantly diminished mature biofilm formation by *L. monocytogenes*, with percentages ranging from 83.82 % on 1/4MIC to 21.80 % on 4MIC (Fig. 8). This suggests that the CFS from *Lpb. pentosus* 6MMl effectively not only prevented early biofilm formation but also disrupted established biofilms. This finding is strongly corroborated by gene expression data (Fig. 9), which indicates that CFS suppressed the transcription of *plcB*, *hly*, and *prfA* genes in *L. monocytogenes*, pointing to a potential reduction in bacterial virulence through decreased release of hemolysins and modulation of phospholipase activity. Probiotics can influence biofilms in numerous ways, including the release of antimicrobial

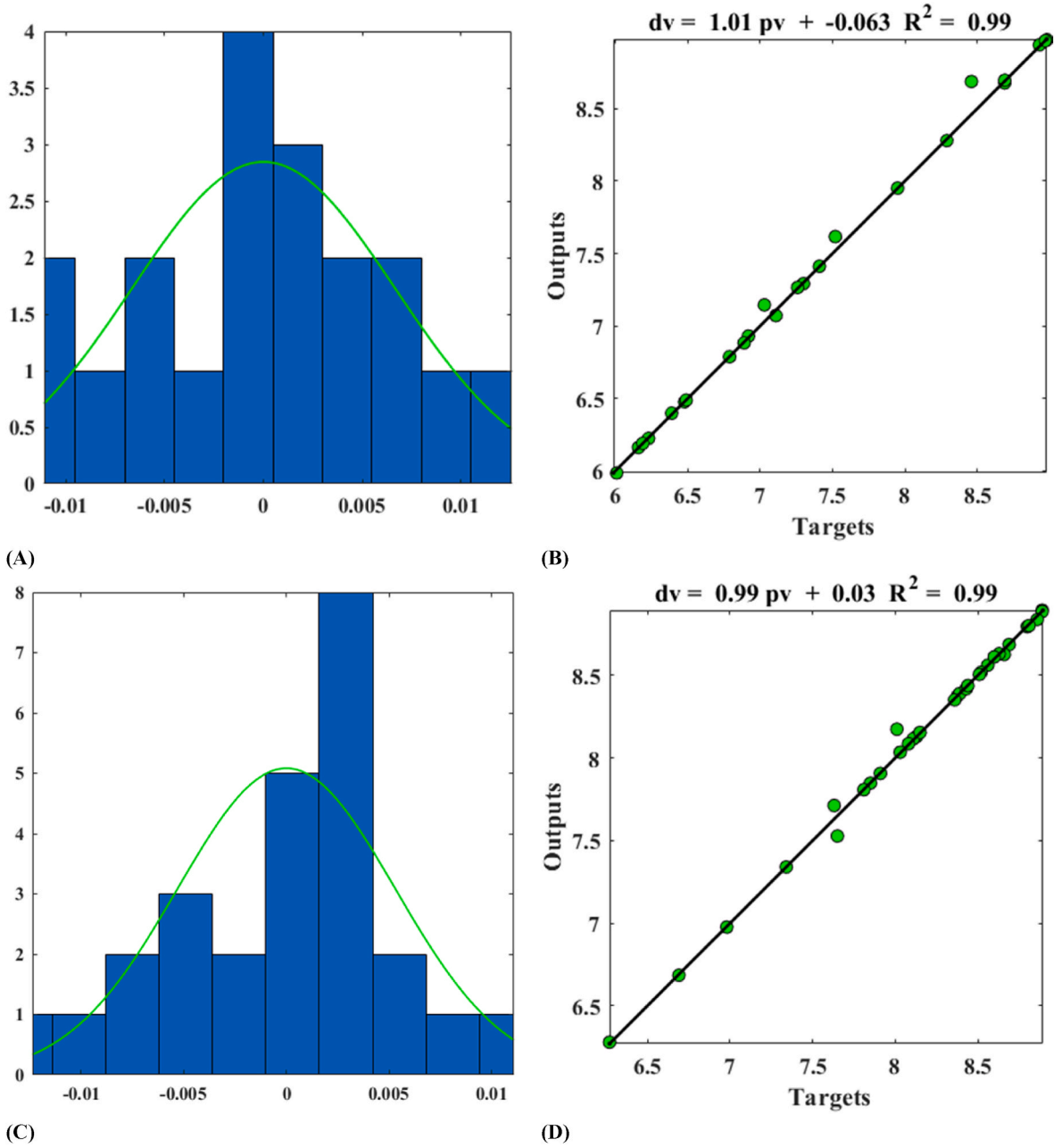

Fig. 13. Error residuals and distribution of actual vs. predicted data in Viability (A and B) and Acidity (C and D) prediction.

substances, competitive exclusion of pathogens, immune response modulation, disruption of cell membranes and proteins, enhancement of the gut barrier, production of biosurfactants, and reduction of biofilm-related gene expression, leading to bacterial DNA degradation [9]. A study conducted by Parvin et al. [44] investigated the antimicrobial and antibiofilm effects of biosurfactants derived from *Lpb. pentosus* MSCIN-24 and *Lpb. pentosus* MSCIN-25 against pathogens responsible for food spoilage and skin infections. These biosurfactants demonstrated broad-spectrum antimicrobial efficacy against both food and topical pathogens, with MIC and antibiofilm values ranging from 5 to 15 mg/mL, resulting in cellular disruption [44].

The antioxidant properties of *Lpb. pentosus* 6MMl were assessed (Fig. 10). This strain demonstrated a DPPH radical scavenging ability of 57.82 %, an ABTS radical scavenging ability of 64.61 %, and a linoleic acid peroxidation inhibition of 42.29 %. Consequently, incorporating *Lpb. pentosus* 6MMl into foods or supplements could be beneficial for health. Notably, the strain also showed substantial cytotoxic effects against a variety of cancer cell lines, with cytotoxicity levels reaching 59.85 mg/mL for HT-29, 63.95 mg/mL for HeLa, and 78.71 mg/mL for MCF-7 cells (Fig. 11). Likewise, a study by Alizadeh Behbahani, Noshad et al. [10] demonstrated that *Lpb. pentosus* SM1 exhibited noteworthy scavenging activities, achieving 57.60 % against DPPH radicals, 60.54 % against ABTS radicals, and 25.90 % inhibition of linoleic acid peroxidation. The cytotoxicity of *Lpb. pentosus* SM1 was recorded at 36.57 mg/mL for HT-29 cells and 38.20 mg/mL for HeLa cells [9]. Enzymatic defenses, including superoxide dismutase, NADH-oxidase, NADH-peroxide, and heterologous non-heme catalase, are recognized as key components in protecting against oxidative stress in LAB [45].

Research has explored the cholesterol-lowering properties of LAB. *Lpb. pentosus* 6MMl demonstrated impressive cholesterol assimilation activity at 50.31 ± 1.15 %. Likewise, *Lpb. pentosus* DSM 20314, which was isolated from fermented rice in China, exhibited a cholesterol assimilation rate of 52.9 % [46]. Various mechanisms have been proposed to explain the cholesterol-lowering properties of LAB, including

cholesterol assimilation, bile salt deconjugation, and micellar sequestration of cholesterol [47]. It is suggested that these probiotic isolates, when taken orally, could have a similar impact in the intestine, aiding in the regulation of plasma cholesterol levels [46].

The strain demonstrated the highest level of resistance to nalidixic acid, with an inhibition zone of 15.20 mm, while showing the lowest resistance to chloramphenicol, which had an inhibition zone of 27.30 mm (Fig. 12). *Lpb. pentosus* 6MMl did not display any hemolytic activity, DNase production, or biogenic amine formation. These findings are consistent with those reported by Ref. [9,18,48]. Therefore, *Lpb. pentosus* 6MMl can be regarded as safe for use in food applications.

In this study, GPR model was employed to predict the acid and bile tolerance parameters, as illustrated in Fig. 13. The results indicate that the GPR model effectively captures both parameters with a high degree of accuracy. The coefficient of determination (R^2) for both predictions was an impressive 0.99, while the MAPE was recorded at 0.33 % for bile tolerance and 0.21 % for acidity. Furthermore, the analysis of residual errors revealed a normal distribution for both predicted parameters, suggesting that the model's predictions are reliable and consistent. The regression equations derived from the model demonstrate that the discrepancies between actual and predicted data are minimal, underscoring the robustness of the GPR approach in this context. These findings highlight the potential of GPR models in accurately predicting critical parameters in various applications, paving the way for enhanced decision-making processes in fields such as food science, environmental monitoring, and quality control.

4. Conclusion

Lpb. pentosus 6MMI exhibits promising probiotic properties and strong anti-biofilm capabilities, demonstrating significant resilience to harsh gastrointestinal conditions and effective competition against *L. monocytogenes*. Its notable characteristics, including high hydrophobicity, auto-aggregation, and antioxidant activity, enhance its potential as a beneficial probiotic. The strain's ability to significantly reduce biofilm formation and interfered with the expression of virulence-related genes in *L. monocytogenes* underscores its potential application in food safety and as a functional probiotic in promoting human health. Overall, *Lpb. pentosus* 6MMI represents a valuable candidate for further investigation in probiotic development and biofilm management strategies. In this study, the GPR model accurately predicted bile tolerance and acid parameters with a high R^2 of 0.99 and minimal MAPE values of 0.33 % and 0.21 %, respectively. The residual errors showed a normal distribution, indicating reliable and consistent predictions. These results underscore the robustness of the GPR approach in predicting critical parameters, enhancing decision-making in fields like food science and quality control.

CRediT authorship contribution statement

Behrooz Alizadeh Behbahani: Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Mostafa Rahmati-Joneidabad:** Writing – original draft, Supervision, Resources, Methodology. **Morteza Taki:** Writing – review & editing, Methodology, Investigation.

Consent for publication

All authors approved the manuscript for publication.

Availability of data and material

All data relevant to the study are included in the article.

Ethical approval

This article does not contain any studies with human or animal subjects.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Grammarly in order to paraphrase

and grammatically check the sentences. After using this tool/service, the authors reviewed and

edited the content as needed and take full responsibility for the content of the published article.

Funding

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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