International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

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Probiotic and antimicrobial characterization of bacterial isolates from poultry waste-contaminated soil

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International Journal of Science and Research Archive, 2025, 16(01), 544-553

Publication history: Received on 25 May 2025; revised on 05 July 2025; accepted on 07 July 2025

Article DOI: https://doi.org/10.30574/ijsra.2025.16.1.2018

Abstract

Soil contaminated with poultry waste may harbor probiotic bacteria with beneficial traits. This study to evaluate proteolytic probiotic bacteria from such soil for potential use in health and biotechnology applications. Six isolates previously isolated, identified and screened for proteolytic potential were used. Probiotic screening included antibiotic susceptibility (disc diffusion), hemolytic activity on blood agar, bile resistance at 0.1–2.0% bile salt, and acid tolerance at pH 1.5 to 3.5. Growth responses were evaluated via CFU count and optical density. *Bacillus subtilis* strain NBT-15, *Bacillus amyloliquefaciens* strain FORCN102, *LactoBacillus plantarum* strains ML05 and B19, and *LactiplantiBacillus plantarum* strains KCB4 and S4 were identified. The acid tolerance test revealed that all isolates were able to survive highly acidic conditions ranging from pH 1.5 to 3.5. The highest viable count was observed in *LactoBacillus plantarum* strain ML05 at pH 3.5 with 1.5×10^6 CFU/mL, while *Bacillus subtilis* strain NBT-15 had 2.3×10^3 CFU/mL at pH 1.5, indicating strong acid resistance. In the bile resistance assay, isolates demonstrated moderate to high survival at bile concentrations from 0.1% to 2.0%. *LactoBacillus plantarum* strain B19 exhibited the highest resistance with 98.99% at 0.1% bile and 44.21% at 2.0%, while *LactiplantiBacillus plantarum* strain S4 showed 94.29% resistance at 0.1% and 28.76% at 2.0%. Antibiotic susceptibility profiling showed that *Bacillus amyloliquefaciens* strain FORCN102 was susceptible to all ten antibiotics tested, with inhibition zones of 16 mm for levofloxacin, 15 mm for rifampicin and erythromycin, and 14 mm for gentamicin, suggesting low antibiotic resistance. In contrast, *LactoBacillus plantarum* strains ML05 and S4 were completely resistant to all antibiotics tested. Hemolysis tests revealed that *LactoBacillus* and *LactiplantiBacillus* strains were non-hemolytic, while *Bacillus subtilis* strain NBT-15 and *Bacillus amyloliquefaciens* strain FORCN102 exhibited alpha-hemolysis. Antibacterial assays showed varying degrees of inhibition against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*. *LactoBacillus plantarum* strains, produced the highest zone of inhibition of 12 mm against *E. coli, S. aureus* and *S. typhi*, while *Bacillus amyloliquefaciens* strain FORCN102 showed inhibition zones of 2 mm against *E. coli* which was the lowest among all. The findings suggest that poultry waste-contaminated soil is a viable source of proteolytic probiotic bacteria with promising functional properties for potential therapeutic and industrial applications.

Keyword: Proteolytic Probiotic Bacteria; Acid Tolerance; *Bacillus subtilis*; *LactoBacillus plantarum*; Antibacterial Activity; Poultry Waste

1. Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host, primarily by maintaining or restoring the natural balance of gut microbiota (Hill *et al*., 2014). These beneficial microbes play vital roles in enhancing immune function, modulating gut microbial ecology, improving nutrient absorption, and providing protection against pathogenic organisms in both humans and animals (Sanders *et al*., 2018). In animal husbandry, probiotics have been explored as effective alternatives to antibiotics, promoting growth performance and

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improving feed conversion efficiency, especially in poultry and livestock farming (Markowiak and Śliżewska, 2018). Among the functional traits desirable in probiotic strains, proteolytic activity which is the ability to break down proteins into peptides and amino acids, is of particular importance. Proteolytic enzymes produced by probiotics aid in protein digestion, enhance nutrient bioavailability, and contribute to the synthesis of bioactive peptides with health-promoting properties such as antimicrobial, antioxidant, and immunomodulatory activities (Cichońska and Złotkowska, 2018). Additionally, the proteolytic system of probiotics supports their adaptation and survival in gastrointestinal environments where dietary proteins serve as nitrogen sources (González-Rodríguez *et al*., 2013).

Soils contaminated with poultry waste represent a unique ecological niche teeming with microbial life, including bacteria capable of surviving and thriving under nutrient-rich, protein-loaded conditions. Poultry waste, comprising of feces, feathers, feed residues, and bedding materials is rich in organic matter, especially nitrogenous compounds, making it a potential reservoir for isolating bacteria with high proteolytic capacity (Al-Masri, 2021). Several studies have demonstrated the presence of *LactoBacillus, Bacillus*, and other probiotic genera in poultry environments, highlighting their adaptability and metabolic versatility (Kabir, 2009).

However, the indiscriminate disposal of poultry waste poses serious environmental challenges, including water and soil pollution, greenhouse gas emissions, and the proliferation of antibiotic-resistant bacteria due to residual antibiotics in poultry litter (Sharma *et al*., 2020). This growing concern has intensified research into microbial bioremediation strategies, where selected bacteria are employed to degrade organic pollutants, neutralize toxic compounds, and restore ecological balance (Sharma and Singh, 2021). Isolating functional microbes, particularly proteolytic probiotics from such waste environments, not only addresses the bioremediation goal but also supports sustainable agriculture and biotechnological innovation. Thus, this study aims to screen for proteolytic probiotic bacteria isolated from poultry waste-contaminated soil, evaluating their potential for use in environmental management and health-related applications.

2. Materials and methods

2.1. Source of test organisms

The proteolytic test organisms were obtained from stock cultures previously isolated and characterized by Makuachukwu and George-Okafor. (2025).

2.2. Probiotic Screening of the Isolates

2.2.1. Standardization of Inoculum

McFarland turbidity standard was prepared by dissolving 1ml of barium chloride ($BaCl_2$) into 9ml of sulphuric acid (H_2SO_4). Then, pure cultures of identified bacterial isolates from a 24hour plate culture were selected. Sterile wire loop was used to pick small colonies of each isolate and emulsified into test tubes containing 5ml of sterile saline, they were vortexed thoroughly. Adjustment was made with extra inoculums or diluents, until 0.5 McFarland turbidity standards were obtained (Meena *et al.,* 2015).

2.2.2. Antibiotics resistance test

The antibiotic susceptibility of the isolates was examined by disc diffusion technique. The 24 hr old culture was swabbed on MRS agar plates and Mueller Hinton Agar for *LactoBacillus* sp. and *Bacillus sp*. Antibiotic impregnated discs were placed onto these inoculated plates. These plates were incubated at 37°C for 24 h. Zone of inhibition was observed after 24 hr. Resistance was assessed against Gentamicin (10 µg), Azithromycin (30 µg), Cefuroxime (30 µg), Amoxil (10 µg), Rifampicin (30 µg), Ciprofloxacin (10 µg), Ceftazidime (30 µg), Erythromycin (10 µg), Streptomycin (10 µg), Levoflaxacin (10 µg) (Bauer *et al*., 1966).

2.2.3. Hemolytic activity

The modified method of Akinjogunla and Enabulele, (2010) was utilized. A loopful 18/24h culture of *each of the isolates* was singly inoculated by spotting the culture onto the blood agar plates and incubated at 37°C for 24hrs. Thereafter, plates were observed for hemolysis by the development of zones of inhibition. The zone of inhibition of 1 mm or above including the diameter of smeared area was taken as a positive result.

2.2.4. Bile Resistance Assay

A modified version of the method described by Belicová *et al*. (2013) was adopted to evaluate the survival rate of six isolates in the presence of bile. The modification involved using a wider range of bile concentrations (0.1–2.0% taurodeoxycholic acid). Each test organism (2 g biomass, ~6.5–8.2 × 10^8 CFU/mL) was grown in basal medium with a single bile concentration and incubated at 37 °C for 18–24 h. Basal media without bile served as controls. Survival was assessed by transferring 10% (v/v) of each treated culture into fresh basal broth and monitoring growth spectrophotometrically at 600 nm.

2.2.5. Acid Tolerance Test

For the acid tolerance assay, nutrient broth (for *Bacillus* spp.) and MRS broth (for *LactoBacillus* spp.) were prepared. Citrate buffer solutions were adjusted to pH 1.5, 2.0, 2.5, 3.0, and 3.5 using 1 N HCl or 1 N NaOH, following the method of Cotter and Hill (2003). Individual isolates were inoculated into their respective broth media and incubated at 30 °C for 3 h. post-incubation, 0.1 mL of culture was plated onto nutrient agar or MRS agar and incubated at 30 °C for 24–48 h. Colonies were counted and expressed as CFU/mL.

3. Result

Table 1 Antibiotic Susceptibility and Antibacterial Effect of the Selected Proteolytic Bacteria

		Antibiotics										Pathogens		
SN	**Isolate**	**CN**	**CEF**	**RD**	**CTZ**	**S**	**AZM**	**AMX**	**CPX**	**E**	**LEV**	***E. coli* (mm)**	***S. aureus* (mm)**	***S. typhi* (mm)**
1	*Bacillus subtilis* strain NBT-15	11	5	13	9	16	10	13	12	13	14	10	8	8
2	*Bacillus amyloliquefaciens* strain FORCN102	14	7	15	12	15	12	13	10	15	16	2	8	5
3	*LactoBacillus plantarum* strain ML05	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	12	9	6
4	*LactoBacillus plantarum* strain B19	13	(0)R	(0)R	(0)R	12	11	(0)R	8	(0)R	16	10	12	8
5	*LactiplantiBacillus plantarum* strain KCB4	11	(0)R	(0)R	(0)R	10	(0)R	9	10	(0)R	15	10	8	12
6	*LactiplantiBacillus plantarum* strain S4	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	(0)R	11	12	8

Key to Antibiotics; CN = Gentamicin; CEF = Cefuroxime; RD = Rifampicin; CTZ = Ceftazidime; S = Streptomycin; AZM = Azithromycin; AMX = Amoxil; CPX = Ciprofloxacin; E = Erythromycin; LEV = Levofloxacin; R = (Resistant)

Table 2 Hemolytic and Bile Concentration Effect of the Selected Proteolytic Bacteria

SN	**Isolate**	**Hemolytic Activity**	**0.1% Bile**	**0.2% Bile**	**0.3% Bile**	**0.6% Bile**	**1.0% Bile**	**2.0% Bile**
1	*Bacillus subtilis* strain NBT-15	+	96.59	94.92	93.26	68.44	60.37	40.72
2	*Bacillus amyloliquefaciens* strain FORCN102	+	97.12	94.42	90.11	63.31	46.04	34.80
3	*LactoBacillus plantarum* strain ML05	-	91.46	84.60	78.66	73.86	67.53	40.78
4	*LactoBacillus plantarum* strain B19	-	98.99	98.15	96.81	80.29	73.41	44.21
5	*LactiplantiBacillus plantarum* strain KCB4	-	96.09	92.53	89.41	84.25	73.31	37.63
6	*LactiplantiBacillus plantarum* strain S4	-	94.29	90.48	87.62	85.90	75.81	28.76

Table 3 Acid Tolerance Effect of the Selected Proteolytic Bacteria

pH	***Bacillus subtilis* strain NBT-15 (CFU/mL)**	***Bacillus amyloliquefaciens* strain FORCN102 (CFU/mL)**	***LactoBacillus plantarum* strain ML05 (CFU/mL)**	***LactoBacillus plantarum* strain B19 (CFU/mL)**	***LactiplantiBacillus plantarum* strain KCB4 (CFU/mL)**	***LactiplantiBacillus plantarum* strain S4 (CFU/mL)**
1.5	2.3×10^3	1.8×10^3	4.1×10^3	3.2×10^3	2.9×10^3	3.5×10^3
2.0	3.5×10^4	2.6×10^4	7.5×10^4	6.0×10^4	5.8×10^4	6.2×10^4
2.5	6.8×10^4	5.0×10^4	8.9×10^4	7.3×10^4	7.1×10^4	8.5×10^4
3.0	9.1×10^5	8.7×10^5	1.4×10^5	1.1×10^5	1.0×10^5	1.3×10^5
3.5	1.1×10^6	1.2×10^6	1.5×10^6	1.3×10^6	1.2×10^6	1.4×10^6

4. Discussion

The results of the antibiotic susceptibility testing and antibacterial activity against common pathogens (*Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*) revealed a differential resistance and inhibition profile among the six proteolytic bacterial isolates obtained from poultry waste-contaminated soil. Notably, *Bacillus subtilis* strain NBT-15 and *Bacillus amyloliquefaciens* strain FORCN102 demonstrated susceptibility to a broad range of antibiotics and exhibited moderate to strong antibacterial activity. Conversely, *LactoBacillus plantarum* strains ML05 and S4 showed complete resistance to all tested antibiotics, although they retained some inhibitory activity against the test pathogens.

From the result *Bacillus* species showed greater antibiotic susceptibility and broader-spectrum antibacterial effects compared to *LactoBacillus* and *LactiplantiBacillus* strains. For example, *Bacillus amyloliquefaciens* FORCN102 showed high susceptibility to Gentamicin (CN, 14 mm), Rifampicin (RD, 15 mm), Levofloxacin (LEV, 16 mm), and Erythromycin (E, 15 mm), which is consistent with the genus's known antimicrobial profile and intrinsic antibiotic production capacity (Mandic-Mulec *et al*., 2015). In contrast, *LactoBacillus plantarum* ML05 exhibited resistance to all antibiotics tested, yet it produced clear inhibition zones against E. coli (12 mm), S. aureus (9 mm), and S. typhi (6 mm), indicating its potential production of bacteriocins or organic acids, which are commonly secreted by lactic acid bacteria (Klaenhammer, 1993; Gänzle, 2015). Strains B19, KCB4, and S4, although partly or fully resistant to antibiotics, showed moderate inhibition zones, particularly against S. aureus and S. typhi. This divergence suggests that antibiotic resistance in probiotic strains does not necessarily correlate with diminished antimicrobial potential, which aligns with reports by Sharma *et al*. (2020), who observed high bile salt- and acid-tolerant *LactoBacillus* isolates with selective antibiotic resistance and antimicrobial activity.

The observed resistance of *LactoBacillus* and *LactiplantiBacillus* strains to most antibiotics, including cefuroxime, ceftazidime, and ciprofloxacin, is both promising and concerning. From a probiotic perspective, intrinsic antibiotic resistance can enable these strains to survive alongside antibiotic therapy, providing continuous gut protection (Imperial and Ibana, 2016). However, their resistance profile raises biosafety concerns about horizontal gene transfer, especially when such strains are applied in food or therapeutic formulations (Mathur and Singh, 2005). The strong inhibition observed among *Bacillus* strains supports their potential for use in bio-control or therapeutic applications. Their dual capacity to act as probiotics and antimicrobial agents makes them ideal candidates for use in poultry farming as alternatives to growth-promoting antibiotics (Elshaghabee *et al*., 2017).

The findings are consistent with previous research on *Bacillus* species showing both high antibiotic sensitivity and antimicrobial activity (Cutting, 2011). For instance, *Bacillus subtilis* has been reported to inhibit Gram-positive and Gram-negative pathogens due to lipopeptide and peptide antibiotic production (Stein, 2005). Similarly, the resistance of *LactoBacillus plantarum* strains to cephalosporins and fluoroquinolones mirrors observations in clinical and food-derived isolates, as described by Nawaz *et al*. (2011). Moreso, the ability of resistant *LactoBacillus* strains to inhibit pathogens aligns with the functional model proposed by Corr *et al*. (2007), where *LactoBacillus*-mediated pathogen exclusion is facilitated by competitive adhesion, immune modulation, and bacteriocin production rather than traditional antibiotic mechanisms.

Table 2 presents result on the hemolytic activity and bile salt tolerance of six proteolytic bacterial isolates. Hemolysis was used to assess safety (non-pathogenicity), while resistance to increasing bile concentrations (0.1% to 2.0%) tested probiotic robustness in gut-like conditions. Among the isolates, only the two *Bacillus* strains (NBT-15 and FORCN102) exhibited positive hemolytic activity, suggesting potential safety concerns. The four *LactoBacillus* and *LactiplantiBacillus* strains showed non-hemolytic activity, which is desirable for probiotics. All strains exhibited decreasing viability with increasing bile concentration, but many retained over 70% resistance at lower concentrations (0.1–1.0%).

The hemolysis test revealed a clear genus-level trend: *Bacillus* strains were hemolytic (+), while *LactoBacillus* and *LactiplantiBacillus* strains were non-hemolytic (−). This is in line with known characteristics of *Bacillus* spp., some of which produce cytolytic toxins (Stein, 2005; Elshaghabee *et al*., 2017), while lactic acid bacteria (LAB) are generally safe and non-pathogenic (FAO/WHO, 2002). Bile tolerance declined progressively with increased bile concentrations in all isolates. For instance, B. subtilis NBT-15 had 96.59% resistance at 0.1% bile, dropping to 40.72% at 2.0%, a similar trend observed in *B. amyloliquefaciens* FORCN102. Interestingly, the *LactoBacillus plantarum* strain B19 showed the highest bile tolerance, maintaining >98% resistance up to 0.3% and still retaining 44.21% at 2.0%, suggesting strong intestinal survivability. Among the LAB, L. plantarum S4 had the lowest bile resistance at 2.0% (28.76%), although still acceptable by probiotic standards. This pattern reflects the expected physiological challenge bile salts impose by disrupting bacterial membranes and proteins (Begley *et al*., 2005). Strains with better tolerance may possess bile salt hydrolase (BSH) activity or cell envelope adaptations enabling survival in gastrointestinal conditions.

From a probiotic development perspective, non-hemolytic behavior and bile resistance are core safety and functional indicators (Sanders *et al*., 2010). The non-hemolytic status of all *LactoBacillus* and *LactiplantiBacillus* strains confirms their biosafety for probiotic use. Moreover, their high bile tolerance, especially up to 1.0%, aligns with the bile salt concentrations found in poultry birds' small intestine (0.3–0.5%) (Gilliland *et al*., 1984), supporting their potential survivability and colonization capacity in vivo. In contrast, *Bacillus* spp., despite good bile resistance, may pose safety issues due to hemolytic activity and should undergo further safety screening before application. However, some *Bacillus subtilis* strains are already GRAS-certified and used in commercial probiotics after thorough toxicological evaluation (Hong *et al*., 2005), indicating that not all hemolytic *Bacillus* strains are necessarily harmful. The observed non-hemolytic behavior of LAB strains aligns with numerous studies reporting *LactoBacillus plantarum* as a non-pathogenic, safe probiotic species (Tamang *et al*., 2020). *LactoBacillus plantarum* strains isolated from fermented foods and gut microbiota have shown comparable bile resistance levels (Sharma *et al*., 2020), further validating these findings.

In comparison, *Bacillus* strains with hemolytic activity have been previously reported by Stein (2005) and should be cautiously evaluated. Nevertheless, Elshaghabee *et al*. (2017) emphasized that hemolysis alone is not definitive of pathogenicity; genetic profiling and toxin testing are needed to confirm safety. Furthermore, the declining trend in viability with increasing bile concentrations is consistent with observations by Hyronimus *et al*. (2000), who noted strain-specific variability among LAB and *Bacillus* in response to bile salts. The exceptional bile tolerance of L. plantarum B19 supports its candidacy as a robust probiotic, similar to findings by Argyri *et al*. (2013), who reported L. plantarum strains from fermented olives with high bile and acid resistance.

Table 3 evaluates the acid tolerance of six proteolytic bacterial isolates across pH levels ranging from 1.5 to 3.5, simulating stomach-like conditions. All isolates showed a progressive increase in total viable count (TVC) with rising pH. At extremely acidic pH (1.5), all isolates survived with reduced counts (10^3 CFU/mL range), while at pH 3.5, they achieved maximum growth (10^6 CFU/mL range). Among the strains, *LactoBacillus plantarum* ML05 and *LactiplantiBacillus plantarum* S4 exhibited the highest tolerance, with superior viability even at low pH values.

A general trend is observable: as pH increased, bacterial viability improved across all isolates. This is expected, as low pH (≤2.0) mimics the harsh gastric environment, which significantly stresses microbial membranes and intracellular components (Charteris *et al*., 1998). However, survival at pH 1.5, although low, confirms acid resistance — a crucial probiotic trait (Lee and Salminen, 1995). L. plantarum ML05 showed 4.1×10^3 CFU/mL at pH 1.5 and steadily increased to 1.5×10^6 CFU/mL at pH 3.5, demonstrating consistent acid resilience. Similarly, L. plantarum B19, KCB4, and S4 maintained growth trends across acidic conditions, with only marginal viability loss at pH 1.5. Notably, *Bacillus* strains such as B. subtilis NBT-15 and B. amyloliquefaciens FORCN102 also tolerated low pH levels well, though their growth at pH 3.0 and 3.5 remained slightly lower than that of LAB strains. These observations suggest that all isolates possess adaptive mechanisms to acidic stress, such as proton extrusion systems, acid-shock proteins, and cell wall integrity preservation (Papadimitriou *et al*., 2016).

The ability of these bacterial isolates to survive at low pH levels highlights their potential to endure gastric transit and reach the intestine alive — a prerequisite for probiotics (FAO/WHO, 2002). This is particularly important for oral probiotic formulations, where stomach acid is the first major physiological barrier. The outstanding acid tolerance of L. plantarum ML05 and S4 implies they may be suitable candidates for probiotic development, especially for use in fermented foods or dietary supplements targeting gut health. Additionally, *Bacillus* strains that survive acid exposure could be leveraged in spore-forming probiotic applications, where resistance to harsh conditions is advantageous (Hong *et al*., 2005). However, while acid tolerance is essential, it must be assessed in conjunction with bile salt tolerance, hemolytic activity, and antimicrobial efficacy to determine overall probiotic fitness and safety.

These findings are consistent with previous studies indicating that *LactoBacillus plantarum* strains possess excellent acid tolerance, particularly those isolated from fermented foods and animal guts (Sharma *et al*., 2020; Tamang *et al*., 2020). For instance, Argyri *et al*. (2013) reported L. plantarum strains from table olives surviving at pH 2.0–3.0 with minimal loss in viability. Furthermore, *Bacillus* strains have been described as moderately acid-tolerant, with some spore-forming species demonstrating remarkable survivability under gastric-like conditions (Cutting, 2011). The data from NBT-15 and FORCN102 support this, although LAB strains still outperformed them in terms of cell viability under increasing pH. Papadimitriou *et al*. (2016) noted that acid-tolerant LAB typically express multiple stress response genes, a feature likely shared by the high-performing isolates in this study.

5. Conclusion

This study highlights the probiotic potential of proteolytic bacterial strains isolated from poultry waste-contaminated soil, particularly members of the *LactoBacillus* and *Bacillus* genera. These isolates demonstrated essential probiotic

characteristics, including tolerance to acidic and bile environments, non-hemolytic activity, and inhibitory effects against common pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*. Such traits suggest their potential for survival and functionality within the gastrointestinal tract. The findings affirm that poultry waste-contaminated soil can serve as a promising reservoir for isolating beneficial microorganisms with both proteolytic and probiotic properties. These isolates may contribute to animal gut health, waste bioconversion, and possibly reduce reliance on synthetic antibiotics. Nonetheless, further molecular and in vivo validation is necessary to establish their safety and effectiveness before commercial or therapeutic application.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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