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Novel Lactic Acid Bacteria from Multi-Age Piglets: In Vitro Probiotic Traits and Antibacterial Effects Against *Escherichia coli*

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Abstract

Lactic acid bacteria (LAB) are important probiotics that support intestinal health and growth performance in pigs through modulation of gut microbiota and production of antimicrobial compounds. This study aimed to isolate and evaluate LAB with probiotic potential from feces of healthy, antibiotic- and probiotic-free piglets of different ages. Forty-two fecal samples were collected from a farm in Lamphun province, yielding 318 LAB isolates, of which 135 gram-positive, catalase-negative bacilli were characterized for probiotic traits. Only two isolates (PMvet212 and PMvet318) survived at pH 3.1 with a viability loss of less than 1 log CFU/mL; both tolerated 0.3% bile salt, and PMvet212 also survived at 0.5% bile salt. Their hydrophobicity values were 7.85% and 12.38%, respectively, indicating low adhesion capacity. Both isolates showed alpha-hemolysis. Cell-free supernatants of these isolates inhibited *Escherichia coli* from diarrheic piglets and *Staphylococcus aureus* ATCC6538, with inhibition zones classified as intermediate. These findings indicate that LAB from piglet feces, particularly PMvet212, possess moderate probiotic potential and antibacterial activity, and may serve as candidates for development as feed additives to promote swine gut health and reduce reliance on antibiotics.

Keywords: piglet feces; lactic acid bacteria (LAB); probiotic traits; antimicrobial effects; *Escherichia coli*

1. Introduction

Escherichia coli (*E. coli*) is one of the most important pathogens causing diarrhea in neonatal and weaned piglets. Piglet diarrhea leads to substantial economic losses in the swine industry due to increased morbidity and mortality, reduced growth performance, and higher veterinary costs [1]. Prevention is generally more effective than treatment, yet traditional control strategies have relied heavily on antibiotics [2]. This dependence has raised serious concerns about antibiotic residues in meat and the global spread of antimicrobial resistance (AMR) [3]. As regulatory authorities and consumers increasingly demand reductions in antibiotic use, there is a growing need for sustainable alternatives that can maintain animal health and productivity without contributing to AMR [4]. Several alternative approaches have been proposed, including organic acids, copper sulfate, zinc oxide, prebiotics, herbal supplements, and probiotics. Among these, probiotics have attracted particular attention for their ability to promote intestinal health, reduce the incidence and severity of enteric diseases, and support growth performance in pigs, while reducing the need for antibiotics [5]. Probiotic supplementation has been shown to modulate the gut microbiota, enhance immune function, and improve nutrient utilization in swine. Recent studies have confirmed that probiotics can serve as viable antibiotic alternatives for preventing bacterial diarrhea in piglets, with benefits

extending to improved feed efficiency and overall health [6]. Moreover, probiotic use aligns with the One Health framework by mitigating AMR risks at the animal–human–environment interface [7].

At birth, the gastrointestinal tract of animals is sterile but rapidly colonized by microorganisms from the mother and the surrounding environment. This early colonization plays a critical role in the development of the digestive system, metabolic functions, and immune competence of neonates [8]. The gut microbiota's composition is shaped by factors such as delivery mode, maternal microbiota, diet, and environmental exposure [9]. In pigs, a diverse and balanced gut microbiota supports resistance to pathogens, adaptation to dietary changes, and improved physiological functions [10]. The porcine gastrointestinal tract is anatomically compartmentalized, with the small intestine— particularly the duodenum, jejunum, and ileum—being the main site for nutrient digestion and absorption. Maintaining a healthy microbiota in these regions is critical for growth and disease prevention, especially in intensive swine production systems.

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits to the host by improving the microbial balance in the gastrointestinal tract (FAO/WHO, 2002). Effective probiotics must survive exposure to gastric acid and bile salts, adhere to intestinal epithelial cells, and exhibit antagonistic effects against pathogens [11]. In swine production, probiotics are used both to support growth and to prevent colonization by enteric pathogens such as Salmonella spp., *E. coli*, and Clostridium perfringens [12]. Lactic acid bacteria (LAB) are among the most widely studied probiotic groups due to their non-pathogenic nature, acid and bile tolerance, and ability to produce antimicrobial substances such as organic acids, hydrogen peroxide, and bacteriocins [13]. These antimicrobial compounds are considered safe and, in some cases, have been linked to reduced pathogen load and improved gut health in pigs [14].

The probiotic properties of LAB are highly strain-specific, even among isolates belonging to the same species [15]. Only a limited number of strains with strong acid and bile tolerance, adhesion ability, and potent antimicrobial activity have been identified, and their functional properties often vary depending on the host and environmental context. Recent advances in microbiome analysis and targeted isolation techniques have enabled the discovery of novel LAB strains from indigenous pig breeds and different rearing environments, some of which show promise for improving gut integrity, modulating immunity, and reducing pathogenic *E. coli* colonization [16]. However, further research is needed to characterize these strains comprehensively and assess their potential as antibiotic alternatives in commercial pig production.

Therefore, the aim of the present study was to isolate and characterize LAB with probiotic potential from fecal samples of pigs at different ages raised without antibiotics or probiotics. The focus was on identifying strains that could survive under gastrointestinal conditions, exhibit inhibitory activity against pathogenic *E. coli*, and demonstrate other desirable probiotic traits. The findings of this work provide a basis for selecting promising LAB strains for development as feed additives to promote swine gut health, reduce reliance on antibiotics, and contribute to the global effort to mitigate AMR in livestock production.

2. Materials and Methods

2.1. Ethical Approval

Since fecal samples were collected without handling or restraining the animals, specific approval for animal use was not required. However, the experimental procedures involved the use of microbial isolates. Therefore, all microbiological work was reviewed and approved by the Institutional Biosafety Committee (IBC) of Chiang Mai University (Approval No. CMUIBC A-0763004).

2.2. Sample Collection

The study population comprised suckling piglets aged 7–30 days. The required sample size was estimated based on an assumed Lactobacillus detection prevalence of 50%, a total piglet population of 2,800, an effect size of 0.3, statistical power $(1 - \beta)$ of 0.98, and an alpha error probability of 0.02,

using G*Power software (version 3.1). The minimum calculated sample size was 40; therefore, 42 fecal samples were collected to ensure adequate statistical power. Samples were obtained from a commercial pig farm in Lamphun province where no antibiotics or probiotics had been used. To maximize diversity, no more than five samples were taken from any single farrowing pen. Freshly voided feces with normal consistency and yellow to brown coloration were collected from the pen floor, placed in sterile 5 mL microcentrifuge tubes (≥2 g each), and transported at 4 °C to the laboratory for processing.

2.3. Isolation of Lactic Acid Bacteria (LAB)

One gram of fecal sample was homogenized in 9 mL of 0.85% sterile saline and serially diluted (10^{-4} to 10^{-8}). Aliquots (100 μL) were spread onto de Man, Rogosa, and Sharpe (MRS) agar supplemented with 0.5% $CaCO_3$. Plates were incubated anaerobically at 37 °C for 48 h. Colonies producing clear zones were subcultured in MRS broth to obtain pure isolates. Gram-positive, non-spore-forming bacteria were retained and tested for catalase activity; only catalase-negative isolates were preserved at –20 °C in MRS broth with 20% (v/v) sterile glycerol [17].

2.4. Evaluation of Probiotic Properties

2.4.1. Acid Tolerance

Colonies grown on MRS agar were suspended in 5 mL phosphate-buffered saline (PBS; pH 7.2) and adjusted to 0.5 McFarland standard (~1.5×10^8 CFU/mL). One milliliter of suspension was mixed with 9 mL sterile PBS adjusted to pH 2.0 or pH 3.1 using 1 N HCl. The mixtures were vortexed for 10 s and incubated at 37 °C for 3 h. Viable cell counts before and after incubation were determined by plate counting on MRS agar. Acid tolerance was expressed as the reduction in bacterial counts (log CFU/mL) compared with the initial value [17].

2.4.2. Bile Salt Tolerance

Bacterial suspensions prepared as above were inoculated (1 mL) into 9 mL MRS broth supplemented with 0.3%, 0.5%, or 1% (w/v) bile salts (Sigma-Aldrich). Control cultures contained no bile salts. Tubes were incubated anaerobically at 37 °C for 24 h. Viable cell counts were determined at 0 and 24 h, and tolerance was evaluated based on survival relative to controls. Each strain was tested in duplicate [18].

2.4.3. Adhesion Ability to Intestinal Epithelium (Surface Hydrophobicity)

Adhesion potential was assessed by the microbial adhesion to hydrocarbons (MATH) method. Bacteria grown on MRS agar were suspended in 5 mL PBS (pH 7.2) and adjusted to 6.0 McFarland standard. Optical density at 600 nm (OD_{600}) was adjusted to 0.6. Three milliliters of suspension was mixed with 1 mL of xylene in a glass tube, vortexed for 90 s, and left at room temperature for 30 min to allow phase separation. The aqueous phase was carefully removed, and OD_{600} was measured again. Surface hydrophobicity (%H) was calculated as:

$$\%H = [(OD_{600} \text{ before mixing} – OD_{600} \text{ after mixing})/OD_{600} \text{ before mixing}] \times 100$$

Controls consisted of PBS and xylene without bacteria. All assays were performed in duplicate [19].

2.4.4. Hemolytic Activity

For safety evaluation, isolates were streaked on tryptic soy agar (TSA) supplemented with 5% (w/v) sheep blood and incubated at 37 °C for 48 h. Hemolysis type was determined visually: β-hemolysis (clear zone), α-hemolysis (green/brown discoloration), and γ-hemolysis (no change). Staphylococcus aureus ATCC 6538 was used as a β-hemolysis positive [20].

2.4.5. Antimicrobial Activity Against E. coli and S. aureus ATCC 6538

The agar well diffusion method was used. LAB cultures grown in MRS broth at 37 °C for 24 h were centrifuged at 4,000 × g for 5 min, and supernatants were filtered through 0.45 μm membranes to obtain cell-free supernatants (CFS). Indicator strains—pathogenic *E. coli* (from diarrheic piglets) and *Staphylococcus aureus* ATCC 6538—were adjusted to 0.5 McFarland standard in PBS and spread onto nutrient agar plates. Wells (7 mm) were bored aseptically, and 80 μL CFS was added per well. Lactic acid (2% v/v) served as the positive control. Plates were incubated at 37 °C for 24 h, and inhibition zones were measured in millimeters. All tests were performed in duplicate [21].

2.5. Data Interpretation

Probiotic Properties

Acid Tolerance

Survival at acidic pH was assessed by comparing bacterial counts (log CFU/mL) before and after incubation at pH 2.0 and pH 3.1. A reduction of less than 1 log CFU/mL at pH 3.1 was considered acceptable for acid tolerance [22].

Bile Salt Tolerance

Bacterial counts (log CFU/mL) at 0 h and 24 h in MRS broth containing bile salts were compared. Strains were classified as tolerant if no significant reduction in viable counts was observed over the incubation period.

Surface Adhesion Ability

Adhesion potential was expressed as surface hydrophobicity (%H) calculated by the MATH assay. Higher %H values indicated stronger cell surface adhesion capabilities.

Hemolytic Activity

Hemolysis was classified into three types: β-hemolysis, indicating complete lysis of red blood cells and the presence of a clear zone around the colony; α-hemolysis, representing partial lysis with a green or brown discoloration surrounding the colony; and γ-hemolysis, showing no lysis or visible change in the medium. According to safety criteria for probiotic use, LAB strains exhibiting α- or β-hemolysis were considered unsuitable, whereas only γ-hemolytic strains were deemed acceptable [23].

Antimicrobial Activity Against *E. coli* and *S. aureus* ATCC 6538

The antimicrobial potential of the cell-free supernatants (CFS) was assessed by measuring the diameter of the inhibition zones against *Escherichia coli* and *Staphylococcus aureus* ATCC 6538. The results were interpreted according to the criteria summarized in Table 1. This approach allowed for a comparative evaluation of the inhibitory effects of different CFS preparations on the selected pathogenic strains.

Table 1. Interpretation of antimicrobial activity based on inhibition zone diameter.

Symbol	Interpretation	Inhibition Zone Diameter (mm)
-	Non-inhibition	<5
+	Weak inhibition	>5
++	Intermediate inhibition	>10
+++	Strong inhibition	>15
++++	Very strong inhibition	>20

2.6. Statistical Analysis

Data were analyzed using descriptive statistics. Means and standard deviations were calculated where appropriate to summarize the results.

3. Results

3.1. Isolation of Lactic Acid Bacteria (LAB) from Swine Feces

From 42 individual fecal samples collected from pigs of different ages, a total of 318 bacterial colonies producing clear zones on MRS agar supplemented with 0.5% $CaCO_3$ under anaerobic incubation at 37 °C for 48 h were initially obtained. These colonies were preliminarily considered as potential lactic acid bacteria. Gram staining revealed that 296 isolates were Gram-positive, of which 146 were rod-shaped (bacilli), 136 were cocci, and 14 were coccobacilli. Nineteen isolates were identified as yeasts based on morphology, and three isolates could not be maintained during subculturing; both groups were excluded from further analysis.

Catalase testing of the 296 Gram-positive isolates showed that 296 were catalase-negative, consistent with typical LAB characteristics, whereas 19 were catalase-positive and thus removed from subsequent screening. Consequently, 135 isolates were confirmed as Gram-positive, rod-shaped, catalase-negative bacteria and retained for probiotic property evaluation. For five isolates with ambiguous Gram-staining results, the test was repeated. Among these, isolates PMvet120, PMvet151, and PMvet183 exhibited budding cells and pleomorphic shapes, which were consistent with yeast morphology rather than bacteria (Figure 1). These isolates also showed poor growth in MRS broth and were therefore excluded.
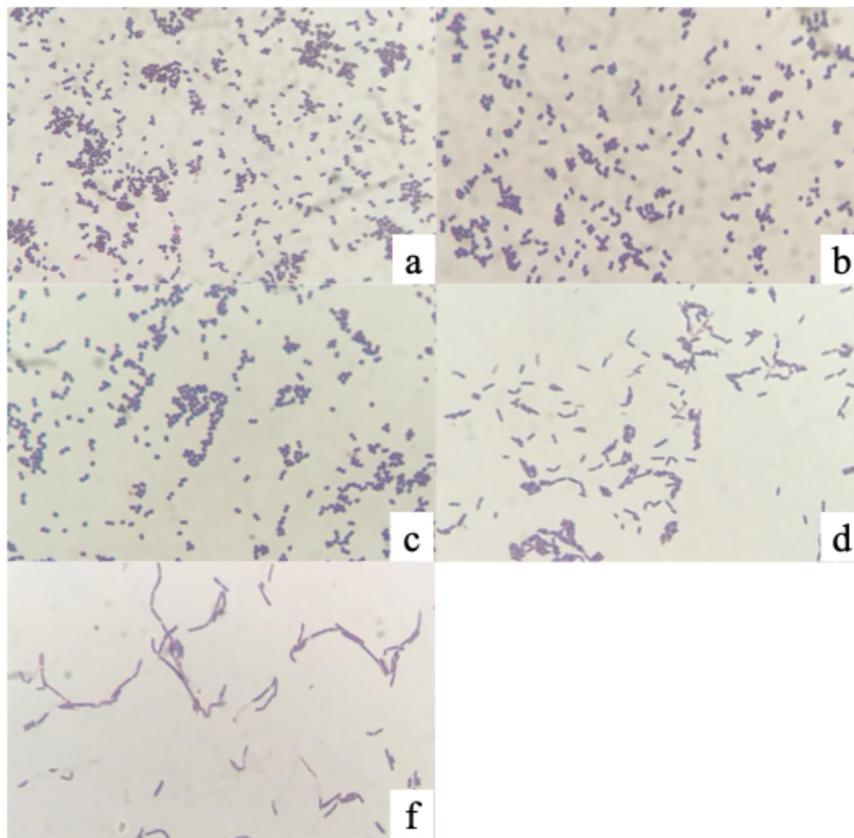

Figure 1. Microscopic images of representative isolates after Gram staining, observed under a light microscope at 1,000 × magnification: (a) PMvet120; (b) PMvet151; (c) PMvet183; (d) PMvet212; and (e) PMvet318.

3.2. Probiotic Properties of Selected LAB Isolates

3.2.1. Acid Tolerance

Among the 135 LAB isolates confirmed as Gram-positive, rod-shaped, and catalase-negative, only five strains—PMvet120, PMvet151, PMvet183, PMvet212, and PMvet318—demonstrated acid

tolerance, with a reduction in viable counts of approximately 1.00 log CFU/mL or less after incubation at pH 3.1 for 3 h. No isolates survived exposure to pH 2.0 under the same conditions. The survival rates of these five isolates at pH 3.1 are summarized in Table 2.

Table 2. Survival of selected LAB isolates at pH 3.1 after 3 h of incubation at 37 °C.

Isolate ID	log CFU/mL	
	Initial Count (average)	pH 3.1
120		5.35
151		6.20
183	6.37	6.15
212		5.60
318		5.56

3.2.2. Bile Salt Tolerance

The five acid-tolerant isolates were subsequently evaluated for their ability to survive in the presence of bile salts. After 24 h of anaerobic incubation at 37 °C, all isolates maintained viable counts above 6.00 log CFU/mL in MRS broth containing 0.3% bile salts. However, when the concentration was increased to 0.5%, only isolate PMvet212 retained a viable count of 6.56 ± 0.04 log CFU/mL, indicating substantial bile tolerance. In contrast, isolates PMvet151, PMvet183, and PMvet318 exhibited counts below the detection limit (<3.00 log CFU/mL) at 0.5% bile concentration, while PMvet120 showed partial survival. At 1.0% bile salts, none of the isolates survived above the detection threshold. The survival profiles are presented in Table 3.

Table 3. Survival of LAB isolates in MRS broth containing different bile salt concentrations after 24 h of incubation at 37 °C.

Isolate ID	0% (0 h)	0% (24 h, Control)	0.3% (24 h)	0.5% (24 h)
120	6.45	9.34	7.35	3.54
151	6.58	9.02	7.46	<3.00
183	6.47	9.12	7.54	<3.00
212	6.81	9.66	7.81	6.56
318	6.00	9.08	7.38	<3.00

3.2.3. Cell Surface Hydrophobicity (MATH Assay)

The hydrophobicity of the five selected LAB isolates was assessed using the microbial adhesion to hydrocarbons (MATH) assay with xylene as the hydrophobic phase. The results revealed considerable variation among isolates. PMvet318 exhibited the highest hydrophobicity (12.38 ± 0.03%), suggesting a relatively better ability to adhere to the intestinal epithelium. This was followed by PMvet212 (7.85 ± 0.02%), while the remaining isolates demonstrated values below 7%, indicating weaker adhesion potential. The hydrophobicity profiles are summarized in Table 4.

Table 4. Cell surface hydrophobicity (%) of selected LAB isolates as determined by the MATH assay using xylene.

Isolate ID	OD$_{600}$ Before	OD After (0 min)	OD After (30 min)	%Hydrophobicity (mean)
120	0.937	1.092	1.014	5.93
151	0.952	1.146	1.042	9.30
183	0.937	1.145	1.062	6.46
212	0.871	1.014	0.924	7.85
318	0.935	1.171	1.010	12.38

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3.2.4. Hemolytic Activity (Safety Evaluation)

Hemolytic activity of the five LAB isolates was assessed on tryptic soy agar (TSA) supplemented with 5% sheep blood. The results indicated that isolates PMvet120, PMvet151, and PMvet183 exhibited γ-hemolysis, characterized by the absence of any clear or discolored zones around colonies, thereby suggesting a non-hemolytic and potentially safe profile for probiotic application. In contrast, isolates PMvet212 and PMvet318 displayed α-hemolysis, evidenced by greenish discoloration surrounding the colonies, which indicates partial red blood cell lysis (Figure 2). The presence of α-hemolysis in these isolates raises potential safety concerns and suggests that they may not be suitable candidates for direct probiotic use without further safety evaluation.
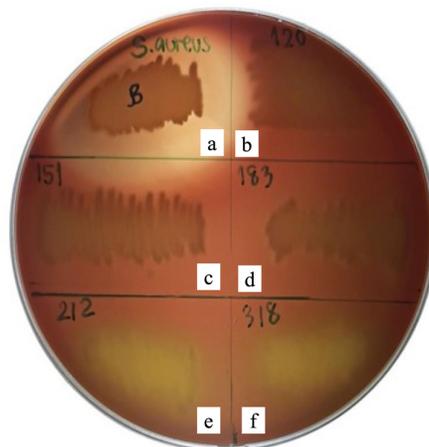

Figure 2. Hemolytic patterns of the five LAB isolates on tryptic soy agar supplemented with 5% sheep blood after 48 h of incubation at 37 °C. Isolates PMvet120 (b), PMvet151 (c), and PMvet183 (d) exhibited γ-hemolysis (no lysis), whereas PMvet212 (e) and PMvet318 (f)showed α-hemolysis (partial lysis, greenish discoloration). *Staphylococcus aureus* ATCC 6538 (a) was included as a positive control, displaying β-hemolysis (complete lysis with a clear zone).

3.2.5. Antimicrobial Activity Against Escherichia coli and Staphylococcus aureus

The antimicrobial activity of the cell-free supernatants (CFS) from the five selected LAB isolates was evaluated using the agar well diffusion assay against *E. coli* (field isolate from diarrheic piglet) and *Staphylococcus aureus* ATCC 6538. Among the tested isolates, only PMvet212 and PMvet318 produced distinct and measurable inhibition zones against both target pathogens. In contrast, isolates PMvet120, PMvet151, and PMvet183 demonstrated incomplete or weak inhibition against E. coli and showed no detectable inhibition against S. aureus. The measured diameters of inhibition zones are summarized in Table 5.

Table 5. Antimicrobial activity of cell-free supernatants (CFS) from selected LAB isolates against *Escherichia coli* (field isolate) and *Staphylococcus aureus* ATCC 6538, determined by the agar well diffusion assay.

Isolate ID	Clear zone (mm) – *E. coli*	Clear zone (mm) – *S. aureus*
PMvet120	Incomplete inhibition (no clear zone)	0
PMvet151	Incomplete inhibition (no clear zone)	0
PMvet183	Incomplete inhibition (no clear zone)	0
PMvet212	12.50	11.75
PMvet318	11.50	10.50
Control (2% lactic acid)	18.50	15.25

4. Discussion

The present study identified two lactic acid bacteria (LAB) isolates of the Bacilli group from fecal samples of suckling piglets (7–30 days old) that demonstrated probiotic potential. These findings are in line with earlier reports by Buasai Petsuriyawong (2011) [22] and Dowarah et al. (2018) [24], who successfully isolated LAB from piglet feces collected around weaning age (28–35 days). The detection of LAB at this early stage supports the well-established observation that piglet intestines are naturally colonized by Lactobacillus spp. before weaning, which are among the most prevalent beneficial microbes in the small intestine [25]. Colonization is strongly influenced by maternal sources, particularly sow feces, the environment, and milk, consistent with evidence from both human and porcine milk showing the transfer of probiotic microorganisms to offspring [26]. Recent investigations further confirm that maternal microbial transfer plays a vital role in shaping early-life gut microbiota composition and disease resilience in piglets [6].

In addition to bacterial isolates, three yeast strains were also identified from the piglet feces in this study. Their ability to withstand acidic pH and bile salts is noteworthy, as yeasts are increasingly recognized as effective probiotics due to their ability to survive harsh gastrointestinal conditions. Previous works have identified *Saccharomyces boulardii* and related yeast genera as promising candidates due to their acid tolerance, bile salt resistance, and ability to inhibit pathogens [27,28]. More recently, novel yeast strains from livestock sources have been shown to modulate immune responses and protect against enteric infections, reinforcing their potential in animal health management [29,30]. This suggests that while LAB remain the dominant focus of probiotic studies, cocci-shaped LAB such as Enterococcus and yeast isolates should not be overlooked in future investigations.

The acid and bile salt tolerance assays conducted here further confirmed strain-specific variability. Out of 135 confirmed LAB isolates, only five survived prolonged exposure at pH 3.1, although none survived at pH 2.0. This is consistent with earlier findings that probiotic survival in gastric conditions is highly strain-dependent [31]. Among these five isolates, PMvet212 demonstrated superior tolerance to 0.5% bile salt, a concentration comparable to the physiological levels encountered in the small intestine. Such tolerance is crucial for probiotic viability after oral administration, as bile salts are known to disrupt microbial membranes [32]. Recent studies have reinforced the role of bile-tolerant LAB in reducing diarrhea incidence and supporting intestinal health in weaning piglets [33,34]. Thus, the resilience of PMvet212 under bile stress highlights its potential as a candidate strain for feed supplementation in piglets facing post-weaning stress.

Other functional traits, including adhesion to intestinal epithelial cells and hemolytic activity, were also investigated. Surface hydrophobicity testing revealed that PMvet318 exhibited the highest percentage adhesion capacity (12.38%), although this was lower than the threshold suggested in some earlier reports (>40%). This finding underscores the complexity of adhesion mechanisms, as hydrophobicity alone does not fully explain epithelial colonization. Instead, multiple attributes such as auto-aggregation, extracellular polysaccharide production, and interactions with mucins contribute to successful colonization [35]. Regarding safety, PMvet120, 151, and 183 were non-hemolytic (γ-hemolysis), while PMvet212 and 318 showed partial α-hemolysis. Although α-hemolysis is often considered less harmful than β-hemolysis, comprehensive genetic and phenotypic evaluations remain essential, as hemolysin genes have been reported in some Lactobacillus strains [36]. Current safety guidelines strongly recommend further in vivo testing, including antibiotic resistance profiling and toxin gene screening, prior to commercial application [37,38].

Finally, antimicrobial activity was detected in isolates PMvet212 and 318, which inhibited both *Escherichia coli* and *Staphylococcus aureus*. These results are significant, as *E. coli* is a primary cause of piglet diarrhea worldwide, and antibiotic-resistant strains remain a pressing concern in swine production. The inhibitory effect observed in this study is consistent with earlier work demonstrating that LAB produce antimicrobial compounds, including organic acids and bacteriocins, which reduce the growth of pathogenic bacteria [21,31]. More recently, studies have confirmed that piglet-derived LAB strains can effectively reduce pathogen colonization, improve intestinal barrier integrity, and

enhance host immunity [39,40]. The moderate inhibition zones observed here suggest that while PMvet212 and 318 are promising, further characterization of their antimicrobial metabolites and verification under in vivo conditions are necessary. These findings open future research directions focusing on multi-strain formulations or co-cultures combining LAB and yeast for synergistic effects in piglet health management.

5. Conclusions

In this study, only two bacilli-shaped LAB isolates obtained from piglet feces demonstrated promising probiotic potential, with tolerance to acidic and bile conditions, moderate antimicrobial activity, and partial adhesion ability. While these results indicate their potential role as feed additives in swine production, the overall efficacy of the isolates remains moderate when compared to the broader functional properties expected of commercially viable probiotics.

Therefore, further comprehensive evaluations are warranted. Future studies should expand the characterization of these isolates to include pathogen inhibition against a wider spectrum of enteric microbes, immunomodulatory activity, antibiotic resistance profiling, and in vivo performance trials. Additionally, integrating LAB with other probiotic candidates such as yeast may provide synergistic effects on gut health and growth performance in piglets. Such efforts would not only support the reduction of antibiotic use in livestock but also contribute to sustainable swine production by mitigating antimicrobial resistance and enhancing animal welfare.

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