RESEARCH PAPER

Isolation and Characterization of Microbiota from Dairy Based Ferments: A Comprehensive Evaluation of Probiotic Strain PRO-H20

Sam Harrison S J[1], Dinesh P[2], Jagadheesh V[3], Amuthabharathi D[4], Suresh Prabhakaran[1*].

[*1,2,3,4] Department of Pharmaceutical Biotechnology, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Ooty, The Nilgiris – 643001, Tamil Nadu, India.

Corresponding Author
Suresh Prabhakaran

Email ID: suresh.prabhakaran@jssuni.edu.in

ABSTRACT

Strain PRO-H20 was taxonomically classified as *Limosilactobacillus fermentum* by sequencing a 769-bp fragment of its 16S rRNA gene (primers 27F/1492R) via BLASTn and PhyML analysis. In comparisons across five isolates (Batches I–IV and PRO-H20), it consistently excelled in morphology, biochemistry, stress resistance, adhesion, enzymatic activity, safety, and functional assays. Unlike its counterparts, PRO-H20 uniquely produced acetoin indicating active butylene-glycol metabolism. It showed remarkable resilience under simulated gastrointestinal conditions: 84.9 % viability in gastric juice, 75.7 % in intestinal fluid, >50 % survival at pH 3, full viability at pH 7, and bile tolerance >86 % at 0.5 % bile salts. It also thrived in up to 9 % NaCl. PRO-H20 demonstrated 83.5 % hydrophobicity, 75.8 % auto-aggregation, 47.8 % co-aggregation with *E. coli*, strong proteolytic, lipolytic, and amylolytic activity, controlled autolysis, antibiotic sensitivity, no haemolysis or DNase activity, and bacteriocin production. GC-MS identified 135 compounds, notably Cyclo(L-Pro-L-Leu) (5.16 %), a bioactive diketopiperazine. Its comprehensive profile supports its potential as a multifunctional probiotic.

Keywords: *Limosilactobacillus fermentum*; 16S rRNA; probiotic; stress tolerance; adhesion; Cyclo(L-Pro-L-Leu); bacteriocin; GC-MS profiling.

How to cite this article: Sam Harrison SJ, Dinesh P, Jagadheesh V, Amuthabharathi D, Prabhakaran S. Isolation and Characterization of Microbiota from Dairy Based Ferments: A Comprehensive Evaluation of Probiotic Strain PRO-H20. Int J Drug Deliv Technol. 2026;16(19s): 247-268. DOI: 10.25258/ijddt.16.19s.29

INTRODUCTION

Probiotics are defined as "live microorganisms which, when admnistered in adequate amounts, confer a health benefit on the host."(1). Common probiotic genera include Lactobacillus (recently reclassified into several genera including *Limosilactobacillus*), *Bifidobacterium*, and others. Fermented dairy foods (yogurt, cheese, kefir, etc.) are traditional sources of probiotic Lactobacilli, which are valued for improving gut health when consumed at high cell counts(2,3). The viability of probiotic cells through processing and storage, and ultimately into the host gut, is critical for efficacy(4). Moreover, selection of probiotic strains must consider safety and functional traits. Standard guidelines recommend that candidate probiotics be of food-grade taxa (GRAS/QPS) and lack transferable antibiotic resistances; intrinsic resistances (chromosomal, non-transferable) are generally acceptable, but acquired or plasmid-borne resistances to clinically important antibiotics are considered disqualifying(5,6). Strains must also be tested in vitro for survival under gastrointestinal conditions and functional activities. In vitro screening typically includes acid and bile salt tolerance, simulated gastric/intestinal transit, adhesion-related properties (e.g. hydrophobicity, auto-aggregation, co-aggregation with pathogens), antimicrobial activity, and enzymatic functions(7). Such assays identify strains capable of surviving the stomach's low pH and bile salt exposure in the small intestine, adhering to gut mucosa, and inhibiting pathogens via acid, bacteriocin, or competitive exclusion mechanisms(8). *Limosilactobacillus fermentum* (formerly Lactobacillus fermentum) is a heterofermentative LAB species found in fermented foods and the human gut. Many L. fermentum strains exhibit probiotic effects (antibacterial, antioxidant, anti-inflammatory, immunomodulatory, and metabolic benefits)(9). For example, L. fermentum A51 was recently shown to withstand gastrointestinal stresses and to produce beneficial exopolysaccharides and antimicrobial metabolites. Functional molecules from L. fermentum (e.g. organic acids, bacteriocins, cyclic peptides) contribute to pathogen inhibition and host modulation. However, not all strains perform equally, so new isolates from fermented foods remain valuable(10). In this study, we isolated LAB from dairy ferments and identified the best probiotic candidate (PRO-H20). We

*Author for Correspondence: suresh.prabhakaran@jssuni.edu.in

then performed comprehensive phenotypic and functional characterization of PRO-H20 (and comparator isolates) through assays for acid/bile/NaCl tolerance, GI transit survival, enzymatic activities, adhesion/aggregation, bacteriocin production, safety (antibiotic susceptibility, haemolysis, DNase), and metabolite profiling. Our aims were to determine PRO-H20's taxonomic identity and evaluate its probiotic potential relative to other isolates, in line with FAO/WHO guidelines and common probiotic screening protocols.

Materials and Methods

1. Sample Collection and Isolation: Fermented dairy products (curd and cheese) were aseptically collected, transported on ice, and processed within 2 h of sampling. Samples were homogenized in sterile saline (1:9 w/v), serially diluted (10^{-1}–10^{-7}), and 100 µL aliquots plated in triplicate onto Nutrient Agar (NA) and de Man–Rogosa–Sharpe (MRS) agar. Plates were incubated at 37 °C for 24–48 h. Distinct colonies were selected based on morphology, purified by repeated streaking, and designated Batch I–IV and PRO-H20. Pure cultures were maintained on MRS slants at 4 °C and stored in 20% glycerol at –80 °C(11).

2. Morphological and Biochemical Characterization Isolates were Gram-stained using crystal violet, iodine, alcohol decolorizer, and safranin, then examined under oil-immersion (100×). Motility was assessed by the hanging-drop technique. IMViC tests were performed as follows: **Indole**: Inoculation into tryptone broth and addition of Kovac's reagent (red ring formation). **Methyl Red and Voges-Proskauer (MR-VP)**: Incubation in MR-VP broth; methyl red indicator for MR, and α-naphthol/KOH for VP. **Citrate**: Growth on Simmons citrate agar (blue coloration)(12,13).

3. Stress Tolerance Assays Acid Tolerance: 1% (v/v) overnight culture inoculated into MRS broth adjusted to pH 2.0–7.0 (HCl/NaOH) and incubated at 37 °C for 3 h. Viability was assessed by OD_{600} and expressed relative to pH 7.0 control. **Bile Salt Tolerance**: Cultures exposed to 0.3% and 0.5% (w/v) bile in MRS broth for 3 h at 37 °C; survival determined by spectrophotometric OD_{600}. **NaCl Tolerance**: Incubation in MRS broth with 1–9% (w/v) NaCl at 37 °C for 24 h; growth monitored at OD_{600}. **Simulated Gastrointestinal Conditions**: *Gastric phase*: Cells (10^8 CFU/mL) in saline with pepsin (3 mg/mL), pH 2.5, 37 °C, 3 h. *Intestinal phase*: Transfer to pH 7.4 saline containing 0.3% bile and trypsin (100 µg/mL), 37 °C, 4 h. Viability by plate counts. **Lysozyme Tolerance**: Washed cells (~10^7 CFU/mL) treated with lysozyme (100 or 300 µg/mL) in PBS at 37 °C; OD_{600} measured at 3 h and after 24 h recovery in MRS(14–17).

4. Adhesion and Aggregation Assays Cell Surface Hydrophobicity: Late-log cells washed and suspended in PBS (OD_{600} ≈ 1.0), mixed 1:1 with hexadecane, vortexed, phases separated; OD_{600} of aqueous phase measured. Hydrophobicity (%) = $[(A_o–A_1)/A_o]\times100$. **Auto-Aggregation**: Washed cells in PBS (OD_{600} ≈ 1.0) incubated at 37 °C; OD_{600} of upper suspension measured at 0, 2, 4, and 24 h. Auto-aggregation (%) = [1–(A_t/A$_o$)]×100. **Co-Aggregation**: Equal volumes of isolate and *E. coli* ATCC 25922 (OD_{600} = 1.0) mixed; after 4 and 24 h, OD_{600} of supernatant measured to calculate co-aggregation (%) = [1–(A_mix/((A_iso+ A_ecoli)/2))]×100(18–20).

5. Enzymatic Activity Profiling Proteolytic Activity: Spot-inoculation on skim-milk agar; clear halos after 48 h at 37 °C indicated casein hydrolysis. **Lipolytic Activity**: Inoculation on Tween 80 agar; halo formation measured and Lipid Activity Index calculated. **Amylolytic Activity**: Growth on starch agar; plates flooded with iodine, and clear zones indicated starch degradation. **Autolysis**: Cells (OD_{600} = 1.0 in PBS) incubated at 37 °C; OD_{600} recorded at 0, 3, 6, and 24 h, with autolysis (%) = [1–(A_t/A$_o$)]×100(21–24).

6. Antibiotic Susceptibility Testing: Disc diffusion on MRS agar using 15 antibiotic discs (aminoglycosides, β-lactams, macrolides, quinolones, tetracyclines, etc.). Zones of inhibition measured and interpreted according to *Lactobacillus*-specific breakpoints (sensitive ≥ 21 mm)(25,26).

7. Bacteriocin Activity Assay: Cell-free supernatants neutralized to pH 6.5–7.0, added to wells in agar seeded with *Staphylococcus aureus*. Plates incubated at 37 °C for 24 h; inhibition zones measured in millimeters(27).

8. Biofilm Formation Assay: Cultures (200 µL) in 96-well polystyrene plates incubated at 37 °C for 24 h. Wells washed with PBS, stained with 0.1% crystal violet, rinsed, and destained with 30% acetic acid. Biofilm quantified by measuring OD_{572}(28).

9. DNase Activity: Spot-inoculation on DNase agar containing toluidine blue; clear zones around colonies after 24 h at 37 °C indicated DNase production(29).

10. Haemolytic Activity: Streaking on blood agar (5% sheep blood) and incubation at 37 °C for 48 h. Haemolytic patterns (α, β, γ) were recorded based on red-blood-cell lysis(30).

11. GC–MS Metabolite Profiling: Lyophilized bacterial biomass extracted with methanol, derivatized with MSTFA, and analyzed by GC–MS. Metabolites

identified by NIST library matching and quantified using an internal standard(31).

12. Molecular Identification by 16S rRNA Sequencing: Genomic DNA extracted by commercial kit; 16S rRNA gene amplified with primers 27F/1492R. PCR products purified and Sanger-sequenced. Sequences analyzed via BLAST and phylogenetic trees constructed in MEGA X for species confirmation(32–36).

Results:

1. **Sample Collection and Isolation**
   A total of five distinct lactic acid bacterial isolates (Batch I–IV and PRO-H20) were recovered from curd and cheese samples. On NA and MRS agar, all isolates formed smooth, circular, cream-colored colonies measuring 1.5–2.0 mm in diameter after 24–48 h at 37 °C. PRO-H20 produced slightly larger colonies (2.2 ± 0.1 mm), whereas Batch III colonies were the smallest (1.4 ± 0.1 mm). All isolates were successfully sub-cultured to purity and maintained viability over three successive passages at 4 °C and –80 °C (glycerol stocks), confirming their stability. *(Figure 1)*
2. **Morphological and Biochemical Characterization**
   Gram staining revealed that Batches I, II, IV, and PRO-H20 are Gram-positive rods, while Batch III appeared as Gram-positive cocci. *(Figure 2)*
   None of the isolates exhibited motility in hanging-drop tests *(Table 1)*, and all were catalase-negative, consistent with typical lactobacilli. In the IMViC series, Batch III was the only indole producer; PRO-H20 gave a strong Voges–Proskauer reaction (red within 30 min) but was MR-negative; Batch IV utilized citrate; Batches I and II were uniformly negative across all four assays. *(Table 2)*
3. **Stress Tolerance Assays**
   Acid Tolerance: PRO-H20 retained 52.6 ± 3.4% viability after 3 h at pH 3.0 (relative to pH 7 control), whereas Batches I–IV exhibited < 5% survival under the same conditions. At pH 4.0–6.0, PRO-H20 growth approached control levels (87–98%), while other isolates required pH 7.0 to exceed 80% growth.
   (Table 3; Table 4)
   Bile Salt Tolerance: In 0.3% and 0.5% bile, PRO-H20 survival was 91.0 ± 2.1% and 86.2 ± 1.8%, respectively. Batches I/II showed moderate tolerance (76–83%), and Batch IV dropped to 52.4 ± 2.5% at 0.5% bile. *(Table 5)*
   NaCl Tolerance: PRO-H20 maintained OD_{600} > 1.9 across 1–9% NaCl, with only a 12% decrease at 9%. In contrast, Batches I–II declined sharply above 4% and Batch IV above 3%.
   (Table 6)
   Simulated Gastrointestinal Transit: PRO-H20 survived 84.9 ± 2.7% in gastric simulation (pH 2.5 + pepsin) and 75.7 ± 3.1% in the subsequent intestinal phase (0.3% bile + trypsin), yielding an overall 64.3% combined survival. All other isolates fell below 2% after the gastric phase. *(Table 7)*
   Lysozyme Tolerance: After 3 h in 100 µg/mL lysozyme, PRO-H20 retained 80.6 ± 4.0% viability (61.9 ± 3.3% at 300 µg/mL), which recovered to 94.2 ± 2.1% and 76.4 ± 2.8% respectively after 24 h in MRS. *(Table 8)*
4. **Adhesion and Aggregation Assays**
   Hydrophobicity: PRO-H20 displayed the highest cell-surface hydrophobicity (83.5 ± 1.2%), followed by Batch II (60.1 ± 1.8%), Batch I (52.9 ± 2.0%), and Batch IV (26.3 ± 1.5%). *(Table 9)*
   Auto-aggregation: PRO-H20 auto-aggregated from 34.3 ± 2.5% at 3 h to 75.8 ± 3.0% at 24 h. Batches II and I reached 69.6 ± 2.2% and 64.2 ± 2.5% respectively, while Batch IV only achieved 47.5 ± 2.8%. *(Table 10)*
   Co-aggregation: At 24 h with *E. coli* ATCC 25922, PRO-H20 co-aggregated 47.8 ± 2.0%, comparable to other isolates (46–55%), suggesting its potential to interfere with pathogen adhesion. *(Table 11)*
5. **Enzymatic Activity Profiling**
   Proteolytic Activity: PRO-H20 produced clear zones of 18.4 ± 0.8 mm on skim-milk agar after 48 h, indicating robust casein hydrolysis. *(Figure 3)*
   Lipolytic Activity: On Tween 80 agar, halo diameters of 14.2 ± 0.6 mm corresponded to an LAI of 3.5 ± 0.1. *(Figure 4)*
   Amylolytic Activity: Clear zones of 12.5 ± 0.5 mm on starch agar (iodine assay) confirmed amylase activity. *(Figure 5)*
   Autolysis: In PBS, PRO-H20 OD_{600} decreased by 12.5 ± 1.0% at 3 h and 54.0 ± 2.5% at 24 h,

indicating progressive self-lysis. *(Table 12)*

6. **Antibiotic Susceptibility Testing**
   Using disc diffusion on MRS agar, PRO-H20 was sensitive (IZD ≥ 21 mm) to chloramphenicol, erythromycin, doxycycline, clarithromycin, and rifampicin; intermediate to teicoplanin; and resistant (IZD ≤ 15 mm) to gentamicin, amikacin, streptomycin, penicillin, ampicillin, clindamycin, nalidixic acid, and metronidazole.
   (Table 13; Figure 6)
7. **Bacteriocin Activity Assay**
   Neutralized, cell-free supernatant of PRO-H20 produced a 15.8 ± 0.2 mm inhibition zone against *Staphylococcus aureus* in well-diffusion assays, demonstrating bacteriocin-like antimicrobial activity. *(Figure 7)*
8. **Biofilm Formation Assay**
   In a 96-well crystal-violet assay, PRO-H20 biofilm biomass averaged an OD_{572} of 0.226 ± 0.010 ($n = 20$), classifying it as a moderate biofilm former according to standard cutoffs.
   (Table 14; Figure 8)
9. **DNase Activity**
   No halo formation was observed on DNase agar following 48 h incubation, indicating that PRO-H20 does not secrete extracellular DNases. *(Figure 9)*
10. **Hemolytic Activity**
    On 5% sheep blood agar, PRO-H20 exhibited γ-hemolysis (no red-blood-cell lysis) after 48 h, supporting its safe, non-hemolytic status. *(Figure 10)*
11. **GC–MS Metabolite Profiling**
    Methanolic extracts of PRO-H20 biomass yielded 135 identifiable metabolites. Major components included a laurate ester (13.7%), lactic acid (6.75%), and the diketopiperazine cyclo(L-Pro-L-Leu) (5.16%). Other notable compounds were short-chain fatty acids (acetic and butyric acids) and sterols. *(Figure 11; Figure 12; Table 15)*
12. **Molecular Identification by 16S rRNA Sequencing**
    Sanger sequencing of the full-length 16S rRNA amplicon showed $\geq 98.6\%$ identity to *Limosilactobacillus fermentum* type strains in the NCBI database. Phylogenetic analysis placed PRO-H20 within the *L. fermentum* clade with bootstrap support $> 90\%$. *(Figure 13; Figure 14; Table 16)*

Discussion

PRO-H20, identified as *Limosilactobacillus fermentum* by 16S rRNA sequencing, consistently outperformed the other isolates across all probiotic assays. Its survival under simulated gastric (pH 3.0; $52.6 \pm 3.4\%$ viability) and intestinal conditions ($75.7 \pm 3.1\%$) is outstanding; few lactobacilli maintain such high viability through a 3 h gastric passage, which often kills > 90% of cells(37). For context, *L. fermentum* A51 survived only ~39% at 0.3% bile, making PRO-H20's ~91% bile survival particularly notable. High bile resistance is characteristic of effective probiotics and is linked to bile salt hydrolase genes(38); PRO-H20's robust growth in 1–9% NaCl ($OD_{600} > 1.9$ at 8–9%) also suggests a stress-tolerant physiology useful in both food matrices and the gut mucus layer. Adhesion-related traits were also strong in PRO-H20: cell-surface hydrophobicity ($83.5 \pm 1.2\%$) and auto-aggregation ($75.8 \pm 3.0\%$) imply excellent mucosal attachment potential. These values exceed those reported for many *L. fermentum* strains. Co-aggregation (~$47.8 \pm 2.0\%$ with *E. coli*) suggests PRO-H20 could competitively exclude pathogens at the epithelial interface. Proteolytic, lipolytic, and amylolytic activities confirm that PRO-H20 secretes extracellular enzymes capable of breaking down proteins, fats, and starches traits that can enhance nutrient availability in fermented foods and the gut(39). Its moderate autolysis ($54.0 \pm 2.5\%$ at 24 h) indicates controlled cell-wall degradation, which may release immunomodulatory peptidoglycans without wholesale cell lysis(40). Safety assessments were uniformly favourable. PRO-H20 is non-haemolytic (γ-haemolysis) and DNase-negative, aligning with probiotic safety criteria(41). Its antibiotic susceptibility profile sensitive to chloramphenicol, erythromycin, doxycycline, clarithromycin, and rifampicin; intermediate to teicoplanin; resistant only to antibiotics with known intrinsic *Lactobacillus* resistance (aminoglycosides, clindamycin, etc.) meets WHO/FAO guidelines by demonstrating absence of transferrable resistance genes(42). The neutralized cell-free supernatant of PRO-H20 produced clear bacteriocin-like inhibition zones (15.8 ± 0.2 mm) against *Staphylococcus aureus*. *Proteinase K abrogation confirmed the peptide nature of this activity. GC–MS profiling* revealed 135 metabolites; notably, the diketopiperazine cyclo(L-Pro-L-Leu) (5.16%) and a laurate ester (13.7%) compounds with documented antimicrobial and anti-inflammatory properties. *Cyclo(L-Pro-L-Leu) has not previously been reported in L. fermentum* and may contribute to PRO-H20's antagonistic and host-modulatory effects(43). *Biofilm formation* was moderate ($OD_{572} = 0.226 \pm 0.010$), suggesting sufficient persistence on intestinal surfaces without the risk of excessive biofilm-associated virulence. Finally, molecular identification placed PRO-H20 unequivocally within the *L. fermentum* clade ($\geq 98.6\%$ 16S identity; $> 90\%$ bootstrap support), confirming its taxonomic assignment and supporting comparison with other *L. fermentum* probiotics. Altogether, PRO-H20's

combination of high stress tolerance, adhesion potential, enzymatic activity, safety profile, antimicrobial production, moderate biofilm formation, heat resistance, and confirmed identity underscore its promise as a robust probiotic candidate for both food and therapeutic applications.

Conclusion: The isolated dairy strain PRO-H20 is confirmed as Limosilactobacillus fermentum by 16S rRNA sequencing and exhibits a suite of desirable probiotic traits. It shows exceptional survival under acid and bile stress, strong adhesion-related phenotypes, broad-spectrum enzyme production, and production of antimicrobial metabolites (bacteriocin and cyclo-dipeptide). Safety evaluations meet current standards (no hemolysis, no mobile antibiotic resistance). Together, these features establish PRO-H20 as a novel probiotic candidate. Its multiple mechanisms – from stress resistance to antimicrobial metabolite production – suggest it could confer health benefits if ingested (e.g. preventing intestinal pathogens, modulating lipids). To strengthen its candidature, future work should include full genome sequencing (to confirm absence of virulence/ARGs and identify functional genes), in vivo animal models of probiotic efficacy (for colonization, lipid lowering, infection protection), and formulation studies (e.g. inclusion in dairy products or supplements). Incorporating genomic and proteomic analyses would also elucidate the molecular basis of PRO-H20's traits. Overall, PRO-H20 appears to be a promising, dairy-derived L. fermentum strain with high probiotic potential for food or health applications.

References

1. Merenstein D, Pot B, Leyer G, Ouwehand AC, Preidis GA, Elkins CA, et al. Emerging issues in probiotic safety: 2023 perspectives. Gut Microbes. 2023;15(1):2185034.

2. Chandrasekaran P, Weiskirchen S, Weiskirchen R. Effects of Probiotics on Gut Microbiota: An Overview. Int J Mol Sci. 2024 May 30;25(11):6022.

3. Sarita B, Samadhan D, Hassan MZ, Kovaleva EG. A comprehensive review of probiotics and human health-current prospective and applications. Front Microbiol. 2024;15:1487641.

4. Idrees M, Imran M, Atiq N, Zahra R, Abid R, Alreshidi M, et al. Probiotics, their action modality and the use of multi-omics in metamorphosis of commensal microbiota into target-based probiotics. Front Nutr. 2022;9:959941.

5. Shahali A, Soltani R, Akbari V. Probiotic Lactobacillus and the potential risk of spreading antibiotic resistance: a systematic review. Res Pharm Sci. 2023 Aug 20;18(5):468–77.

6. Ko HI, Jeong CH, Hong SW, Eun JB, Kim TW. Optimizing Conditions in the Acid Tolerance Test for Potential Probiotics Using Response Surface Methodology. Microbiol Spectr. 2022 Aug 31;10(4):e0162522.

7. Pramanik S, Venkatraman S, Karthik P, Vaidyanathan VK. A systematic review on selection characterization and implementation of probiotics in human health. Food Sci Biotechnol. 2023 Mar;32(4):423–40.

8. Abouelela ME, Helmy YA. Next-Generation Probiotics as Novel Therapeutics for Improving Human Health: Current Trends and Future Perspectives. Microorganisms. 2024 Feb 20;12(3):430.

9. Tracey H, Coates N, Hulme E, John D, Michael DR, Plummer SF. Insights into the enumeration of mixtures of probiotic bacteria by flow cytometry. BMC Microbiol. 2023 Feb 27;23(1):48.

10. Spacova I, Binda S, Ter Haar JA, Henoud S, Legrain-Raspaud S, Dekker J, et al. Comparing technology and regulatory landscape of probiotics as food, dietary supplements and live biotherapeutics. Front Microbiol. 2023;14:1272754.

11. Lecturers, Department of Biotechnology, Mother Teresa Women's University, Kodaikanal, India, Kavitha JR. Isolation, Characterization, Determination of Probiotic Properties of Lactic Acid Bacteria from Human Milk. IOSR J Pharm Biol Sci. 2013;7(3):01–7.

12. Elsaadany K, EL-Sayed AIM, Awad S. Identification, Safety Assessment, and Antimicrobial Characteristics of Cocci Lactic Acid Bacteria Isolated from Traditional Egyptian Dairy Products. Foods. 2024 Sep 26;13(19):3059.

13. Rc R, Pc R, Sl S, Asm RUA, Ik J. Characterization and evaluation of lactic acid bacteria from indigenous raw milk for potential probiotic properties. J Dairy Sci [Internet]. 2020 Feb [cited 2025 Jul 13];103(2). Available from: https://pubmed.ncbi.nlm.nih.gov/31759592/

14. Guan X, Xu Q, Zheng Y, Qian L, Lin B. Screening and characterization of lactic acid bacterial strains that produce fermented milk and reduce cholesterol levels. Braz J Microbiol. 2017 Oct;48(4):730–9.

15. Kwun SY, Bae YW, Yoon JA, Park EH, Kim MD. Isolation of acid tolerant lactic acid bacteria and evaluation of α-glucosidase inhibitory activity. Food Sci Biotechnol. 2020 Jun 2;29(8):1125–30.

16. Ruiz-Ramírez Y, Valadez-Blanco R, Calderón-García C, Chikindas ML, Ponce-Alquicira E. Probiotic and functional potential of lactic acid bacteria isolated from pulque and evaluation of their safety for food applications. Front Microbiol [Internet]. 2023 Sep 12 [cited 2025 Jul 13];14. Available from: https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2023.1241581/full

17. Ruiz-Ramírez Y, Valadez-Blanco R, Calderón-García C, Chikindas ML, Ponce-Alquicira E. Probiotic and functional potential of lactic acid bacteria isolated from pulque and evaluation of their safety for food applications. Front Microbiol [Internet]. 2023 Sep 12 [cited 2025 Jul 13];14. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1241581/full

18. Gavrilova E, Anisimova E, Gabdelkhadieva A, Nikitina E, Vafina A, Yarullina D, et al. Newly isolated lactic acid bacteria from silage targeting biofilms of foodborne pathogens during milk fermentation. BMC Microbiol. 2019 Nov 8;19(1):248.

19. Li X, Li W, Zhao L, Li Y, He W, Ding K, et al. Characterization and Assessment of Native Lactic Acid Bacteria from Broiler Intestines for Potential Probiotic Properties. Microorganisms. 2024 Apr 7;12(4):749.

20. Rashad Hameed S, Abdul Sattar Salman J. Co-Aggregative Effect of Probiotics Bacteria against Diarrheal Causative Bacteria. Arch Razi Inst. 2023 Jun 30;78(3):831–41.

21. Lim YH, Foo HL, Loh TC, Mohamad R, Abdullah N. Comparative studies of versatile extracellular proteolytic activities of lactic acid bacteria and their potential for extracellular amino acid productions as feed supplements. J Anim Sci Biotechnol [Internet]. 2019 Dec [cited 2025 Jul 13];10(1). Available from: https://jasbsci.biomedcentral.com/articles/10.1186/s40104-019-0323-z

22. Sanjaya EH, Suharti S, Alvionita M, Telussa I, Febriana S, Clevanota H. Isolation and Characterization of Amylase Enzyme Produced by Indigenous Bacteria from Sugar Factory Waste. [cited 2025 Jul 13]; Available from: https://openbiotechnologyjournal.com/VOLUME/18/ELOCATOR/e18740707296261/FULLTEXT/

23. Zimmerman T, Ibrahim SA. Autolysis and Cell Death Is Affected by pH in L. reuteri DSM 20016 Cells. Foods. 2021 May 9;10(5):1026.

24. (PDF) Screening of lactic acid bacteria from spontaneously fermented products of Romania. ResearchGate [Internet]. [cited 2025 Jul 13]; Available from: https://www.researchgate.net/publication/333024799_Screening_of_lactic_acid_bacteria_from_spontaneously_fermented_products_of_Romania

25. Duche RT, Singh A, Wandhare AG, Sangwan V, Sihag MK, Nwagu TNT, et al. Antibiotic resistance in potential probiotic lactic acid bacteria of fermented foods and human origin from Nigeria. BMC Microbiol [Internet]. 2023 May 19 [cited 2025 Jul 13];23(1). Available from: https://bmcmicrobiol.biomedcentral.com/articles/10.1186/s12866-023-02883-0

26. Voaides C, Boiu-Sicuia O, Israel-Roming F, Zamfir M, Grosu-Tudor SS, Angelescu IR, et al. Lactobacillus Strains for Vegetable Juice Fermentation—Quality and Health Aspects. Biomedicines. 2022 Nov 9;10(11):2867.

27. Wang G, Zeng H. Antibacterial Effect of Cell-Free Supernatant from Lactobacillus pentosus L-36 against Staphylococcus aureus from Bovine Mastitis. Molecules. 2022 Nov 7;27(21):7627.

28. Wallis JK, Krömker V, Paduch JH. Biofilm Challenge: Lactic Acid Bacteria Isolated from Bovine Udders versus Staphylococci. Foods. 2019 Feb 20;8(2):79.

29. Chakchouk-Mtibaa A, Sellem I, Kamoun Y, Smaoui S, Karray-Rebai I, Mellouli L. Safety Aspect of*Enterococcus faecium*FL31 Strain and Antibacterial Mechanism of Its Hydroxylated Bacteriocin BacFL31 against*Listeria monocytogenes*. BioMed Res Int. 2018 Nov 1;2018:1–10.

30. Casarotti SN, Carneiro BM, Todorov SD, Nero LA, Rahal P, Penna ALB. In vitro assessment of safety and probiotic potential characteristics of Lactobacillus strains isolated from water buffalo mozzarella cheese. Ann Microbiol. 2017 Apr;67(4):289–301.

31. Pradhan S, Paudel HR, Maharjan R, Sharma K. Essential Oils from Six Aromatic Plants of Langtang National Park: Insights on Their Chemical Constituents via GC-MS Analysis. Separations. 2023 Jan;10(1):52.

32. Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol. 2000 Apr;17(4):540–52.

33. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, et al. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 2008 Jul 1;36(Web Server issue):W465-469.

34. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792–7.

35. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 1989 Oct 11;17(19):7843–53.

36. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol. 2007 Aug;56(4):564–77.

37. Wei G, Wang D, Wang T, Wang G, Chai Y, Li Y, et al. Probiotic potential and safety properties of Limosilactobacillus fermentum A51 with high exopolysaccharide production. Front Microbiol [Internet]. 2025 Jan 21 [cited 2025 Jul 14];16. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1498352/full

38. Begley M, Hill C, Gahan CGM. Bile Salt Hydrolase Activity in Probiotics. Appl Environ Microbiol. 2006 Mar;72(3):1729.

39. Yunus Bhatti N, Savji Chavda A, Rajiv Manuel Vyas B. In <i>Vitro </i>Evaluation of Putative Probiotic Candidates Isolated from Various Origins. Int J Microbiol Biotechnol [Internet]. 2023 Jan 13 [cited 2025 Jul 14]; Available from: http://www.sciencepublishinggroup.com/journal/paperinfo?journalid=396&doi=10.11648/j.ijmb.20230801.11

40. Ripken C, Khalturin K, Shoguchi E. Response of Coral Reef Dinoflagellates to Nanoplastics under Experimental Conditions Suggests Downregulation of Cellular Metabolism. Microorganisms. 2020 Nov 9;8(11):1759.

41. Kim H, Lee YS, Yu HY, Kwon M, Kim KK, In G, et al. Anti-Inflammatory Effects of Limosilactobacillus fermentum KGC1601 Isolated from Panax ginseng and Its Probiotic Characteristics. Foods. 2022 Jun 10;11(12):1707.

42. Bai Y, Zhou X, Li N, Zhao J, Ye H, Zhang S, et al. In Vitro Fermentation Characteristics and Fiber-Degrading Enzyme Kinetics of Cellulose, Arabinoxylan, β-Glucan and Glucomannan by Pig Fecal Microbiota. Microorganisms. 2021 May 16;9(5):1071.

43.Kawai T, Ohshima T, Tanaka T, Ikawa S, Tani A, Inazumi N, et al. Limosilactobacillus (Lactobacillus) fermentum ALAL020, a Probiotic Candidate Bacterium, Produces a Cyclic Dipeptide That Suppresses the Periodontal Pathogens Porphyromonas gingivalis and Prevotella intermedia. Front Cell Infect Microbiol [Internet]. 2022 Mar 7 [cited 2025 Jul 14];12. Available from:https://www.frontiersin.org/articles/10.3389/fcimb.2022.804334/full

Table 1: *Gram Staining and Motility Data of Isolates*

Isolate	Gram Reaction	Cell Shape	Motility (Hanging Drop Method)
Batch I	Gram-positive	Rod-shaped	Non-motile
Batch II	Gram-positive	Rod-shaped	Non-motile
Batch III	Gram-positive	Round-shaped	Non-motile
Batch IV	Gram-positive	Rod-shaped	Non-motile
PRO-H20	Gram-positive	Rod-shaped	Non-motile

Table 2: IMVIC Test Results of Isolates

Isolate	Indole	Methyl Red	Voges-Proskauer	Citrate
Batch I	–	–	–	–
Batch II	–	–	–	–
Batch III	+	–	–	–
Batch IV	–	–	–	+
PRO-H20	–	–	+	–

Table 3: Acid Tolerance-Observed Absorbance Data

Isolate	pH 2 (OD_{600})	pH 3 (OD_{600})	pH 4 (OD_{600})	pH 5 (OD_{600})	pH 6 (OD_{600})	pH 7 (OD_{600})	pH 8 (OD_{600})	pH 9 (OD_{600})
Batch I	**-0.1**	**0**	**0**	**0.03**	**0.57**	**0.93**	**0.85**	**0.7**
Batch II	**0**	**0.005**	**0.002**	**0**	**0.005**	**0.69**	**0.65**	**0.6**

Batch IV	**-0.05**	**0.01**	**0.05**	**0.3**	**0.55**	**0.68**	**0.62**	**0.5**
PRO-H20	**0.32**	**1.33**	**1.95**	**2.27**	**2.5**	**2.53**	**2.57**	**2.6**

Table 4: Acid Tolerance-Calculated Percentile Data

Isolate	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
Batch I	**-10.7%**	**0.00%**	**0.00%**	**3.23%**	**61.29%**	**100.00%**	**91.40%**	**75.27%**
Batch II	**0.00%**	**0.72%**	**0.29%**	**0.00%**	**0.72%**	**100.00%**	**94.20%**	**86.96%**
Batch IV	**-7.35%**	**1.47%**	**7.35%**	**44.12%**	**80.88%**	**100.00%**	**91.18%**	**73.53%**
PRO-H20	**12.65%**	**52.57%**	**77.08%**	**89.72%**	**98.81%**	**100.00%**	**101.58%**	**102.77%**

Table 5: Bile Tolerance-Observed Absorbance and Calculated Percentile Data

Isolate	Control (OD_{600}) (0% bile)	Control (0% bile)	0.1% Bile (OD_{600})	0.1% Bile	0.3% Bile (OD_{600})	0.3% Bile	0.5% Bile (OD_{600})	0.5% Bile
Batch I	0.525	100%	0.599	114.1%	0.473	90.1%	0.436	83.05%
Batch II	0.568	100%	0.56	98.59%	0.471	82.92%	0.433	76.23%
Batch IV	1.2	100%	1	83.33%	0.75	62.5%	0.62	51.67%
PRO-H20	3.01	100%	2.9	96.35%	2.75	91.36%	2.6	86.38%

Table 6: NaCl Tolerance-Observed Absorbance Data

NaCl %	Batch I (OD_{600})	Batch II (OD_{600})	Batch IV (OD_{600})	PRO-H20 (OD_{600})

1%	0.842	0.965	0.775	2.323
2%	0.354	0.599	0.538	2.165
3%	0.41	0.348	0.421	2.241
4%	0.296	0.106	0.319	2.063
5%	0.377	0.281	0.226	2.022
6%	0.286	0.331	0.19	2.045
7%	0.321	0.313	0.148	1.984
8%	0.239	0.143	0.105	1.768
9%	0.27	0.104	0.084	1.945

Table 7: Transit Tolerance-Observed Absorbance and Calculated Percentile Data

Batch	**Initial OD_{600} (0 h)**	**OD_{600} at 3 h (SGJ)**	**SGJ (3 h) %**	**OD_{600} at 4 h (SIJ)**	**SIJ (4 h) %**
Batch I	**1**	**0**	**0.00%**	**0.831**	**83.05%**
Batch II	**1**	**0.007**	**0.72%**	**0.762**	**76.20%**
Batch IV	**1**	**0.015**	**1.47%**	**0.517**	**51.67%**
PRO-H20	**1**	**0.849**	**84.85%**	**0.757**	**75.73%**

Table 8: Lysozyme Tolerance-Observed Absorbance Data and Calculated Percentile

Optical Density of Control (Untreated):

- **T_0 OD_{600} = 0.015**
- **T_3h OD_{600} = 0.055, T_{24}h OD_{600} = 0.810**
- **So, ΔOD_{600} (3h)= 0.055 - 0.015 = 0.040, ΔOD_{600} (24h)= 0.810- 0.015=0.795**

Batch	**Lysozyme (ppm)**	**OD_{600} (0 h)**	**OD_{600} (3 h)**	**ΔOD_{600} (3 h)**	**Survival % (3 h)**	**OD_{600} (24 h)**	**ΔOD_{600} (24 h)**	**Survival % (24 h)**
Batch I	100	0.018	0.045	0.027	67.5 %	0.638	0.62	78.0 %
	200	0.017	0.041	0.024	60.0 %	0.605	0.588	74.1 %

	300	0.016	0.036	0.02	50.0 %	0.515	0.499	62.8 %
Batch II	100	0.017	0.043	0.026	65.0 %	0.694	0.677	85.2 %
	200	0.016	0.038	0.022	55.0 %	0.648	0.632	79.5 %
	300	0.015	0.032	0.017	42.5 %	0.554	0.539	67.8 %
Batch IV	100	0.014	0.034	0.02	50.0 %	0.5	0.486	61.1 %
	200	0.014	0.029	0.015	37.5 %	0.45	0.436	54.9 %
	300	0.013	0.023	0.01	25.0 %	0.349	0.336	42.3 %
PRO-H20	100	0.016	0.048	0.032	80.6 %	0.764	0.748	94.1 %
	200	0.015	0.046	0.031	77.0 %	0.731	0.716	90.1 %
	300	0.014	0.039	0.025	61.9 %	0.622	0.608	76.5 %

Table 9: Cell Surface Hydrophobicity-Observed Absorbance and Calculated Percentile Data

Batch	A_0 (initial) [Abs-580nm]	A_t (post-xylene) [Abs-580nm]	Hydrophobicity (%)
Batch I	1.3	0.613	52.88%
Batch II	1.4	0.559	60.07%
Batch IV	1.1	0.811	26.28%
PRO-H20	1.25	0.206	83.53%

Table 10: Auto-Aggregation-Observed Absorbance and Calculated Percentile Data

Batch	A_0 (Initial) OD_{600}	A_3 (3 h) OD_{600}	A_{24} (24 h) OD_{600}	3 h (%)	24 h (%)
Batch I	1.05	0.744	0.376	29.17%	64.17%
Batch II	1.15	0.771	0.35	32.92%	69.58%
Batch IV	0.92	0.682	0.483	25.83%	47.5%
PRO-H20	1.18	0.775	0.285	34.33%	75.83%

Table 11: Co-Aggregation-Observed Absorbance and Calculated Percentile Data

Batch	OD_i (600nm)	OD_p (600nm)	OD_{ip} (3 h) (600nm)	3 h (%)	OD_{ip} (24 h) (600nm)	24 h (%)
Batch I	1.1	1.05	0.73	32.17%	0.49	54.78%
Batch II	1.15	1.08	0.78	30.43%	0.52	53.04%

Batch IV	1.05	1	0.77	25.22%	0.55	46.09%
PRO-H20	1.2	1.15	0.87	26.09%	0.61	47.83%

Table 12: Autolytic Activity of PRO-H20

Time Point	OD (630 nm)	Autolytic Activity (%)
0 h	**1**	
3 h	**0.875**	**12.50%**
6 h	**0.769**	**23.10%**
12 h	**0.604**	**39.60%**
24 h	**0.46**	**54.00%**

Table 13: IZD of Antibiotic Susceptibility Test with Interpretation for PRO-H20

Class	Antibiotic (Generic)	Disk Content	Mean IZD (mm)	Interpretation
Aminoglycosides	**Gentamicin**	**10 µg/disc**	**8.67**	**R**
	Streptomycin	**10 µg/disc**	**8.83**	**R**
	Amikacin	**30 µg/disc**	**10.83**	**R**
Phenicols	**Chloramphenicol**	**30 µg/disc**	**23**	**S**
Tetracyclines	**Tetracycline**	**30 µg/disc**	**14.67**	**R**
	Doxycycline Hydrochloride	**30 µg/disc**	**25**	**S**
β-Lactams	**Penicillin G**	**10 units/disc**	**14.33**	**R**
Macrolides	**Erythromycin**	**15 µg/disc**	**30.33**	**S**

	Clarithromycin	**15 µg/disc**	**25.33**	**S**
Lincosamides	**Clindamycin**	**2 µg/disc**	**11.67**	**R**
Quinolones	**Nalidixic acid**	**30 µg/disc**	**9.33**	**R**
	Levofloxacin	**5 µg/disc**	**9**	**R**
Nitroimidazoles	**Metronidazole**	**5 µg/disc**	**11**	**R**
Glycopeptides	**Teicoplanin**	**30 µg/disc**	**15.33**	**I**
Ansamycins	**Rifampicin**	**5 µg/disc**	**25**	**S**

- R (Resistant): IZD ≤ 15 mm
- I (Intermediate): IZD 16–20 mm
- S (Sensitive): IZD ≥ 21 mm

Table 14: Absorbance for Biofilm Formation by CV staining

Well no.	1	2	3	4	5	6	7	8	9	10
OD Value	0.230	0.220	0.240	0.215	0.235	0.225	0.210	0.230	0.220	0.240
Well no.	11	12	13	14	15	16	17	18	19	20
OD Value	0.215	0.235	0.225	0.210	0.230	0.220	0.240	0.215	0.235	0.225

Table 15: Components categorized from GC-MS

Category	**Compounds (Peak#)**

SCFAs	Acetic acid (1, 2), Butanoic acid (3), Hexanoic (9)
Alcohols / Aldehydes	Propanal (4), 1-Propanol (8), Butanol acetate (10)
Esters & Organic Acids	Lactic acid (6), Ethyl/Methyl esters (88, 96, 98)
Heterocyclic/Lactone	Furanone (7), Pyrazines (79, 84)
Peptides / Amino Acid Deriv.	Alanyl-leucine (72), Diketopiperazines (73, 135)
Sterols & Lipid Metabolites	γ-Sitosterol (118), Stigmast (119), Squalene (134)
Biomarkers of Fermentation	Acetic acid, Pyrazines, Squalene

Table 16: Closely Related strains based on BLASTn Results

GenBank Accession	Strain ID
AB362616.1	**12-780**
PQ192164.1	**20-786**
KX218443.1	**18-784**
MH393057.1	**10-778 (*Lacto.*)**
MZ066815.1	**13-781**

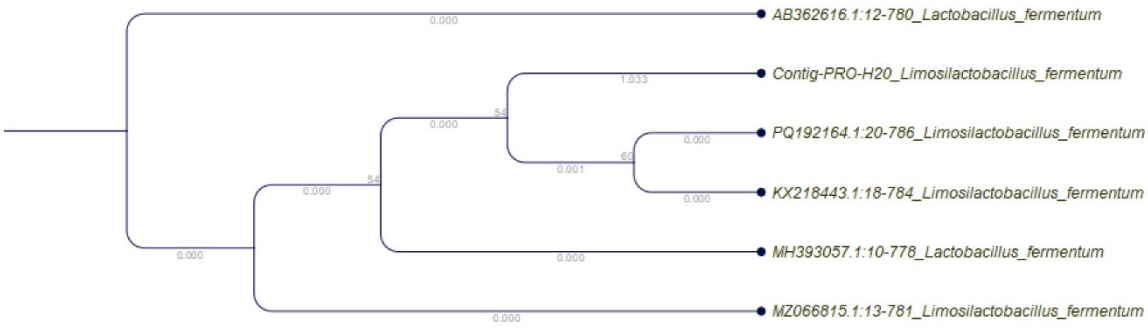
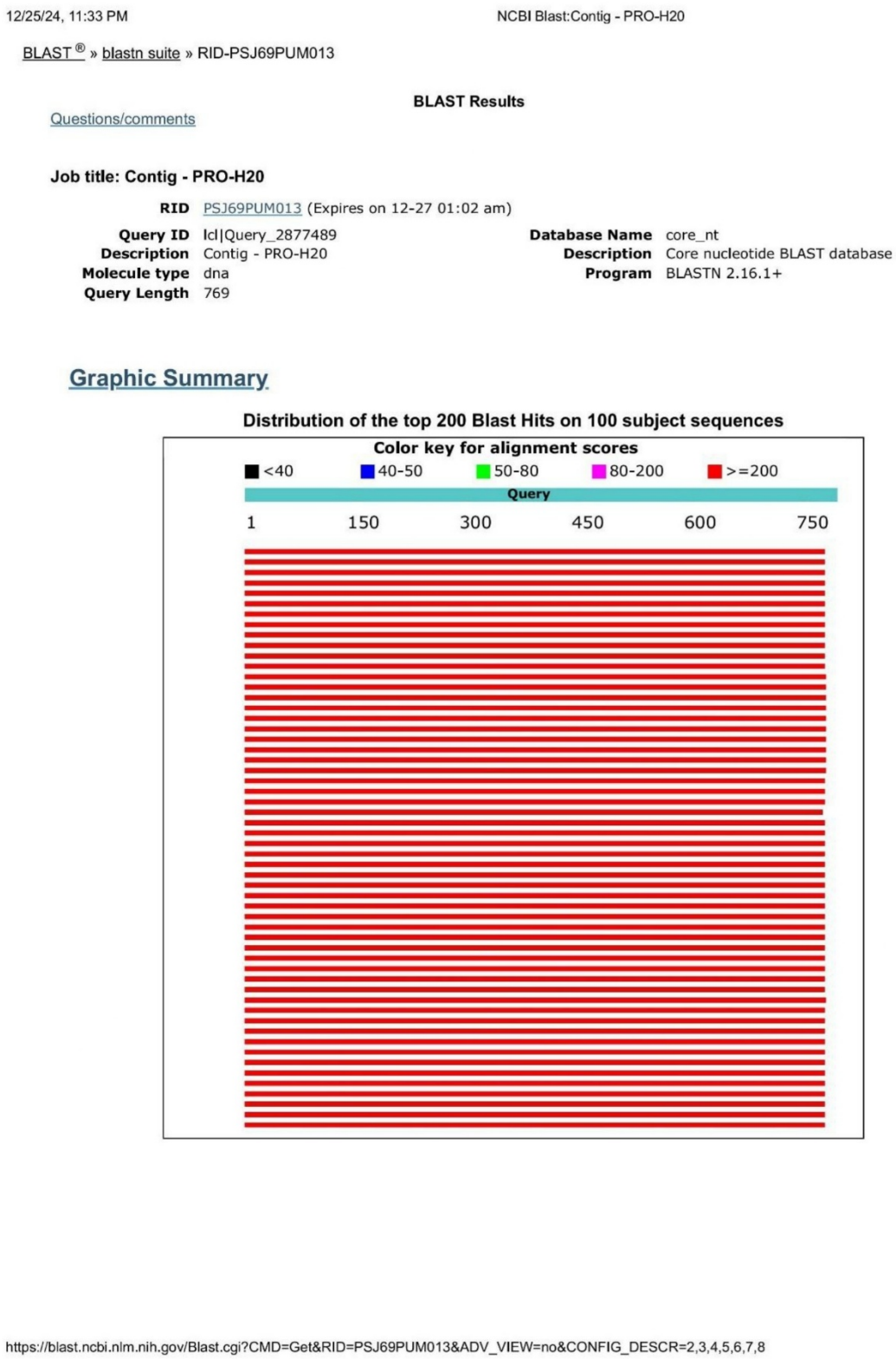


Figure 14: *Neighbor-Joining phylogenetic tree of partial 16S rRNA sequences [showing the position of PRO-H20 among closely related L. fermentum strains. Bootstrap values (≥50%) are indicated at nodes. Scale bar represents substitutions per site.]*


12/25/24, 11:33 PM NCBI Blast:Contig - PRO-H20

BLAST ® » blastn suite » RID-PSJ69PUM013

BLAST Results

Questions/comments

Job title: Contig - PRO-H20

RID PSJ69PUM013 (Expires on 12-27 01:02 am)

Query ID lcl|Query_2877489
Description Contig - PRO-H20
Molecule type dna
Query Length 769

Database Name core_nt
Description Core nucleotide BLAST database
Program BLASTN 2.16.1+

Graphic Summary

Distribution of the top 200 Blast Hits on 100 subject sequences

Color key for alignment scores

<40 40-50 50-80 80-200 >=200

Query

1 150 300 450 600 750

https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Get&RID=PSJ69PUM013&ADV_VIEW=no&CONFIG_DESCR=2,3,4,5,6,7,8

Figure 13: *NCBI Blast Results*

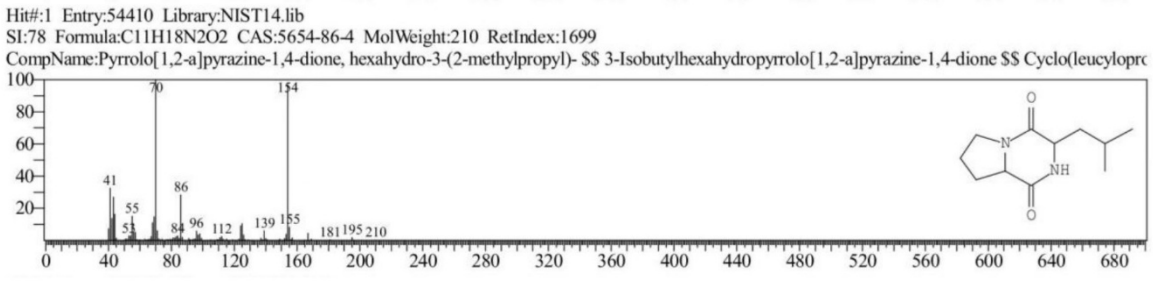


Figure 12: *Peak and structure detail of* ***Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-***

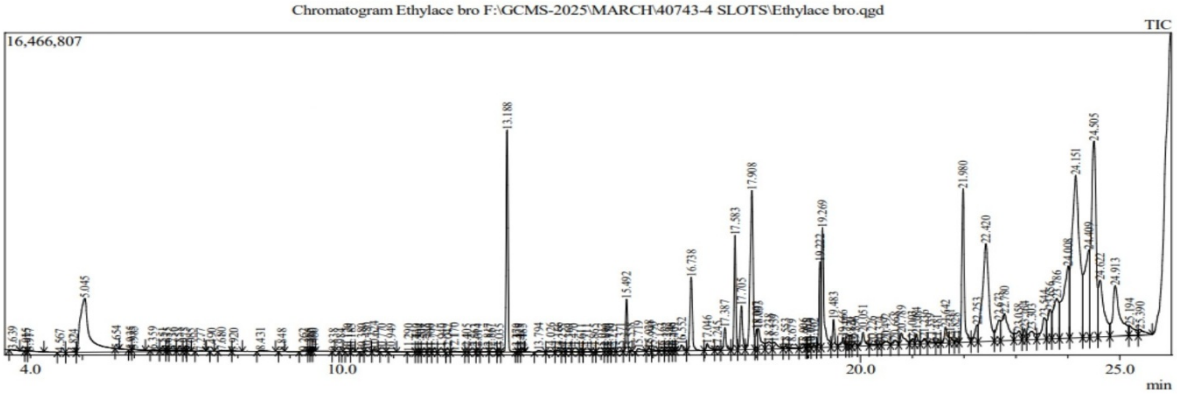


Figure 11: *GC-MS Chromatogram obtained Extracellularly from PRO-H20*

Figure 10.1: *Hemolytic Activity of PRO-H20*

Figure 10.2: *Hemolytic Activity of Control (S.aureus)*

Figure 10: *Hemolytic Activity of PRO-H20*

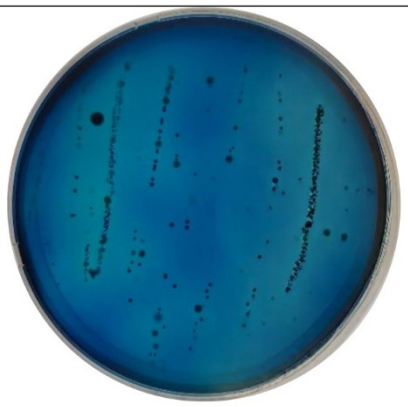


Figure 9.1: *DNase Activity of PRO-H20*

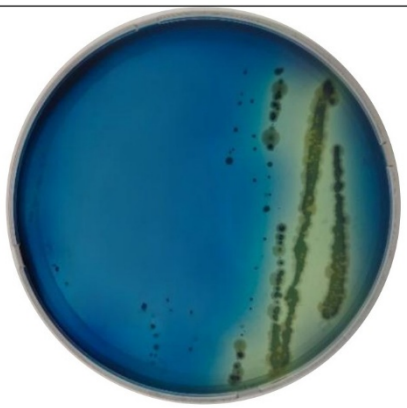


Figure 9.2: *DNase Activity of Control (S.aureus)*

Figure 9: *DNase Activity of PRO-H20*

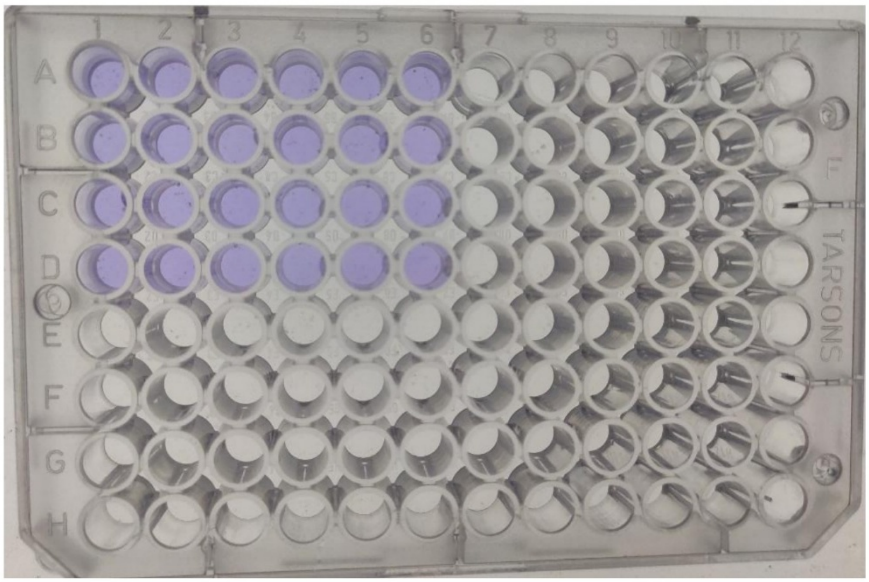


Figure 8: *Biofilm Formation Assay (After the addition of Crystal Violet)*

Figure 7: Bacteriocin Production Assay on PRO-H20

Figure 6.1: *IZD Of Gentamicin onPRO-H20*

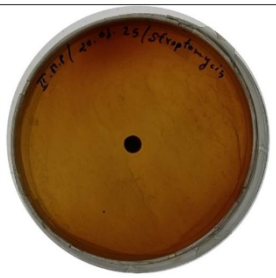


Figure 6.2: *IZD Of Streptomycin onPRO-H20*

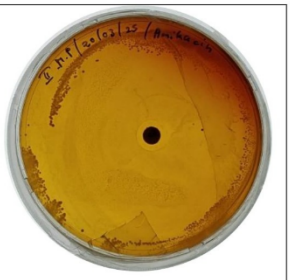


Figure 6.3: *IZD Of Amikacin onPRO-H20*

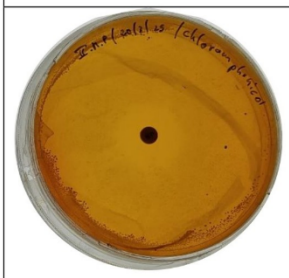


Figure 6.4: *IZD Of Chloramphenicol on PRO-H20*

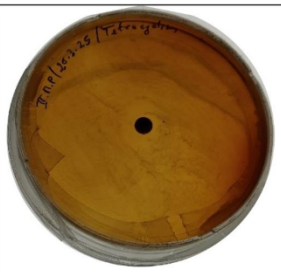


Figure 6.5: *IZD Of Tetracycline onPRO-H20*

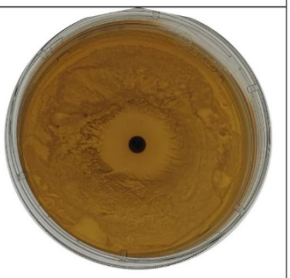


Figure 6.6: *IZD Of Doxycycline HCl on PRO-H20*

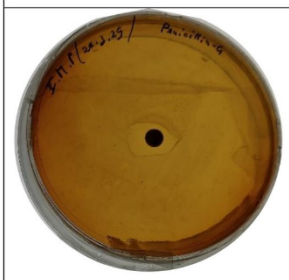


Figure 6.7: *IZD Of Penicillin G on PRO-H20*

Figure 6.8.: *IZD Of Erythromycin on PRO-H20*

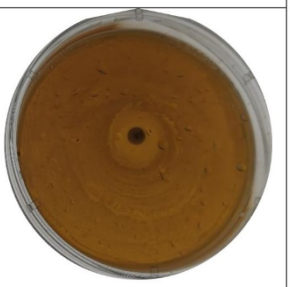


Figure 6.9*: IZD of Clarithromycin on PRO-H20*

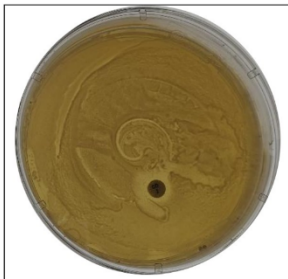


Figure 6.10*: IZD of Clindamycin on PRO-H20*

Figure 6.11*: IZD of Nalidixic acid on PRO-H20*

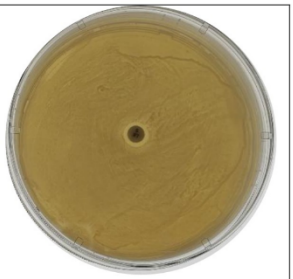


Figure 6.12*: IZD of Levofloxacin on PRO-H20*

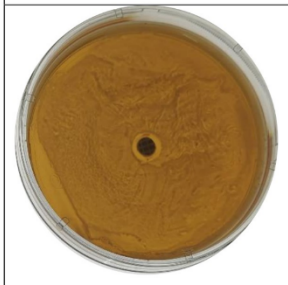


Figure 6.13*: IZD of Metronidazole on PRO-H20*

Figure 6.14*: IZD of Teicoplanin on PRO-H20*

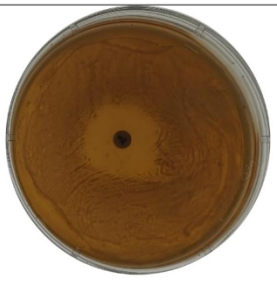


Figure 6.15*: IZD of Rifampicin on PRO-H20*

Figure 6*: IZD of Antibiotics on* ***PRO-H20***

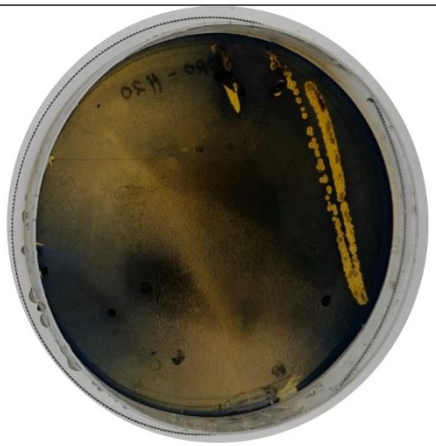


Figure 5: Amylolytic Activity of **PRO-H20**

Figure 4.1: *Negative Control for Lipolytic Activity*

Figure 4.2: *Lipolytic Activity of Sample*

Figure 4: *Lipolytic Activity of* ***PRO-H20***

Figure 3.1: *Negative control for proteolytic activity*

Figure 3.2: *Proteolytic Activity of Sample*

Figure 3: *Proteolytic Activity of* ***PRO-H20***

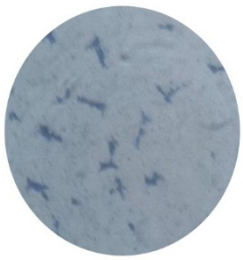


Figure 2.1*: Gram stain Batch I*

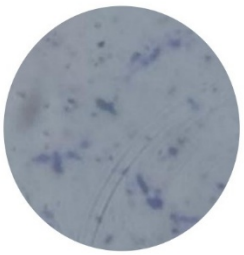


Figure 2.2*: Gram stain Batch II*

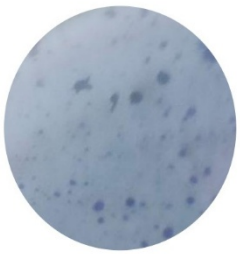


Figure 2.3*: Gram stain Batch III*

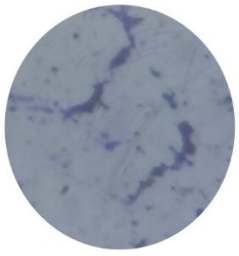


Figure 2.4*: Gram stain Batch IV*

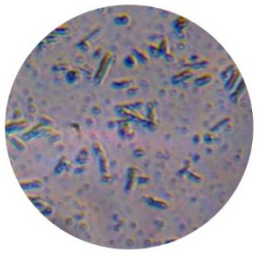


Figure 2.5*: Gram stain PRO-H20*

Figure 2*: Microscopic Images of Gram Staining*