

Characterization of *Weissella cibaria* isolates from fermented curd and evaluation of their probiotic potential

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Abstract

In this study, two bacterial isolates were obtained from curd samples fermented using lemon (*Citrus limon*) and imli (*Tamarindus indica*) and identified as *Weissella cibaria* strains GCC_24LM and GCC_24IM through biochemical and 16S rRNA gene sequencing. In vitro assays demonstrated notable tolerance to acidic gastric juice, bile salts, and pancreatin, suggesting their ability to survive gastrointestinal transit. The strain also displayed moderate cell surface hydrophobicity, cellular autoaggregation, and biofilm formation, enhancing their potential for gut colonization. Both strains exhibited negative results for hemolytic, DNase, and gelatinase activity, indicating safety, while variable resistance profiles have been observed in antibiotic susceptibility testing. Importantly, GCC_24LM showed a high cholesterol assimilation rate of 96.66%. Although both strains fail to exhibit antagonistic activity against pathogens, their overall probiotic traits suggest promising applications. These findings support the potential of *W. cibaria* strains as emerging probiotic candidates for functional food development.

1. Introduction

The human gastrointestinal (GI) tract hosts a highly diverse and dense microbial ecosystem, often referred to as the gut microbiota, which plays a critical role in maintaining overall health and homeostasis. Among these, lactic acid bacteria (LAB) are especially valued for their contributions to digestion, immune modulation, and protection against pathogens. Probiotics, defined as live microorganisms that confer health benefits when administered in adequate amounts, are increasingly gaining attention for their role in gastrointestinal health and beyond. LAB such as *Lactobacillus* and *Bifidobacterium* have traditionally dominated the probiotic landscape (Nath et al., 2020; Miri et al., 2022; Kang et al., 2025), while lesser-known genera like *Weissella* have begun to draw significant interest for their promising attributes and underexplored potential. *Weissella cibaria*, a Gram-positive, non-spore-forming, obligately heterofermentative LAB, has been isolated from a range of sources including fermented foods (Lee et al., 2013), human saliva (Kang et al., 2006), animal faeces (Xia et al., 2019) and clinical samples (Björkroth et al., 2002). This species has recently emerged as a notable probiotic candidate due to its ability to produce bacteriocins, exopolysaccharides (EPS), and short-chain fatty acids, as well as its resilience under gastrointestinal (GI) conditions (Ahmed et al., 2022; Zammouri et al., 2024). Certain strains of *W. cibaria* have demonstrated strong acid and bile tolerance, cholesterol-lowering activity, antioxidant capacity, and immunomodulatory effects, making them attractive for both food and therapeutic applications (Teixeira et al., 2021).

Despite its promising features, *W. cibaria* remains underrepresented in commercial probiotic formulations, likely due to the relatively limited number of comprehensive characterizations compared to traditional probiotics. An integrated phenotypic-genotypic analysis of strains SP7 and SP19 revealed strong acid and bile tolerance, robust adhesion to human epithelial cell lines, and antimicrobial Activity against *Escherichia coli* and *Salmonella* strains, which were attributed to organic acid production rather than bacteriocins (Patrone et al., 2021). Additionally, genome-based investigations have confirmed the absence of virulence factors and acquired antibiotic resistance genes in *W. cibaria*, suggesting a favorable safety profile. In a study of the strain UTNGt210, genome sequencing revealed genes associated with stress resistance, folate

biosynthesis, and antimicrobial peptide production, underscoring its functional robustness and biotechnological potential (Tenea and Hurtado, 2021).

Another study investigating *W. cibaria* JW15 from kimchi reported significant enhancement of immune responses in both in vitro and clinical settings. This strain survived simulated gastric conditions and bile exposure, enhanced natural killer (NK) cell activity, and regulated inflammatory cytokines in human trials (Lee et al., 2018). These immune-boosting effects highlight the strain's potential beyond digestive health, particularly in the modulation of systemic immunity. Moreover, *W. cibaria* has been shown to influence cholesterol metabolism. In a study comparing strains isolated from fermented batter, one *W. cibaria* strain demonstrated up to 78% cholesterol removal in vitro, suggesting possible roles in cardiovascular health (Lakra et al., 2020). Importantly, the EPS produced by *W. cibaria* also function as a prebiotics, promoting the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*. These polysaccharides have been found to resist degradation in the GI tract, further enhancing the strain's functional benefits (Park et al., 2023).

Given this wide array of biofunctional traits, the current study focuses on isolating and characterizing *W. cibaria* strains from lemon and tamarind fermented curd, aiming to evaluate their probiotic potential through in vitro assessments. The isolates underwent extensive screening for acid, bile, and phenol tolerance, autoaggregation, cell surface hydrophobicity, cholesterol assimilation, antimicrobial activity, biofilm formation, and safety markers including hemolytic and DNase Activity. The goal is to compare these traits with benchmarks set by previous studies to determine whether these native strains could be suitable for functional food development or clinical application.

2. Materials and methods

2.1. Isolation and characterization of bacteria

2.1.1. Collection of samples and isolation of bacteria

Curd prepared using Imli (*Tamarindus indica*) and Lemon (*Citrus limon*) was aseptically collected in pre-sterilized containers and promptly transported to the laboratory under controlled conditions. The samples were serially diluted up to a 10^{-3} dilution, and 0.1 ml of each diluted aliquot was spread onto freshly prepared de Man, Rogosa, and Sharpe (MRS) agar plates. The inoculated plates were incubated at 37°C for 24 hr to promote bacterial growth. Distinct bacterial colonies were then selected and sub-cultured on fresh MRS agar plates to obtain pure isolates for subsequent analysis.

2.1.2. Morphological and biochemical characterization of bacteria

Preliminary identification of bacterial isolates was conducted based on colony morphology, cultural characteristics, and microscopic examination. The isolates were observed for colony color, surface texture, Gram reaction, and cell shape. Biochemical tests including the indole production test, methyl red (MR) test, Voges–Proskauer (VP) test, citrate utilization test, oxidase test, catalase test, starch hydrolysis test, and

triple sugar iron (TSI) test were performed following standard protocols described by Holt et al. (1994) and Cappuccino and Sherman (2005).

2.1.3. Molecular Identification

Genomic DNA isolation was performed using the Xploreagen gDNA Extraction Buffer™ method. The extracted DNA was purified by centrifugation at 10,000 rpm and quantified at a concentration of 168 ng/μl. Following isolation, the 16S rRNA gene was amplified using polymerase chain reaction (PCR). The amplification reaction was set up in a 50 μl mixture containing 1 μl of template DNA, 2 μl each of 16S forward primer (5'-GGATGAGCCCGCGGCCTA-3') and reverse primer (5'-CGGTGTGTACAAGGCCCGG-3'), 4 μl of 2.5 mM dNTPs, 10 μl of 10X Taq DNA polymerase buffer, 1 μl of Taq DNA polymerase (3U/μl), and 30 μl of Milli-Q water. The PCR cycling conditions included an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes, and a final extension at 72°C for 7 minutes.

The amplified PCR products were then subjected to sequencing using the Sanger method. The sequencing reaction was prepared in a 10 μl volume containing 4 μl of Big Dye Terminator Ready Reaction Mix, 1 μl of template DNA (100 ng/μl), 2 μl of primer (10 pmol/μl), and 3 μl of Milli-Q water. The sequencing PCR was carried out for 25 cycles with an initial denaturation at 96°C for 5 minutes, followed by denaturation at 96°C for 30 seconds, hybridization at 50°C for 30 seconds, and elongation at 60°C for 90 seconds. The sequencing was performed using the ABI 3130xl Genetic Analyzer with Big Dye Terminator v3.1 and a POP_7 polymer capillary array. Data analysis was conducted using SeqScape v5.2 software, and a consensus sequence 16S rRNA gene sequence was generated. The sequence was used to perform BLAST to find the closest homologous sequence present in the non-redundant database. Based on the maximum identity score, 16S rRNA gene sequences were downloaded and aligned using Clustal W. A phylogenetic tree was constructed using the maximum likelihood method under the GTRGAMMA model in RAxML version 7.2.8. All analyses were conducted using the Geneious R8 software package, developed by Biomatters Ltd., Auckland, New Zealand (Nath et al., 2019).

2.2. Screening of probiotic properties of isolated bacteria

2.2.1. Simulated gastric juice tolerance test

To evaluate bacterial resistance under acidic gastric conditions, simulated gastric juice was prepared containing 3 g/L pepsin, 7 mM KCl, 45 mM NaHCO₃, and 125 mM NaCl, with the pH adjusted to 3.0 using 1 M HCl and 1 M NaOH, as described by Archer and Halami (2015). Phosphate-buffered saline (PBS) was used as the control medium. Overnight-grown bacterial cultures were centrifuged at 5000 rpm for 15 minutes at 5°C, and the resulting pellets were resuspended in 10 ml of PBS. These bacterial suspensions were then incubated separately in simulated gastric juice and PBS. Bacterial growth was monitored hrly by measuring optical density (OD) at 600 nm to assess viability under both test and control conditions.

2.2.2. Bile tolerance test

Bile tolerance of the bacterial isolates was assessed according to the method of Sreenadh et al. (2022). A 500 µl aliquot of overnight-grown bacterial culture was inoculated into freshly prepared MRS broth containing 0.3% bile salts (Himedia Pvt. Ltd). The same isolates were also inoculated into MRS broth without bile to serve as the control. Both test and control cultures were incubated at 37°C for 4 hr. Bacterial growth was measured at specific time intervals by recording absorbance at 600 nm, and the percentage of bile resistance was calculated based on the difference in growth between the treated and control samples.

2.2.3. Pancreatin tolerance test

Pancreatin tolerance of the isolates was assessed by inoculating 100 µl of overnight-grown bacterial culture into 10 ml of MRS broth supplemented with 0.5% (w/v) pancreatin. A control was maintained using MRS broth without pancreatin. The cultures were incubated at 37°C in a shaker incubator for 48 hr. Bacterial growth and tolerance were evaluated by measuring the optical density at 600 nm at 0, 24, and 48 hr (Nath et al., 2021).

2.2.4. Assessment of cell surface hydrophobicity

Cell surface hydrophobicity, which reflects the bacterial ability to adhere to epithelial surfaces, was evaluated using hydrocarbon adhesion assays. A 20-hr-old bacterial culture was centrifuged at 12,000 rpm for 5 minutes, and the resulting pellets were washed twice with phosphate-buffered saline (PBS, pH 7.2) before being resuspended in 6 ml of PBS. The initial absorbance at 600 nm was recorded. Then, 3 ml of the suspension was separately mixed with 1 ml of n-hexadecane and toluene, vortexed for 2 minutes, and incubated for 1 hr to allow phase separation. The aqueous phase was carefully collected, and the final absorbance was measured at 600 nm (Nath et al., 2020). The percentage of cell surface hydrophobicity was calculated using the following formula:

$$\text{Rate of hydrophobicity (\%)} = \frac{OD_{initial} - OD_{final}}{OD_{initial}} \times 100$$

2.2.5. Cellular autoaggregation assay

Autoaggregation was evaluated following the method described by Nath et al. (2020), which assesses the ability of bacterial cells to adhere to each other. Overnight-grown bacterial cultures were centrifuged at 5,000 rpm for 10 minutes to collect the cell pellets. These were washed thoroughly with PBS (pH 7.2) and resuspended in PBS. The initial OD at 600 nm was recorded. The suspension was then incubated at 37°C for 2 hr. After incubation, the upper phase was carefully collected, and its final OD was measured at 600 nm. The autoaggregation percentage was calculated using the formula:

$$\text{Autoaggregation rate (\%)} = \frac{OD_{initial} - OD_{final}}{OD_{initial}} \times 100$$

2.2.6. NaCl tolerance test

The tolerance of bacterial isolates to different salt concentrations was evaluated by streaking them on MRS agar plates supplemented with varying concentrations of NaCl (0%, 0.5%, 1%, 5%, 7.5%, 10%, 12.5%, and 15%). The inoculated plates were incubated at 37°C for 24 hr. Bacterial growth was observed, and the effect of NaCl concentration on growth inhibition was recorded.

2.2.7 Phenol tolerance test

The tolerance of bacterial isolates to phenol was adjudged upon inoculating 500 µl aliquot of overnight-grown bacterial culture to MRS broth supplemented with 0.2%, 0.4% and 0.6% (v/v) of phenol (SRL, India), and incubating at 37°C for 48 hr. The tubes without phenol were considered as control. The bacterial growth inferring tolerance to phenol was measured spectrophotometrically at 600nm after 24 hr and 48 hr of incubation.

2.3. *In vitro* biofilm assay

2.3.1. Congo red agar (CRA) method

Biofilm formation by the isolated strain was assessed using the CRA method, following the protocol described by Yumnam et al. (2025). Briefly, Brain Heart Infusion (BHI) agar (37 g/L) and sucrose (2 g/L) were combined to prepare the CRA medium. The mixture was autoclaved, after which Congo red stain (0.8 g/L) was added. The medium was then poured into Petri plates and allowed to solidify. Test organisms were inoculated onto the plates and incubated aerobically at 37°C for 24 hr. The colonies that appeared black, dry, and crystalline were classified as strong biofilm producers, whereas red or pink colonies indicated weak biofilm formation. Dark colonies lacking a crystalline structure were categorized as intermediate biofilm producers.

2.3.2. Tissue culture plate (TCP) method

Biofilm formation was further assessed using the TCP method (Hassan et al., 2011; Yumnam et al., 2025). Briefly, test organisms were inoculated into tryptic soy broth (TSB) supplemented with 1% glucose and incubated at 37°C for 24 hr. After incubation, 200 µL of the TSB-glucose culture containing the bacterial isolates was transferred into separate wells of a sterile, flat-bottomed 96-well polystyrene plate, followed by a second incubation at 37°C for another 24 hr. Uninoculated broth served as the negative control. Following incubation, the wells were gently tapped to discard the contents and washed 2–3 times with 0.2 mL of PBS (pH 7.2) to remove non-adherent cells. The remaining attached biofilm was fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was removed by rinsing the wells with distilled water, and the plates were left to air-dry. The OD of the extracted stain was then measured at 600 nm using a microplate reader. The biofilm formation ability of each isolate was determined by comparing the OD values of the test isolates with that of the negative control (uninoculated broth). Based on this comparison, isolates with OD values less than or equal to OD_{control} were classified as non or weak biofilm producers, those with OD values greater than $2 \times OD_{\text{control}}$ but less than or equal to $4 \times OD_{\text{control}}$ were categorized as moderate biofilm producers, and isolates with OD values exceeding $4 \times OD_{\text{control}}$ were identified as strong biofilm producers (Hassan et al., 2011).

2.4. Safety assessment and antimicrobial activity

2.4.1. Antibiotic susceptibility test

The antibiotic susceptibility test was conducted using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA) medium (Baccer et al., 1966). Bacterial samples were spread evenly on freshly prepared MHA plates, and antibiotic discs were placed equidistantly on the surface. The plates were incubated at 37°C for 24–48 hr, and the diameter of the inhibition zone was measured. The antibiotic resistance profile of the isolate was classified according to the Clinical and Laboratory Standards Institute (CLSI, 2025) guidelines. The antibiotics used in this study were procured from 'HiMedia'.

2.4.2. Haemolytic activity

Hemolytic activity is a crucial factor in determining the safety of probiotic bacteria, as the absence of hemolysis indicates non-virulence. To assess this, bacterial isolates were streaked onto 5% sheep blood agar plates and incubated at 37°C for 48 hr. Following incubation, the formation of zones around the colonies was examined and classified into three categories: β -hemolysis (clear zone indicating complete hemolysis), α -hemolysis (green-hued zone indicating partial hemolysis), or γ -hemolysis (no zone, indicating no hemolysis) (Gerhardt et al., 1981).

2.4.3. *DNase activity*

The test isolates were streaked onto DNase agar plates (Himedia, Mumbai, India) and incubated anaerobically at 40°C for 72 hr, following the method described by Rastogi et al. (2019). After incubation, the plates were treated with 3% HCl for 8 minutes, and the colonies were observed for the presence of a clear zone, indicating DNase activity.

2.4.4 Gelatinase activity

Gelatinase activity was determined by the protocol established by Dela Cruz and Torres (2012). The test isolates were streaked onto the gelatin agar (HiMedia, India) comprising 3% gelatin and incubated anaerobically for 48 hr at 37°C. After incubation, the plates were flooded with saturated solution of ammonium sulphate and kept at -20°C for observing clear zone around the colonies. Isolates producing clear zones indicate positive results for gelatinase activity.

2.4.5 Cholesterol assimilation test

The ability of bacterial isolates to assimilate cholesterol was determined upon inoculating 500 μ l bacterial aliquot in MRS thio broth (supplemented with 0.3% bile salt) and filter sterilized 1% w/v cholesterol (HiMedia, India) under anaerobic conditions for 72 hr at 37°C. Following incubation, bacterial supernatant was collected upon centrifuging the growth medium at 5000rpm for 10 minutes at 4°C. Thereafter, 500 μ l supernatant was mixed with 3ml of 95% ethanol (v/v) and 2ml 45% KOH (w/v) and heated in the water bath for 10 minutes at 60°C. After cooling the mixture, 5ml n-hexane and 3ml distilled water was added and allowed to stand for 15 min at room temperature for phase separation. Upon decanting the hexane layer, 4ml o-phthalaldehyde reagent was added and allowed to stand for 10 minutes, followed by the addition of 2ml concentrated sulphuric acid (98%), which was further allowed to stand for 10 minutes. Cholesterol

assimilation was calculated upon measuring the absorbance of uninoculated and spent broth at 550 nm while the following formula calculated the percentage of cholesterol assimilation:

$$\text{Cholesterol removal \%} = \frac{(\text{Cholesterol uninoculated} - \text{Cholesterol inoculated})}{\text{Cholesterol uninoculated}} \times 100$$

2.4.4. Extraction of antibacterial agents and evaluation of their antagonistic activity

The isolation of antibacterial agents was carried out following the protocol described by Sreenadh et al. (2022). Each bacterial isolate, cultured for 48 hr, was mixed with an equal volume (5 mL) of ethyl acetate and subjected to shaking at 20 rpm for 10 minutes using a rotary shaker. The mixture was then centrifuged, and the supernatant was carefully transferred into a fresh tube, allowing the ethyl acetate to evaporate. The residual content was used to assess its antagonistic effect against the selected test pathogens.

The test pathogens included *E.coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27852. The antagonistic potential of bacterial metabolites was assessed using the well-diffusion method. Mueller-Hinton agar (MHA) plates were inoculated with each test pathogen, and wells were created using a sterile cork borer. Each well was loaded with 60 µL of the extracted supernatant, followed by incubation at 37°C for 24–48 hr. Dimethyl sulfoxide (DMSO) served as a negative control. The inhibition zones were measured and categorized based on their diameter: highly sensitive (> 20 mm), moderately sensitive (10–20 mm), or resistant (< 10 mm) (Prabhurajeshwar and Chandrakanth, 2017).

2.5. Evaluation of technological properties

2.5.1. Proteolytic activity

Proteolytic activity was determined using an agar medium containing 10% skimmed milk powder and 2% agar. Wells were carefully created in the agar, and 20 µL of bacterial cultures were introduced into each well. The plates were then incubated at 37°C for 48 to 72 hr. The formation of a clear zone around the wells indicated positive proteolytic Activity (Raveschot et al., 2020; Sreenadh et al., 2022).

2.5.2. Lipolytic activity

To assess lipolytic activity, bacterial isolates were streaked onto tributyrin agar plates and incubated at 37°C for 72 to 96 hr. The appearance of a clear zone around the colonies was considered a positive indicator of lipolytic Activity (Aspri et al., 2017).

2.5.3. Amylolytic activity

Amylolytic activity was evaluated by spot-inoculating the test isolates onto the surface of Luria-Bertani (LB) agar (Himedia, Mumbai, India) supplemented with 20 g/L of soluble starch. The plates were incubated at 37°C for 72 hr, after which a 1% iodine solution was applied. The formation of a halo zone around the colonies indicated positive amylolytic Activity (Espirito-Santo et al., 2014; Sreenadh et al., 2022).

2.4. Statistical analysis

All experiments were conducted in triplicate, and the results were presented as mean values $\pm$ standard deviations from three independent replicates. All data were analyzed using Student's t-test to compare the values between the control and test groups for each isolates. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Identification of bacteria

3.1.1. Biochemical identification

The biochemical characteristics of bacterial isolates obtained from different curd samples are presented in Table 1. The isolates recovered from the curd prepared with Lemon (*Citrus limon*) and Imli (*Tamarindus indica*), designated as GCC_24LM and GCC_24IM, respectively, displayed similar morphological and biochemical profiles. Both isolates formed yellow, smooth-surfaced colonies on MRS agar and were identified as Gram-positive bacilli. Biochemical characterization revealed that both isolates were indole-negative, MR-positive, VP-negative, and citrate-positive. Both tested isolates were positive for catalase and oxidase activity, while showing negative results for TSI reactions. Based on the morphological and biochemical traits, both GCC_24LM and GCC_24IM were presumptively identified as *Weissella* species.

Table 1
Biochemical characteristics of bacterial isolates

Characteristics	Bacterial isolates	
	GCC_24LM	GCC_24IM
Morphological characteristics		
Colour	Yellow colonies	Yellow colonies
Surface	Smooth	Smooth
Gram staining	Gram-positive	Gram-positive
Cell shape	Bacilli	Bacilli
Biochemical characteristics		
Indole	-	-
MR	+	+
VP	-	-
Citrate	+	+
Starch hydrolysis	-	-
Catalase	+	+
Oxidase	+	+
Triple sugar iron	-	-
'+' indicates positive results and '-' indicates negative results		

3.1.2. Molecular identification

The alignment of isolates GCC_24IM and GCC_24LM with database sequences contained 208 distinct patterns, with 12.53% of positions consisting of gaps or undetermined characters. Both isolates clustered closely together, forming a distinct clade most closely related to *Weissella cibaria* strain II-I-59 (NR_036924.1). This suggests that the isolates are genetically similar and likely belong to *W. cibaria* (Fig. 1). Other *Weissella* species, such as *W. coleopterorum*, *W. kandleri*, and *W. confusa*, formed separate clades, reflecting the genetic diversity within the genus.

3.2. Screening of probiotic properties of isolated bacteria

3.2.1. Simulated gastric juice tolerance test

The growth of bacterial isolates under control (PBS buffer, pH 7.2) and test (simulated gastric juice, pH 3) conditions was monitored at hourly intervals using OD measurements. The results indicate a time-dependent increase in bacterial growth for both conditions (Fig. 2), with control groups consistently showing higher OD values compared to the test groups, suggesting that acidic conditions hinder bacterial

proliferation. The growth rate was initially slow in the first hr, followed by a significant increase between 1 and 2 hr, where isolate GCC_24IM exhibited the highest percentage increase of 80.5% and 69.6% in the test and control group, respectively. Statistical analysis using a paired t-test showed no significant differences at 0–1 hr ($p = 0.12$ for GCC_24LM, $p = 0.09$ for IM). However, a significant reduction in bacterial growth was observed in the test groups between 1 and 2 hr ($p = 0.03$ for GCC_24LM, $p = 0.01$ for IM), confirming the inhibitory effect of gastric acidity. Between 2 and 3 hr, bacterial growth continued to increase in both conditions, but the percentage increase was lower compared to the 1–2 hr interval. The highest bacterial growth was observed in the control groups, exhibiting 69.7% for the isolate GCC_24IM and 51.5% for the isolate GCC_24LM. The isolate inoculated in the simulated gastric juice exhibited a lower trend, which was observed as 43.9% for the isolate GCC_24IM and 37.2% for the isolate GCC_24LM. Despite the continued growth, the difference between control and test groups remained statistically significant at 2–3 hr ($p = 0.04$ for GCC_24LM, $p = 0.02$ for IM), indicating a persistent inhibitory effect of acidic conditions. These overall results suggest that acidic conditions strongly suppress bacterial proliferation, particularly during the rapid growth phase, before a potential adaptation reduces the inhibitory effect at later time points.

3.2.2. Bile tolerance test

The effect of bile on bacterial growth was assessed by comparing the OD values in control and bile-treated groups for GCC_24LM and GCC_24IM isolates over time. A paired t-test revealed a significant decrease in bacterial growth in the bile-treated group compared to the control at 1 hr (Fig. 3). The isolate GCC_24IM showed a 20.98% growth in the control group while the bile-treated group exhibited 3.92% growth, indicating the inhibitory effect of bile. Similarly, for GCC_24LM, the control group showed a 55.91% increase, whereas the treated group had a significantly lower increase of 16.96%. At 2 hr, the inhibitory effect of bile persisted. The isolate GCC_24IM in the control group showed a significant growth of 55.09%, while the treated group showed a lower increase of 33.89% ($t = 4.21$, $p = 0.010$). The GCC_24LM control group exhibited a 52.68% increase, whereas the treated group had a reduced increase of 32.68% ($t = 3.87$, $p = 0.020$). At 3 hr, bacterial growth continued to be lower in the bile-treated groups, but the difference was relatively reduced compared to earlier time points. The growth of isolate GCC_24IM was found to be 31.32% and 34.58% in the control group and bile-treated group, respectively, showing some adaptation to bile stress ($t = 2.98$, $p = 0.020$). On the other hand, the isolate GCC_24IM showed an increased growth of 55.91% and 44.00% in the control and treated groups ($t = 4.56$, $p = 0.010$). These findings indicate that bile significantly inhibits bacterial growth, particularly at 1 hr and 2 hr, with a persistent but slightly reduced effect at 3 hr. However, both isolates demonstrated the ability to tolerate bile stress over time, suggesting their potential for survival in the gastrointestinal tract, a crucial trait for probiotic efficacy.

3.2.3. Pancreatin tolerance test

The effect of pancreatin on bacterial growth was assessed for the isolates by comparing their OD values in treated and control groups over time. The results indicate that at 0 hr, there was no significant difference between the control and pancreatin-treated groups for both GCC_24IM ($t = 1.98$, $p = 0.081$) and GCC_24LM ($t = 1.67$, $p = 0.109$), confirming similar initial bacterial densities before treatment. At 24 hr, a statistically significant reduction in bacterial growth was observed in the pancreatin-treated group. The mean OD of the

isolate GCC_24LM was significantly lower in the treated group compared to the control, with a mean difference of 0.842 ($t = 4.99$, $p = 0.003$). Similarly, for IM, the OD was significantly lower in the treated group than in the control, with a mean difference of 0.812 ($t = 5.87$, $p = 0.001$). These results suggest a strong inhibitory effect of pancreatin on bacterial proliferation at 24 hr (Fig. 4). At 48 hr, the inhibitory effect of pancreatin persisted but was relatively reduced compared to 24 hr. The OD for GCC_24IM in the treated group was significantly lower than in the control, with a mean difference of 0.242 ($t = 3.01$, $p = 0.018$). Similarly, the treated group showed significantly lower OD for IM than the control, with a mean difference of 0.528 ($t = 4.76$, $p = 0.005$). These findings confirm that pancreatin significantly suppresses bacterial growth ($p < 0.05$), with the most potent inhibition occurring at 24 hr and a sustained effect at 48 hr.

3.2.4. Assessment of cell surface hydrophobicity

Cell surface hydrophobicity is an essential trait influencing bacterial adhesion to host epithelial cells and interaction with hydrophobic surfaces. The results showed that isolate GCC_24IM exhibited higher hydrophobicity in n-hexadecane (67.77%) compared to xylene (51.94%), whereas isolate GCC_24LM demonstrated greater affinity for xylene (60.81%) than n-hexadecane (47.40%). These variations suggest differences in the surface characteristics of the isolates, likely influenced by the composition of their outer membrane structures, including proteins and exopolysaccharides. The higher hydrophobicity of GCC_24LM in n-hexadecane indicates a stronger interaction with aliphatic hydrocarbons, while the preference of GCC_24IM for xylene suggests a greater affinity for aromatic hydrocarbons. These findings highlight the potential of both isolates in colonizing and adhering to host epithelial surfaces, an essential feature for effective probiotic function. Further studies on adhesion to intestinal cells and biofilm formation could provide more insights into their probiotic potential.

3.2.5. Cellular autoaggregation assay

The autoaggregation ability of probiotic bacteria plays a crucial role in colonization and adherence to the intestinal epithelium, which is essential for probiotic functionality. In this study, isolates GCC_24IM and GCC_24LM exhibited significant autoaggregation percentages, with GCC_24IM showing higher aggregation ability (67.16%) compared to GCC_24LM (56.75%). The higher autoaggregation percentage of GCC_24IM suggests a stronger ability to form cell clusters, possibly contributing to enhanced adhesion and competitive exclusion of pathogens. The observed variations between the isolates could be attributed to differences in cell surface properties, such as hydrophobicity and exopolysaccharide production. These results indicate that both isolates possess substantial autoaggregation capacity, a key factor in their potential probiotic effectiveness.

3.2.6. NaCl tolerance test

The ability of probiotic bacteria to tolerate high salt concentrations is essential for their survival in various environments, including food processing and the gastrointestinal tract. In this study, both the isolated strains demonstrated tolerance to 15% NaCl, indicating their adaptability to osmotic stress. This characteristic enhances their potential application in fermented food products and ensures their viability in challenging conditions. The observed salt tolerance suggests that these isolates can maintain their metabolic Activity and growth despite the high osmolarity, further supporting their probiotic suitability.

3.2.7. Phenol tolerance test

The ability of the isolates to tolerate phenol was evaluated by monitoring their growth in MRS broth supplemented with increasing concentrations of phenol (0.2%, 0.4%, and 0.6%) over 48 hr (Fig. 5). Both isolates (GCC_24IM and GCC_24LM) showed a concentration- and time-dependent reduction in growth. At 0 hr, all isolates showed comparable OD values across concentrations, with only minor declines in the presence of phenol. After 24 hr, isolate GCC_24IM exhibited relatively higher growth ($OD = 0.73 \pm 0.02$) in 0.2% phenol than GCC_24LM ($OD = 0.40 \pm 0.02$), while at 0.4% and 0.6% phenol, both isolates showed substantial inhibition, with OD values declining to 0.21 ± 0.01 and 0.13 ± 0.01 for IM, and 0.18 ± 0.02 and 0.13 ± 0.02 for GCC_24LM, respectively.

At 48 hr, further differences were observed. GCC_24LM showed the highest tolerance in 0.2% phenol ($OD = 1.10 \pm 0.05$), followed by GCC_24IM ($OD = 0.60 \pm 0.02$). However, in 0.4% phenol, growth remained suppressed for both isolates (IM: 0.22 ± 0.03 ; GCC_24LM: 0.28 ± 0.02), and in 0.6% phenol, OD values dropped further (IM: 0.11 ± 0.01 ; GCC_24LM: 0.22 ± 0.03). These results indicate that both isolates can tolerate phenol up to 0.4%, with significantly higher resistance shown by isolate GCC_24IM at all concentrations and time points, particularly in 0.2% phenol, suggesting better survivability under phenolic stress.

3.3. In vitro biofilm assay

In this study, both tested isolates produced black, dry, and crystalline colonies on CRA medium, indicating strong biofilm-forming ability phenotypically. Quantitative assessment using the TCP method revealed that *W. cibaria* strain GCC_24IM exhibited an OD of 0.901, while *W. cibaria* strain GCC_24LM showed an OD of 0.503. Based on the biofilm production interpretation criteria ($OD_{\text{control}} = 0.218$), strain GCC_24IM was classified as a strong biofilm producer, whereas strain GCC_24LM was identified as a moderate biofilm producer. This difference in biofilm production methods of CRA and TCP was observed among the isolates and may be due to strain-specific characteristics, environmental factors, and varying adhesion mechanisms.

3.4. Safety Assessment and antimicrobial Activity

3.4.1. Antibiotic susceptibility test

Antibiotic susceptibility testing was performed for the isolates GCC_24LM and GCC_24IM using the disc diffusion method in accordance with CLSI and EUCAST guidelines, with *Escherichia coli* ATCC 25922 as the internal control strain (Table 2). The results revealed that GCC_24LM was resistant to most β -lactam antibiotics, including penicillin G, methicillin, oxacillin, and all cephalosporins tested. However, it showed susceptibility to ampicillin and an intermediate response to ceftriaxone. It also exhibited resistance to carbapenems, monobactams, and β -lactamase inhibitor combinations like ceftazidime/avibactam. Among aminoglycosides, GCC_24LM was susceptible to gentamicin, streptomycin (both 120 μg and 300 μg discs), and minocycline but resistant to others like amikacin, kanamycin, neomycin, netilmicin, and tobramycin. The strain was fully resistant to fluoroquinolones and tetracycline but showed susceptibility to polymyxins.

(polymyxin B and colistin sulphate), vancomycin, rifampicin, and partially to macrolides (resistant to azithromycin and clarithromycin, susceptible to erythromycin). It exhibited intermediate resistance to chloramphenicol and bacitracin.

Table 2
AST Profile of isolate GCC_24LM and GCC_24IM as compared with *Escherichia coli* ATCC 25922

Sl. No.	Class	Sub-Class	Antibiotics	Isolate GCC_24LM	Isolate GCC_24IM	<i>Escherichia coli</i> ATCC 25922
1	—	—	Sterile Disc (Control)	0	0	0
2	β-Lactam	Penicillin	Penicillin G (P 10 units)	20 [R]	11 [R]	12 [R]
3			Methicillin (MET 5mcg)	14 [R]	33 [S]	--
4			Ampicillin (AMP 10 mcg)	15 [S]	28 [S]	11 [R]
5			Oxacillin (OX 1 mcg)	15 [R]	42 [S]	--
6		Cephalosporin	Cefdinir (CDR 5 mcg)	0 [R]	11 [R]	19 [R]
7			Cefixime (CFM 5 mcg)	0 [R]	0 [R]	19 [R]
8			Cefepime (CPM 30 mcg)	0 [R]	10 [R]	26 [R]
9			Cefotaxime (CTX 30 mcg)	0 [R]	24 [I]	24 [R]
10			Ceftriaxone (CTR 30 mcg)	27 [I]	23 [R]	20 [R]
11			Ceftazidime (CAZ 30 mcg)	9 [R]	29 [S]	22 [R]
12			Cefoxitin (CX 30 mcg)	11 [R]	25 [S]	19 [R]
13		Carbapenems	Imipenem (IPM 10 mcg)	0 [R]	6 [R]	11 [R]
14			Meropenem (MRP 10 mcg)	0 [R]	11 [R]	22 [R]
15		Monobactam	Aztreonam (AT 30 mcg)	0 [R]	0 [R]	22 [R]
16	β-Lactam β-Lactamase	Ceftazidime/Avibactam (CZA 30/20 mcg)	0 [R]	6 [R]	24 [R]	
17		Amoxicillin/Clavulanic acid (AMC 20/10 mcg)	11 [R]	42 [S]	16 [R]	
18		Piperacillin/Tazobactam (PTZ 100/10 mcg)	17 [R]	26 [S]	23 [S]	
19	Aminoglycosides	Amikacin (AK 30 mcg)	9 [R]	24 [S]	14 [R]	
20		Gentamicin (HLG 120 mcg)	16 [S]	33 [S]	20 [S]	
21		Kanamycin (K 30 mcg)	9 [R]	18 [S]	0 [R]	
<i>R: Resistant; I: Intermediate; S: Susceptible</i>						

Sl. No.	Class	Sub-Class	Antibiotics	Isolate GCC_24LM	Isolate GCC_24IM	<i>Escherichia coli</i> ATCC 25922
22			Neomycin (N 10mcg)	10 [R]	13 [R]	13 [R]
23			Streptomycin (HLS 300 mcg)	11 [S]	18 [S]	13 [S]
24			Streptomycin (S 10 mcg)	8 [R]	31 [S]	13 [S]
25			Netilmicin (NET 30 mcg)	0 [R]	0 [R]	11 [R]
26			Tobramycin (TOB 10 mcg)	0 [R]	0 [R]	17 [R]
27			Fluroquinolones	Ciprofloxacin (CIP 5 mcg)	0 [R]	0 [R]
28			Ofloxacin (OF 5 mcg)	0 [R]	24 [R]	21 [R]
29			Norfloxacin (NX 10 mcg)	9 [R]	26 [I]	28 [S]
30			Levofloxacin (LE 5 mcg)	7 [R]	30 [S]	13 [R]
31			Moxifloxacin (MO 5 mcg)	9 [R]	22 [I]	28 [S]
32			Levonadifloxacin (LND 10 mcg)	10 [R]	0 [R]	10 [R]
33	Tetracyclines		Tetracycline (TE 30 mcg)	16 [R]	45 [S]	13 [R]
34			Tigecycline (TGC 15 mcg)	10 [R]	0 [R]	11 [R]
35			Minocycline (MI 30 mcg)	24 [S]	10 [R]	9 [R]
36	Glycopeptides		Teicoplanin (TEI 30 mcg)	0 [R]	6 [R]	--
37			Vancomycin (VA 30 mcg)	10 [R]	17 [S]	--
38	Polypeptide		Bacitracin (B 10 mcg)	12 [I]	14 [S]	--
39	Macrolides		Azithromycin (AZM 15 mcg)	12 [R]	40 [S]	--
40			Clarithromycin (CLR 15 mcg)	10 [R]	10 [R]	--
<i>R: Resistant; I: Intermediate; S: Susceptible</i>						

Sl. No.	Class	Sub-Class	Antibiotics	Isolate GCC_24LM	Isolate GCC_24IM	<i>Escherichia coli</i> ATCC 25922
41			Erythromycin (E 15 mcg)	11 [R]	26 [S]	17 [I]
42	Polymixins		Polymyxin B (PB 300 mcg)	21 [S]	18 [S]	10 [R]
43			Colistin sulphate (CS 10 mcg)	11 [S]	12 [S]	0 [R]
44	Sulfonamides		Co-Trimaxazole (COT 25 mcg)	8 [R]	13 [R]	15 [R]
45	Rifampicin		Rifampicin (RIF 5 mcg)	10 [S]	39 [S]	0 [R]
46	Nitronidazole		Metronidazole (MT 4 mcg)	0 [R]	10 [R]	0 [R]
47	Lincosamides		Clindamycin (CD 2 mcg)	13 [R]	14 [R]	--
48	Chloramphenicol		Chloramphenicol (C 30 mcg)	14 [I]	20 [I]	20 [I]
49	Nitrofurantoin		Nitrofurantoin (NIT 300 mcg)	0 [R]	0 [R]	0 [R]
50	Fosfomicin		Fosfomicin (FO 200 mcg)	0 [R]	0 [R]	0 [R]
<i>R: Resistant; I: Intermediate; S: Susceptible</i>						

In contrast, GCC_24IM showed a broader susceptibility profile. It was susceptible to penicillin substitutes like methicillin, oxacillin, ampicillin, and β -lactam combinations such as amoxicillin/clavulanic acid and piperacillin/tazobactam. It also demonstrated susceptibility to multiple aminoglycosides which includes amikacin, gentamicin, kanamycin, and streptomycin, and to fluoroquinolones such as levofloxacin and norfloxacin (intermediate), although it was resistant to ciprofloxacin. Among glycopeptides and macrolides, GCC_24IM was susceptible to vancomycin, bacitracin, azithromycin, and erythromycin. It was also susceptible to polymyxins, rifampicin, and exhibited intermediate response to chloramphenicol. However, it remained resistant to carbapenems, monobactams, tetracycline, tigecycline, and several other antibiotic classes such as lincosamides, nitrofurans, and fosfomicin. Overall, GCC_24IM demonstrated a more favorable antibiotic sensitivity profile compared to GCC_24LM, indicating potential as a safer probiotic candidate in terms of lower antimicrobial resistance.

3.4.2. Haemolytic Activity

The haemolytic activity test is crucial for assessing the safety of probiotic strains, as haemolysis production can indicate potential pathogenicity. In this study, the isolates GCC_24LM and GCC_24IM exhibited no haemolytic activity, confirming their non-pathogenic nature and suitability for probiotic applications.

3.4.3. DNase Activity

DNase production is associated with virulence in certain bacterial strains. The absence of DNase activity in both isolates supports their safety profile, as they do not exhibit extracellular DNase production, which is a characteristic of some opportunistic pathogens

3.4.4. Gelatinase Activity

In the present study, none of the isolates revealed the formation of clear zones around the streaked colonies, indicating the inability to produce gelatinase production.

3.4.5. Cholesterol Reduction Activity

The ability of the isolates to assimilate cholesterol was assessed, revealing a significant difference between the two. Isolate GCC_24LM demonstrated a high cholesterol reduction capacity of 96.66%, whereas GCC_24IM exhibited a lower assimilation rate of 33.33%.

3.4.6. Extraction of antibacterial agents and evaluation of their antagonistic Activity

Probiotics are often known to produce inhibitor chemicals like bacteriocins and naturally produce several organic acids, which inhibit the growth and propagation of pathogenic strains. However, upon testing against the pathogens, isolates GCC_24LM and GCC_24IM did not show any zone of inhibition, revealing the absence of antagonistic properties.

3.5. Evaluation of technological properties

In this study, isolates GCC_24LM and GCC_24IM showed no proteolytic, lipolytic, or amylolytic Activity. The absence of these enzymatic activities suggests that these strains may not contribute to the degradation of proteins, lipids, or starch during fermentation. While enzymatic Activity is desirable in some applications, the lack of these functions can be advantageous in preventing undesirable breakdown of food components, ensuring stability and consistency in probiotic formulations.

4. Discussion

In this study, bacterial isolates from curd samples fermented with lemon and imli were characterized and identified as *W. cibaria* strains (GCC_24LM and GCC_24IM). A series of in vitro evaluations was conducted to assess their probiotic potential. Both isolates showed the ability to survive in simulated gastric conditions, with *Weissella cibaria* strain GCC_24LM exhibiting higher survivability. This trait is essential for probiotic efficacy as it ensures the bacteria can transit through the stomach and reach the intestine. Similar tolerance was reported in *W. cibaria* JW15, which survived at pH 3.0 for 2 hr and 0.3% bile salts exposure (Lee et al., 2013). Acid and bile tolerance are critical for surviving the harsh GI tract environment, and *W. cibaria* SP19 has demonstrated similar resilience (Patrone et al., 2021). In bile salt tolerance assays, the isolated strains endured 0.3% oxgall, again with GCC_24LM showing greater resilience. Tolerance to bile is necessary for colonization in the small intestine. Cai et al. (2022) reported that *W. cibaria* 018 survived bile

salt concentrations of up to 3.0 g/L, and this tolerance was linked to the HigBA toxin-antitoxin system activation under stress. GCC_24LM also exhibited higher tolerance to pancreatin and simulated intestinal fluids, highlighting its robustness throughout the digestive tract. These findings align with *W. cibaria* strains isolated from kimchi and dairy cows, demonstrating high pancreatin tolerance and enzymatic resistance (Yu et al., 2019).

The isolates also showed good NaCl and phenol tolerance, with GCC_24LM outperforming GCC_24IM. This indicates their ability to survive food processing conditions. In previous studies, *W. cibaria* MD2 exhibited high phenol and NaCl tolerance and was suggested for use in food formulations due to its stability under such stresses (Lakra et al., 2020). GCC_24LM showed higher cell surface hydrophobicity and aggregation, which facilitates adhesion to intestinal epithelial cells and biofilm formation. *W. cibaria* MD2 demonstrated autoaggregation and strong hydrophobic interactions that support mucosal adherence (Lakra et al., 2020). Similarly, strains like JW15 adhered well to Caco-2 cells, exhibiting properties comparable to commercial strains like *L. rhamnosus* GG (Ahn et al., 2013). In the present study, biofilm formation was prominently observed in GCC_24LM, thereby enhancing probiotic persistence in the GI tract. *W. cibaria* strains have been noted to form biofilms as a survival and colonization mechanism, as shown by (Lakra et al., 2020).

Antibiotic susceptibility tests indicated sensitivity to most commonly used antibiotics, confirming their safety. This aligns with findings from *W. cibaria* strains D29 and D30, which exhibited susceptibility to a broad range of antibiotics and lacked hemolytic Activity (Yu et al., 2019). Both isolates were non-hemolytic, DNase, and gelatinase-negative, reaffirming their non-pathogenic and safe nature. Safety assessments from *W. cibaria* P71 and SP19 studies also confirmed a lack of virulence genes and hemolytic behavior (Elavarasi et al., 2014; Lakra et al., 2020). In cholesterol assimilation assays, GCC_24LM showed 96.66% reduction, substantially higher than GCC_24IM. This is notably greater than values reported in *W. cibaria* MD2 and KTSMBNL 28 strains, which reduced cholesterol by 78% and approximately 50%, respectively (Elavarasi et al., 2014; Lakra et al., 2020). Despite these beneficial properties, no antibacterial activity was observed against the tested pathogens by either isolate, which is a deviation from earlier findings. Studies have reported that *W. cibaria* JW15 and MD2 produced bacteriocins effective against pathogens like *Listeria monocytogenes* and *E. coli* (Lee et al., 2013; Lakra et al., 2020). This might be attributed to strain-specific metabolic differences or the absence of bacteriocin-producing genes.

5. Conclusion

The present study successfully isolated and identified two strains of *W. cibaria* (GCC_24LM and GCC_24IM) from lemon- and imli-fermented curd, and comprehensively evaluated their probiotic properties. Both strains demonstrated considerable tolerance to acidic gastric conditions, bile salts, and pancreatin, suggesting strong survival potential through the gastrointestinal tract. They also exhibited desirable cell surface characteristics such as moderate to strong hydrophobicity and autoaggregation ability, which are important for mucosal adhesion. The isolates showed moderate biofilm formation, which may enhance colonization and persistence in the gut. Additionally, both strains were non-hemolytic, gelatinase- and DNase-negative, indicating safety for probiotic use. Notably, *W. cibaria* GCC_24LM showed a high cholesterol-lowering capacity (96.66%), supporting its potential in cardiovascular health management.

However, neither strain exhibited antagonistic activity against the tested pathogens, highlighting the need for further optimization or combination with other strains. Future research should focus on in vivo validation of these probiotic properties, exploring their interactions with the host microbiome, and assessing health benefits through clinical trials. Genome-based studies could provide deeper insights into their functional genes and safety profiles. The promising characteristics of these isolates indicate their potential application in functional foods or dietary supplements, contributing to novel probiotic formulations targeting gut health and metabolic disorders.

Declarations

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Consent to publish declaration: Not applicable.

Availability of data and materials: All data generated or analysed during this study are included in this article.

Author Contribution

S.N: Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing. P.P, M.P and P.C: Formal analysis, Investigation, Writing – original draft. A.N, H.Y and I.S: Methodology, Validation, Visualization.

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Figures

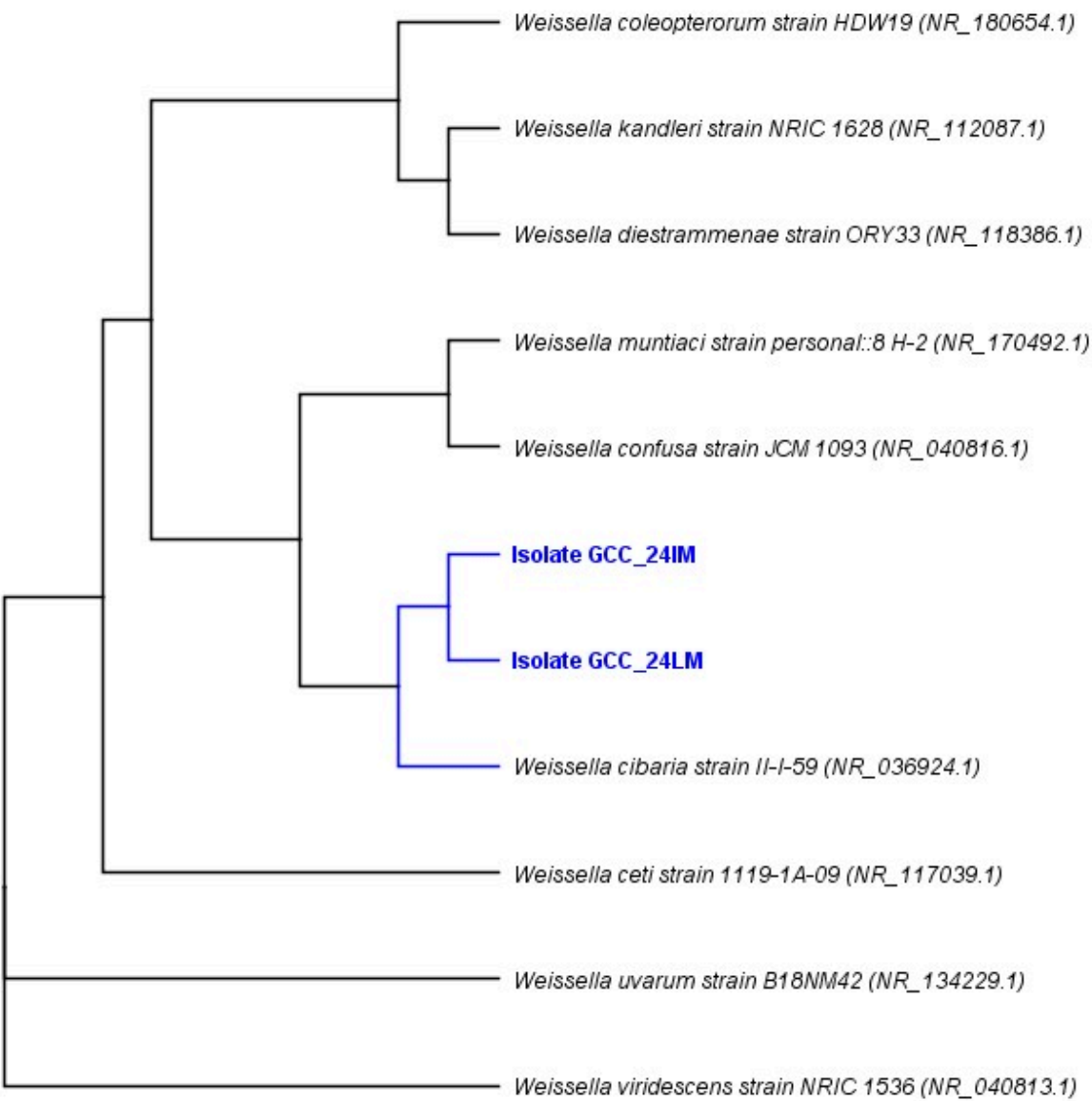


Figure 1

Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of isolates GCC_24IM and GCC_24LM with reference strains of the genus *Weissella*.

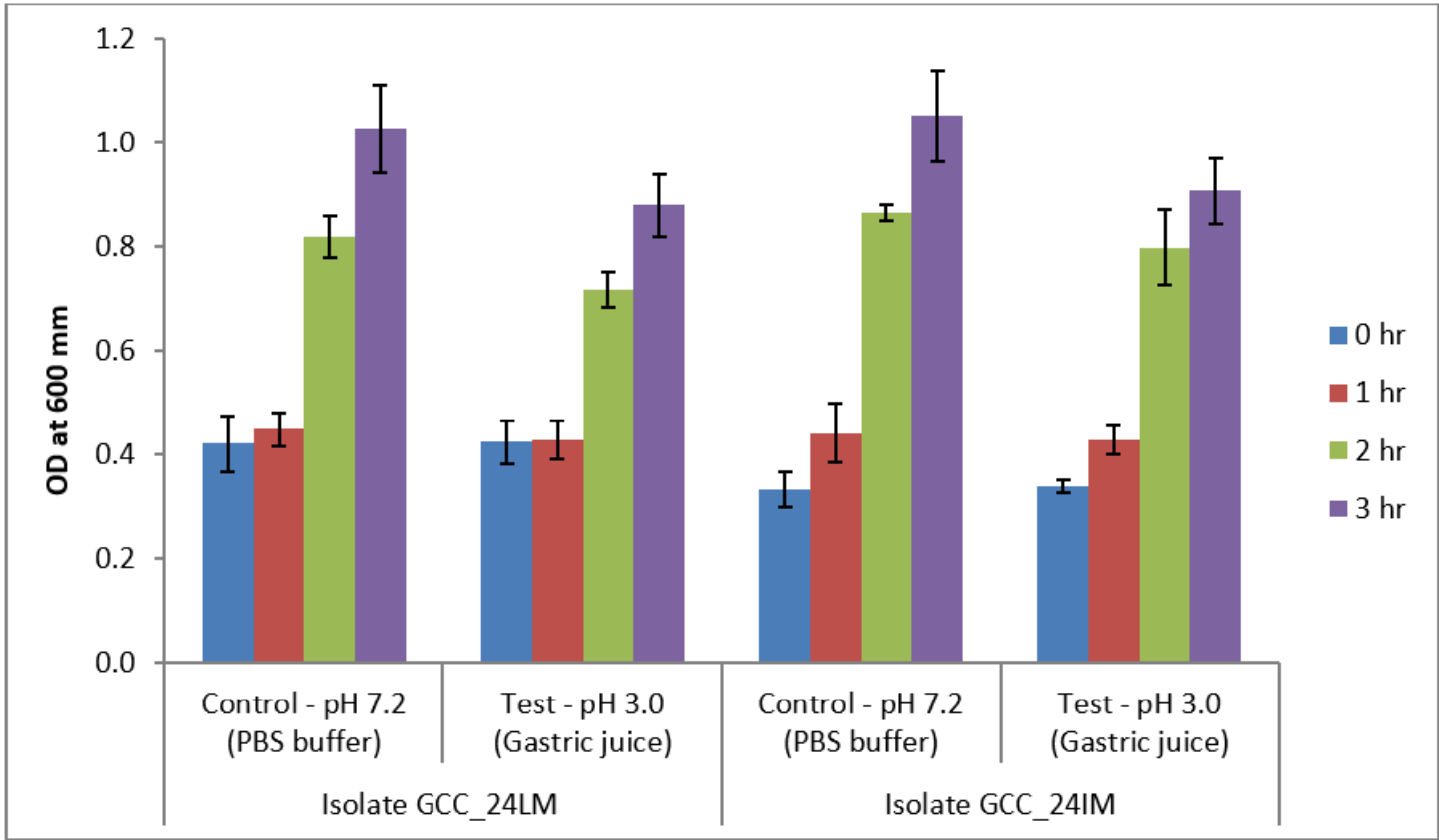


Figure 2

Growth of bacterial isolates GCC_24LM and GCC_24IM in control (pH 7.2) and simulated gastric juice (pH 3.0) over 3 hr, measured by OD at 600 nm. Data are mean $\pm$ SD (n=3). Growth was time-dependent and significantly lower in acidic conditions, especially between 1–2 h and 2–3 h ($p < 0.05$).

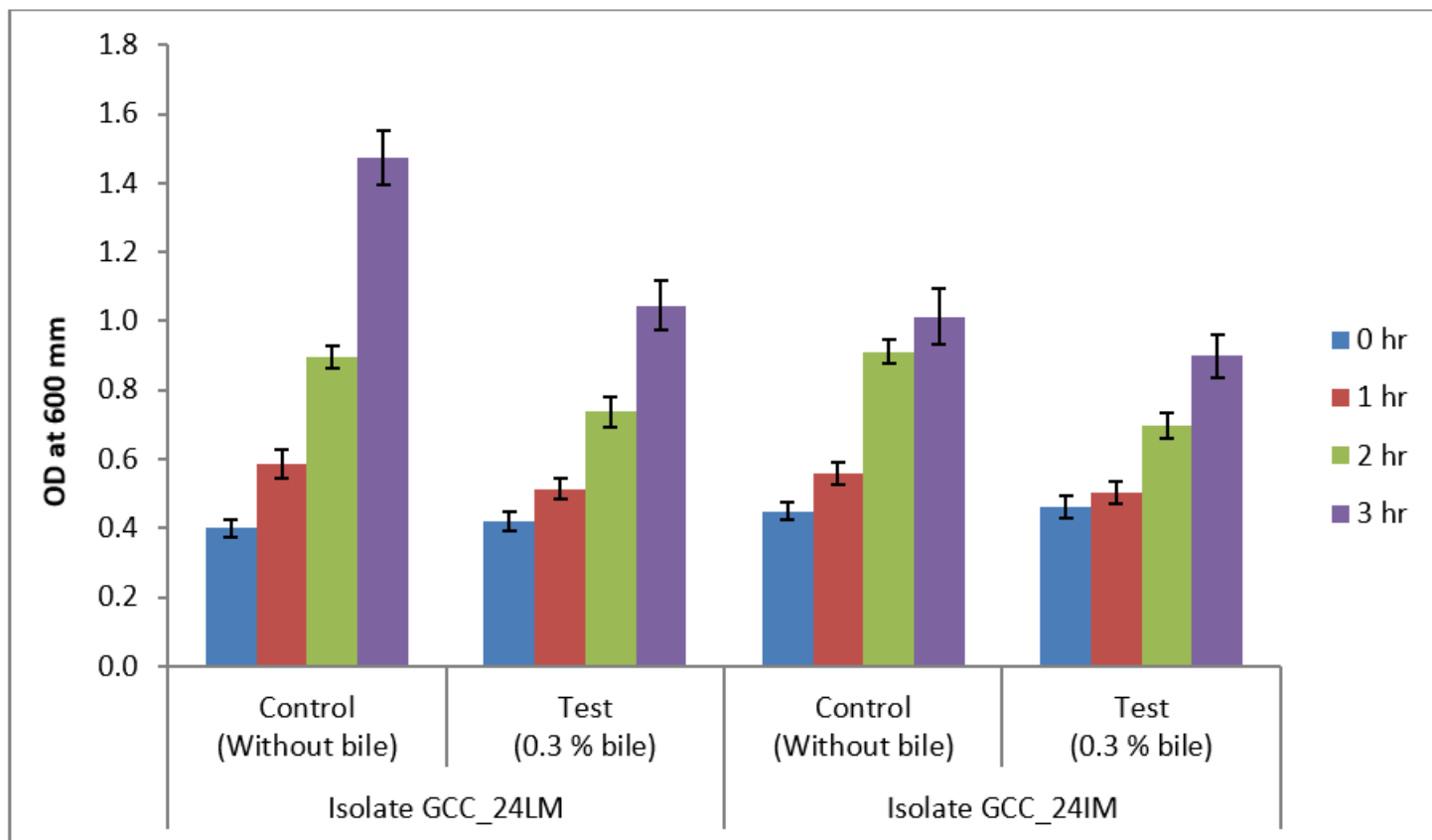


Figure 3

Effect of 0.3% bile on growth of bacterial isolates GCC_24LM (lemon) and GCC_24IM (imli) over 3 hr, measured by OD at 600 nm. Data represent mean $\pm$ SD (n=3). Bile significantly inhibited growth at 1–2 h ($p < 0.05$), with partial recovery by 3 h, indicating time-dependent bile tolerance.

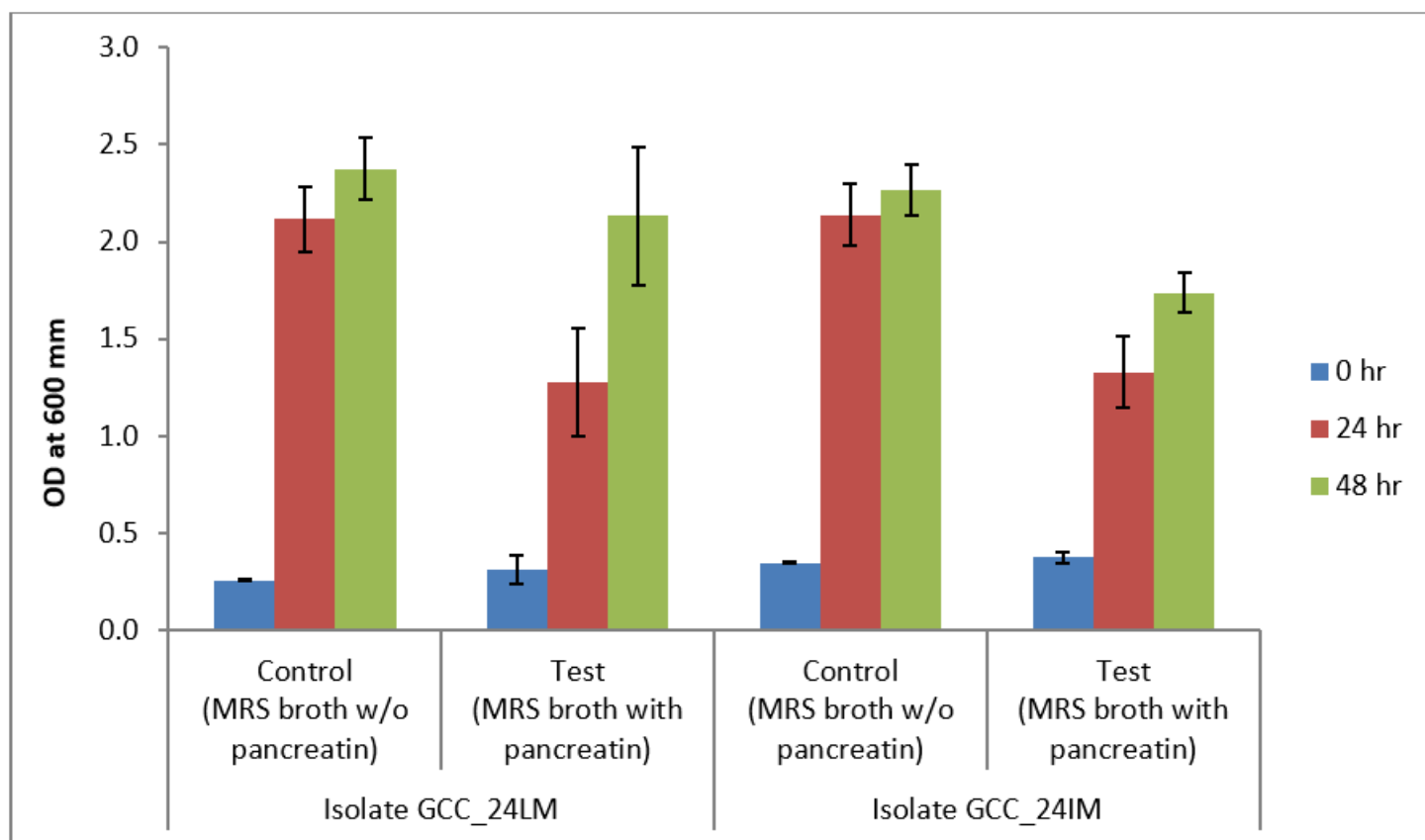


Figure 4

Effect of pancreatin on the growth of bacterial isolates GCC_24LM and GCC_24IM over 48 hr, measured by OD at 600 nm. Control samples (MRS broth without pancreatin) showed continuous growth, while pancreatin-treated groups exhibited significantly reduced growth at 24 hr ($p < 0.05$), with partial recovery by 48 hr. Data represent mean $\pm$ SD ($n=3$).

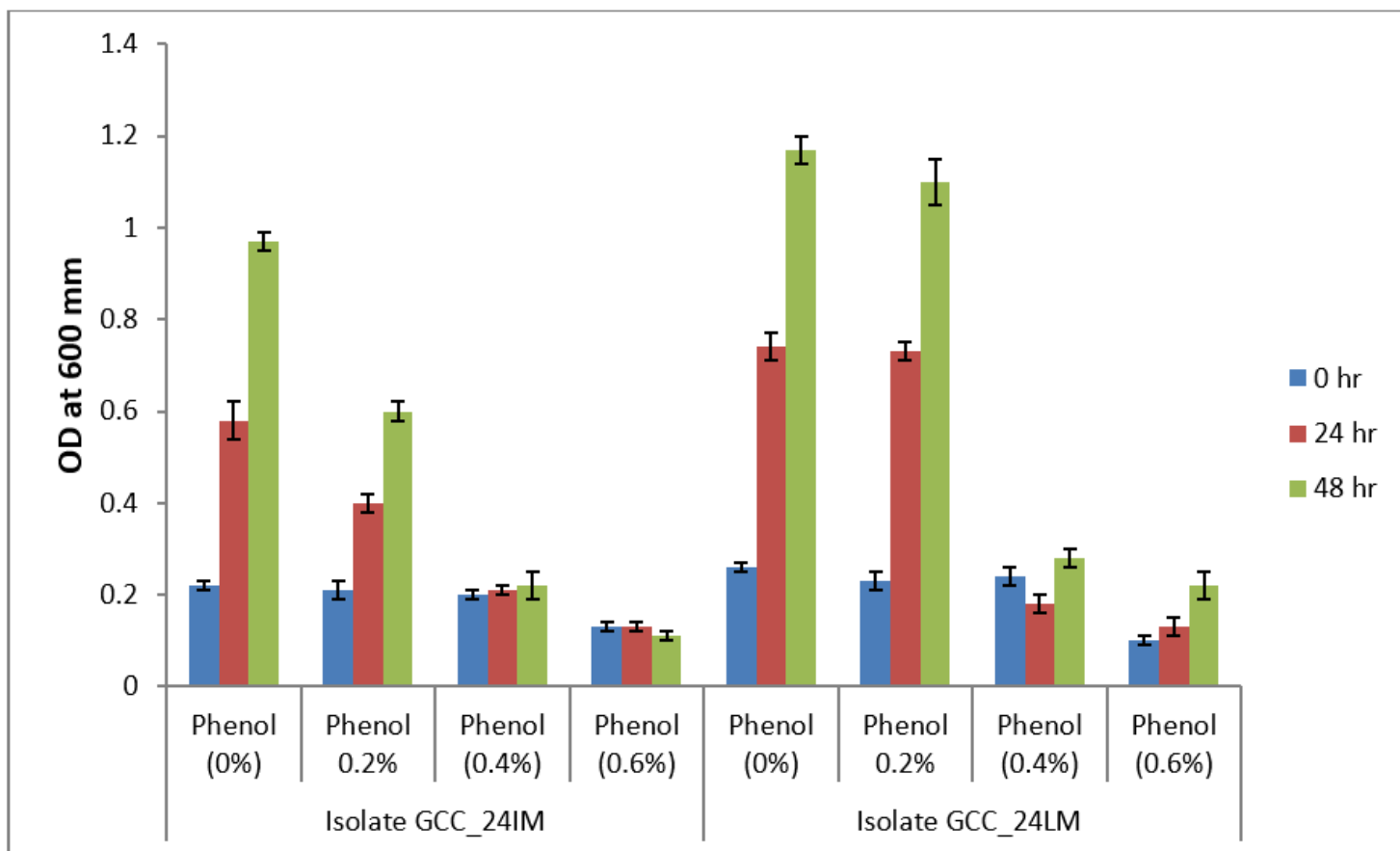


Figure 5

Effect of different phenol concentrations (0%, 0.2%, 0.4%, and 0.6%) on the growth of bacterial isolates GCC_24IM and GCC_24LM over 48 hr.