

EFFICACY OF BIOSYNTHESIZING FOLATE, RIBOFLAVIN AND TYPICAL PROBIOTIC TRAITS REVEAL THE POTENTIAL USE OF *Lactobacillus plantarum* LCN13 AS A FEED ADDITIVE FOR SWINE FARMING

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ABSTRACT

Lactic acid bacteria have been advocated as probiotics to replace antibiotic growth promoters, improve growth performance, and reduce cost production in swine farming. The aim of this study is to identify and evaluate the probiotic traits of strain LCN13 isolated from traditionally fermented feed for swine. Thirty-five bacterial isolates with different morphological characteristics were isolated, among which 9 isolates showed the ability to produce lactic acid and antibacterial activity against pathogenic bacteria. Among them, isolate LCN13 exhibited a strong capacity to produce lactic acid (18.5 ± 0.31 g/L), inhibited gastrointestinal pathogens such as *Salmonella typhimurium* ATCC 14028 (18.3 ± 1.52 mm), *Escherichia coli* ATCC 11105 (24.7 ± 2.14 mm), and *Staphylococcus epidermidis* ATCC 35984 (31.6 ± 2.93 mm), and produced 182 ng/mL folate and 233 ng/mL riboflavin as measured by LC-MS/MS after 24 hours of incubation. Based on morphological, biochemical, and 16S rRNA gene analysis, the isolate LCN13 was identified as *Lactobacillus plantarum*. Phenotypic analysis revealed that *L. plantarum* LCN13 showed remarkable resistance to 1.2% ox-bile salt, 2.0 mM H₂O₂, and pH 3.0. In addition, the ability to produce high levels of folate (253.6 ± 10.7 ng/mL) and riboflavin (312.0 ± 12.2 ng/mL) after 48 hours was exploited for the first time in the *L. plantarum*. Taken together, *L. plantarum* LCN13 might serve as a potential probiotic candidate for animal farming.

Keywords: Lactic acid bacteria, *Lactobacillus plantarum*, probiotics, riboflavin, swine farming.

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INTRODUCTION

Probiotics have been considered “living micro-organisms” that have positive effects on gut microbiota and immunological systems in humans and livestock (Kook et al., 2019). In animal farming, the administration of probiotics in animal feed is much more effective in reducing serum cholesterol and antibiotic usage, preventing pathogen colonization and improving growth performance (Huang et al., 2013; Popova, 2017). The probiotic concept has been extended as the growth promoter for pets, insects, and aquatic animals. Due to the ban on in-feed antibiotics by the European legislation in 2006, the use of probiotics as additives has drawn more attention over the world (Xiao et al., 2016).

Lactic acid bacteria (LAB) are known as a major group of probiotics. They have been mainly isolated in foods and fermented products such as milk products and beverages (Tambekar & Bhutada, 2010). The genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus* are common members of LAB, in which the genus *Lactobacillus* contributes significantly to the natural microbiota of the animal alimentary tract. The addition of *L. salivarius* and *L. plantarum* to animal feed led to a significant reduction of pathogens such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhimurium*, offering protection of piglets against diarrhoea (Corr et al., 2007; Popova, 2017; Lin et al., 2018). To be a successful probiotic candidate, a strain has to colonize the gastrointestinal tract, suffer from oxidative stress burst and acidic pH, and avoid severe damage of bile salts. Since humans and animals are not able to synthesize riboflavin (vitamin B2) and folate (vitamin B9), the production of riboflavin is also an important probiotic property (Quach et al., 2021b). The enrichment of probiotic bacteria producing B-vitamins in swine farming can replace chemically synthesized vitamins that have widely been used in animal farming. However, only a few studies

evaluated systematically the probiotic traits and vitamin B production of lactic acid bacteria in swine farming.

In Vietnam, several studies have been carried out to evaluate the human health benefits of probiotic strains derived from fermented food (La Anh, 2015). However, the research on LAB with remarkable probiotic properties and vitamin B production used for swine farming is still sparse. In this study, we focused on isolation and screening for LAB with remarkable antibacterial activity from traditionally fermented feed for swine. Moreover, probiotic traits of potent *L. plantarum* LCN13, including lactic acid production, secondary metabolite, oxidative stress, acidic pH, bile salt tolerance, and vitamin B production also were studied. Bacterial candidates holding both capabilities of producing vitamin B and probiotic characteristics will provide large benefits to the livestock industry.

MATERIAL AND METHODS

Isolation and preservation of lactic bacteria

Four samples of traditionally fermented feed for swine were collected from different areas of Bac Giang province, Vietnam in September 2018. All samples were stored in an icebox and then transported rapidly to the laboratory. LAB were isolated on Man Rogosa Sharp (MRS) agar (LAB-M, Bury, UK) at 37 °C for 48 hours. The Gram-staining and catalase tests were conducted to screen the grown colonies with different morphological properties. Only Gram-positive and catalase-negative colonies were streaked out several times on MRS agar to obtain pure isolates. All isolates were stored at -80 °C in glycerol stock (40%, v/v) for further studies.

Screening of the LAB for high acid-producing and anti-pathogenic capacities

The isolates were cultivated on MRS agar supplemented with 0.5% CaCO₃ and incubated at 37 °C for 48 hours. After that, the calcium-dissolving zones were measured in triplicate (Yelnetty et al., 2020). The lactic acid in the supernatant of each isolate was

determined using a spectrophotometric method as described previously (Borshchevskaya et al., 2016).

The anti-pathogenic activity of the LAB culture supernatant was tested according to the agar diffusion method (Quach et al., 2021a). The following tested pathogenic bacteria including *E. coli* ATCC 11105, *S. typhimurium* ATCC 14028, and methicillin-resistant *Staphylococcus epidermidis* ATCC 35984 (MRSE) were used in this study. The diameters of the inhibition zones were measured using a zone scale and expressed in millimeters.

Riboflavin and folate production

The strain LCN13 was cultivated on MRS medium at 37 °C overnight. The overnight culture was transferred to MRS medium and adjusted to an optical density at 600 nm (OD_{600}) of 0.1 at 37 °C. At 24 hours, 48 hours, and 72 hours of incubation time, samples were taken for vitamin B measurement. The concentration of riboflavin and folate was measured using LC-MS/MS following AOAC Official Method 2013.13 and 2015.15, respectively (Salvati et al., 2016; Meisser-Redeuil et al., 2019).

Tolerance to oxidative stress, acidic pH, and bile salt

The strain LCN13 was propagated in MRS medium at 37 °C overnight under vigorous agitation. Oxidative stress tolerance of strain LCN13 was investigated by the preparation of MRS broth supplemented with 0, 1.0, 2.0, 3.0 mM hydrogen peroxide (H_2O_2). The tolerance to acidic pH was conducted on the fresh MRS medium adjusted to pH 6.0, pH 5.0, pH 4.0, pH 3.0, and pH 2.0. As for bile salt tolerance, the MRS broths supplemented with 0, 0.4, 0.8 and 1.2% (w/v) of ox-bile salt were prepared. All experiments were started by the addition of LCN13 overnight culture with an OD_{600} of 0.1 and the cultures were then incubated at 37 °C with continuous shaking. At intervals, cell growth was observed spectrophotometrically at 600 nm every 2 hours (Quach et al., 2021b).

Identification and phylogenetic tree analysis of the potentially probiotic strain

Bacterial morphology of strain LCN13 was observed on the MRS agar and under a light microscope. Physiological and biochemical characteristics were conducted following the modified methods of Karami et al. (2017). The antibiotic susceptibility of strain LCN13 was measured using a disc diffusion test (Das et al., 2016). The test discs were kanamycin (30 µg), nalidixic acid (30 µg), tetracycline (100 µg), chloramphenicol (30 µg), amikacin (30 µg), ampicillin (10 µg), ceftazime (30 µg), streptomycin (10 µg), bacitracin (0.04 U), cefoxitin (30 µg).

For molecular identification using 16S rRNA sequencing, genomic DNA was extracted using the G-spin™ Total DNA Extraction Mini Kit (Intron Bio) according to the manufacturer's instructions. The universal primer pair of 27F (5'-TAACACATGCAAGTTCGAACG-3') and 1429R (5'-GGTGTGACGGGCGGTGTGTA-3') were used for amplification of the 16S rRNA gene. The PCR product was purified with DNA Purification Kit (Promega, Madison, USA) and then sent to First BASE Laboratories Sdn. Bhd. (Malaysia) for Sanger sequencing. The 16S rRNA sequence of isolated strain LCN13 was then compared to 9 reference bacterial strains including *L. plantarum* JCM 1149^T (NR 115605), *L. plantarum* NRBC 15891^T (NR 113338), *L. plantarum* DSM 10667^T (NR 025447), *L. fabifermentans* DSM 21115^T (NR 113339), *L. brevis* ATCC 14869^T (NR 116238), *L. koreensis* DCY50^T (NR116854), *L. yonginensis* THK-V8 (NR 109452), *L. kimchicus* DCY51 (NR 116411), *L. rhamnosus* NBRC 3425^T (NR 113332) in GenBank (NCBI) using the Basic Local Alignment Search Tool program (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic tree was constructed with MEGA version 7.0 using Kimura 2-parameter model. Numbers at nodes indicate the percentages of 1000 bootstrap re-samplings and *Bacillus amyloliquefaciens* BCRC 11601^T was used as the outgroup branch.

Statistical analysis

The data were expressed as mean \pm standard deviation using Excel 2010 and XLSTAT 2016 software for analysis of one-site deviation (ANOVA). The P value ≤ 0.05 was statistically significant.

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria

A total of 35 colonies with different morphological characteristics were isolated from traditionally fermented feed for swine. Out of 35 isolates, 9 were found to be Gram-positive, cocci- or rod-shaped, and catalase-negative, which were selected for further

studies. In addition, all isolates exhibited strong growth on MRS media.

Screening of high acid- and antibiotic-producing lactic acid bacteria

As shown in Table 1, a calcium-dissolving ring was observed in all isolates when tested on MRS agar containing 0.5% CaCO₃. Out of these isolates, 4 including LCN13, LCN16, LCN29, and LCN30 were found to be good acid producers with calcium-dissolving zones > 12 mm. The presence of clear halos surrounding the colonies suggested that these obtained LAB metabolized sugar to produce acid such as lactic acid, which reacted with calcium carbonate to yield soluble calcium lactate.

Table 1. Acid production and antibacterial activity of LAB strains against pathogenic bacteria

Isolates	Calcium-dissolving zones (mm)	Lactic acid (g/L)	Activity against pathogenic bacteria (mm)		
			<i>S. typhimurium</i> ATCC 14028	<i>E. coli</i> ATCC 11105	MRSE ATCC 35984
LCN4	4.2 ^a \pm 0.32	4.7 ^a \pm 0.18	13.8 ^d \pm 2.51	10.1 ^{a,b} \pm 2.53	11 ^b \pm 1.12
LCN9	6.1 ^b \pm 0.21	5.5 ^b \pm 0.38	3.8 ^b \pm 1.43	8.1 ^a \pm 1.76	9.8 ^b \pm 1.51
LCN13	13.1 ^f \pm 0.19	18.5 ^h \pm 0.31	18.3 ^e \pm 1.52	24.7 ^e \pm 2.14	31.6 ^e \pm 2.93
LCN16	12.3 ^e \pm 0.16	16.2 ^g \pm 0.32	21.1 ^g \pm 2.10	17.9 ^c \pm 2.22	20.1 ^d \pm 1.85
LCN22	6.2 ^b \pm 0.19	5.3 ^{a,b} \pm 0.18	6.9 ^c \pm 0.87	12.5 ^b \pm 0.94	0 ^a
LCN23	8.1 ^c \pm 0.34	9.1 ^c \pm 0.24	0 ^a	16.9 ^c \pm 1.09	9.7 ^b \pm 1.14
LCN29	12.5 ^e \pm 0.27	14.2 ^e \pm 0.40	18.9 ^f \pm 1.21	17.2 ^c \pm 1.11	21.5 ^d \pm 1.98
LCN30	15.3 ^g \pm 0.11	15.2 ^f \pm 0.35	16.9 ^e \pm 1.47	21.4 ^d \pm 1.75	17.3 ^c \pm 1.64
LCN31	9.1 ^d \pm 0.21	13.9 ^d \pm 0.12	22.1 ^h \pm 1.87	17.8 ^c \pm 1.41	15.9 ^c \pm 1.21

Note: Values with different letters are significantly different according to Fisher LCD test (P < 0.05).

To support this result, an analysis of lactic acid produced during fermentation was carried out. LCN13 isolate produced the highest amount of lactic acid (18.5 \pm 0.31 g/L) followed by LCN16 (16.2 \pm 0.32 g/L), and LCN30 (15.2 \pm 0.35 g/L) (Table 1). As proved previously, strong acid-producing LAB falls into 4 main genera including *Pediococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* (Tilahun et al., 2018). The addition of high acid-producing lactic acid

bacteria to animal feed was reported to prevent mouldy feed, shorten the fermentation process, enrich nutrients, and improve growth performance (Yeh et al., 2018; Nguyen et al., 2020). Of note, only isolate LCN13 could produce 182 ng/mL folate and 233 ng/mL riboflavin and after 24 hours of incubation (Fig. 1).

Antibacterial activity experiments showed that most isolates had inhibitory effects against 3 tested pathogenic bacteria

although isolate LCN22 and LCN23 were not effective in inhibiting MRSE ATCC 35984 and *S. typhimurium* ATCC 14028, respectively (Table 1). *E. coli* ATCC 11105 was the most sensitive pathogen to the inhibitory effect of 9 LAB isolates. Among tested isolates, LCN13 proved to have significant activity against *S. typhimurium* ATCC 14028 (18.3 ± 1.52 mm), *E. coli* ATCC 11105 (24.7 ± 2.14 mm), and MRSE

ATCC 35984 (31.6 ± 2.93 mm). This result was in agreement with previous studies showing that *L. plantarum* and *L. pentaceus* had the ability to inhibit the growth of 3 tested pathogenic bacteria (Tambekar & Bhutada, 2010; Amin et al., 2020). The mode of action of LAB is attributed to H₂O₂ production, organic acids, and especially secondary metabolites such as bacteriocin (Hütt et al., 2006).

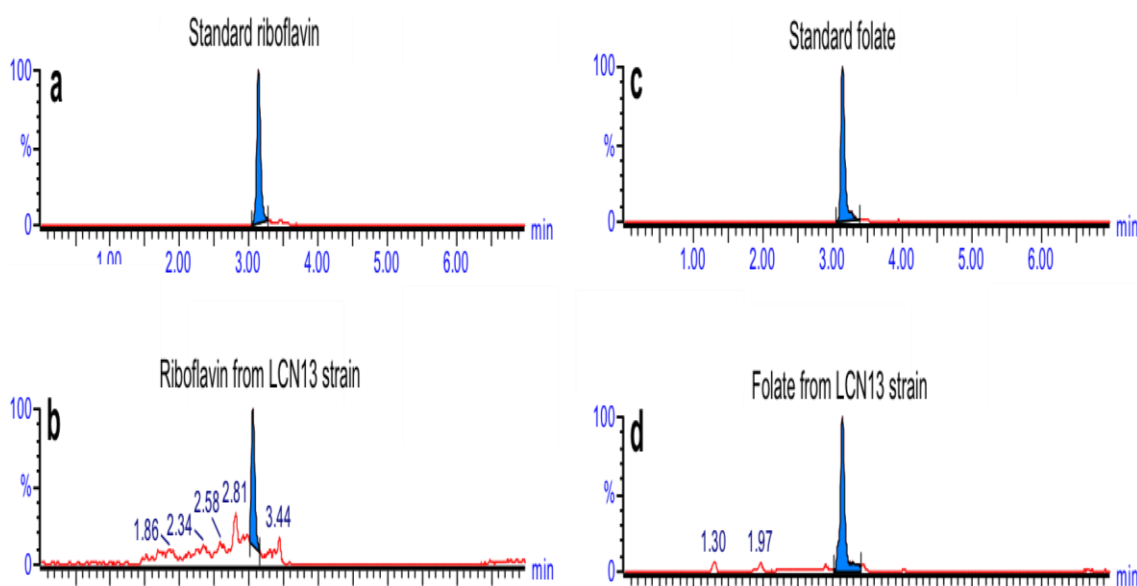


Figure 1. Chromatogram analysis of standard riboflavin (a), riboflavin produced by LCN13 strain (b), standard folate (c), and folate produced by LCN13 strain (d)

Identification of LCN13 strain

The colonies grown on MRS agar were rough, dry irregular colonies with a jelly-like surface (Table 2). The growth characteristics were observed at the temperature range of 30–40 °C, pH range of 2.0–8.0, and NaCl range of 0–5.0%. The isolate LCN13 was able to ferment various sugar sources including mannitol, glucose, lactose, maltose, sucrose, xylose, melibiose, sorbitol, mannose, and fructose. Moreover, LCN13 was susceptible to 7 antibiotics such as vancomycin, nalidixic acid, kanamycin, ampicillin, ceftazime, streptomycin, and cefoxitin. These results indicated that the isolate LCN13 belonged to the genus *Lactobacillus*.

Analysis of the 16S rRNA sequence showed that strain LCN13 was around 99% identical to that of *L. plantarum* JCM 1149^T (NR 115605) and *L. plantarum* NRBC 15891^T (NR 113338). The 16S rRNA gene sequence of strain LCN13 was deposited onto the GenBank (NCBI) under the accession number OK067384. Furthermore, the neighbour-joining phylogenetic tree was constructed to show that strain LCN13 had the closest relationship with *L. plantarum*, which was in accordance with that of conventional identification (Fig. 2). Based on the phenotypic and molecular identification, strain LCN13 was identified as *L. plantarum* LCN13.

Table 2. Phenotypic characteristics and antibiotic sensitivity of *L. plantarum* LCN13

Characteristics	Result	Lactose	+
Morphological characteristics		Maltose	+
Shape	Rod	Sucrose	+
Motility	-	Ramnose	-
Gram staining	+	Xylose	+
Spore formation	-	Melibiose	+
Physiological properties		Sorbitol	+
Temperature range for growth	30–40 °C	Manose	+
Optimum temperature	37 °C	Fructose	+
pH range for growth	2.0–8.0	Antibiotic sensitivity	
Optimum pH	6.0	Vancomycin (30 µg)	S
NaCl range for growth	0–5.0%	Nalidixic acid (30 µg)	S
Optimum NaCl	2.0%	Kanamycin (30 µg)	S
Biochemical properties		Tetracycline (100 µg)	R
Catalase	-	Chloramphenicol (30 µg)	S
Amylase	-	Amikacin (30 µg)	R
Protease	+	Ampicillin (10 µg)	S
Cellulase	-	Ceftazime (30 µg)	S
Acid production from		Streptomycin (10 µg)	S
Mannitol	+	Bacitracin (0.04 U)	R
Glucose	+	Cefoxitin (30 µg)	S

Note: (+), positive test; (-), negative test; R, resistance; S, sensitive.

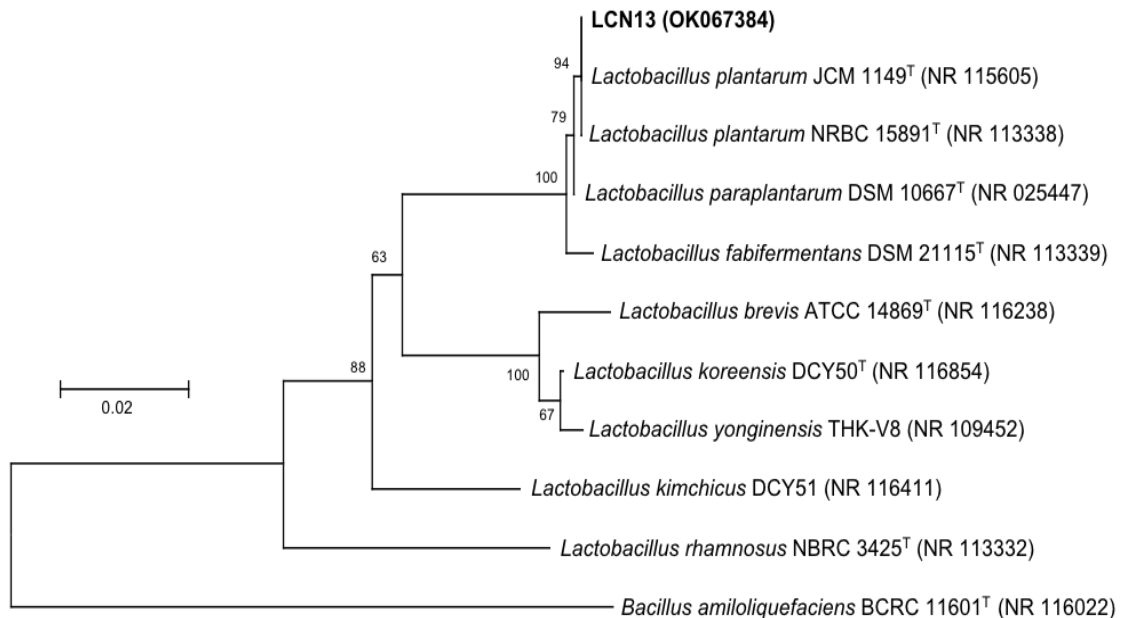


Figure 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of LCN13 strain with other closely related species

Tolerance to bile salt, oxidative stress, and acidic pH

Resistance to bile salts produced in the gastrointestinal tract is one of the important probiotic properties since bile severely damages lipids and fatty acids of the cell membrane, leading to cell death (Amin et al., 2020). Surprisingly, *L. plantarum* LCN13 could survive well in 0.8% ox-bile salt after 14 hours of incubation. Increasing ox-bile concentration to 1.2% resulted in much lower

survival of strain LCN13, which was the highest ox-bile salt concentration recorded in *L. plantarum* strains (Fig. 3a). A previous study showed that *L. plantarum* LCN13 isolated from Chinese traditional fermented acid beans could suffer up to 0.45% ox-bile salt after 12 hours of incubation. *L. plantarum* could prevent the negative effects of bile salts due to bile salt hydrolase and deconjugation activities (Tambekar & Bhutada, 2010; Huang et al., 2013).

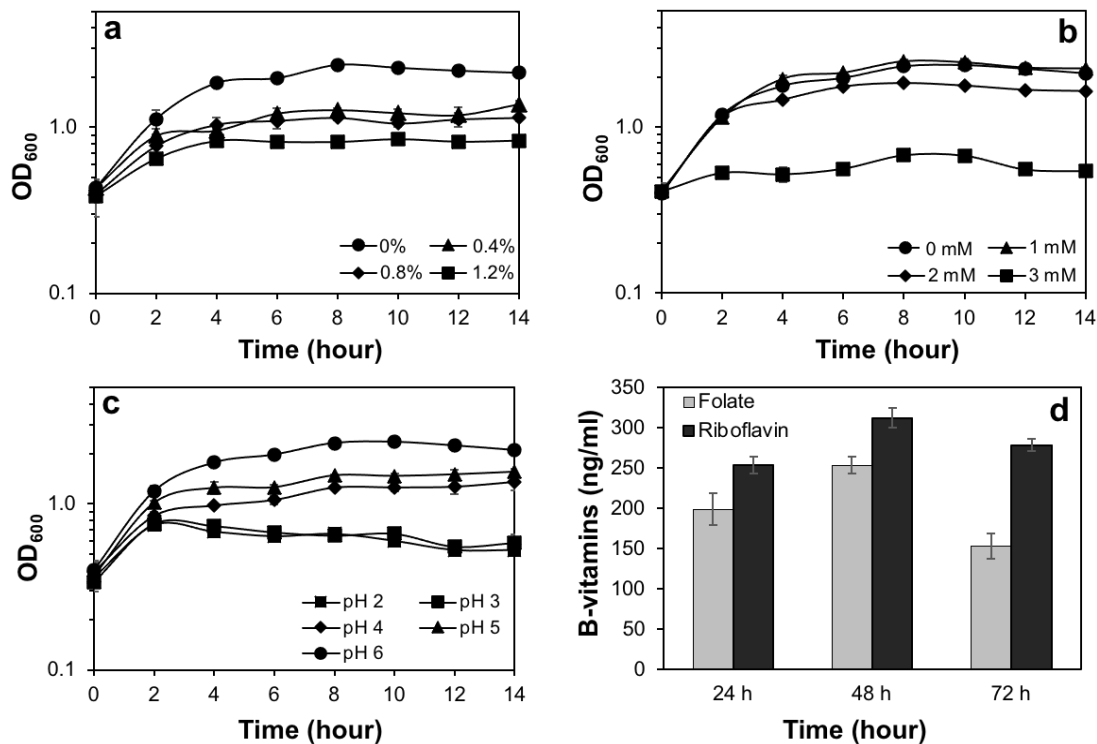


Figure 3. Growth curves under different stress conditions including ox-bile salt (a), H₂O₂ (b), acidic pH (c), and B-vitamin production (d) of strain LCN13

Probiotic bacteria are believed to have encountered oxidative stress generated from prolonged luminal oxidant exposure, exogenous stimuli, and imbalanced microbiota. As shown in Figure. 3b, *L. plantarum* LCN13 conferred strong resistance to 2.0 mM H₂O₂. Upon increasing the H₂O₂ concentration to 3.0 mM, the growth was significantly reduced without recovery after 14 hours. Lin et al. (2018) reported that *L. plantarum* AR113 could tolerate up to 1.5

mM H₂O₂, which was not comparable to this study, further confirming the potential of this strain in resisting oxidative stress generated in the gastrointestinal tract of swine.

Resistance to acid pH is also an important indicator of probiotic activity. Strain LCN13 showed fairly good tolerance to pH 5.0 and pH 4.0, at which the viability was slightly reduced (Fig. 3c). However, exposure to pH 2.0 and pH 3.0 resulted only in a growth delay

that could not be recovered after 14 hours. The potential capability to survive in acidic environments could be due to the distribution of nonenzymatic and enzymatic antioxidants encoded in the genome. The survival of *L. plantarum* LCN13 at acidic pH was similar to other studied probiotic strains that were isolated from traditional food products for humans and animals (Tambekar & Bhutada, 2010; Karami et al., 2017).

Folate and riboflavin production

Besides common probiotic activities, the ability to produce folate and riboflavin is also another important property of *L. plantarum* LCN13. After 24 hours of incubation, strain LCN13 yielded 199.1 ± 19.5 ng/mL folate (Fig. 3d). The prolongation of incubation time to 48 hours increased significantly folate production (253.6 ± 10.7 ng/ml). After 72 hours, folate production was significantly reduced to 153.4 ± 15.7 ng/mL. As described previously, *L. plantarum* ATCC 14917 produced the highest folate concentration of 63.2 µg/ml in the optimized medium (Wu et al., 2017), which was 252-fold higher than that of *L. plantarum* LCN13. By contrast, *L. plantarum* GG and G72 isolated from kimchi yield only 50.1 ± 1.3 ng/ml and 55.5 ± 0.2 ng/mL folate, respectively (Hye-Ji et al., 2021). They indicated that folate productivity varied strongly at species level depending on isolation source and medium.

On the other hand, the riboflavin concentration of 254.0 ± 10.3 ng/mL was produced by *L. plantarum* LCN13 after 24 hours. The highest riboflavin concentration, 312.0 ± 12.2 ng/mL, was observed after 48 hours, which was equal to *L. plantarum* CRL 725 (309 ± 9 ng/mL) (Sabo et al., 2020). Increasing incubation time to 72 hours reduced relatively riboflavin production (278.6 ± 7.7 ng/mL) of strain LCN13. These results suggested that *L. plantarum* LCN13 was a producer of folate and riboflavin. Since animals are not able to synthesize vitamin B such as B2 and B9, feeding diets supplemented with both vitamins are required to decrease stress effects, increase growth

performance, and improve the lean meat percentage of growing-finishing swines (Han et al., 2017).

CONCLUSION

In this current study, thirty-five unique bacterial isolates were isolated from traditionally fermented feed for swine, among which 9 isolates were characterized for their lactic acid production and antipathogenic activity. Among them, the isolate LCN13 produced the highest lactic acid concentration (18.5 ± 0.31 g/L) and showed great inhibitory effects against *S. typhimurium* ATCC 14028, *E. coli* ATCC 11105, and MRSE ATCC 35984. Especially, LCN13 was able to yield simultaneously folate (182 ng/ml) and riboflavin (233 ng/mL). The isolate LCN13 was then identified as *Lactobacillus plantarum* LCN13 based on morphological and molecular analysis. *L. plantarum* LCN13 was much more resistant to bile salt, H₂O₂, and acidic pH. For the first time, the probiotic traits and production of B-vitamins were exploited in *L. plantarum* LCN13. These results proved for the first time that *L. plantarum* LCN13 comprises distinctive characteristics including probiotic traits and B-vitamin production, serving as a promising probiotic candidate for swine farming.

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