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PRIMARY STUDY ON THE PROBIOTIC PROPERTIES OF *Lactobacillus casei* LC 304.08 FOLLOWING INTERNATIONAL STANDARDS

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Abstract. Products containing beneficial bacteria for humans have been widely used recently. The bacteria in these products generally belong to the genus *Lactobacillus*. Among the species of this genus, *Lactobacillus casei* attracts a lot of interest due to their valuable properties. With these properties, many *L. casei* strains can be used as probiotics. Based on the recommendation of FAO/WHO for probiotic bacteria, in our study strain LC 304.08 was identified based on morphology and biochemical properties as well as nucleotide sequence of 16S rDNA. The strain was then evaluated for its properties *in vitro* such as the ability to survive at low pH and in the presence of bile salt, the ability to produce antibacterial substances and enzyme as well as its safety regarding hemolytic and antibiotic resistance. The results showed that the strain was identified as *Lactobacillus casei*. It survived well at low pH and in the presence of bile salt, and it produced β-galactosidase with the activity of 81 ± 1.1 (U/mL). In addition, the strain was safe regarding its hemolytic activity and antibiotic resistance.

Keywords: β-galactosidase, hemolytic activity, *Lactobacillus casei*, probiotic.

Classification numbers: 1.2.1, 1.2.4, 1.3.2

1. INTRODUCTION

Probiotics are defined as live microorganisms or a product containing viable microorganisms in sufficient numbers to alter the microflora which, when administered in adequate amounts, confer a health benefit on the host. The most extensively used probiotics are *Lactobacilli*, *Bifidobacteria*, *Bacilli*, and yeasts [1]. Among them, *Lactobacillus casei* attracts a lot of interest due to its valuable properties. *L. casei* exists in many products used in our life [2]. Despite their long history of safe use, for being used in human, the bacterial strain should be assessed carefully both *in vitro* and *in vivo* according to FAO/WHO recommendation [1]. Although many studies report characteristics of bacterial strains used as probiotics in Viet Nam,

most of them are not done following FAO/WHO recommendation [3, 4]. Therefore, using FAO/WHO recommendation is needed for assessing bacteria used as probiotics strains in Viet Nam.

In our study, we assessed whether the strain meets the requirements *in vitro* for being a probiotic according to FAO/WHO recommendation. At first, the strain was identified as *Lactobacillus casei* based on the morphology and biochemical properties as well as the nucleotide sequence of 16S rDNA. Survival of the strain at low pH (pH 1, 2 and 3) and in the presence of different bile salt concentrations (from 0.062 % to 8 %) were investigated. In addition, the strain was capable of producing β-galactosidase (81 ± 1.1 U/ml) and antibacterial substances against *Escherichia coli* VTCC 11775, *Salmonella enterica* VTCC 12277, *Shingella flexneri* VTCC 12060 and *Bacillus cereus* VTCC 11289. Lastly, the safety aspects of the strain, regarding its hemolytic activity and antibiotic resistance, were assessed.

2. MATERIALS AND METHODS

2.1. Materials

Strain LC 304.08 was isolated from fermented pork roll (Thanh Hoa province, Viet Nam).

Pathogens for testing antibacterial activities were provided by Viet Nam Type Culture Collection (VTCC), Institute of Microbiology and Biotechnology, Vietnam National University Hanoi, including *Escherichia coli* VTCC 12272, *Salmonella enterica* VTCC 12277, *Bacillus cereus* VTCC 11289, and *Shingella flexneri* VTCC 12060.

Antibiotics used in the study were Chloramphenicol (30 µg), Vancomycin (30 µg), Penicillin (10 µg), Kanamycin (30 µg), Tetracycline (30 µg), Erythromycin (15 µg), Streptomycin (10 µg), Ampicillin (10 µg), and Gentamicin (10 µg).

All chemicals were purchased from Sigma Aldrich (USA) and Merk (Germany); MRS and other media were purchased from LAB (Neogen company, USA) or from Microbiol (Italy).

2.2. Methods

2.2.1. Morphological observation

The morphology of colony and cell of strain LC 304.08 was examined and observed under a Primo Star ZESSI microscope at 100-fold magnification.

2.2.2. Biochemical properties

The fermentation ability of the strain was studied using API CH50 Kit (Biomerieux, USA) following the manufactory instructions.

2.2.3. PCR amplification, sequencing, and phylogenetic analysis

The 16S rDNA was amplified using 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) primers. The reaction mixture (50 µl) contained 25 µl master mix, 50 pmol of each primer, and 1 µl of template DNA. Thermocycles of PCR included 5 minutes of heat shock at 95 °C, followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1.30 minutes, and a final extension at 72 °C for 7 minutes. The PCR products were then analyzed by electrophoresis on 1% agarose gel, purified with QIA quick gel extraction kit (Qiagen), and the sample was sent to 1st Base company (Singapore) for sequencing. The 16S rDNA sequences were compared with sequences available on the Gen

Bank/EMBL/DDBJ databases by using the BLAST Search tool. The alignment with corresponding sequences was performed by using Bioedit version 7.2. A phylogenetic tree was constructed by MEGA version 10 and the neighbor-joining method [5]. Topography of the constructed tree was evaluated by bootstrap analysis with 1000 replicates [6].

2.2.3. Antibiotic susceptibility

Antibiotic susceptibility/resistance was tested by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [7]. Antibiotic discs (Oxoid) were placed on the surface of the plate, containing the LC 304.08 strain. After 48 h incubation at 37 °C the results interpreted as susceptible (S), or resistant (R) according to the diameter of inhibition zones measured in mm (means ± SD of 3 trials).

2.2.4. Agar well-diffusion antimicrobial assay

The supernatant, obtained after 2 growing days of the strain, was put into wells on the plates containing 1 of the 4 pathogen strains. Antibacterial activity was determined by antibacterial zone around the wells [8].

2.2.5. β-galactosidase assay

The green colour of the strain was tested on an MRS agar plate containing 60 µL of X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) with a concentration of 20 mg/mL. After that, β-galactosidase activity was determined as follows: 1 mL of bacteria in the stationary phase was centrifuged at 12000 × g for 5 min at 5 ^{o}C. The cell pellet after washing twice in phosphate buffer, containing 60 mM $Na_2HPO_4{\times}7H_2O$ and 40 mM NaH_2PO_4 with pH 7.0, was permeabilized with toluene/acetone (1:9 v/v) solution, and vortexed for 7 min. Then 900 µL of phosphate buffer and 200 µL of ONPG (O-nitrophenyl-β-D-galactopyranoside) with a concentration of 4 mg/mL were added to 100 µL of the permeabilized cell suspension and placed in a water bath at 37 ^{o}C for 15 min. Next, 0.5 mL of 1 M Na_2CO_3 was added to stop the reaction. The absorbance values of the solution, obtained after centrifuging, were measured at both wave lengths of 420 and 560 nm, and β-galactosidase value was calculated in Miller units using the following formula:

$$MU = 1000 \times \frac{A\ (420) - 1.75 * A2\ (560)}{\frac{15min}{1\ ml} * A1(560)}$$

where A1(560) was the absorbance just before assay and A2(560) was the absorbance value of the reaction mixture [9].

2.2.6. Survival at the medium with low pH values (1, 2, 3) and the presence of bile salt

0.2 ml of cells grown at 37 ^{o}C for 48 h were centrifuged, washed twice in phosphate buffer and resuspended in 2.0 ml of normal MRS (pH 6.8 ± 0.2) and MRS with the pH of 1.0, 2.0, and 3.0, respectively (normal MRS medium adjusted to each pH value with HCl) to obtain final counts between 10^7 - 10^8 CFU/mL. After 1.5 h and 3 h, 0.1 ml of MRS medium with different pH values was plated out on normal MRS agar plates. Results were expressed as percentage decreases in log CFU/mL, wherein the log CFU/mL in a normal MRS medium was assumed as 100 % after incubation. Experiments were made in duplicate [10].

To assess the survival of the strain in the presence of bile salt, 10 µl of the culture broth with 10^7 - 10^8 CFU/mL after treating with 0.062 - 8 % bile salt for 48 h was dropped in MRS

agar plate. The plate was incubated at 37 ^{o}C and the growth of the strain was recorded after 48 h [11].

2.2.7. Hemolytic activity

The hemolytic activity was determined by streaking the strain on Columbia Blood (Microbiol) agar plates supplemented with 5 % defibrinated sheep blood after 48 h of incubation at 37 ^{o}C. The hemolytic reaction was recorded by observing a clear zone of hydrolysis around the colonies (β-hemolysis), a partial hydrolysis and greenish zone (α-hemolysis), or no reaction (γ-hemolysis) [12].

3. RESULTS AND DISCUSSION

According to FAO/WHO recommendations for bacterial strains to be used as a probiotic, the strain should be identified and then its characteristics, including the ability to survive at low pH and in the presence of bile salt, the ability to produce antibacterial substances and enzyme as well as its safety regarding hemolytic and antibiotic resistance should be assessed.

3.1. Identification, morphology and biochemistry properties of the strain

3.1.1. Morphology properties

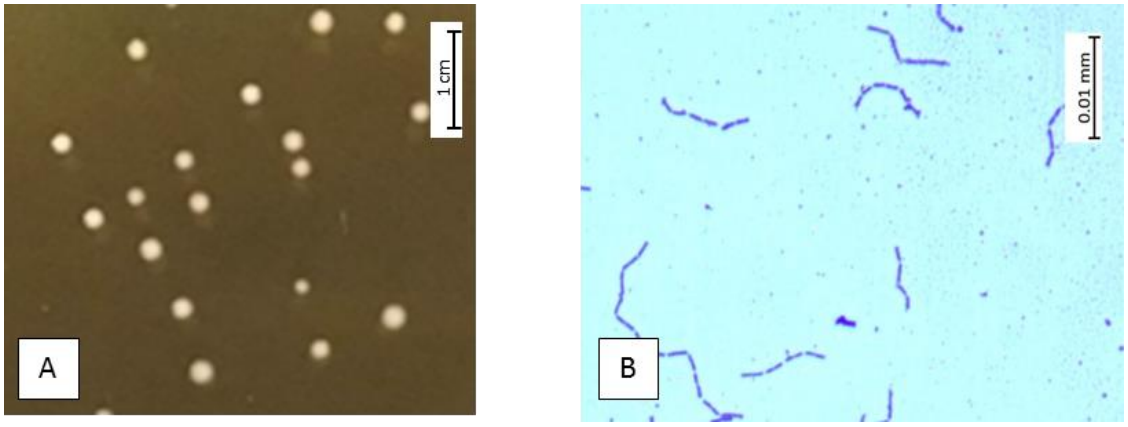


Figure 1. Image of the morphology of colony (A) and cell (B) of the strain.

The colony of LC 304.08 was convex/domed with entire margin, smooth and shiny surface (Figure 1A). Under microscope, its cell was rod-shaped with the size range around 0.7 - 1.1 × 2.0 - 4.0 micrometer (Figure 2B). The morphology of colony and cell of the strain was characterized for *L. casei* as reported in the study of Sutula *et al*. (2012) [13].

3.1.2. Biochemical properties

The strain ability to use carbohydrate was assessed using API CH50 Kit (Biomerieux, USA), and the results are presented in Table 1. The results in Table 1 were consistent with the results for *L. casei* of the manufactory attached in the kit.

Table 1. The strain ability to use carbohydrate by using API CH50 Kit (Biomerieux, USA).

No	Carbohydrate		No	Carbohydrate	
0	Control		25	Esculin	+++
1	Glycerol	+	26	Sacilin	++
2	Erythritol	-	27	Cellobiose	+++
3	D-anabinose	-	28	Maltose	+++
4	L-anabinose	-	29	Lactose	+++
5	Ribose	+++	30	Mellibiose	-
6	D-xylose	-	31	Saccharose	+++
7	L-xylose	-	32	Trehalose	+++
8	Adonitol	-	33	Inulin	+
9	B-methyl xyloside	-	34	Melizitose	+++
10	Galactose	+++	35	D-rafinose	-
11	D-glucose	+++	36	Amidon	-
12	D-fructose	+++	37	Glycogen	-
13	D-mannose	+++	38	Xylitol	-
14	L-sorbose	+++	39	B-gentiobiose	++
15	Rhamnose	-	40	D-turanose	+++
16	Dulcitol	-	41	D-lyxose	+
17	Inositol	++	42	D-tagatose	+++
18	Mannitol	+++	43	D-fucose	-
19	Sorbitol	+++	44	L-fucose	-
20	α-methyl-D-mannoside	-	45	D-arabitol	-
21	α-methyl-D-glucoside	-	46	L-arabitol	-
22	N-acetyl-glucosamine	++	47	Gluconate	++
23	Amygdalin	++	48	2-ceto-gluconate	-
24	Arbutin	++	49	5-ceto-gluconate	+

(+): Fermentation percent was 10 - 30 %; (++): Fermentation percent was 30 - 60 %;
(+++): Fermentation percent was > 60 %; (-): Fermentation percent was < 10 %.

3.1.3. Identification of the strain by the nucleotide sequences of 16S rDNA

After checking the PCR product of amplification of 16S rDNA (Figure 2), its nucleotide sequences were obtained and deposited in Genebank with the accession number of MZ555940. The sequences were analyzed, and the phylogenetic tree of the strain LC 304.08 was established (Figure 3).

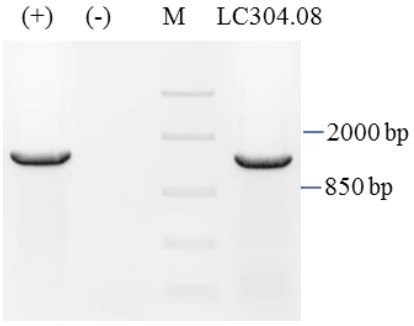


Figure 2. Image of electrophoresis result of 16S rDNA amplification.(+): positive control, 16S rDNA from which was amplified successfully; (-): negative control, in which DNA template was replaced by water; (M): FastRuler Middle Range DNA Ladder (Thermo Scientific).

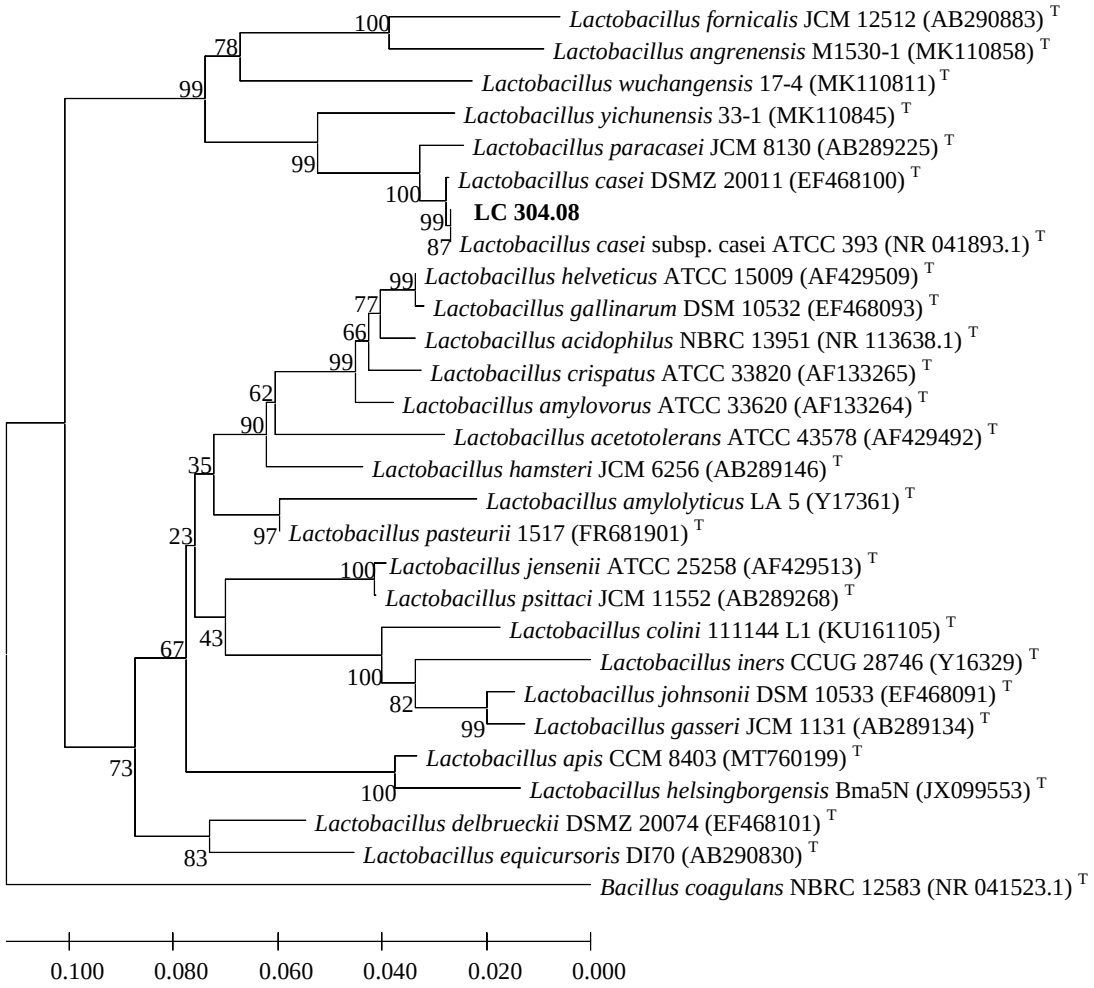


Figure 3. Phylogenetic tree of strain LC 304.08.

Neighbour-joining tree based on 16S rRNA gene sequences showed the position of strain LC 304.08 among related species in the *Lactobacillus* genus. *Bacillus coagulans* NBRC 12583 T was used as an outgroup. Bootstrap values of above 50 % based on 1000 replications were given at nodes. Bar was 2 % sequence difference.

The results based on the morphology, biochemical properties, and the sequence of 16S rDNA showed that LC 304.08 was identified as *Lactobacillus casei*, which was listed among safe strains that can be used for probiotic according to FAO/WHO recommendations.

3.2. Probiotic characteristics

3.2.1. The ability of the strain to survive at low pH and in the presence of bile salt

Among the probiotic characteristics, the viability of strain, especially in the acidic environment of the stomach and bile secreted in the duodenum is one of many important

parameters which affect desired benefits of the strain. The survival rate of the strain in low pH media and in the presence of bile salt was presented in Figure 4 and Figure 5, respectively. The results showed that strain LC 304.08 survived well at low pH and in the presence of bile salt.

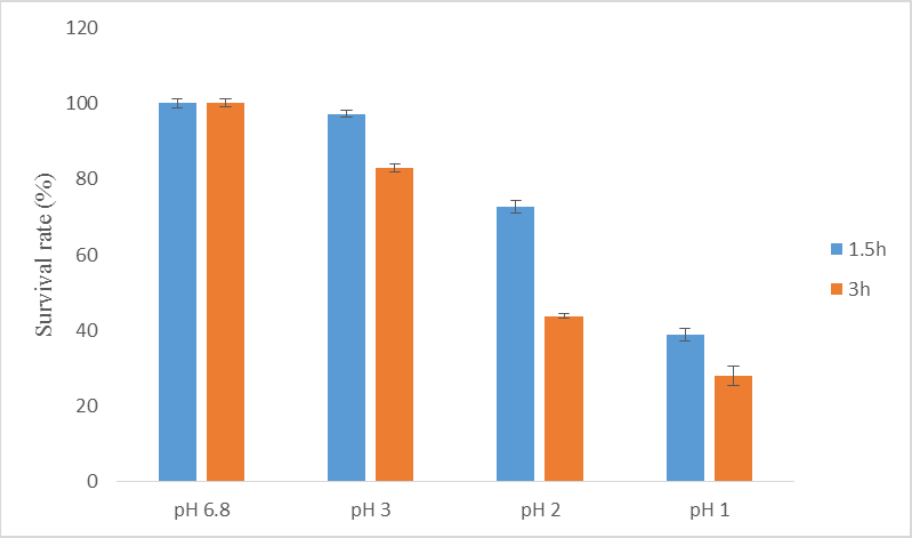


Figure 4. The survival rate of the strain at low pH after 1.5 and 3 hours.

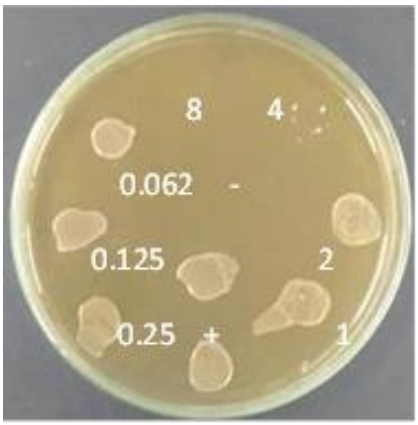
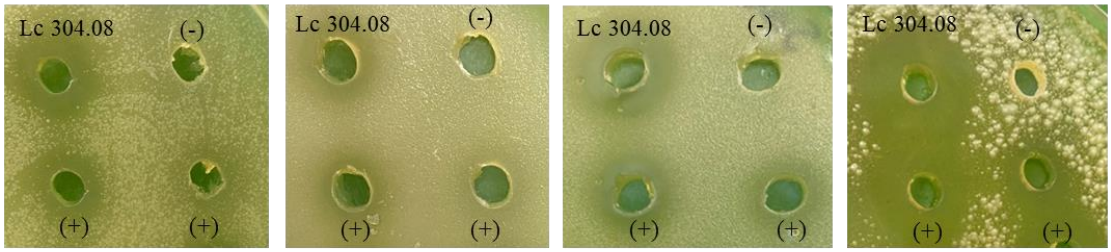


Figure 5. Image of the growth of the strain treated with different concentrations of bile salt for 48 hours on MRS agar medium.

3.2.2. The ability of the strain to produce β-galactosidase and antibacterial substances

In addition, the ability of the strain to produce β-galactosidase and antibacterial substances against some pathogens is presented in Figures 6 and 7, respectively. After screening in the agar plate containing X-gal, β-galactosidase activity of strain LC 304.08 was determined with a value of 81 ± 1.1 (U/mL). Lactose intolerance is a common problem in children and most adults in the world, therefore, adding probiotic capable of producing β -galactosidase could help lactose intolerance symptoms. Furthermore, the strain was capable of producing antibacterial activity against *E. coli* VTCC 12272, *S. enterica* VTCC 12277, *S. flexneri* VTCC 12060, and *B. cereus* VTCC 11289.

Figure 6. Image of ability of the strain to produce β-galactosidase.



E. coli VTCC 12272 | *S. flexneri* VTCC 12060 | *S. enter*ica VTCC 12277 | *B. cereus* VTCC 11289

Figure 7. Image of ability of the strain to produce antibacterial substances against *E. coli* VTCC 12272, *S. enterica* VTCC 12277, *S. flexneri* VTCC 12060, and *B. cereus* VTCC 11289.

(-): negative control, in which the medium without bacterium is used; (+): positive control is a culture broth of the bacteria capable of producing antibacterial substances against the pathogen strains.

In the research of Widodo *et al.* (2012), the survival rate of *L. casei* 1AF at pH 2.0 for 90 minutes is 60.4 % and the strain inhibits the growth of *E. coli* and *B. cereus* [14]. *L. casei* KL14 in the research of Sharma *et al.* (2016) shows the survival rate of 96 % at pH 2.0 for 90 minutes [15]. In our case, the survival rate of *L. casei* 304.08 was around 76 % and it inhibited 4 pathogen strains, including *E. coli* VTCC 12272, *S. flexneri* VTCC 12060, *S. enterica* VTCC 12277, and *B. cereus* VTCC 11289. Based on the results, the strain *L. casei* 304.08 possessed the probiotic properties such as i) survival at low pH (pH 1, 2 and 3) and in the presence of 2 % bile salt; ii) ability to produce antibacterial substances and β-galactosidase. These characteristics were important for probiotic strains to exert their useful functions in intestinal tract such as balancing microflora, defending against pathogenic bacteria and increasing immunity.

3.3. Safety assessment

Although there is evidence that probiotics can play a role in disease prevention and health promotion, their safety should be considered carefully. In our study, safety assessment, including hemolytic activity and antibiotic resistance of the strain, was examined.

3.3.1. Hemolytic activity

The hemolytic activity of the strain LC 304.08 was shown in Figure 8. Based on the result, hemolytic activity of the strain was γ, therefore, the strain was safe in term of its hemolytic activity [12].

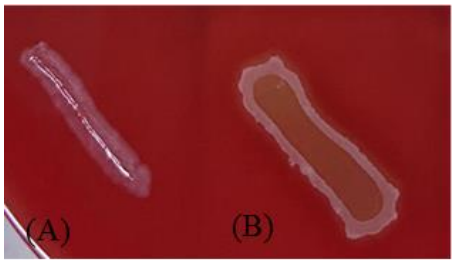


Figure 8. Image of hemolytic activity of the strain (A) and positive control (B).

3.3.2. Antibiotic susceptibility

The results of the strain's antibiotic susceptibility are shown in Table 2.

Table 2. Antibiotic susceptibility of strain LC 304.08.

No.	Antibiotic	Amout (µg/disc)	**LC 304.08**	
			Diameter of antibacterial zone (mm)	**Resistant (R) or sensitive (S) antibiotic**
1.	Chloramphenicol	30	49 ± 0.16	S
2.	Vancomycin	30	10 ± 0.23	R
3.	Penicillin	10	56 ± 0.45	S
4.	Kanamycin	30	0 ± 0.81	R
5.	Tetracycline	30	46 ± 0.53	S
6.	Erythromycin	15	37 ± 0.53	S
7.	Streptomycin	10	15 ± 0.18	S
8.	Ampicillin	20	51 ± 0.61	S
9.	Gentamicin	10	13 ± 0.21	S

The results in the Table 2 showed that the strain was resistant against vancomycin and kanamycin. Intrinsically resistance of L. casei to vancomycin is reported in many studies due to the replacement of the terminal D-alanine residue by D-lactate or D-serine in muramyl-pentapeptide molecule 16, 17]. Moreover, it is well documented that most Lactobacillus species are intrinsically resistant to aminoglycosides, including gentamicin, kanamycin, streptomycin, and neomycin [18]. Recently, Campedelli *et al.*, 2019 indicate that twenty different predicted sequences encoding for aminoglycoside-modifying enzymes are identified among the 161 Lactobacillus genomes [19]. This resistance is considered intrinsic and originates from the low impermeability of lactobacilli cell surface for aminoglycosides [20]. Therefore, in general the strain LC 304.08 met all requirements regarding in vitro assessment of a potential probiotic strain following FAO/ WHO recommendation. The strain was deposited at the VTCC with the code of VTCC 60014.

4. CONCLUSIONS

Strain LC 304.08 was identified as *Lactobacillus casei* based on morphology and biochemical properties as well as nucleotide sequence of 16S rDNA. It survived well at low pH and in the presence of bile salt. In addition, the strain was capable of producing antibacterial

activity against *E. coli* VTCC 11775, *S. enterica* VTCC 12277, *S. flexneri* VTCC 12060, and *B. cereus* VTCC 11289. β-galactosidase activity of the strain was 81 ± 1.1 (U/mL). Also, the strain was safe regarding its hemolytic activity and antibiotic resistance.

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Credit authorship contribution statement. Nguyen Ngoc Hong: Investigation, Van Huong Giang: Investigation, Nguyen Thi Phuong: Investigation, Dao Ngoc Ha: Investigation, Nguyen Duy Ha: Formal analysis, Le Huy Hoang: Formal analysis, Nguyen Quynh Uyen: Methodology, Hoang Van Vinh: Supervision.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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