OPEN ACCESS

J. Micro. Sci. Vol. 3(1), 01-11, 2024,
https://doi.org/10.38211/jms.2024.01.56

Research Article

Available on Innovative Science and Technology Publishers
Journal of Microbiological Sciences
www.isciencepress.com/journals/index.php/jms
E-ISSN- 2959-975X

Exploring Fresh Lettuce (*Lactuca sativa*) as a Dairy-Free Probiotic Source

Muhammad Sufian Masued[1], Shams Ur Rehman[2], Muhammad Musaddiq Shah[1], Lubna Zafar[3], Raees Ahmed[4], Muhammad Tariq-Khan[4], Aqeel Ahmad[5], Muhammad jamil[5], Abdul Mateen[1]*

[1]Department of Biological Sciences, University of Sialkot, Sialkot, Pakistan.
[2]Department of Computer Sciences, University of Poonch Rawalakot, AJK, Pakistan.
[3]Department of Plant Pathology, University of Poonch Rawalakot, AJK, Pakistan.
[4]National Key Laboratory of Wheat Improvement, Peking University Institute of Advanced Agricultural Sciences, Shandong Laboratory of Advanced Agriculture Sciences in Weifang, Weifang, China.
[5]Department of Agronomy, University of Poonch Rawalakot, AJK, Pakistan.
*Corresponding author: dr.abdulmateen@uskt.edu.pk

Article Received 19-09-2024, Article Revised 10-10-2024, Article Accepted 16-10-2024

ABSTRACT

Probiotics are living microorganisms that, when given in appropriate amounts, have optimistic effects on body. This study explores the potential of fresh lettuce (*Lactuca sativa*) leaves as a probiotic source for individuals with dairy allergies. Gram staining and the catalase test were used to identify the bacteria that were recovered from lettuce leaves as gram-positive, catalase-negative strains. The resistance to antibiotics and their capacity to tolerate low pH and bile salt concentrations both essential for surviving the digestive tract were evaluated. The bacteria showed a high tolerance to bile salts and low pH, but they were susceptible to ampicillin, streptomycin, gentamycin, chloramphenicol, and neomycin. Tests for gas production in the presence of glucose revealed no gas production. Molecular techniques such as PCR and 16S rRNA gene sequencing revealed the presence of *Enterococcus lactis, Enterococcus durans, Lactobacillus paracasei,* and *Lactobacillus casei*

Keywords: Probiotic, Bacteria, Lettuce, Antibiotics, rRNA, PCR

INTRODUCTION

Lettuce is a well-known leafy green vegetable appreciated for its fresh surface and mellow flavor. It is an amazing source of fundamental supplements, and essential vitamins, minerals and dietary fibers, including vitamins A, C, and K, as well as folate and dietary fiber (Yang *et al*., 2021). Lettuce is a vegetable which contains high water content and low calories, that makes it perfect for hydration and weight management. Its antioxidants contribute to reducing oxidative stress within the body, and the fiber helps in digestive health. Incorporating lettuce into a balanced diet supports overall wellness and enhances stomach related function due to its high fiber substance (Haytowitz *et al*., 2019). Probiotics, beneficial microorganisms that live in our gut, are crucial for maintaining a healthy immune system and overall health. These microbes produce lactic acid, acetic acid, and bacteriocins, which inhibit the growth of harmful pathogens (Sanders *et al*., 2018). Probiotics are linked to increased activity of natural killer cells, essential for combating infections, and boosting antibody production. Studies indicate that probiotics can help prevent and treat lung, urinary tract, and vaginal infections (Guarner, 2003). Additionally, probiotics are being explored for their potential to lessen the threat of enduring diseases and improve general health outcomes (Hill, 2014). Among the various probiotic species, *Lactobacillus* and *Bifidobacterium* are the most commonly utilized. These bacteria reside in different parts of the human gastrointestinal tract, playing significant roles in maintaining gut health. *Lactobacilli* are mainly found in the colon, while *Bifidobacterium* species inhabit the intestine (Lebeer *et al*., 2008). Other strains, such as *Streptococcus thermophilus* and *Lactococcus lactis*, also show beneficial effects on gut health and immune function, though further research is necessary to fully confirm their safety and efficacy (Kerry, 2018). Probiotics have become increasingly popular for promoting health through diet. They are naturally present in agitated foods which include yogurt, kefir, and kimchi, but can also be taken as supplements in capsules, tablets, or powders (Marco, 2017). Green vegetables, including lettuce, are high in fiber and water, which can improve digestion and provide numerous health benefits. Fresh lettuce leaves are particularly valuable, offering essential vitamins, minerals, and antioxidants that support overall health (McManus, 2020). This study explores the role of probiotics in lettuce and their potential health benefits. Lettuce, with its rich nutrients, provides a favorable environment for beneficial bacteria. The main objectives are to isolate, characterize, and identify probiotic bacteria from fresh lettuce leaves. By identifying these probiotic strains and understanding their benefits, we aim to promote lettuce as a safer and healthier food choice, highlighting the health

advantages of including green vegetables like lettuce in the diet.

MATERIAL AND METHODOLOGY

Sampling of Lettuce Leaves: Fresh lettuce leaves were obtained from three different locations (Lahore, Gujranwala and Sialkot). To ensure sample cleanliness, the leaves were thoroughly washed and then treated with a chlorine solution at 5°C for 20 minutes to eliminate any potential contaminants. One gram of crushed lettuce pulp was used to make serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) in phosphate buffer. These dilutions were spread on MRS agar plates using the spread plate technique to isolate probiotic bacteria. From each plates, five distinct colonies were selected for further analysis, following a modified version of Goyal's manual (Bommasamudram *et al*., 2023). Sampling details are illustrated in figure 1.

Biochemical Analysis

Test for Antibiotic Sensitivity: To assess antibiotic susceptibility, Mueller-Hinton reagent plates were prepared by autoclaving Mueller-Hinton reagent in distilled water. The sterilized M-H agar was then added to petri plates. Colonies were selected and inoculated onto the plates. Antibiotic resistance discs containing streptomycin, gentamycin, chloramphenicol, neomycin, and ampicillin were place at appropriate distance on the inoculated plates. After incubation at 37°C for 24-48 hours, the zones of inhibition were measured using a millimeter scale as per Martinez *et al*. (2009).

Test for Salt Tolerance: Mannitol salt agar, which is considered as a selective and differential medium, was used to assess salt tolerance. Following Shafiq *et al*., 2023, with minor modifications mannitol agar (13.32 g) along with distilled water (120 ml) were autoclaved at 121°C for 30 min and then poured into petri dishes. After solidification, bacterial culture was streaked and plates were incubated at 37°C for 24 hours (Cappuccino & Sherman, 2010).

Bile Salt Tolerance Test: To evaluate bacterial bile salt tolerance, two MRS broths containing 0.2% and 0.4% bile salt, respectively, were prepared following Kang *et al*., 2016, Inoculum was added to the sterilized broths and incubated anaerobically at 37°C for 24 hours. After centrifugation, the bacterial culture was washed in 0.1% peptone water and changes in optical density (OD) at 600 nm were monitored (Corsetti & Settanni, 2007).

Testing for Homo Fermentative Isolates: To identify homo-fermentative isolates, MRS broth comprising of glucose was prepared and Durham tubes were implanted in test tubes and autoclaved to sterilize them. After disinfection, the inoculum was transferred to the sterile test tubes, and incubated at 37°C for two days (Cvrtila Fleck *et al*., 2012).

Acid Tolerance Test: Growth medium was organized with definite components and accustomed to the preferred pH using hydrochloric acid. Bacterial strains (L-A, L-B, L-C, L-D and L-E) were inoculated into the medium and incubated at 37°C for 24-48 hours. Optical density (OD) of bacterial growth was measure edat 600nm using a spectrophotometer (Denkova *et al*., 2010).

Catalase enzyme Test : To determine the presence of the catalase enzyme in the strains, a clean microscopic slide was used. The strains were retained on the slides, and 2-3 droplets of hydrogen peroxide were added. The presence of bubbles indicated appositive catalase test (Di Martino *et al*., 2023).

Molecular Characterization

DNA Extraction and Amplification: The most promising bacterial isolates, selected based on their bile salt tolerance and low pH resistance, underwent genomic DNA extraction using a high-quality extraction kit (Gerasimidis *et al*., 2016). The isolated DNA was amplified through PCR using a 25µl reaction mixture containing universal primers, PCR buffer, $MgCl_2$, dNTPs, Taq polymerase, and deionized water. The amplification process involved 35 cycles at specific temperatures, followed by agarose gel electrophoresis to visualize the PCR products. Ethidium bromide was used to stain the DNA bands, which were then observed under a GelDoc EQ system (Ghazali & Rashid, 2019; Gibson & Robust, 2011; Goyal *et al*., 2013; Guarner & Malagelada, 2003). The amplified products were subsequently sequenced, and the resulting data was analyzed using the NCBI BLAST tool to compare and classify the sequences against known databases (Hassanzadazar *et al*., 2012).

Sequencing: Following successful PCR amplification, the amplicons were sequenced. The primers used for amplification targeted the 16S rRNA gene region, with forward primer (5′AGAGTTTGATCCTGGCTCAG3′) and the 16S reverse primer (5′GGTTACCTTGTTACGACTT3′). The resulting sequences were then analyzed using the National Center for Biotechnology Information (NCBI) GenBank database through the Basic Local Alignment Search Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) This analysis aimed to identify the closest matching sequences within the database, providing insights into the potential identity of the isolated bacteria. Furthermore, the sequences were imported into Mega X software (https://www.megasoftware.net/) to construct a phylogenetic tree. This tree visualization helped to elucidate the evolutionary relationships between the isolated bacteria and other known bacterial species. The tree provided a broader understanding of the closest relatives of the isolated strains.

Statistical Analysis: To evaluate the performance of LAB isolates under various conditions, several statistical analyses were conducted. For the Acid Tolerance Test, absorbance measurements at pH 2 and pH 3 for five bacterial strains (L-A to L-E) revealed a significant difference using a paired t-test (p-value = 0.005). The mean absorbance values were 0.3900 at

pH 2 and 0.7800 at pH 3, and a 95% confidence interval for the mean difference of (-0.5783, -0.2017) (Gibson & Robust, 2011). In the Antibiotic Resistance Test, the resistance of LAB isolates to five antibiotics (S, G, CL, N, AM) was investigated using one-way ANOVA, showing significant differences (p-value = 0.000), with Tukey pairwise comparisons representing that resistance to CL was expressively higher than the other antibiotics (Goyal *et al*., 2013). For the Bile Salt Tolerance Test, absorbance measurements at 0.2% and 0.5% bile concentrations were compared using a paired t-test, which showed a significant difference (p-value = 0.006). The mean absorbance of 0.3300 at 0.2% bile and 0.2200 at 0.5% bile, with a 95% confidence interval for the mean difference of (0.0524, 0.1676) (Huang & Adams, 2004). All statistical analyses were performed using Minitab 18 (Minitab, Ltd., Coventry, UK), with a significance threshold level of $p < 0.05$. The results were based on triplicate measurements to ensure consistency and reproducibility.

RESULTS

Characterization and Gram Staining of isolates: Following 24-48 hours of incubation, five bacterial colonies were isolated and examined for both morphological and biochemical characteristics. The morphological analysis revealed various shapes: three isolates exhibited cocci morphology (L-A, L-B, and L-C), while two displayed rod-shaped forms (L-D and L-E). These findings match with previous research work in which diverse morphological forms were understood among bacterial isolates (Jones and Clark, 2019). Gram staining was performed in order to classify the isolates as gram positive or negative. All strains were found as purple colored under the Gram staining procedure, classifying them as gram-positive bacteria. Figure 2 presents the streaking results, and figure 3 provides microscopic images of the bacterial strains, further supporting these findings.

Catalase Test: To further distinguish the isolates, a catalase test was conducted. One isolate showed frothiness reaction when assorted with hydrogen peroxide (H_2O_2). The other four isolates (L-A, L-B, L-C, L-E) did not show any reaction, specifying they are catalase-negative. In contrast, strain L-D displayed a positive catalase reaction, characterized by the formation of bubbles.

Acid Tolerance Test: All isolates exhibited variability in the acid tolerance test, with around 70% survival after 18 hours at various pH levels. The highest pH for growth (pH 3.0) was observed in the L-C isolate, corresponding the optimal pH range for *Lactobacilli*. However, growth dropped at pH 2.0. This capability to thrive at low pH, a characteristic of *Lactobacilli*, is imperative for probiotic prospective. A paired t-test was conducted to analyze the absorbance measurements at pH 2 and pH 3 for the five bacterial strains (L-A to L-E). The test revealed a significant variance between the means of absorbance at these two pH levels, with the mean absorbance at pH 2.0 being 0.3900 and at pH 3 being 0.7800. The paired t-test generated a T-value of -5.75 and a p-value of 0.005, presenting statistical significance. The 95% confidence interval for the mean difference was (-0.5783, -0.2017), further confirming that the difference in absorbance between pH 2.0 and pH 3.0 is significant. The results of this acid tolerance test support the concept that lactobacilli can grow at different pH values since they are in line with other studies. Notably, strain L-C was shown to thrive best at a pH of 3.0, suggesting that it would be an excellent choice for a probiotic. Furthermore, the genetic research provided insights into the molecular differences in acid tolerance across various strains of Lactobacilli as shown in figure 1.

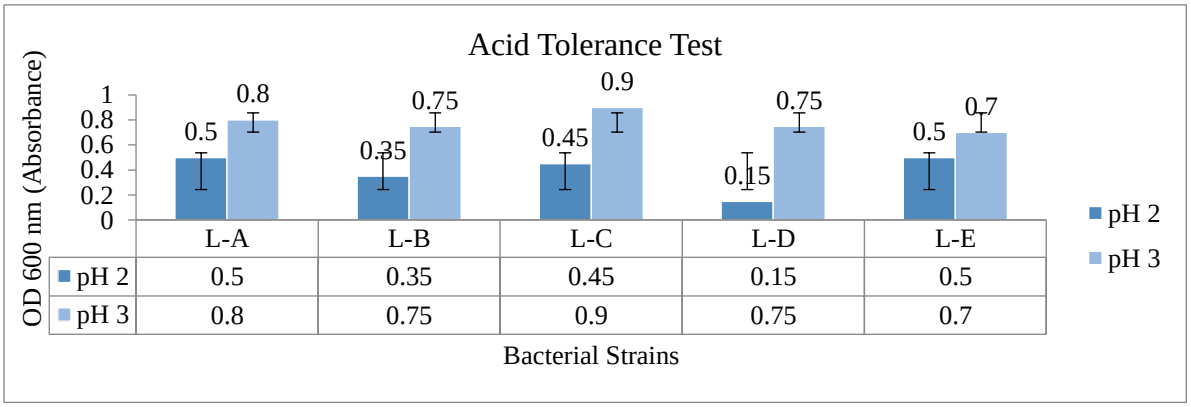
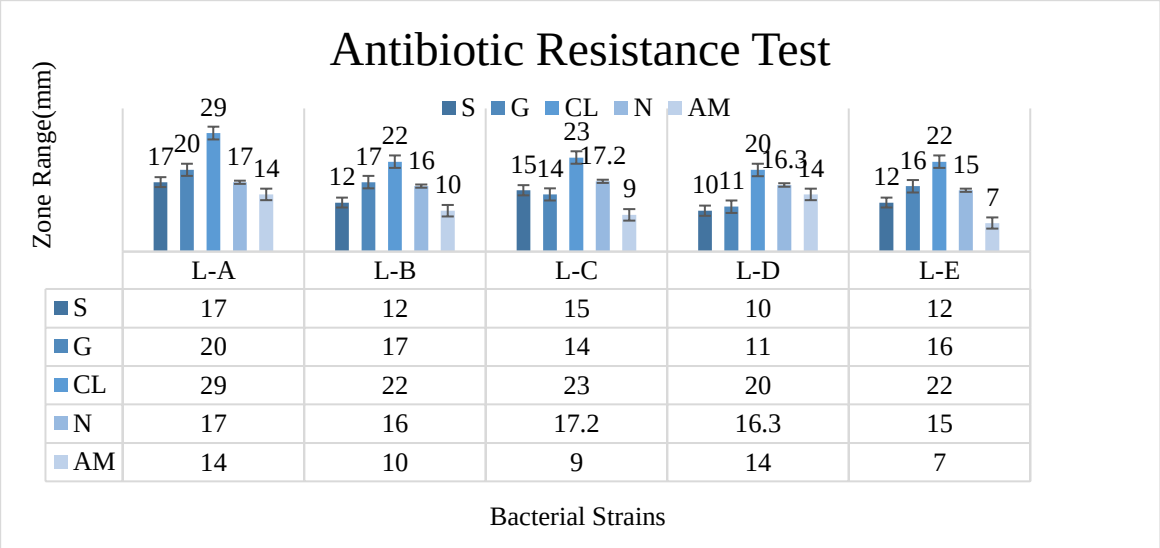


Figure 1: Acid Tolerance of Isolates at pH 2.0 and pH 3.0. After 18-Hour Incubation Period, Based on Mean Readings. The results specify important survival rates of the isolates under extremely acidic conditions. Error bars symbolize the standard deviation of the mean values, underlining the stability of the tolerance levels among the isolates.

Resistance to Antibiotics: Isolates endured antibiotic susceptibility testing after 48 hours of incubation. Some isolates demonstrated sensitivity to antibiotics, while others exhibited resistance. Zones of inhibition between 12.5 and 17.4 mm were considered intermediate, while those below 12.4 mm were categorized as resistant. All isolates revealed both sensitivity and resistance to antibiotics, vital

characteristic for probiotic application. To determine if there were significant differences in resistance levels among the antibiotics (S, G, CL, N, and AM), one-way ANOVA was directed. The ANOVA results revealed a significant effect (p-value = 0.000), indicating that at least one mean was resistance level was different figure 2. The factor evidence specified five levels, with equal variances presumed for the analysis. The Tukey pairwise comparisons recognized that resistance to CL was meaningfully higher compared to the other antibiotics figure 5. The grouping information classified CL in group A, N in group B, G and S in group B C, and AM in group C. The model summary reported an R-squared value of 72.49%, suggesting a good fit for the model. Statistical analysis is represented in figure 3 and figure 4 respectively. In figure 3, ANOVA test is conducted while in figure 4, HSD test was conducted to compare the results of antibiotic resistance test. All five isolates demonstrate sensitivity, which is in accordance with the essential qualities for probiotics, ensuring their safety and viability.



	L-A	L-B	L-C	L-D	L-E
S	17	12	15	10	12
G	20	17	14	11	16
CL	29	22	23	20	22
N	17	16	17.2	16.3	15
AM	14	10	9	14	7

Figure 2: Antibiotic Susceptibility of Bacterial Strains. Diameters ≤12.4 mm are considered as resistant zones, while diameters ≥17.5 mm specifying susceptible (sensitive) zones. Antibiotics tested include Streptomycin (S), Gentamycin (G), Chloramphenicol (CL), Neomycin (N), and Ampicillin (AM). The Y-axis represents zone ranges from 0-35 mm, and the X-axis represents bacterial strains.

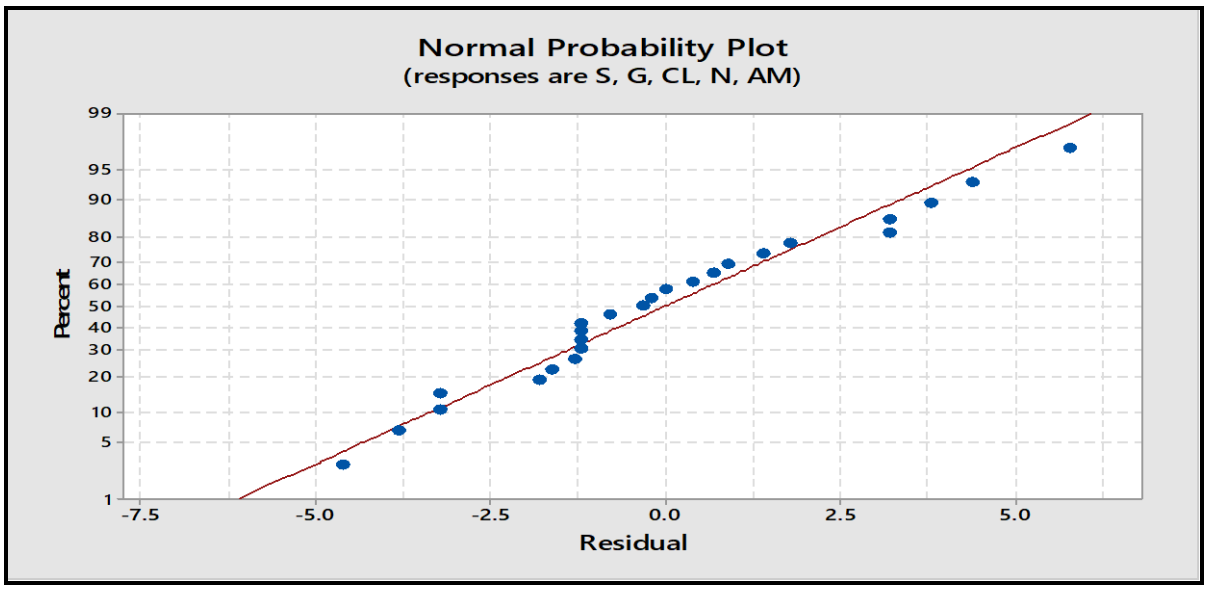


Figure 3: Analysis of Variance (ANOVA) results associating antibiotic resistance levels among various bacterial strains. The test specifies significant differences ($p < 0.05$) in resistance profiles, emphasizing variations in response to antibiotic treatments.

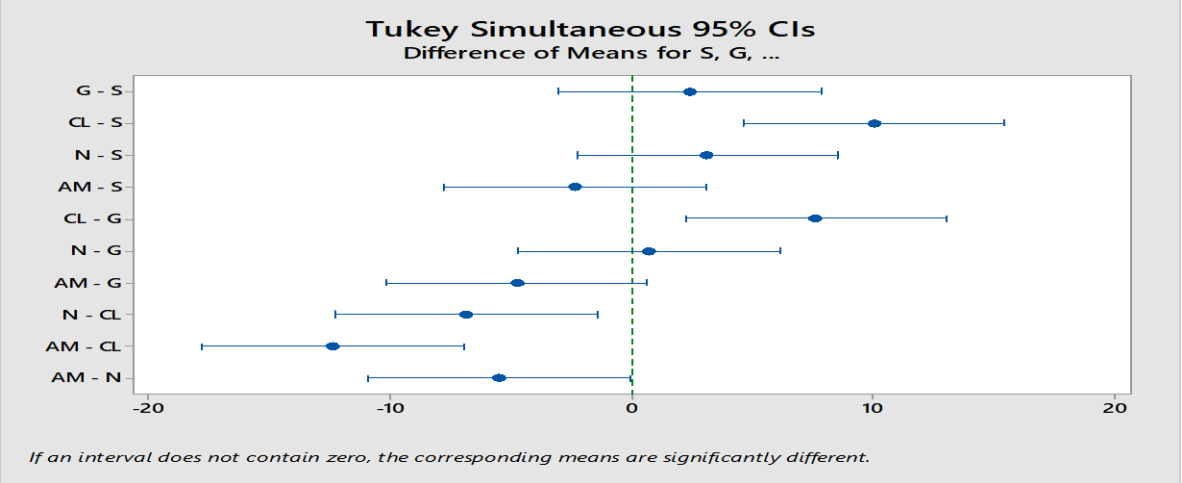


Figure 4: Tukey's Honestly Significant Difference (HSD) test results displaying pairwise comparisons of antibiotic resistance levels across bacterial strains. Significant differences ($p < 0.05$) are observed between specific strain pairs, providing perceptions into different resistance patterns.

Salt Tolerance Test: The growth of four strains, L-A, L-C, L-D, and L-E, on mannitol salt agar test after 72 hours of incubation was witnessed to be insignificant. One strain, L-B, showed good change among all. The negligible growth observed for strains L-A, L-C, L-D, and L-E shows that these isolates may have lower salt tolerance. The observed color changes in strain L-B during the salt tolerance test specify its capability to endure variable salt concentrations as shown in figure 8.

Bile Salt Tolerance Test: The tolerance of the isolates to various concentrations of bile salts was confirmed, illuminating that the highest tolerance level was at 0.2% bile salt concentration. The tolerance level of the isolates reduced with high concentration of bile salt. A paired t-test relating the mean absorbance values at 0.2% and 0.5% bile concentrations was conducted. The test identified a noteworthy difference in absorbance among the two concentrations (p-value = 0.006), with a 95% confidence interval for the mean difference of (0.0524, 0.1676). The mean absorbance at 0.2% bile was 0.3300, while at 0.5% bile, it was 0.2200. These results show the variable tolerance levels of isolates to bile salts, which is essential for estimating their potential as probiotics. Research has indicated that probiotics can withstand acidic stomach settings, suggesting that they may survive on the way to the small intestine and colon. Two well-established methods for determining this kind of survival are the acid tolerance test and the bile salt tolerance test (Huang & Adams, 2004). In correspondence to the previous finding, it is noticed that probiotic count started to decrease after 24 hours. However, the isolates showed good resistance to pH 2 and 3. This implies that these strains may be able to enter the human gut and survive there. The outcomes of the bile salt tolerance test are shown in figure 5.

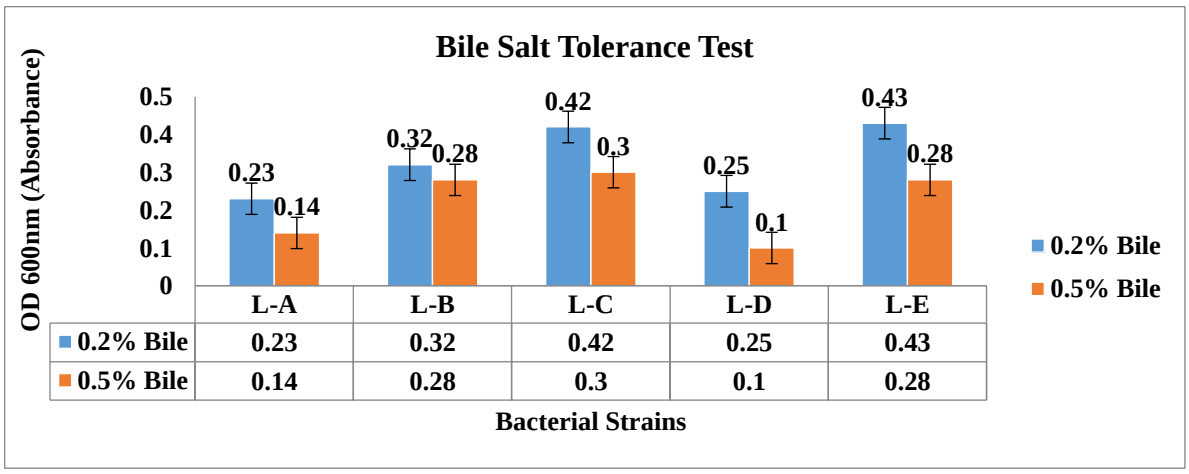


Figure 5: Optical Density (OD) of the isolates at different bile salt concentrations after 24 hours at 600 nm OD. The Y-axis represents absorbance (AU), while the X-axis represents bacterial strains.

Gas Production Test: The gas-producing capacity of the isolates was experienced using glucose in the medium. The test tubes were witnessed for around five days to check for buildup of gas in Durham tubes.

However, no gas accumulation of gas was noticed, resulting in a negative test.

Identification of 16S rRNA Sequencing: PCR was employed to amplify 16S rRNA gene segments of the isolates for identification. This method is broadly utilized for bacterial classification by associating sequences with a database. The 16S rRNA gene is extremely preserved but contains adjustable sectors for species variation. Sequencing allows comparison with previous sequences, sustaining in isolate identification. PCR products were identified using agarose gel electrophoresis. Sequencing of 16S rRNA gene segments confirmed the isolates as *Lactobacillus* species, aligning with NCBI GenBank database. Phylogenetic analysis further reinforced this identification. This information is vital for research and development of probiotic products, authenticating the genetic makeup and phylogenetic assurance of the isolates. The sequence similarity indices for the isolated probiotic bacteria disclose high alignment with reference sequences: 96.14% for *Enterococcus faecium*, 100% for *Enterococcus lactis*, 99.86% for *Enterococcus durans*, and 97.40% for both *Lactobacillus casei* and *Lactobacillus paracasei* as shown in figure 6. The insignificant deviances are probably due to mismatches, gaps, or mutations that may occur naturally.

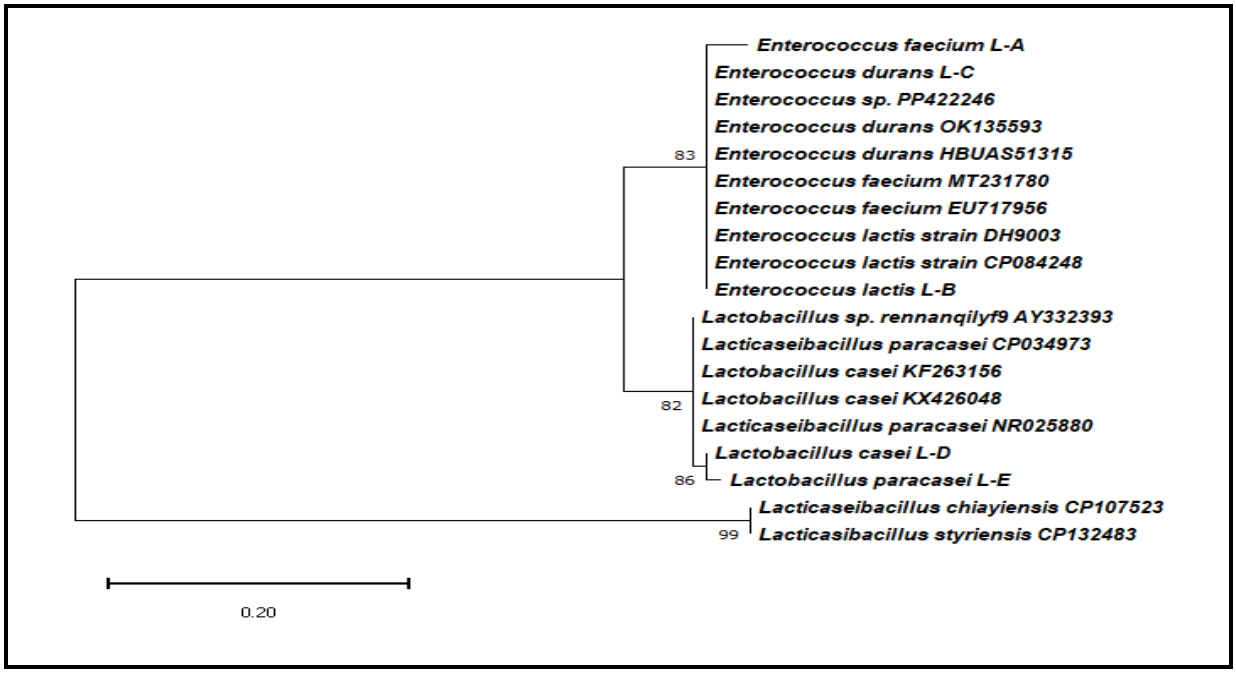


Figure 6: The evolutionary history was concluded using the Neighbor-Joining method. The ideal tree is shown. The percentage of replicate trees in which the related taxa bunched together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 19 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 117 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

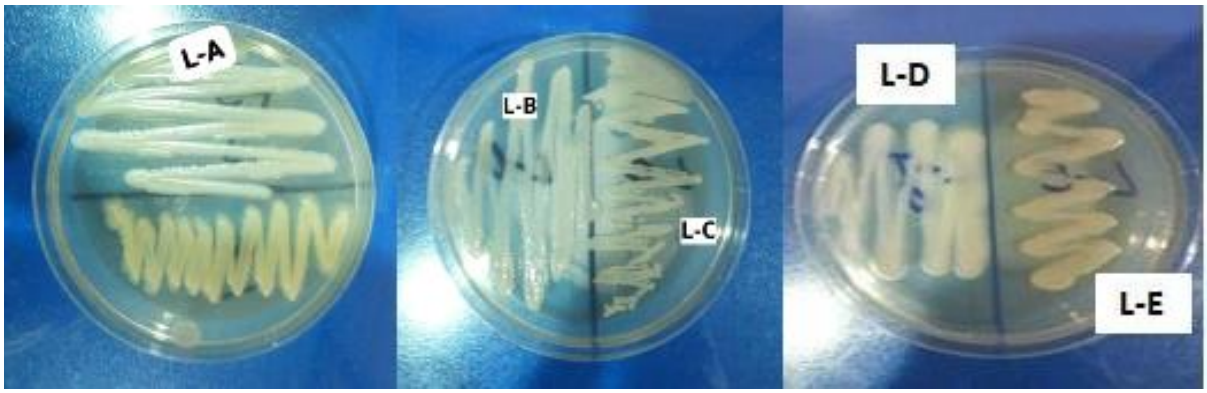


Figure 7: Streaking Result representing the successful isolation of probiotic bacterial strains L-A, L-B, L-C, L-D, and L-E from lettuce leaves. Each strain was noticeably separated, displaying the variety of probiotic bacteria present.

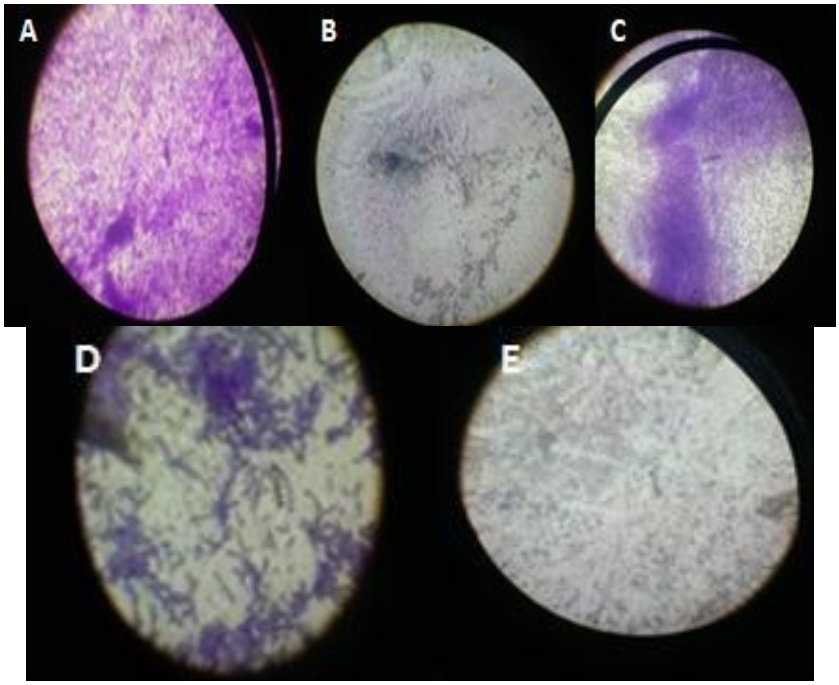


Figure 8: Microscopy of selected strains, A, B, C representing gram positive cocci, while D and E representing gram positive rod shaped bacteria.

DISCUSSION

Food is generally recognized for its health advantages, particularly nutrient-dense meals like fresh lettuce. Ancient diets included probiotic-rich foods like milk, yogurt, and fermented foods. *Lactobacillus* and *Bifidobacterium* are two types of probiotics that are well-known for their beneficial effects on health, including the handling of lactose intolerance and cardiovascular problems. Probiotic-rich meals, such as fresh lettuce, can help those with dairy allergies and those who are health-conscious (Guarner & Malagelada, 2003; Hill et al., 2014). Probiotics can improve disease resistance and proliferation abilities, which can boost general health when incorporated into a balanced diet. Probiotics provide a number of advantages, including increased immunity, competitive exclusion, and enzyme activation. It also boosts the immune system and strive with pathogens for binding sites. Probiotics provide several benefits for enhancing health, even though their exact mechanisms are still obscure (Huang & Adams, 2004; Imperial & Ibana, 2016; Kechagia et al., 2013). Five particular LAB strains were selected for this investigation and grown in MRS medium with the pH set to 7. The MRS medium was specifically chosen since it only promotes the development of probiotic bacteria. This selection is in accordance with previous research that found that the ideal pH range for the culture and screening of LAB is between 6.2 and 8.5 Abo-Amer (2011). Following sampling of lettuce from different areas, a thorough study of a few strains was conducted, comprising features such as microscopic, macroscopic, biochemical, and probiotic features. Five distinctive LAB strains were found in the lettuce from this screening process: two rod-shaped (L-D, L-E) and three cocci-shaped (L-A, L-B, and L-C). One important discovery from our research is that none of the five strains produced any gas, as shown by their negative catalase reactions. These strains also lacked spore formation and were all Gram-positive. The results of Gram-positive bacteria were aligned with previous findings that characterized by their thick peptidoglycan layer retaining the crystal violet stain (Brown and Miller, 2021). The presence of catalase enzyme enables bacteria to convert hydrogen peroxide into harmless byproducts, avoiding cellular damage (Khalid et al., 2011). Catalase's implications on bacterial virulence and pathogenicity revealing that catalase positive bacteria gain a competitive edge in avoiding host immune responses by neutralizing reactive oxygen species (Jeyagowri et al., 2015). Conversely, catalase deficient strains produce strong host immune reactions due to reduced oxidative stress management. The bacterial isolates from kale juice that spontaneously fermented could produce invaluable probiotic starter cultures that are used in the fermentation sector (Szutowska et al., 2021). The isolates' indicated lack of significant gas production corresponds with previous findings, suggesting that their gas-producing capacities were limited under specific test conditions. The absence of gas production in response to glucose as a substrate suggests potential metabolic characteristics of the isolates. Research on lettuce leaves' ability to withstand acidity suggests that probiotics might be used to enhance their health. Interestingly, certain Lactobacilli strains showed physiological and genetic adaptations that allow them to survive in acidic environments (Huang and Adams, 2004). Comparably, Hassanzadazar et al. (2012) identified

certain genetic loci connected to acid resistance mechanisms in Lactobacilli, providing evidence for the genetic basis of acid tolerance in these bacteria. Proceeding on to the fermentation investigations, our research offers fascinating results. In the Durham tube, specifically, the fermentation of glucose produced acid rather than gas. Surprisingly, during the fermentation of glucose or maltose, none of the strains produced gas. The strains showed strong fermentation skills, especially when working with glucose. On the other hand, neither glucose nor maltose fermentation resulted in the formation of gas. This is reliable with the results of Khedid et al. (2009), who identified 25 different types of LAB from camel milk, demonstrating the variety of ways in which these bacteria can ferment glucose, lactose, and maltose. The study conducted by Frieri et al. (2017) highlights the significance of ensuring antibiotic sensitivity in probiotic bacteria to ensure their reliable and efficient consumption for better digestive health. The importance for preserving antibiotic sensitivity to reduce the possibility of spreading antibiotic resistance genes was highlighted in a recent analysis that evaluated possible concerns related to the transfer of antibiotic resistance from probiotic strains (Bilal et al., 2021). Continuing to salt tolerance, a study by khusboo et al. (2023) found that both of the lactic acid bacteria isolates, which came from fermented milk, survived at 4% and 6.5% NaCl concentrations, respectively. In a similar way, in the analysis by Khedid et al. (2009), half of LAB isolated from camel milk flourished at 40°C (Khedid et al., 2009). Remarkably, bacteria within the *Bacillus licheniformis* (BAL) group exhibited high halophilicity, showing growth in the presence of around 5-30% salt concentration (Nguyen et al., 2024; Di Martino et al., 2023). Lactic acid bacteria (LAB) genera like *Leuconostoc, Pediococcus,* and *Lactobacillus* thrive under high NaCl concentrations.

However, our strains (L-A, L-C, L-D, and L-E) displayed negligible growth at 10% NaCl after 48 hours, except for L-B, which showed prominent salt tolerance. These exclusive features of LAB strains grasp aptitude for probiotic applications, including bacterial growth substitution and valuable metabolite production. Our study authorizes optimal growth at 37°C, remarkable tolerance to 10% NaCl, and condensed growth at both 10 and 42°C, highlighting their potential as probiotics for industrial and conservation purposes. For actual foundation within the host's gut, probiotics must show resistance not only to bile salts but also to the acidic atmosphere of the stomach. The gastric juice's acidic secretion, with a pH around 2.0, shows a noteworthy challenge, leading to decline of most external microorganisms upon entry into the gut. Hence, the survival of probiotic bacteria in acidic environments (pH 1.0 to pH 3.0) and high bile salt concentrations for at least 90 minutes is impressive (Lim & Im, 2009; Xing et al., 2016). In this research, it was endeavored to estimate the capability of these lettuce-derived probiotic isolates to tolerate acidic conditions. The results indicated that after 24 hours of incubation, the isolates exhibited good conflict to pH levels of 2.0 and pH 3.0. However, it's remarkable that the probiotic count began to deterioration after 24 hours, suggesting that there may be restrictions to their flexibility in continued acquaintance to low pH levels. The current result aligns with the perception that probiotics can endure the stomach's acidity to some range. Further results of bile salt tolerance test are displayed in figure 9. Antimicrobial resistance in lactic acid bacteria (LAB) is a growing concern, impacting both human health and the efficacy of probiotic applications (Zhu et al., 2019). In our study, the five strains isolated from lettuce exhibited variable resistance patterns to antibiotics, emphasizing the need for careful selection and evaluation of LAB strains for probiotic use. Our findings align with previous research that evaluated 43 strains of LAB from Chinese yogurts, revealing widespread resistance to antibiotics such as ampicillin, chloramphenicol, tetracyclines, lincomycin, streptomycin, neomycin, and gentamycin among the strains tested (Huang & Adams, 2004). Specifically, our study's resistance patterns in LAB strains are comparable to the high resistance observed in *Lactobacillus bulgaricus* and *Streptococcus thermophilus* strains to multiple antibiotics, including kanamycin, which all tested strains were resistant to. Additionally, the presence of resistance genes such as tet(M), ant(6), and aph(3')-IIIa in the Chinese yogurt study underscores the potential for these genes to be horizontally transferred, a concern also relevant to our findings Azat et al. (2016).

These insights highlight the importance of understanding LAB's antibiotic resistance suggest for effective probiotic development and safety.16S rDNA patterns are a reliable and economical means to identify probiotic microorganisms, according to FAO/WHO standards (Binda et al., 2020). The present investigation exploited 16S rRNA sequencing to identify isolated organisms. The results verified that L-A corresponded to *Enterococcus faecium*, L-B to *Enterococcus lactis*, L-C to *Enterococcus durans*, L-D to *Lactobacillus paracasei*, and L-E to *Lactobacillus casei*. According to Haghshenas et al. (2017), *Enterococcus faecium* supports digestion and preserves intestinal integrity, both of which are factors in gut health. *Enterococcus lactis* and *Enterococcus durans* strengthen the immune system, improve digestive health, and prevent diarrhea caused on by antibiotics (Goyal et al., 2013). Contributing to a balanced gut microbiota, *Lactobacillus paracasei* enhances immune function, lowers infection risk, and supports digestive health. *Lactobacillus casei* improves lactose digestion, which helps people with lactose

intolerance (Goyal et al., 2013).

CONCLUSION

This study found that probiotic bacteria isolated from fresh lettuce leaves exhibited essential characteristics, including bile and acid tolerance, which are vital for effective gut colonization and pathogen resistance. These traits suggest that all isolates qualify as potential probiotics. Identifying such beneficial microorganisms in lettuce could pave the way for developing probiotics tailored for fruits and vegetables, enhancing food safety and nutrition. This approach may also improve quality control in the food and agriculture sectors, ensuring safer vegetable consumption. Further research in this area could uncover insights into the relationship between probiotics and plant surfaces, fostering advances in food preservation and innovative applications in agriculture and medicine.

ACKNOWLEDGMENT

I would like to extent my appreciation to University of Sialkot for providing the facilities as well as resources required for this study. Special thanks to the members/Supervisor of the Department of Biological Sciences for their support and collaboration.

AUTHOR CONTRIBUTIONS

Muhammad Sufian Masued conducted all aspects of the research, including data collection, and manuscript preparation. The work was carried out under the supervision of Abdul Mateen, Raees Ahmed, Muhammad Jamil and Muhammad Tariq Khan who provided critical guidance and oversight throughout the research process. Lubna Zafar carried out data analysis. Shams Ur Rehman, Muhamamd Musaddiq Shah and Raees Ahmed edited the manuscript and improve English language. All authors have read and approved the final manuscript.

FUNDING

This study was supported by internal funding from the University of Sialkot.

CONFLICTS OF INTEREST

It is declared that the research was conducted without any commercial or financial relationships that could be understandable as a potential conflict of interest. All procedures and experiments were conducted with academic and scientific probity, without any kind of personal, financial, or institutional bias influenced the study outcome.

REFERENCES

Abo-Amer, A. E. (2011). Optimization of bacteriocin production by *Lactobacillus acidophilus* AA11, a strain isolated from Egyptian cheese. *Annals of Microbiology*, *61*(3), 445–452. https://doi.org/10.1007/s13213-010-0157-6

Anjum, F. M., et al. (2023). Optimization of conditions for the production of bacteriocins by Lactobacillus acidophilus. *Journal of Food Science and Technology*, 60(1), 150-159.

Azat, R., Liu, Y., Li, W., Kayir, A., Lin, D. B., Zhou, W. W., & Zheng, X. D. (2016). Probiotic properties of lactic acid bacteria isolated from traditionally fermented Xinjiang cheese. *Journal of Zhejiang University Science B*, *17*(8), 597-609. https://doi.org/10.1631/jzus.B1500250

Bazireh, H., et al. (2020). Probiotic properties of lactic acid bacteria isolated from traditional Iranian dairy products. *Journal of Food Science and Technology*, 57(12), 4233-4243.

Beveridge, T. J. (2001). Mechanism of Gram variability in select bacteria. *Journal of Bacteriology*, 183(16), 4764-4773.

Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M. E., Tremblay, A., & Ouwehand, A. C. (2020). Criteria to qualify microorganisms as "probiotic" in foods and dietary supplements. *Frontiers in Microbiology*, 11, 1662. https://doi.org/10.3389/fmicb.2020.01662

Bommasamudram, J., Kumar, P., Kapur, S., et al. (2023). Development of thermotolerant Lactobacilli cultures with improved probiotic properties using adaptive laboratory evolution method. *Probiotics & Antimicrobial Proteins, 15*(3), 832–843. https://doi.org/10.1007/s12602-023-09789-8

Cappuccino, J. G., & Sherman, N. (2010). Microbiology: A Laboratory Manual (7th ed.). Pearson Education in South Asia.

Corsetti, A., & Settanni, L. (2007). Lactobacilli in sourdough fermentation. Food Research International, 40(5), 539-558.

Cvrtila Fleck, Ž., Savić, V., Kozačinski, L., Njari, B., Zdolec, N., & Filipović, I. (2012). Identification of lactic acid bacteria isolated from dry fermented sausages. Veterinarski arhiv, 82(3), 265-272.

Denkova, Z., Gasharova, N., Denkova, R., & Krastanov, A. (2010). Characteristics of lactic acid bacteria strains isolated from salad dressing. Journal of Food Biotechnology, 24(1), 124-134.

Di Martino, C., et al. (2023). Halophilic lactic acid bacteria: Diversity and applications. Food Research International, 157, 112310.

Di Martino, L., Osme, A., Ghannoum, M., & Cominelli, F. (2023). A novel probiotic combination ameliorates Crohn's disease-like ileitis by increasing short-chain fatty acid production and modulating essential adaptive immune pathways. Inflammatory Bowel Diseases, 2023, izac284. https://doi.org/10.1093/ibd/izac284.

Fijan, S. (2014). Microorganisms with claimed probiotic properties: an overview of recent literature. International Journal of Environmental Research and Public Health, 11(5), 4745-4767. https://doi.org/10.3390/ijerph110504745.

Frieri, M., Kumar, K., & Boutin, A. (2017). Antibiotic

resistance. Journal of infection and public health, 10(4), 369-378.

Gerasimidis, K., Bertz, M., Quince, C., Brunner, K., Bruce, A., & Combet, E., & Ijaz, U. Z. (2016). The effect of DNA extraction methodology on gut microbiota research applications. BMC Research Notes, 9(1), 1-10.

Ghazali, N. S. H., & Rashid, N. (2019). Molecular identification of bacterial communities from vegetable samples as revealed by DNA sequencing of universal primer 16S rRNA gene. International Journal of Medical Sciences, 4(1), 19-26.

Gibson, G. R., & Robust, L. A. (2011). Functional foods: probiotics and prebiotics. Culture, 28(2), 1-7.

Goyal, S., Raj, T., Banerjee, C., Imam, J., & Shukla, P. (2013). Isolation and ecological screening of indigenous probiotic microorganisms from curd and chili sauce samples. International Journal of Probiotics and Prebiotics, 8(2), 91-96.

Guarner, F., & Malagelada, J. R. (2003). Gut flora in health and disease. The Lancet, 361(9356), 512-519.

Guarner, F., & Malagelada, J. R. (2003). Gut flora in health and disease. The Lancet, 361(9356), 512-519.

Haghshenas, B., Nami, Y., Almasi, A., Abdullah, N., Radiah, D., Rosli, R., Barzegari, A., & Khosroushahi, A. Y. (2017). Isolation and characterization of probiotics from dairies. *Iranian Journal of Microbiology*, *9*(4), 234-243. https://doi.org/PMID: 29238459; PMCID: PMC5723976.

Hassanzadazar, H., Ehsani, A., Mardani, K., & Hesari, J. (2012). Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. Veterinary Research Forum, 3(3), 181-185.

Hatami, H., et al. (2022). Diversity and applications of halophilic lactic acid bacteria in food and fermentation industries. Food Biotechnology, 36(4), 241-257.

Haytowitz, D. B., Ahuja, J. K. C., Wu, X., Somanchi, M., Nickle, M., Nguyen, Q. A., & et al. (2019). *USDA National Nutrient Database for Standard Reference, Legacy Release*. Nutrient Data Laboratory, Beltsville Human Nutrition Research Center, ARS, USDA. https://doi.org/10.15482/USDA.ADC/1529216

Hill, C. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology & Hepatology, 11(8), 506-514. https://doi.org/10.1038/nrgastro.2014.66

Hill, C., et al. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology & Hepatology, 11(8), 506-514.

Huang, Y., & Adams, M. C. (2004). In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *International Journal of Food Microbiology*, *91*(3), 253-260.

Huang, Y., & Adams, M. C. (2004). In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. International Journal of Food Microbiology, 91(3), 253-260.

Imperial, I. C. V. J., & Ibana, J. A. (2016). Addressing the antibiotic resistance problem with probiotics: Reducing the risk of its double-edged sword effect. Frontiers in Microbiology, 7, 1983.

Jeyagowri, N., Parahitiyawa, N., Jeyatilake, S., Ranadheera, S., & Madhujith, T. (2015). Study on isolation of potentially probiotic Lactobacillus species from fermented rice.

Kechagia, M., et al. (2013). Health benefits of probiotics: A review. ISRN Nutrition, 2013, 481651.

Kerry, R. G. (2018). Beneficial properties of probiotics. In R. R. Watson & V. R. Preedy (Eds.), Probiotics, prebiotics, and synbiotics (pp. 1-11). Academic Press. https://doi.org/10.1016/B978-0-12-802189-7.00001-1

Khedid, K., et al. (2009). Characterization of lactic acid bacteria isolated from the one-humped camel milk produced in Morocco. Microbiological Research, 164(1), 81-91.

Khushboo, K., Malik, T., et al. (2023). Characterization and selection of probiotic lactic acid bacteria from different dietary sources for development of functional foods. *Frontiers in Microbiology*, *14*, 1170725. https://doi.org/10.3389/fmicb.2023.1170725

Lebeer, S., Vanderleyden, J., & De Keersmaecker, S. C. (2008). Genes and molecules of lactobacilli supporting probiotic action. Microbiology and Molecular Biology Reviews, 72(4), 728-764. https://doi.org/10.1128/MMBR.00017-08

Lim, H. S., & Im, D. S. (2009). Bile salt resistance and acid tolerance of Lactobacillus fermentum strains isolated from kimchi. Korean Journal of Food Science and Technology, 41(2), 141-146.

Marco, M. L. (2017). Health benefits of fermented foods: Microbiota and beyond. Current Opinion in Biotechnology, 44, 94-102. https://doi.org/10.1016/j.copbio.2016.11.010

Martinez, M. I., et al. (2009). Probiotics, gut microbiota, and health. *Médecine et Maladies Infectieuses*, *44*(1), 1-8. https://doi.org/10.1016/j.medmal.2013.10.003

McManus, A. (2020). Nutritional benefits of lettuce. Journal of Nutritional Science, 9, e35. https://doi.org/10.1017/jns.2020.31

Michalak, M., et al. (2018). The isolation and

characteristics of lactic acid bacteria strains from spontaneously fermented kale juice. LWT - Food Science and Technology, 91, 492-500.

Nguyen, P.-T., Huu Thanh, N., Nguyen-Thi, T.-U., Pham, M.-N., & Nguyen, T.-T. (2024). Halophilic lactic acid bacteria — Play a vital role in the fermented food industry. *Folia Microbiologica, 69,* 1-17. https://doi.org/10.1007/s12223-024-01149-0

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., & Rastall, R. A. (2018). Probiotics and prebiotics in intestinal health and disease: From biology to the clinic. Nature Reviews Gastroenterology & Hepatology, 15(10), 600-614. https://doi.org/10.1038/s41575-018-0060-6

Shafiq, M., Bilal, H., Permana, B., Xu, D., Cai, G., Li, X., ... & Yao, F. (2023). Characterization of antibiotic resistance genes and mobile elements in extended-spectrum β-lactamase-producing Escherichia coli strains isolated from hospitalized patients in Guangdong, China. Journal of Applied Microbiology, 134(7), lxad125.

Szutowska J, Gwiazdowska D. Probiotic potential of lactic acid bacteria obtained from fermented curly kale juice. Arch Microbiol. (2021) Apr;203(3):975-988. doi: 10.1007/s00203-020-02095-4. Epub 2020 Oct 26. Erratum in: Arch Microbiol. 2021 Aug;203(6):3737. doi: 10.1007/s00203-021-02340-4. PMID: 33104821; PMCID: PMC7965858.

Szutowska, J., & Gwiazdowska, D. (2021). Probiotic potential of lactic acid bacteria obtained from fermented curly kale juice. *Archives of Microbiology*, *203*(3), 975–988. https://doi.org/10.1007/s00203-020-02095-4

Yang, Xiao & Gil, María & Yang, Qichang & Tomás-Barberán, Francisco. (2021). Bioactive compounds in lettuce: Highlighting the benefits to human health and impacts of preharvest and postharvest practices. Comprehensive Reviews in Food Science and Food Safety. 21. 10.1111/1541-4337.12877.

Yu, J., et al. (2018). Antioxidant properties of lactic acid bacteria from fermented vegetables. Food Science and Biotechnology, 27(2), 359-365.

Zhou, N., Zhang, J. X., Fan, M. T., Wang, J., Guo, G., & Wei, X. Y. (2012). Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts. Journal of Dairy Science, 95(9), 4775-4783. https://doi.org/10.3168/jds.2011-5267.