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Exploring Beneficial Microbes for Antibiotic Discovery, Enzyme Production, and Gut Health Improvement

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Abstract

The present study aimed to isolate, characterize, and evaluate beneficial bacterial isolates from medicinal plant rhizosphere soils and fermented dairy products for their potential applications in antibiotic discovery, enzyme production, and gut health improvement. Soil isolates designated as S4, S5, and S6 and a probiotic isolate (D1) from curd were obtained using conventional microbiological techniques and characterized based on their morphological and biochemical properties. The soil isolates were presumptively identified as Bacillus spp., while the probiotic isolate was identified as Lactobacillus spp.
The antimicrobial activity of the isolated microorganisms was assessed against selected bacterial pathogens. Among the soil-derived isolates, S6 displayed the highest antibacterial potential. In general, crude culture extracts produced stronger inhibitory effects than the corresponding ethyl acetate extracts, suggesting that the active antimicrobial compounds were more effective in their crude form. In addition to their antibacterial properties, the selected soil isolates were capable of producing the commercially important enzymes L-asparaginase and streptokinase, as demonstrated through qualitative screening and enzyme activity assays. These observations indicate that these isolates possess considerable potential for applications in the pharmaceutical and biotechnology sectors. The probiotic isolate D1 exhibited several desirable probiotic characteristics. It showed antagonistic activity against Staphylococcus aureus, tolerated acidic conditions and bile salts, and remained viable under simulated gastrointestinal conditions, indicating its potential to survive passage through the human digestive tract. However, under the conditions used in this study, D1 did not exhibit detectable production of either L-asparaginase or streptokinase. The results suggest that medicinal plant rhizosphere soils and fermented dairy products serve as valuable reservoirs of beneficial microorganisms with antimicrobial, enzymatic, and probiotic capabilities. To fully explore their practical potential, future investigations should focus on molecular identification, detailed characterization of bioactive metabolites, and large-scale validation studies. Such research would provide a stronger foundation for the development of these microorganisms for applications in healthcare, food biotechnology, pharmaceutical manufacturing, and other industrial biotechnological processes.

Keywords: *Medicinal plant rhizosphere, Bacillus spp., Lactobacillus spp., antimicrobial activity, L-asparaginase, Streptokinase, Probiotics, Enzyme production, Fermented dairy products, Industrial biotechnology, pharmaceutical biotechnology, Beneficial microorganisms.*

1. INTRODUCTION

Microorganisms represent one of the most diverse and essential groups of living organisms, occupying a vast range of habitats including soil, aquatic environments, extreme ecosystems, and the human gastrointestinal tract [1]. Their exceptional metabolic versatility allows them to produce an extensive array of bioactive compounds and enzymes that have significantly advanced the fields of medicine, biotechnology, agriculture, and industrial manufacturing [2]. In addition to maintaining ecological stability through nutrient recycling and the decomposition of organic matter, beneficial microorganisms contribute to host well-being and serve as valuable sources of antibiotics, industrial enzymes, probiotics, and numerous other biologically important metabolites. [3]. Owing to these unique capabilities, microbial resources continue to attract significant scientific interest as sustainable alternatives for addressing global healthcare and industrial challenges [4].

The discovery of microbial antibiotics revolutionized modern medicine by dramatically reducing mortality associated with infectious diseases [5]. The widespread and often indiscriminate use of antibiotics has contributed significantly to the rapid development of antimicrobial resistance (AMR), which is currently regarded as one of the most critical challenges to global public health [6].

The increasing prevalence of multidrug-resistant pathogens has diminished the effectiveness of many existing antibiotics, emphasizing the need to discover new antimicrobial agents with alternative modes of action. Diverse natural habitats, including agricultural and forest soils, compost, marine environments, and fermented food products, contain rich and largely untapped microbial populations that represent promising sources of novel bioactive compounds [7]. Among these, actinomycetes, particularly members of the genus *Streptomyces*, along with several species of *Bacillus*, *Pseudomonas*, and other environmental bacteria, have demonstrated exceptional ability to produce structurally diverse secondary metabolites possessing antibacterial, antifungal, antiviral, and anticancer activities [8]. Recent advances in microbial biotechnology, including genome mining, metagenomics, and molecular characterization, have further enhanced the discovery of previously unknown biosynthetic pathways, offering new opportunities for developing effective antimicrobial agents against resistant pathogens [9].

Besides their pharmaceutical importance, microorganisms represent the most efficient producers of industrially valuable enzymes [10]. Microbial enzymes such as amylases, proteases, cellulases, lipases, pectinases, L-asparaginase, and streptokinase are extensively used in food processing, textiles, detergents, pharmaceuticals, paper manufacturing, biofuel production, and environmental bioremediation [11]. Compared with enzymes derived from plants and animals, microbial enzymes offer several advantages, including rapid growth of producer organisms, high catalytic efficiency, cost-effective large-scale production, easier genetic manipulation, greater stability under diverse environmental conditions, and eco-friendly processing. Continuous improvements in fermentation technology, strain optimization, metabolic engineering, and downstream processing have significantly increased enzyme productivity and commercial viability [12]. Consequently, identifying novel enzyme-producing microorganisms remains a major objective in industrial biotechnology for developing sustainable and economically feasible bioprocesses.

Equally important is the role of beneficial microorganisms in maintaining human health through modulation of the gut microbiota [13]. The human gastrointestinal tract contains a diverse and dynamic community of microorganisms that plays a vital role in digestion, nutrient assimilation, vitamin production, immune system development, and defense against pathogenic microbes. Disruptions in this microbial community, referred to as dysbiosis, have been linked to a wide range of health conditions, including inflammatory bowel disease, obesity, diabetes, allergic disorders, cardiovascular diseases, and neurological disorders. Probiotic microorganisms, especially lactic acid bacteria from the genera *Lactobacillus*, *Lacticaseibacillus*, *Bifidobacterium*, and related groups, contribute to the restoration of gut microbial balance by producing organic acids, bacteriocins, and other antimicrobial substances. They also strengthen the intestinal barrier and help regulate immune responses, thereby promoting overall gut health [14].

Fermented foods and dairy products are valuable sources of probiotic microorganisms that can tolerate acidic gastric conditions and bile salts, allowing them to survive passage through the gastrointestinal tract. These characteristics make them attractive candidates for use in functional foods and therapeutic formulations. In addition, recent advances in pharmacomicrobiomics have demonstrated that the gut microbiota can affect drug metabolism, bioavailability, and treatment outcomes, underscoring its growing significance in the advancement of personalized medicine [15].

The increasing convergence of antimicrobial discovery, enzyme biotechnology, and probiotic research underscores the importance of exploring beneficial microorganisms from diverse ecological niches. Soil ecosystems remain one of the richest sources of antibiotic-producing bacteria, whereas fermented foods and dairy products provide diverse probiotic microorganisms with significant health-promoting properties. Simultaneously, these microbial resources possess immense potential for producing industrially important enzymes through environmentally sustainable fermentation processes. Integrating conventional microbiological techniques with modern molecular and biochemical approaches enables comprehensive screening and characterization of microbial isolates possessing multiple beneficial traits, thereby expanding their potential applications in medicine, industry, agriculture, and environmental sustainability [16].

In this context, the present study, "Exploring Beneficial Microbes for Antibiotic Discovery, Enzyme Production, and Gut Health Improvement," aims to isolate, screen, and characterize beneficial microorganisms from diverse natural sources for their pharmaceutical, industrial, and probiotic potential. The study focuses on identifying microbial isolates capable of producing antimicrobial compounds against pathogenic bacteria, synthesizing valuable enzymes with industrial and therapeutic applications, and exhibiting probiotic characteristics that support gut health. Through systematic microbiological and biochemical evaluation, this research seeks to contribute to the discovery of novel bioactive microorganisms while providing a scientific foundation for the development of sustainable antimicrobial agents, efficient enzyme production systems, and next-generation probiotic formulations [17]. Ultimately, the findings are expected to advance microbial biotechnology and support innovative solutions to global challenges related to antimicrobial resistance, industrial sustainability, and human health.

2. Materials and Methods

2.1 Sample Collection and Isolation of Microorganisms

Rhizosphere soil samples were aseptically collected from the root regions of *Aloe vera*, *Ocimum sanctum* (Tulsi), and *Phyllanthus emblica* (Amla). The collected samples were transferred to the laboratory in sterile containers and processed without delay to preserve microbial viability. Serial dilutions were prepared using sterile physiological saline, and suitable dilutions were inoculated onto Nutrient Agar plates by the Crowded Plate Technique (CPT). The plates were incubated at 37°C for 5 days, after which colonies displaying clear inhibition zones against adjacent microorganisms were identified as potential antibiotic-producing isolates.

These colonies were purified through repeated streak plating to obtain pure cultures and subsequently maintained on Nutrient Agar slants for further characterization and experimental analysis [18].

2.2 Screening of Antibiotic-Producing Isolates

Purified bacterial isolates were cultured in Nutrient Broth and incubated at 37°C under shaking conditions for 72 hours to facilitate the production of extracellular metabolites. Following incubation, the cultures were centrifuged at 8000 rpm for 15 minutes, and the cell-free supernatant was collected. A portion of the supernatant was subjected to ethyl acetate extraction, while the remaining portion was retained as the crude culture extract. The antimicrobial activity of both preparations was assessed against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus* spp. using the agar well diffusion technique. After incubation, the diameters of the inhibition zones were measured to determine the antibacterial effectiveness of each extract [19].

2.3 Isolation and Characterization of Probiotic Bacteria

Fermented dairy products, including curd and yogurt, were aseptically collected from local sources for the isolation of probiotic bacteria. Serial dilutions of the samples were prepared and spread onto de Man, Rogosa and Sharpe (MRS) agar plates. The inoculated plates were incubated at 30–37°C for 48 hours to allow bacterial growth. Morphologically distinct colonies were selected, purified by repeated streaking, and characterized based on colony morphology, Gram staining, catalase activity, and carbohydrate fermentation patterns [20].

2.4 Evaluation of Probiotic Properties

Selected isolates were evaluated for probiotic potential by determining their tolerance to acidic pH and bile salts under simulated gastrointestinal conditions. Antimicrobial activity against selected pathogenic bacteria was assessed using the agar well diffusion method. Auto-aggregation ability was determined by measuring the reduction in optical density of bacterial suspensions after incubation, indicating the ability of isolates to adhere and aggregate [21].

2.5 Screening for Enzyme Production

Selected microbial isolates were screened for the production of L-asparaginase and streptokinase using substrate-specific agar media containing L-asparagine and fibrin, respectively. Isolates producing distinct halo zones around colonies were considered positive enzyme producers and selected for further analysis.

2.6 Enzyme Production and Activity Assay

Positive isolates were cultivated by submerged fermentation under optimized conditions. Following incubation, cultures were centrifuged to obtain cell-free supernatants containing crude extracellular enzymes. No purification procedures were performed, and crude enzyme extracts were used directly for activity determination. L-asparaginase activity was estimated using Nessler's reagent, which detects ammonia released during hydrolysis of L-asparagine. Streptokinase activity was determined using a fibrin clot lysis assay or fibrin plate assay.

Preliminary enzyme characterization included evaluation of optimum pH, temperature, and thermal stability [22].

2.7 Evaluation of Antimicrobial Activity

The antibacterial activity of the microbial extracts was evaluated using the agar well diffusion assay. Standardized bacterial inocula were uniformly spread on Mueller–Hinton agar plates, after which wells were filled with the test extracts along with appropriate positive and negative controls. The plates were incubated at 37°C for 24 hours, and the diameters of the inhibition zones were recorded to assess antibacterial activity [23]. The Minimum Inhibitory Concentration (MIC) of the extracts was determined by the broth microdilution method using a series of two-fold serial dilutions. To determine the Minimum Bactericidal Concentration (MBC), aliquots from MIC wells showing no visible bacterial growth were subcultured onto fresh agar plates, and the absence of colony formation after incubation was considered indicative of bactericidal activity [24].

2.8 Assessment of Probiotic-Mediated Drug Metabolism

Selected probiotic isolates were cultured in MRS broth under anaerobic conditions and standardized to approximately 1×10^8 CFU/mL. The cultures were sequentially exposed to simulated gastric fluid (pH 2.0) and simulated intestinal fluid (pH 6.8) containing bile salts and pancreatin. Experimental groups included drug alone, probiotic alone, and drug–probiotic combinations. Samples were collected at predetermined intervals to evaluate microbial growth, pH variation, and possible drug transformation under simulated gastrointestinal conditions [25].

3. Results

3.1 Isolation and Screening of Antibiotic-Producing Bacteria

Rhizosphere soil samples obtained from the medicinal plants *Aloe vera*, *Ocimum sanctum* (Tulsi), and *Phyllanthus emblica* (Amla) were screened for the presence of antibiotic-producing bacteria using the crowded plate technique. A total of six morphologically distinct bacterial isolates were recovered during the screening process. Isolates that produced distinct zones of inhibition around their colonies, indicating antagonistic activity against neighboring microorganisms, were identified as potential antibiotic producers and selected for subsequent characterization and evaluation.

Figure 1. Isolation of antibiotic-producing bacterial colonies showing zones of inhibition by the crowded plate technique

3.2 Antimicrobial Activity of Soil Bacterial Isolates

The antimicrobial potential of the six soil bacterial isolates (S1–S6) was assessed against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis* using the agar well diffusion method. Among the tested isolates, S6 showed the highest antibacterial activity, producing an inhibition zone of 3 mm against *Staphylococcus aureus*. A comparatively weak inhibitory effect was observed against *Bacillus subtilis*, whereas no antibacterial activity was detected against *Escherichia coli* for any of the isolates. Furthermore, the crude culture supernatant exhibited stronger antimicrobial activity than the corresponding ethyl acetate extract, indicating that the bioactive compounds were more effective in their unextracted form under the conditions of this study.

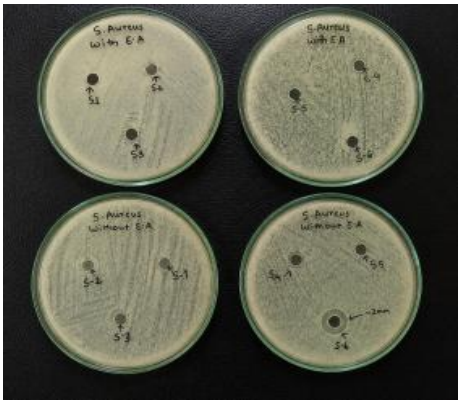

Figure 2. Antimicrobial activity of soil isolate extracts against Staphylococcus aureus by agar well diffusion assay

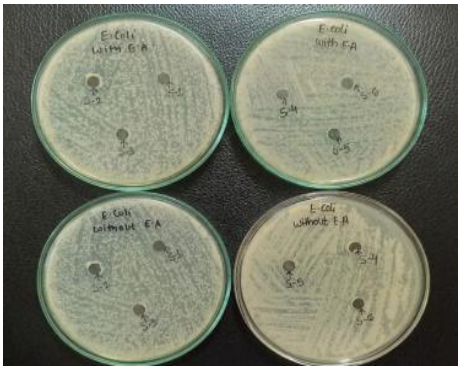

Figure 3. Antimicrobial activity of soil isolate extracts against Escherichia coli by agar well diffusion assay

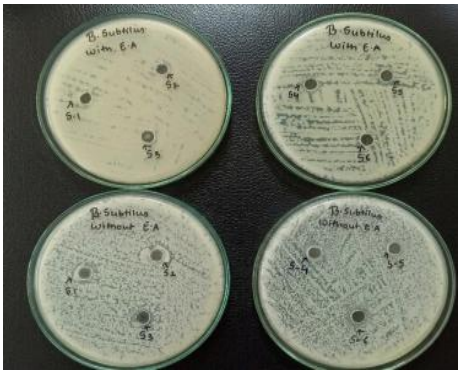

Figure 4. Antimicrobial activity of soil isolate extracts against Bacillus subtilis by agar well diffusion assay

Table No.01: Antimicrobial activity of soil bacterial isolates against test organisms

Test Organism	Zone of inhibition (mm) Crude extract	Zone of inhibition (mm) Ethyl acetate extract
Escherichia coli	No zone observed	No zone observed
Staphylococcus aureus	Zone only in S6 (3mm)	No zone observed
Bacillus subtilis	Weak inhibition zone in S6	No zone observed

Based on the antimicrobial screening results, isolates S4, S5, and S6 were selected for further characterization.

3.3 Morphological and Biochemical Characterization of Soil Isolates

Microscopic examination of the selected isolates following Gram staining showed that S4, S5, and S6 were Gram-positive, rod-shaped bacteria occurring either singly or in short chains. Endospore staining demonstrated the presence of centrally positioned endospores within the vegetative cells, confirming that all three isolates were endospore-forming bacteria. Biochemical analysis revealed that the isolates were positive for indole production, methyl red, Voges–Proskauer reaction, citrate utilization, catalase activity, starch hydrolysis, and glucose fermentation (TSI K/A), while all tested negative for oxidase activity. Based on the combined morphological and biochemical characteristics, the isolates were tentatively identified as *Bacillus subtilis.*

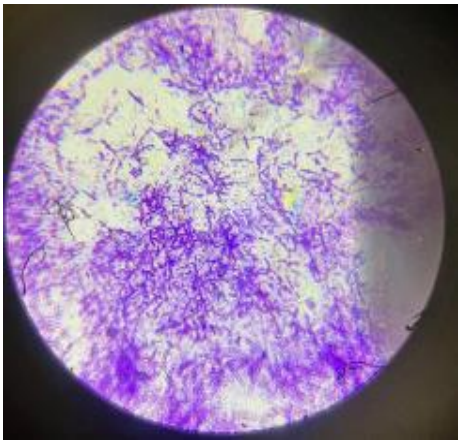

Figure 5. Gram staining of soil isolate S4

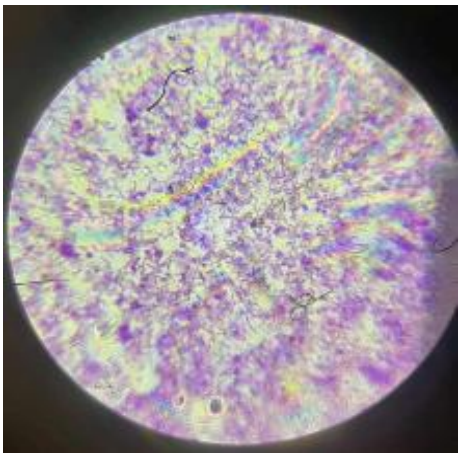

Figure 6. Gram staining of soil isolate S5

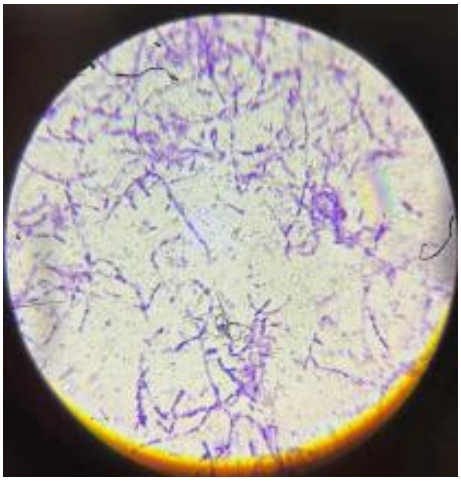

Figure 7. Gram staining of soil isolate S6

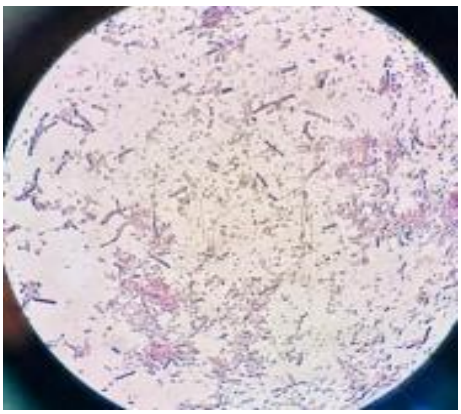

Figure 8. Endospore staining of soil isolate S4

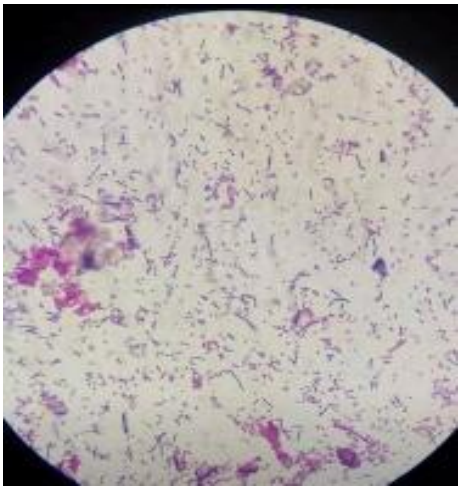

Figure 9. Endospore staining of soil isolate S5

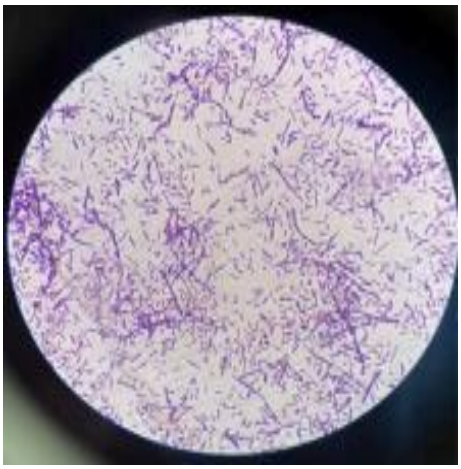

Figure 10. Endospore staining of soil isolate S6

Table 2. Biochemical Characterization of Soil Bacterial Isolates (S4, S5, and S6)

Biochemical Test	S4	S5	S6	Interpretation
Indole Test	+	+	+	Production of indole from tryptophan (tryptophanase positive)
Methyl Red (MR) Test	+	+	+	Mixed-acid fermentation of glucose
Voges–Proskauer (VP) Test	+	+	+	Acetoin production (butanediol fermentation pathway)
Citrate Utilization Test	+	+	+	Ability to utilize citrate as the sole carbon source
Triple Sugar Iron (TSI) Test	K/A*	K/A*	K/A*	Glucose fermentation only (alkaline slant/acid butt)
Catalase Test	+	+	+	Presence of catalase enzyme
Oxidase Test	–	–	–	Absence of cytochrome c oxidase enzyme

Note: + = Positive reaction; – = Negative reaction; K/A = Alkaline (red) slant/Acid (yellow) butt, indicating glucose fermentation only.

3.4 Minimum Inhibitory Concentration (MIC) of Soil Isolates

The antimicrobial potency of the selected isolates was further evaluated by determining the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Bacillus subtilis*. Isolates S4 and S5 inhibited the growth of *Staphylococcus aureus* at 75 µL/mL, whereas isolate S6 exhibited a lower MIC value of 50 µL/mL, indicating stronger antibacterial activity. Against *Bacillus subtilis*, isolate S6 exhibited an MIC of 100 µL/mL.

Figure 11. Minimum inhibitory concentration (MIC) assay of soil isolate S4 against Staphylococcus aureus.

Table No. 03: Minimum inhibitory concentration of isolate S4 against Staphylococcus aureus.
Observation: *The MIC was observed at 75 µL/mL, where no visible turbidity was observed*

Tube No.	Dilution	Concentration (ug/ml)	Amount Of Extract (ml)	Amount Of Diluent (ml)	Amount Of Culture (ml)	Observation
1.	Undiluted	100	4.0	_	0.1	_
2.	1:2	75	2.0	2.0	0.1	_
3.	1:4	50	2.0	2.0	0.1	+
4.	1:8	25	2.0	2.0	0.1	++
5.	1:16	12.5	2.0	2.0	0.1	++
Positive	_	_	_	2.0	0.1	+++
Negative	_	_	_	2.0	_	_
Media Control	_	_	_	2.0	_	_

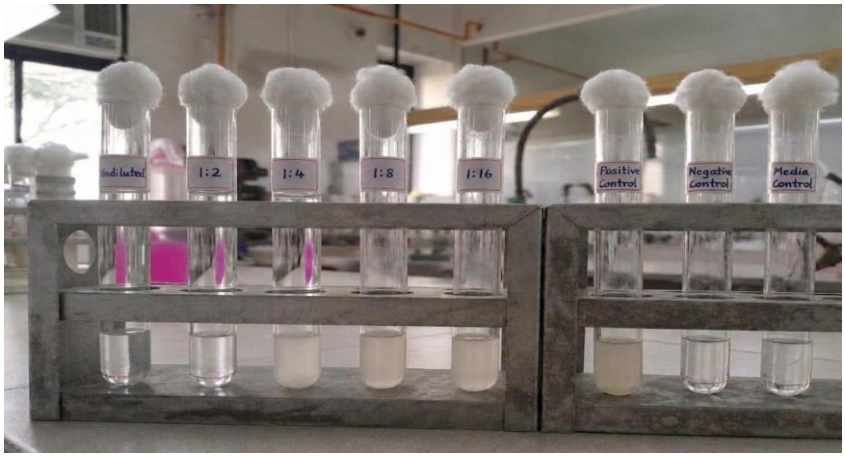

Figure 12. Minimum inhibitory concentration (MIC) assay of soil isolate S5 against Staphylococcus aureus

Table No:04: Minimum inhibitory concentration of isolate S5 against Staphylococcus aureus
Observation: *The MIC was observed at 75 µL/mL, where no visible turbidity was observed*

Tube No.	Dilution	Concentration (ul /ml)	Amount of Extract (ml)	Amount of Diluent	Culture (ml)	Observation
1	Undiluted	100	4	_	0.1	_
2	01:02	75	2	2	0.1	_
3	01:04	50	2	2	0.1	+
4	01:08	25	2	2	0.1	+
5	01:16	12.5	2	2	0.1	++
Positive	_	_	_	2	0.1	+++
Negative	_	_	_	2	_	_

Figure 13. Minimum inhibitory concentration (MIC) assay of soil isolate S6 against Staphylococcus aureus

Table No: 05: Minimum inhibitory concentration of isolate S6 against Staphylococcus aureus.
Observation: *The MIC was observed at 50ul/ml, where no visible turbidity was observed.*

Tube No.	Dilution	Concentration (ul/ml)	Amount of Extract (ml)	Amount of Diluent (ml)	Culture (ml)	Observation
1	Undiluted	100	4	_	0.1	_
2	01:02	75	2	2	0.1	_
3	01:04	50	2	2	0.1	_
4	01:08	25	2	2	0.1	++
5	01:16	12.5	2	2	0.1	++
Positive	_	_	_	2	0.1	+++
Negative	_	_	_	2	_	_
Media control	_	_	_	2	_	_

Figure 14. Minimum inhibitory concentration (MIC) assay of soil isolate S6 against Bacillus subtilis

Table No.06: Minimum Inhibitory Concentration (MIC) assay of isolate S6 against Bacillus subtilis.
Observation: *The MIC was observed at 100ug/ml, where no visible turbidity was observed*

Tube No.	Dilution	Concentration (µg/ml)	Amount of Extract (ml)	Amount of Diluent (ml)	Culture (ml)	Observation
1.	Undiluted	100	4.0	–	0.1	–
2.	1:2	75	2.0	2.0	0.1	+
3.	1:4	50	2.0	2.0	0.1	++
4.	1:8	25	2.0	2.0	0.1	++
5.	1:16	12.5	2.0	2.0	0.1	+++
Positive	–	–	–	2.0	0.1	+++
Negative	–	–	–	2.0	–	–
Media Control	–	–	–	2.0	–	–

3.5 Isolation, Screening and Characterization of Probiotic Isolate

Probiotic bacteria were isolated from fermented dairy products, including curd and milk, using MRS, Rogosa, and Tomato agar media. Colonies exhibiting typical characteristics of lactic acid bacteria were selected and purified for further analysis.

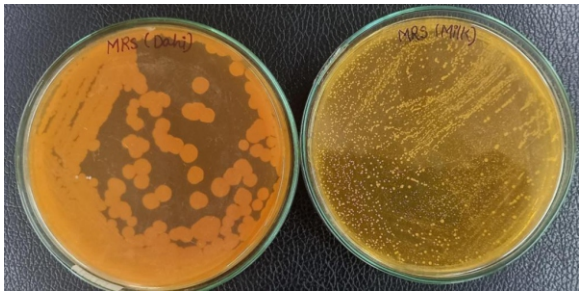

Figure 15. Isolation of probiotic bacteria from curd and milk on MRS agar

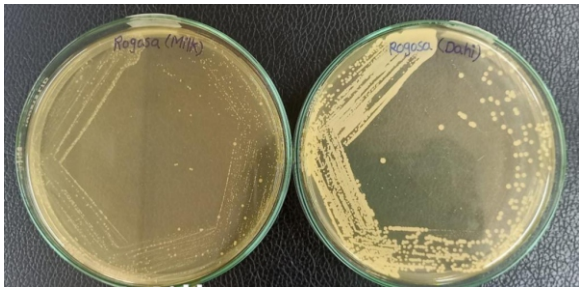

Figure 16. Isolation of probiotic bacteria from curd and milk on Rogosa agar

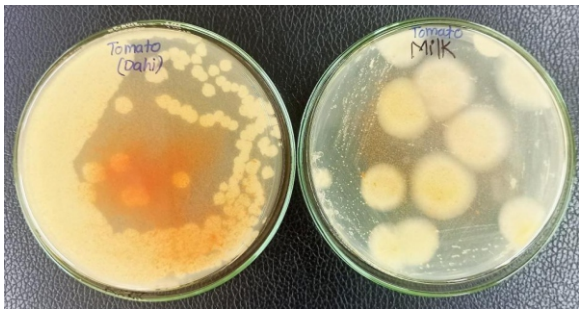

Figure 17. Isolation of probiotic bacteria from curd and milk on Tomato agar

The purified probiotic isolates were evaluated for their antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* using the agar well diffusion assay. Among the tested isolates, only D1 demonstrated measurable antimicrobial activity, producing a 1 mm zone of inhibition against *Staphylococcus aureus*. No inhibitory effect was observed against *Escherichia coli* or *Bacillus subtilis*. Similarly, the ethyl acetate extract of the isolate did not exhibit antibacterial activity against any of the test organisms. Owing to its observed antimicrobial activity, isolate D1 was selected for further characterization and assessment of its probiotic attributes.

3.6 Antimicrobial activity

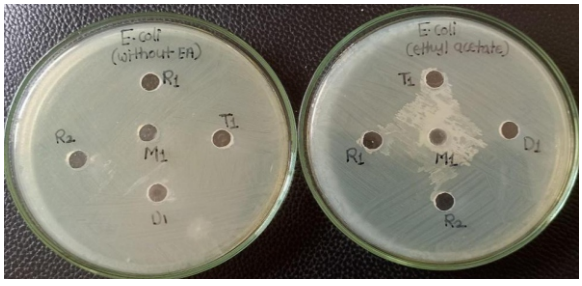

Figure 18. Antimicrobial activity of probiotic isolate D1 against Escherichia coli

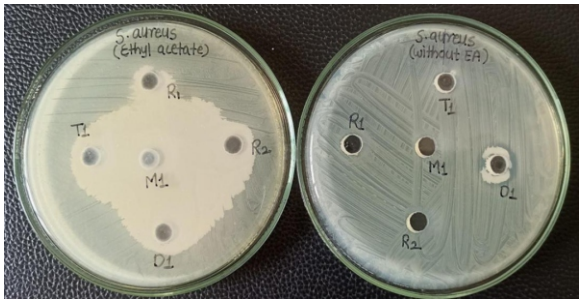

Figure 19. Antimicrobial activity of probiotic isolate D1 against Staphylococcus aureus

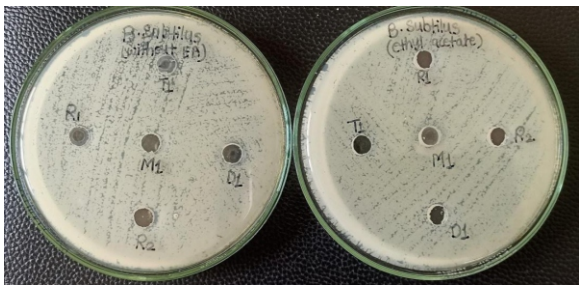

Figure 20. Antimicrobial activity of probiotic isolate D1 against Bacillus subtilis

The selected isolate D1 was subjected to morphological and biochemical characterization for preliminary identification. Gram staining revealed that the isolate was Gram-positive, rod-shaped, and arranged either singly or in short chains, consistent with the typical characteristics of lactic acid bacteria. Biochemical testing indicated positive results for the Indole, Methyl Red, Voges–Proskauer, and Triple Sugar Iron (TSI) tests, whereas the Catalase, Oxidase, and Citrate Utilization tests were negative. Based on the combined morphological and biochemical findings, isolate D1 was tentatively identified as *Lactobacillus* spp.

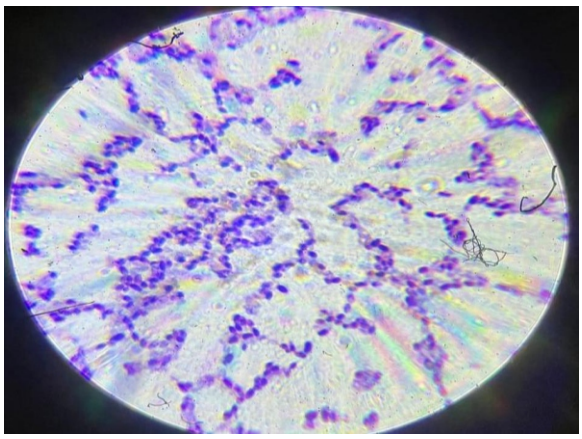

Figure 21. Gram staining of probiotic isolate D1

Table no 07: Biochemical characterization of probiotic isolate D1

Biochemical Test	Observation	Result
Gram Staining	Gram-positive, rod-shaped cells observed	Gram-positive rods
Indole Test	Red ring developed after addition of Kovac's reagent	Positive
Methyl Red (MR) Test	Red colour developed after addition of methyl red indicator	Positive
Voges–Proskauer (VP) Test	Cherry-red colour developed after addition of VP reagents	Positive
Citrate Utilization Test	No colour change observed; medium remained green	Negative
Triple Sugar Iron (TSI) Test	Red slant and yellow butt (K/A) observed	Positive (glucose fermentation)
Catalase Test	No bubble formation after addition of hydrogen peroxide	Negative
Oxidase Test	No purple colour developed	Negative

3.7 Probiotic Properties

The probiotic potential of isolate D1 was evaluated by testing its tolerance to acidic pH and bile salts. The isolate showed strong growth at pH 4.0, moderate growth at pH 3.0, and remained viable at pH 2.0, indicating good acid tolerance. It also survived in bile salt concentrations up to 1.0%, although growth gradually decreased as bile concentration increased.

After exposure to simulated gastrointestinal conditions, D1 successfully formed colonies on MRS agar, confirming its ability to survive conditions similar to those of the human gastrointestinal tract and supporting its potential as a probiotic strain.

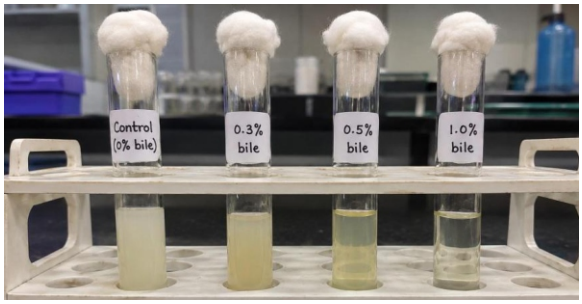

Figure 22. Bile salt tolerance assay of probiotic isolate D1.

Table No. 08: Bile salt tolerance of probiotic isolate D1

Tube No.	MRS broth(ml)	Bile salt concentration %	Inoculum D1 sample	Incubation	Observation (Turbidity)
Control	5.0	0.0%	0.1ml	37°C, 24h	+++
2.	5.0	0.3%	0.1ml	37°C, 24 h	++
3.	5.0	0.5%	0.1ml	37°C, 24h	+
4.	5.0	1.0%	0.1ml	37°C, 24 h	Slight turbidity ±

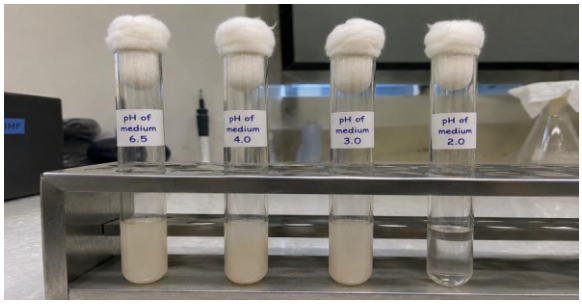

Figure 23. Acid tolerance assay of probiotic isolate D1

Table No.09: Acid tolerance of probiotic isolate D1

Tube No.	pH of Medium	MRS Broth (mL)	Inoculum (mL)	Incubation Conditions	Observation	Result
Control	6.5	5.0	0.1	37°C for 24 h	Heavy turbidity	+++
2	4.0	5.0	0.1	37°C for 24 h	Heavy turbidity	+++
3	3.0	5.0	0.1	37°C for 24 h	Moderate turbidity	++
4	2.0	5.0	0.1	37°C for 24 h	Slight turbidity	±

3.8 Minimum Inhibitory Concentration of Probiotic Isolate

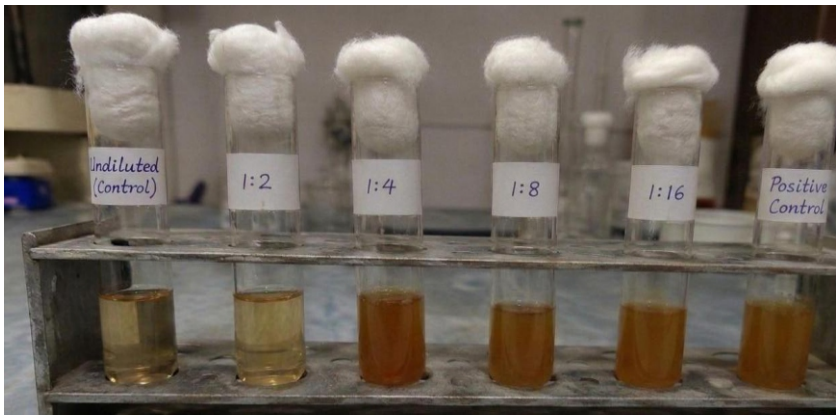

Figure 24. Minimum inhibitory concentration (MIC) assay of probiotic isolate D1 against Staphylococcus aureus.

Observation: The antimicrobial effectiveness of probiotic isolate D1 against *Staphylococcus aureus* was evaluated by broth dilution assay. The isolate exhibited complete inhibition of bacterial growth at 75 µL/mL, which was considered the MIC value.

Table No.10: Minimum inhibitory concentration (MIC) of probiotic isolate D1 against Staphylococcus aureus.

Tube No	Dilution	Concentration ul/ml	Amount of extract (ml)	Amount of diluent (ml)	Amount of Culture (ml)	Observation
1	Undiluted	100	4	_	0.1	_
2	01:02	75	2	2	0.1	_
3	01:04	50	2	2	0.1	+
4	01:08	25	2	2	0.1	++
5	01:16	12.5	2	2	0.1	++
Positive Control	_	_	2	2	0.1	+++
Negative control	_	_	2	2	_	_
Media control	_	_	2	2	_	_

3.9 Screening for L-Asparaginase and Streptokinase Production

For enzyme production studies, selected microbial isolates were screened on substrate-specific media containing L-asparagine and fibrin to detect L-asparaginase and streptokinase production, respectively. Colonies showing clear halo zones were selected and subjected to submerged fermentation. After incubation, the culture broth was centrifuged, and the crude enzyme present in the supernatant was directly used for enzyme activity assays without further purification. Enzyme activity was measured using Nessler's reagent assay for L-asparaginase and fibrin clot lysis or fibrin plate assay for streptokinase.

L-Asparaginase Production on L- Asparagine Agar Plate:

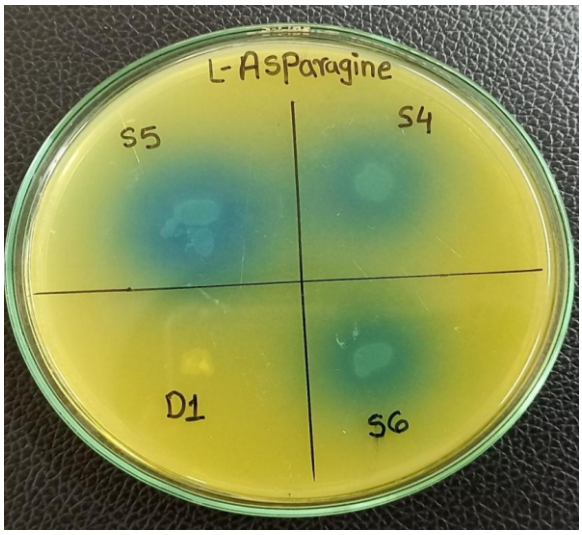

Figure 25. Screening of bacterial isolates for L-asparaginase production on L-asparagine agar

Observation: The selected soil isolates were screened for extracellular enzyme production. All three isolates (S4, S5, and S6) produced distinct blue zones on L-asparagine agar, indicating positive L-asparaginase activity. In contrast, probiotic isolate D1 did not produce any blue zone.

Streptokinase production:

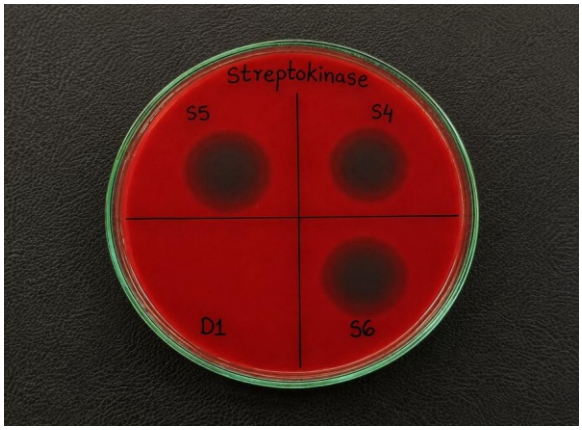

Figure 26. Screening of bacterial isolates for streptokinase production on blood agar

Observation: Similarly, all three soil isolates exhibited clear hemolytic zones on blood agar, confirming streptokinase production. Among them, isolate S6 produced the largest zone of clearance (12 mm), followed by S5 (10 mm) and S4 (8 mm). No streptokinase acti'vity was detected in isolate D1.

3.10 Quantitative Estimation of L-Asparaginase Activity

L-asparaginase activity was further confirmed using Nessler's reagent assay. Progressive colour development was observed with increasing ammonia concentration in the standard solutions. The crude enzyme extract produced an absorbance value of 0.592 at 425 nm, corresponding to an estimated ammonia concentration of approximately 75–80 µg/mL, confirming significant extracellular L-asparaginase activity.

15. Nessler's reagent assay for L- Asparaginase:

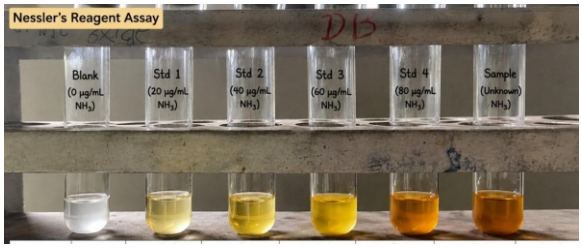

Figure 27. Nessler's reagent assay for L-asparaginase activity

Table No.11: Standard curve and absorbance values for the Nessler's reagent assay

Tube	Ammonia concentration ug/ml	Absorbance at 425nm
Blank	0	0.000
Standard 1	20	0.115
Standard 2	40	0.248
Standard 3	60	0.392
Standard 4	80	0.538
Unknown	Unknown	0.592

3.11 Streptokinase Activity

Streptokinase activity was confirmed by the fibrin clot lysis assay. Complete clot lysis was observed in the positive control, whereas the negative control remained intact. Among the bacterial isolates, S4 exhibited the highest fibrinolytic activity, followed by S5 and S6. No clot degradation was observed with probiotic isolate D1.

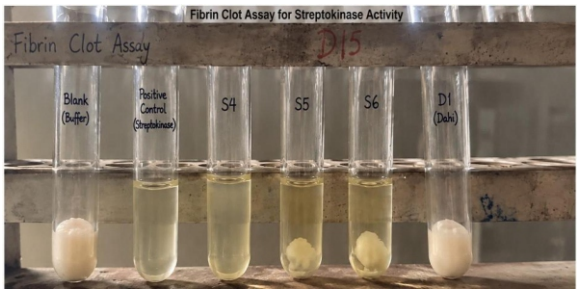

Figure 28. Fibrin clot assay for streptokinase activity

Table No.12: Fibrin clot lysis assay for streptokinase activity

Tube No.	Treatment	Observation after incubation for 90min at 37C	Interpretation
1	Positive Control	Complete clot lysis	Positive
2	Negative Control	Clot remained intact	Negative
3	S4	Maximum clot lysis	Strong positive
4	S5	Moderate clot lysis	Positive
5	S6	Slight to moderate clot lysis	Positive
6	D1	No clot lysis	Negative

3.12 Survival Under Simulated Gastrointestinal Conditions

To evaluate the gut survival potential of the selected probiotic isolate, D1 was exposed sequentially to simulated gastric (pH 2.0–3.0) and intestinal (0.3–1.0% bile salt) conditions. The results of the acid tolerance assay (Figure 50; Table 10) and bile salt tolerance assay (Figure 49; Table 9) demonstrated that isolate D1 remained viable under both acidic and bile salt stress.

Following exposure to the simulated gastrointestinal conditions, the treated culture was streaked onto MRS agar plates to assess bacterial viability. Visible colony growth was observed after incubation, confirming that isolate D1 survived the sequential exposure to acidic pH and bile salts and retained its viability under simulated gastrointestinal conditions.

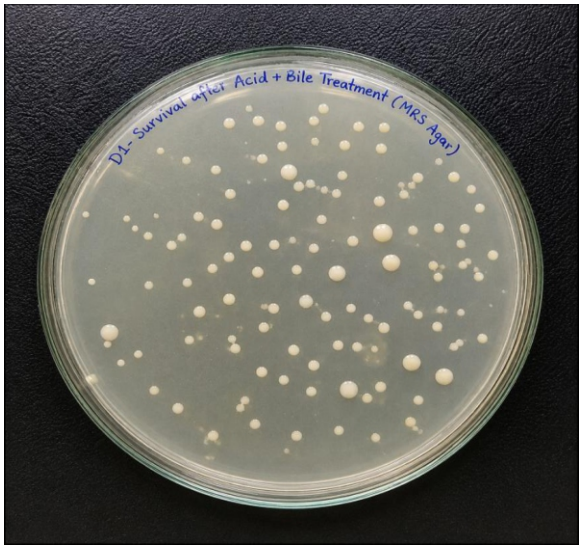

Figure 29. Survival of probiotic isolate D1 on MRS agar after exposure to simulated gastrointestinal conditions

The ability of isolate D1 to survive under simulated gastric and intestinal conditions indicates its potential to withstand passage through the human gastrointestinal tract. This characteristic is an essential criterion for probiotic microorganisms and suggests that isolate D1 may contribute to gut health improvement by surviving gastrointestinal transit and maintaining viability in the intestinal environment.

4. Conclusion

This study demonstrated that medicinal plant rhizosphere soils and fermented dairy products are rich sources of beneficial microorganisms with promising pharmaceutical, industrial, and probiotic applications. The isolated *Bacillus spp.* showed effective antimicrobial activity against *Staphylococcus aureus* and produced the valuable enzymes L-asparaginase and streptokinase, highlighting their biotechnological potential. The probiotic isolate, presumptively identified as *Lactobacillus* spp., exhibited desirable probiotic traits, including tolerance to acidic pH and bile salts, antimicrobial activity, and survival under simulated gastrointestinal conditions.

These findings emphasize the potential of naturally occurring microorganisms as sustainable sources of novel antimicrobial compounds, industrial enzymes, and probiotic cultures. Exploring diverse natural habitats can provide valuable microbial resources to address challenges such as antimicrobial resistance and the growing demand for eco-friendly bioproducts.

Since the isolates were identified using morphological and biochemical methods, molecular identification through 16S rRNA gene sequencing is recommended for accurate taxonomic confirmation. Future research should focus on characterizing the bioactive compounds, optimizing enzyme production, evaluating probiotic safety and efficacy through in vivo studies, and developing scalable production processes.

The results indicate that *Bacillus spp.* and *Lactobacillus spp*. isolated from natural sources possess significant antimicrobial, enzymatic, and probiotic properties, making them promising candidates for applications in healthcare, food biotechnology, pharmaceuticals, and industrial biotechnology.

5. Future Perspectives

Although the present study demonstrated the potential of Bacillus spp. and Lactobacillus spp. as sources of antimicrobial compounds, industrial enzymes, and probiotic candidates, further investigations are required to validate and expand these findings. Molecular identification using 16S rRNA gene sequencing and whole-genome analysis should be performed to confirm the taxonomic identity of the selected isolates and identify genes associated with antimicrobial metabolite production, enzyme synthesis, and probiotic functionality.

Future studies should focus on the purification and structural characterization of antimicrobial metabolites, optimization of fermentation conditions for enhanced enzyme production, and evaluation of enzyme stability and kinetics. In addition, comprehensive safety assessment, antibiotic susceptibility profiling, intestinal adhesion studies, and in vivo evaluation of the probiotic isolate are necessary before therapeutic or commercial applications can be considered.

The antimicrobial activity of the isolates should also be investigated against a broader range of clinically relevant multidrug-resistant pathogens. Furthermore, large-scale fermentation, downstream processing, and formulation studies are required to assess the commercial feasibility of these microbial strains. Exploring diverse ecological niches using advanced genomic and metabolomic approaches may facilitate the discovery of novel microorganisms with enhanced biotechnological potential and contribute to the development of sustainable products for healthcare, pharmaceutical, food, and industrial applications.

6. Declaration of Language Editing

The authors used Grammarly solely for language editing to improve the grammar, clarity, readability, and overall presentation of the manuscript. The tool was not used for experimental design, data collection, data analysis, interpretation of results, or generation of scientific content. All scientific work, including data interpretation, manuscript preparation, and final approval, was carried out by the authors, who take full responsibility for the accuracy and integrity of the study.

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