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Probiotic Characteristics of Lactic Acid Bacteria Isolated from Fermented Foods, Animal Intestines, and Raw Milk

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Abstract

This study was conducted to determine the probiotic characteristics of lactic acid bacteria isolated from various sources. A total of 282 LAB strains were isolated from 400 samples of fermented foods, chicken and swine intestines, and raw milk. Among these, 92 isolates (32.62%) exhibited inhibitory activity against enterotoxigenic *Escherichia coli*, and 10 strains were selected for further characterization due to their strong antibacterial abilities. MALDI-TOF MS revealed that the selected isolates belonged to *Lactiplantibacillus plantarum, Levilactobacillus brevis, Pediococcus acidilactici, Pediococcus pentosaceus,* and *Enterococcus faecium*. They were non-hemolytic and tolerant to acidic conditions and bile salts, with high survival rates (48.48-97.67%), auto-aggregation abilities (41.96-67.52%), and co-aggregation abilities (25.85-45.06%). These findings identified several LAB isolates with promising *in vitro* probiotic-related properties. However, further genomic, biosafety, and *in vivo* studies are required before practical applications.

Keywords

Lactic acid bacteria, probiotic, enterotoxigenic *Escherichia coli*, antibacterial activity

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Introduction

Lactic acid bacteria (LAB) have been demonstrated to possess probiotic characteristics and are among the most widely applied microorganisms in the production of biological products for disease prevention in animal husbandry (Arsène *et al*., 2021). LAB are ubiquitous in nature, particularly in animal gastrointestinal tracts, fermented foods, and milk. LAB isolated from these sources are usually classified into the genera *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Lactococcus* (Abushelaibi *et al*., 2017). Most LAB strains are recognized as safe and exhibit the abilities to resist acidic

pH and bile salt conditions, and adhere effectively to the intestinal epithelium (Zapaśnik *et al*., 2022). In addition, LAB can tolerate adverse conditions during food processing, storage, and probiotic production. Furthermore, LAB can produce a variety of antibacterial substances that inhibit pathogenic microorganisms, including organic acids, hydrogen peroxide (H_2O_2), bacteriocins, and enzymes (Castellano *et al*., 2008). Other mechanisms include competition for adhesion sites and nutrients, enhancement of the immune system, and the inhibition and prevention of pathogenic bacteria (Halder *et al*., 2017).

The probiotic potential of LAB is strain-dependent; therefore, the isolation and characterization of new LAB strains from different natural sources remain important. Fermented foods, animal intestines, and raw milk are considered valuable reservoirs of LAB with promising functional properties (Abushelaibi *et al*., 2017). Among the desirable probiotic characteristics, antagonistic activity against enteric pathogens is particularly important because it may help maintain intestinal microbial balance and reduce pathogen proliferation. Enterotoxigenic *Escherichia coli* (ETEC) K88 is a common enteric pathogen in pigs and was selected as an indicator bacterium in the present study to screen the antibacterial activity of LAB. Previous studies have demonstrated that certain LAB strains exhibit inhibitory activity against pathogenic *E. coli* and possess characteristics desirable for probiotic applications (Rodriguez *et al*., 2009; Tejero *et al*., 2012). However, information regarding LAB isolated from fermented foods, animal intestines, and raw milk with antagonistic activity against ETEC K88 in Vietnam remains limited. Therefore, this study aimed to isolate LAB from these sources and evaluate their probiotic-related characteristics, including antibacterial activity against ETEC K88, tolerance to acidic and bile salt conditions, auto-aggregation ability, and safety-related properties.

Materials and Methods

Materials

The study used samples of fermented foods, animal gastrointestinal contents, and raw milk for the isolation of LAB. The ETEC K88 strain, previously recovered from a piglet with diarrhea, was preserved in our laboratory. This bacterial strain carried both fimbriae (F4) and enterotoxin (STa). In addition, the ETEC K88 bacteria were resistant to multiple antibiotics.

Methods

Sample collection

A total of 400 samples, namely pickled mustard greens, pickled eggplants, kimchi, pickled cucumbers, fermented pork, chicken intestines, swine intestines, and raw milk, were collected in accordance with TCVN 5522:1991, TCVN 10782:2015 (ISO 13307:2013), and TCVN 6400:2010 (ISO 707:2008) in Gia Lam district, Hanoi city, with 50 samples for each sample type. Milk samples were collected from milk collection stations in Phu Dong commune, Gia Lam district, from different farms in the morning (6 am-7 am). Other sample types were randomly purchased from 10 markets in Gia Lam district in the morning (7 am-8 am), with five samples obtained from each market from different retail counters. Samples were kept in ice boxes and immediately transported to the laboratory within 24h for LAB isolation.

Isolation of LAB from various sample types

The LAB isolation was conducted as follows: Samples of pickled mustard greens, pickled eggplants, kimchi, pickled cucumbers, fermented pork, chicken intestines, and swine intestines were aseptically cut into small pieces and homogenized in De Man–Rogosa–Sharpe (MRS) broth at a ratio of 1:9 (w/v) using a stomacher. Milk samples were serially diluted. From each dilution, 100µL of the sample suspension was plated onto MRS agar and then incubated at 37°C for 24-48h. LAB on MRS agar formed milky white or grayish-white, round, convex colonies. One to three typical LAB colonies were picked up for Gram staining and biochemical identification. Positive isolates were preserved in MRS broth supplemented with 20% glycerol (v/v) at -80°C. To avoid duplicate isolates, only one presumptive LAB isolate from each positive sample was selected and stored for subsequent analyses.

Antibacterial activity of LAB isolates

The antagonistic activity of the LAB isolates against ETEC K88 was screened using the agar well diffusion method as described by Geis *et al*. (1983) and Gupta *et al*. (2021) with minor modifications. The LAB isolates were cultured in MRS broth at 37°C for 24h. The LAB culture was then centrifuged at 6000 × g, 4°C, for 20min. The supernatant was collected and passed through a 0.22-µm membrane filter to produce cell-free supernatant (CFS). A portion of the CFS (1mL) was adjusted to pH 6.5 using 0.1N NaOH to obtain pH-neutralized CFS (nCFS). The ETEC K88 culture (10^7 CFU mL^{-1}) was mixed with soft brain heart infusion (BHI) agar (0.8% agar powder) and evenly overlaid onto the surface of MRS agar plates. After incubation at 37°C for 6h, 5 mm-diameter wells were punched into the agar surface. Subsequently, 50µL of CFS/nCFS from the LAB isolates was added to each well, while wells containing 50 µL of MRS broth served as negative controls. The plates were then kept at 4°C for 2-3h to allow diffusion of active compounds into the agar, followed by incubation at 37°C for 18h. Antibacterial activity was assessed by the formation of inhibition zones around the wells. The whole experiment was replicated three times.

Species identification of LAB isolates

LAB isolates exhibiting strong antagonistic activity were identified at the species level by MALDI-TOF mass spectrometry using the Bruker MALDI Biotyper system (Bruker Daltonics, Bremen, Germany). Briefly, a fresh colony grown on MRS agar was plated onto a MALDI target plate and overlaid with 1µL of formic acid, and dried at room temperature. After that, 1µL of matrix solution was applied on top. After air-drying, spectra were acquired using a MALDI Biotyper instrument and analyzed with MBT Compass software version 4.1.100 using the Bruker Daltonics library version 7.0. Species identification was assigned according to the manufacturer's recommended score values. The similarity between MALDI-TOF MS spectra obtained from the bacterial isolates and the reference spectra in the database was evaluated based on Log (score) values and corresponding color indicators (green, yellow, and red). A Log (score) higher than 2.3 (green) represented highly reliable identification at the species level. Scores ranging from 2.0 to 2.3 indicated confident genus-level identification and probable species-level identification. Scores between 1.7 and 2.0 (yellow) suggested only tentative genus identification, while scores below 1.7 (red) reflected insufficient similarity between the unknown spectrum and the reference database profiles.

Hemolytic activity of LAB isolates

The LAB isolates were cultured onto a blood agar plate (containing 5% sheep red blood cells), then incubated at 37°C for 24h. The presence of greenish zones or clear zones indicated α-hemolysis (partial hemolysis) or β-hemolysis (complete hemolysis), while no zone around the colonies was regarded as γ-hemolysis (non-hemolysis). *Staphylococcus aureus* ATCC 25923 was employed as the positive control. The experiment was conducted in triplicate (Halder *et al*., 2017).

Stability of LAB isolates

The tolerance of the LAB isolates under stress conditions was investigated following the protocol of Huligere *et al*. (2023), with slight modifications. The overnight-cultured LAB suspension (approximately 10^9 CFU mL^{-1}) was inoculated into phosphate-buffered saline (PBS) adjusted to different pH values (2.0, 3.0, and 6.4). The pH levels of 2.0 and 3.0 were used to simulate acidic conditions in an animal stomach, while pH 6.4 was employed as a control, representing a favorable environment for the survival of LAB, thereby providing a basis for evaluating the effects of an acidic environment. In parallel, the LAB suspension was introduced into PBS supplemented with different concentrations of bile salts (0.3%, 0.5%, and 1.0% ox bile) to evaluate bile tolerance in the small intestine. All samples were then incubated at 37°C for 3h. After incubation, suspensions were serially diluted and spread on MRS agar, then incubated at 37°C for 24-48h for viable counts enumeration. The whole process was repeated three times. The stability of the LAB under different conditions was determined as follows:

$$\text{Survival rate (\%)} = \frac{Viable\ counts\ at\ 3h}{Viable\ counts\ at\ 0h} \times 100\%$$

Aggregation of LAB isolates

** Auto-aggregation assay*

The auto-aggregation of the LAB isolates was tested as previously described by Yadav et al. (2016). The LAB isolates were cultured in MRS broth (5mL) at 37°C for 16-18h. Cultures were then centrifuged (6000×g, 20min, 4°C) to remove supernatants, and cell pellets were washed and resuspended in PBS. The bacterial suspensions were adjusted to reach an OD_{600} of 0.5 (OD_0) before incubating at 37°C for 2h. Following the incubation, a portion of the upper suspension (1mL) was withdrawn, and the optical density was measured at 600nm (ODt). Auto-aggregation ability was assessed based on the proportion of cells remaining in suspension before and after incubation.

** Co-aggregation assay*

The co-aggregation assay was performed as previously described by Gupta et al. (2021), with some modifications. The LAB isolates were inoculated into MRS broth, while ETEC K88 was grown in brain heart infusion (BHI) broth at 37°C for 24h. After the cultivation period, the cells were collected by centrifugation, then washed and resuspended in PBS. The suspension was then adjusted to OD_{600} = 0.5, mixed with ETEC K88 (OD_{600} = 0.5) at a 1:1 ratio (OD_0), and incubated at 37°C for 2h. Subsequently, 1mL of the upper suspension was collected for the OD_{600} measurement (ODt).

The auto-aggregation and co-aggregation of the LAB isolates were calculated using the following formula:

$$\text{Aggregation (\%)} = (OD_0\text{-}ODt)/(OD_0)\times 100\%$$

Statistical analysis

Data analysis was performed using one-way analysis of variance (one-way ANOVA) in SPSS software (version 26.0; IBM, USA) to compare mean values, with statistical significance set at $P < 0.05$. When significant differences were detected, Tukey's post hoc test was subsequently applied to each dataset, namely the inhibition zone diameters, survival rates under various acid and bile salt conditions, and aggregation assays, to identify pairwise differences among the LAB strains under the same experimental conditions. All data were compiled, processed, and visualized using Microsoft Excel software (2021, Microsoft, USA).

Results and Discussion

Isolation of LAB from various sample types

The results of LAB isolation are presented in **Table 1**. A total of 400 samples were purchased and subjected to LAB isolation; 282 (70.50%) were positive for presumptive LAB. A total of 694 LAB colonies exhibiting typical morphology (**Figure 1**), Gram-positive characteristics (**Figure 2**), and negative catalase activity (**Figure 3**) were preserved in 20% glycerol (v/v) at -80°C. To avoid duplicates, only 282 colonies were selected for culturing in MRS broth and then kept at -80°C for further characterization, with one isolate per each positive sample.

Growth performance

The fermented food samples showed the highest isolation rates of LAB, with pickled mustard greens (92%) having the highest rate, followed by pickled cucumbers (84%), pickled eggplants (80%), fermented pork (78%), and kimchi (76%). For other sample types, the isolation rates of LAB were lower than those observed in the fermented foods. Specifically, positive rates were 44% in chicken intestines, 36% in swine intestines, and 74% in raw milk.

The occurrence of different bacterial morphologies reflects the species diversity of the isolated LAB strains. Rod-shaped bacteria are commonly associated with genera such as *Lactobacillus*, whereas cocci-shaped bacteria are frequently observed in *Lactococcus*, *Pediococcus*, *Enterococcus*, or *Streptococcus* (Giraffa *et al*., 2010). In addition, the successful isolation of a large number of LAB strains from fermented foods, animal gastrointestinal tracts, and milk indicates that these environments are favorable for LAB growth. This has also been reported in previous studies, which have reported that LAB are widely distributed in intestines, milk, and various fermented products such as pickled vegetables and fermented pork, and

Table 1. Isolation of LAB from various sample types

Samples	No. of samples	No. of positive samples	Percentage (%)	No. of strains
Pickled mustard greens	50	46	92	46
Pickled eggplants	50	40	80	40
Kimchi	50	38	76	38
Pickled cucumbers	50	42	84	42
Fermented pork	50	39	78	39
Chicken intestines	50	22	44	22
Swine intestines	50	18	36	18
Raw milk	50	37	74	37
Total	400	282	70.5	282

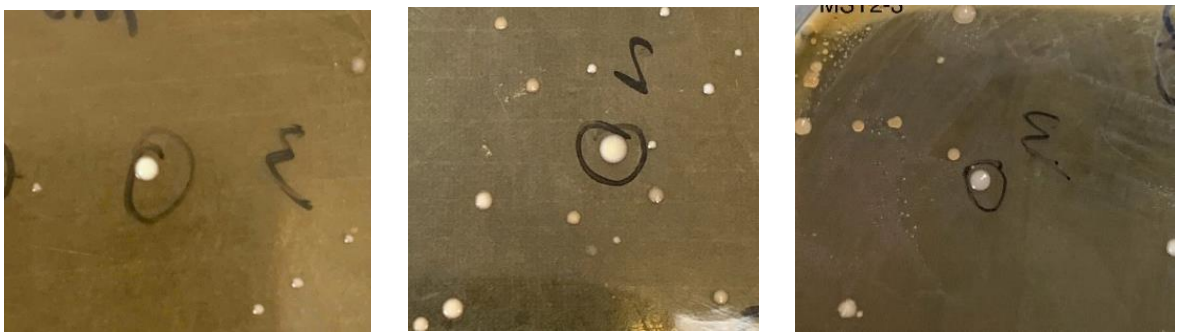


Figure 1. Colony morphology of LAB isolates on MRS agar

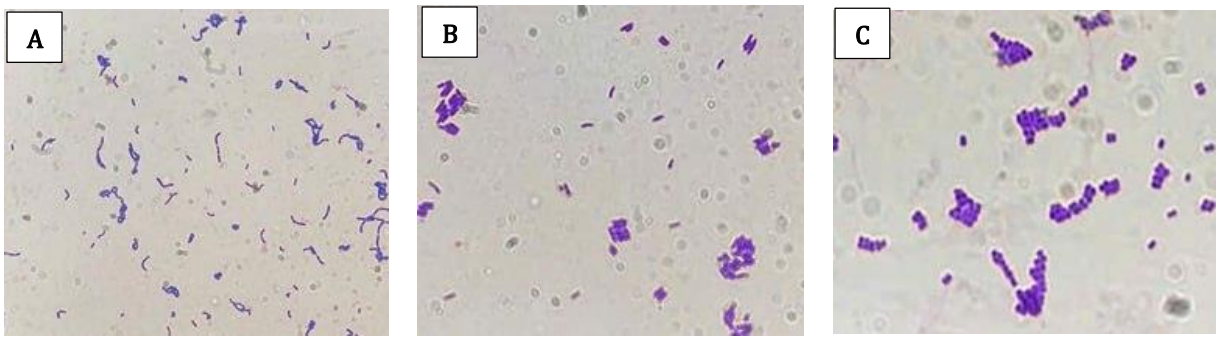


Figure 2. Microscopic morphology of LAB isolates
(Rod-shaped morphology (A, B); Coccoid morphology (C))

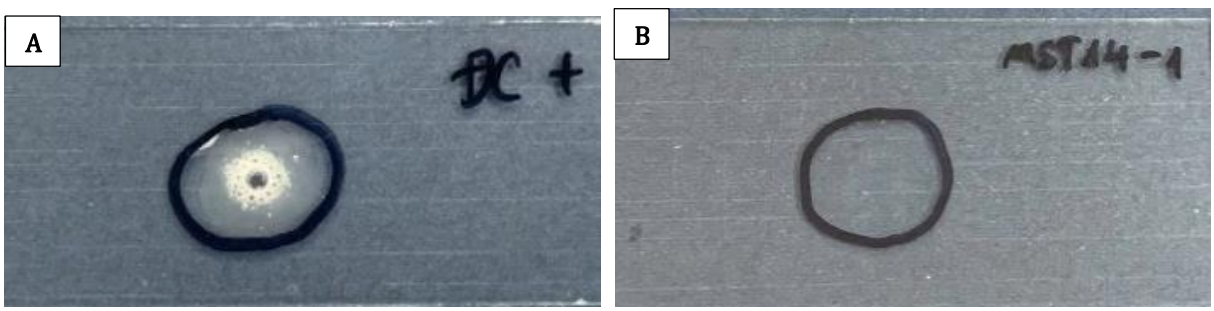


Figure 3. Catalase reaction of LAB isolates
(Positive control showing bubble formation (A); LAB isolate showing no bubble formation (B))

belong to the genera *Lactobacillus, Leuconostoc, Pediococcus, Enterococcus, Lactococcus,* and *Streptococcus* (Abushelaibi *et al*., 2017).

Antagonistic activity of LAB isolates against ETEC K88

The antagonistic activity of the LAB isolates against ETEC K88 was evaluated using the agar well diffusion method, employing CFS and nCFS. The results showed that 92 out of 282 LAB isolates (32.62%) exhibited antagonistic activity against ETEC K88. Among these, the 10 LAB strains that produced the largest inhibition zones against ETEC K88 were selected for further evaluation of probiotic characteristics, as shown in **Table 3**.

The selected LAB isolates showed different degrees of inhibitory activity against ETEC K88, as evidenced by statistically significant differences (P <0.05). For the cell-free supernatants (CFS) of the LAB strains, inhibition zone diameters ranged from 16.5 ± 0.5mm to 21.33 ± 0.58mm. Among them, strain LRL9 showed the strongest antagonistic activity, with the largest inhibition zone diameter of 21.33 ± 0.58mm. LDM6, LDC17, LNC24, LRG15, and LRL18 also exhibited strong inhibitory activity, with inhibition zone diameters larger than 19mm. In contrast, strains LKC32 and LST28 produced the smallest inhibition zones of 16.5 ± 0.5mm and 17.67 ± 1.53mm, respectively. Regarding nCFS, only a subset of the LAB isolates retained inhibitory activity against ETEC K88. Specifically, LNC24, LRG15, LRL9, and LRL18 produced inhibition zones with diameters ranging from 8.67 ± 1.53mm to 13.83 ± 1.26mm, among which LRL9 exhibited the strongest antibacterial activity. Compared with their initial CFS activity, the inhibitory activity after neutralization decreased significantly, indicating that organic acids were the major contributors to antibacterial activity, although other inhibitory compounds may also be involved.

The inhibitory activity observed in this study was higher than that reported by Rodriguez *et al*. (2009), who reported that CFS from 7.6% (8/106) of LAB inhibited the proliferation of all tested *E. coli* strains. According to Markowiak & Ślizewska (2017), *Lactobacillus* spp. exert protective effects against intestinal pathogens, including *E. coli*. Similarly, Hong *et al*. (2019) demonstrated that LAB exhibited strong inhibitory activity against *E. coli* K99, a causative agent of diarrhea in piglets. Furthermore, Tejero *et al*. (2012) showed that *Lactobacillus* spp., particularly *Lactiplantibacillus plantarum*, possess notable antibacterial activity against *E. coli*; however, this activity gradually decreases over 8-48h and is lost after pH neutralization of the CFS.

Antibacterial activity is largely dependent on the production of lactic acid by LAB. Lactic acids can penetrate bacterial cells and exert bactericidal effects by lowering intracellular pH (Ogawa *et al*., 2001). Although low pH and lactic acid negatively affect pathogenic bacteria, previous studies have shown that under acidic conditions (pH 4.0), pathogenic bacteria may still survive and proliferate in the absence of lactic acid (Presser *et al*., 1997). In addition, LAB can produce other antimicrobial compounds, particularly bacteriocins, which have been reported to inhibit a broad range of Gram-negative bacteria (Field *et al*., 2016). Overall, the significant reduction in antibacterial activity following neutralization of the cell-free supernatant suggests that organic acids contributed substantially to the inhibition of ETEC K88. However, the potential role of other antimicrobial compounds produced by LAB, such as bacteriocins and hydrogen peroxide, cannot be excluded.

Species identification of LAB isolates

The identification of the selected LAB isolates using MALDI-TOF MS is shown in **Table 4**. The LAB strains exhibiting strong antagonistic activity against ETEC K88 were identified as belonging to five species: *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*), *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Enterococcus faecium*.

Among the identified strains, *L. plantarum* was the predominant species, with three strains isolated from pickled mustard greens, fermented pork, and swine intestines. Two strains belonging

Table 3. LAB strains exhibiting the largest inhibition zones against ETEC K88

Strain ID	ZDI (mm) ± SD for indicator ETEC K88	
	CFS	*nCFS*
LDM6	19.67^{abc} ± 1.53	-
LKC32	16.5^{c} ± 0.5	-
LDC17	19.83^{abc} ± 0.76	-
LNC24	19.17^{abc} ± 1.04	8.67^{d} ± 1.53
LRG8	18^{abc} ± 1.00	-
LRG15	19.33^{abc} ± 0.76	8.83^{d} ± 1.04
LRL9	21.33^{a} ± 0.58	13.83^{e} ± 1.26
LRL12	18.67^{abc} ± 1.53	-
LRL18	20.5^{ab} ± 1.8	9.33^{d} ± 0.76
LST28	17.67^{bc} ± 1.53	-

Note: ZDI: inhibition zone diameter. SD: standard deviation. (–) no inhibition zone. Different lowercase letters (a–e) indicate statistically significant differences (P <0.05).

Table 4. Identification of selected LAB isolates using MALDI-TOF MS

Strain ID	Samples	Species
LDM6	Pickled mustard greens	*Lactiplantibacillus plantarum*
LKC32	Kimchi	*Pediococcus acidilactici*
LDC17	Pickled cucumbers	*Pediococcus acidilactici*
LNC24	Fermented pork	*Lactiplantibacillus plantarum*
LRG8	Chicken intestines	*Pediococcus pentosaceus*
LRG15	Chicken intestines	*Enterococcus faecium*
LRL9	Swine intestines	*Lactiplantibacillus plantarum*
LRL12	Swine intestines	*Levilactobacillus brevis*
LRL18	Swine intestines	*Enterococcus faecium*
LST28	Raw milk	*Levilactobacillus brevis*

to *L. brevis* were isolated from swine intestines and raw milk. The *Pediococcus* genus was represented by the two strains of *P. acidilactici* isolated from kimchi and pickled cucumbers, and one strain of *P. pentosaceus* isolated fromchicken intestines. In addition, two strains were identified as *E. faecium*, originating from chicken intestines and swine intestines. These results indicate that the LAB isolates exhibiting strong antagonistic activity against ETEC K88 belong to multiple species and originate from diverse sources, including both fermented foods and samples of animal origin. Previous studies have reported that LAB strains with high stability to acidic and bile salt conditions, aerobic environments, and strong inhibitory activity against *E. coli* predominantly belong to *L. plantarum*, *P. pentosaceus*, and *L. brevis* (Brashears *et al*., 2003; Rodriguez *et al*., 2009).

Hemolytic activity of LAB isolates

The safety assessment of the selected LAB isolates based on hemolytic activity is presented in **Table 5**. All 10 LAB isolates showed γ-hemolysis on blood agar plates, characterized by the absence of hemolytic zones surrounding the colonies, indicating no red blood cell lysis. Therefore, all the selected LAB isolates have no hemolytic activity and fulfil one of the fundamental safety criteria for potential probiotics. Our results are in line with previous studies reporting that most LAB, particularly

those belonging to the genera *Lactobacillus* and *Pediococcus*, do not exhibit hemolytic activity and are classified as generally recognized as safe (GRAS) microorganisms or have qualified presumption of safety (QPS) status for applications in food and probiotic products (Yadav *et al*., 2016; Somashekaraiah *et al*., 2019). On the other hand, previous studies have suggested that *E. faecium* strains may harbor certain virulence factors that could adversely affect animal health. According to EFSA (2018), it is necessary to confirm the absence of virulence genes (*IS16*, *hylEfm*, and *esp*) in these strains before they can be applied in probiotic production.

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Stability of LAB isolates

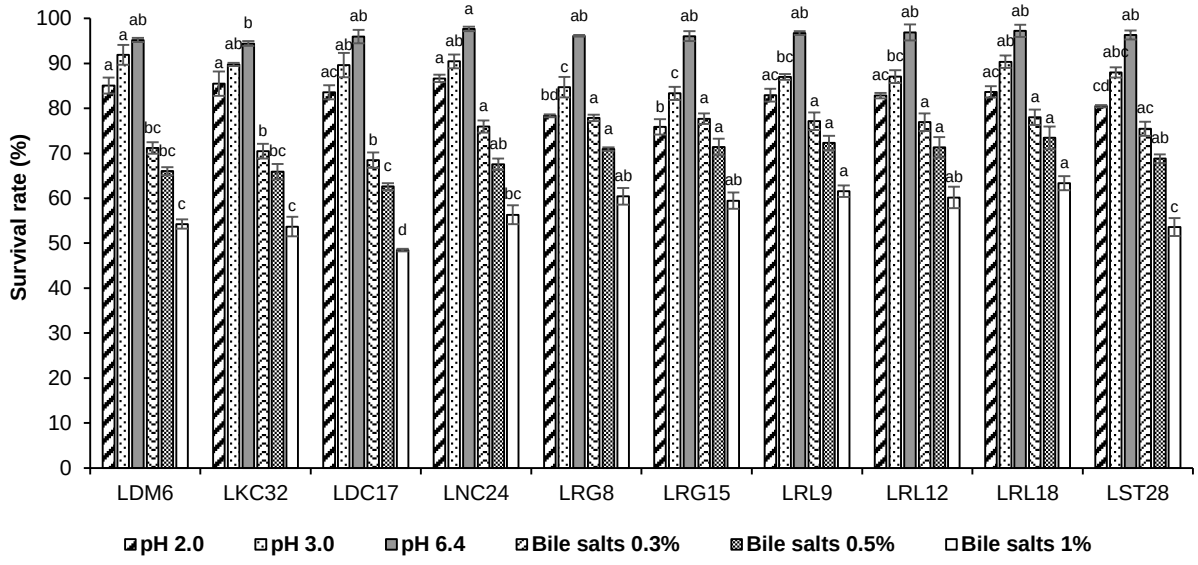
Figure 4 shows the tolerance of the LAB under stress conditions. Overall, all tested LAB isolates exhibited great adaptability to acidic environments (pH 2.0, 3.0, and 6.4) and bile salt concentrations (0.3%, 0.5%, and 1.0%), with survival rates ranging from 48.48% to 97.67%. Statistically significant differences in stability were observed among the LAB isolates (P <0.05).

Under acidic conditions at pH 2.0, the survival rates of the LAB isolates ranged from 75.86% to 86.68%. LNC24 (86.68%), LKC32 (85.57%), and LDM6 (85.07%), isolated from fermented food samples, exhibited significantly higher survival rates than the remaining isolates, indicating great adaptation to highly acidic environments. When the pH increased to 3.0, survival rates markedly increased for all the isolates, ranging from 83.35% to 91.9%.

Table 5. Safety assessment of selected LAB isolates

Isolate ID	Species	Hemolysis		
		α	β	γ
LDM6	*Lactiplantibacillus plantarum*	-	-	+
LKC32	*Pediococcus acidilactici*	-	-	+
LDC17	*Pediococcus acidilactici*	-	-	+
LNC24	*Lactiplantibacillus plantarum*	-	-	+
LRG8	*Pediococcus pentosaceus*	-	-	+
LRG15	*Enterococcus faecium*	-	-	+
LRL9	*Lactiplantibacillus plantarum*	-	-	+
LRL12	*Levilactobacillus brevis*	-	-	+
LRL18	*Enterococcus faecium*	-	-	+
LST28	*Levilactobacillus brevis*	-	-	+

Note: (–) negative; (+) positive.



Note: Different lowercase letters (a–d) above the chart bars show a significant difference (P <0.05); error bars show SD of means (n = 3).

Figure 4. Acid and bile salt stabilities of the selected LAB isolates

Notably, LDM6, LNC24, and LRL18 maintained survival rates above 90%, which were significantly greater than those of LRG8 and LRG15 (P <0.05). At pH 6.4, most strains showed very high survival rates (> 94%), suggesting that near-neutral pH is optimal for LAB isolates. Overall, these results demonstrate that all the LAB isolates possess strong acid tolerance, with particularly enhanced survival capacity observed in strains derived from fermented food sources. However, the survival rate of the LAB isolates tended to decrease as pH became more acidic, with lower viability observed at pH 2.0 and pH 3.0 compared with that at pH 6.4.

In the medium supplemented with bile salts, survival rates gradually decreased as bile salt concentrations increased from 0.3% to 1.0%. At a bile salt concentration of 0.3%, the LAB isolates maintained relatively high survival rates (68.47-78.00%). The LAB strains isolated from animal intestines, namely LRL18 (78.00%), LRG8 (77.87%), LRG15 (77.69%), LRL9 (77.14%), and LRL12 (76.94%), exhibited significantly greater bile tolerance than strains isolated from fermented foods. At 0.5% bile salts, survival rates declined, ranging from 62.61% to 73.44%; however, isolates originating from chicken and swine intestines still maintained survival rates above 70%, which were significantly higher than those of other isolates (P <0.05). At a bile salt concentration of 1.0%, a significant reduction in survival was observed across all the isolates, ranging from 48.48% to 63.33%. Among them, LRL18 (63.33%), LRL9 (61.59%), LRG8 (60.42%), and LRL12 (60.18%) exhibited superior stability, while LDC17, isolated from pickled cucumber, showed the lowest survival rate (48.48%). In this study, the higher bile salt concentrations (up to 1.0%) were used to evaluate the stability of the LAB under extreme gastrointestinal stress conditions rather than physiological levels.

These findings indicate that the acid and bile salt stability of the LAB isolates is strongly influenced by their origin. Isolates recovered from fermented foods (pickled vegetables, kimchi, fermented pork) tended to exhibit higher adaptation to acidic conditions, while isolates of intestinal origin showed greater tolerance to bile salts, likely due to their prolonged exposure and adaptation to the gastrointestinal environment.

Previous studies conducted in Vietnam have also reported similar stability patterns of LAB under acidic and bile salt conditions. Nguyen Thi Hong Nhung *et al*. (2019) observed relatively

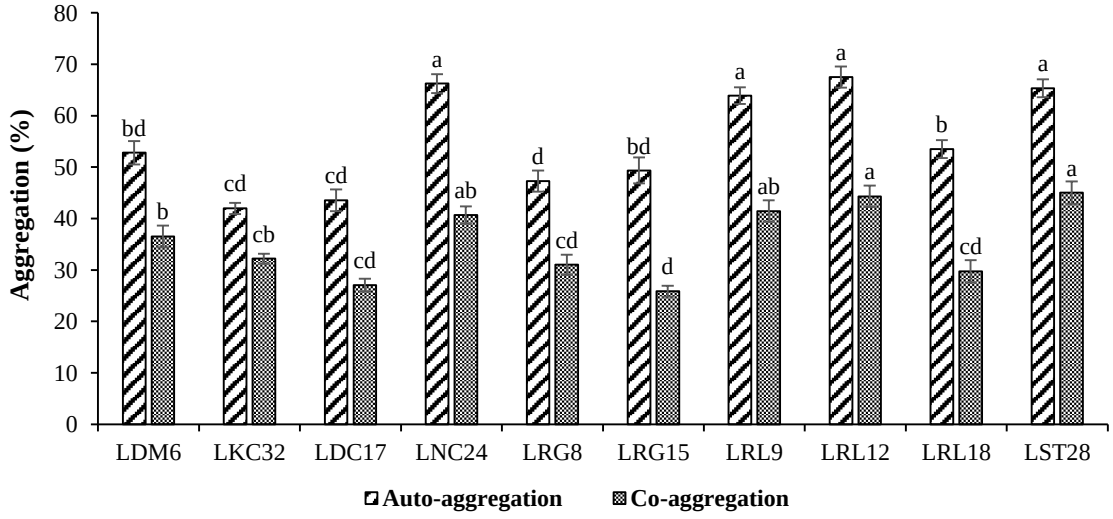
high survival rates of LAB recovered from fermented foods after 3h of incubation at pH 2, 3, and 4, with survival rates exceeding 45%, 60%, and 70%, respectively. At a bile salt concentration of 0.5%, survival degrees reached approximately 90% but declined markedly at a bile salt concentration of 3%, remaining above 50%. Similarly, *Lactobacillus* strains isolated from duck intestines in the study by Nguyen Xuan Canh *et al*. (2018) were able to tolerate pH values ranging from 1 to 4 and bile salts at 0.3%.Rodriguez-Palacios *et al*. (2009) reported that LAB survival rates at pH 2 ranged from 35% to 100%, with 13.2% (at pH 4) and 39.6% (at 0.3% bile salts) of LAB isolates exhibiting survival rates above 80%. Brashears *et al*. (2003) reported that LAB isolates capable of inhibiting *E. coli* tolerated pH values between 2 and 7 and bile salt concentrations ranging from 0.05% to 0.3%.

Aggregation of LAB isolates

Auto-aggregation is linked with the capacity of LAB to attach to intestinal epithelial cells, whereas co-aggregation influences their interactions with pathogenic microorganisms (Collado *et al.*, 2007; Hojjati *et al.*, 2020). Moreover, co-aggregation with other bacteria may prevent pathogen adhesion to the intestinal mucosa and inhibit biofilm formation (Hojjati *et al.*, 2020). In the present study, the auto-aggregation and co-aggregation abilities with ETEC K88 of the 10 selected LAB isolates are illustrated in **Figure 5**.

The auto-aggregation ability of the selected LAB isolates ranged from 41.96% to 67.52%, showing significant differences among strains (P <0.05). Among them, *L. brevis* LRL12 (67.52%), *L. plantarum* LNC24 (66.25%), L. brevis LST28 (65.33%), and *L. plantarum* LRL9 (63.89%) exhibited the highest auto-aggregation capacities. In contrast, *P. acidilactici* LKC32 (41.96%) and LDC17 (43.55%) showed the lowest auto-aggregation abilities among the tested isolates. In addition, the co-aggregation ability with ETEC K88 ranged from 25.85% to 45.06% and was generally lower than the auto-aggregation capacity. The highest co-aggregation was observed in *L. brevis* LST28 (45.06%), followed by *L. brevis* LRL12 (44.32%) and *L. plantarum* LRL9 (41.44%). These strains showed statistically significant differences (P <0.05) compared to strains with a lower co- aggregation ability, such as *E. faecium* LRG15 (25.85%) and *P. acidilactici* LDC17 (27.05%).

The findings in this study are in agreement with previous reports. Zawistowska *et al.* (2022) showed that *L. plantarum* and *L. brevis* strains generally exhibited higher adhesion capacities



Note: Different lowercase letters (a–d) above the chart bars show a significant difference (P <0.05); error bars show SD of means (n = 3)

Figure 5. Aggregation ability of the selected LAB strains

compared to other LAB species. Similarly, Somashekaraiah *et al.* (2019) reported that the auto-aggregation ability of LAB strains ranged from 51.2% to 78.95%. Collado *et al.* (2007) indicated that all tested *L. plantarum* and *E. faecium* strains were capable of co-aggregating with *E. coli* K2. In a study by Hojjati *et al*. (2020), *L. brevis* exhibited co-aggregation and auto-aggregation rates of 48.3% and 40.2%, respectively. Adhesion capacity was strongly dependent on incubation time and tended to increase over a 24h incubation period (Zawistowska-Rojek *et al*., 2022).

In this study, *L. plantarum* and *L. brevis* strains exhibited higher auto-aggregation and co-aggregation abilities with ETEC K88 than isolates belonging to the genera *Pediococcus* and *Enterococcus*. These findings are consistent with previous reports indicating stronger adhesion-related behavior in certain *Lactobacillus* strains (Hojjati *et al*., 2020). However, it should be noted that auto-aggregation and co-aggregation are indirect indicators and cannot be directly equated with actual intestinal colonization or pathogen exclusion. Auto-aggregation and co-aggregation characteristics are affected by bacterial cell morphology and length. Longer bacterial cells possess a larger surface area, resulting in stronger aggregation compared to shorter or coccoid cells. Moreover, adhesion ability is closely associated with the production of exopolysaccharides (EPS), which form a mucous layer facilitating biofilm formation (Rajab *et al*., 2020). These factors play important roles in bacterial interactions and may contribute to the prolonged persistence of LAB in the gastrointestinal tract.

Conclusions

This study showed the high prevalence of LAB in fermented foods, animal gastrointestinal tract samples, and raw milk. LAB isolates exhibiting strong antagonistic activity against ETEC K88 belonged to the species *L. plantarum*, *L. brevis*, *P. acidilactici*, *P. pentosaceus*, and *E. faecium*. Among the selected isolates, *L. plantarum* LRL9 and LNC24 showed the most promising *in vitro* probiotic-related characteristics. However, a comprehensive safety assessment, genomic characterization, and *in vivo* validation are required before considering their application in piglets.

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