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Isolation of *Weizmannia coagulans* and probiotic characterisation of strains

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Abstract:

***Weizmannia coagulans* is a potential bacterial candidate for probiotics because of their advantageous characteristics in endospore formation, antibacterial production, and other beneficial human health effects. In this study, we isolated *W. coagulans* strains from different sample types collected in Hanoi and examined them for probiotic characteristics. From 40 samples, 5 strains were isolated from cow faeces. All *W. coagulans* strains had non-haemolytic activity and were susceptible to seven tested antibiotics. Cell-free supernatants of *W. coagulans* strains cultured in MRS medium could inhibit some bacterial pathogens such as *Enterococcus faecalis*, *Listeria monocytogenes, Vibrio vulnificus,* and *Aeromonas dhakensis*. The strains showed antioxidant activity with scavenging DPPH rates ranging from 72 to 82%. Three strains were able to adhere to the HT-29 cell line with adhesion rates ranging from 46 to 62.59%. The spores and vegetative cells of the three strains also showed great stress tolerance (survival rates >90%) against simulated gastrointestinal fluid. In liquid media containing 0.1% pancreatin and 0.3% bile salt, spores of VTCC 12782 and VTCC 12779 could germinate at rates of 25 and 2%, respectively. With the most beneficial characteristics, *W. coagulans* VTCC 12782 is suggested as a potential candidate for the development of probiotic products in the future.**

Keywords: **beneficial properties, probiotic, Vietnam, *W. coagulans.***

Classification numbers: **3.4, 3.5**

1. Introduction

Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [1]. Recent studies on probiotic microorganisms mainly focus on some genera of *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Streptococcus*, and *Saccharomyces* [2]. These microorganisms are not able to survive in hostile conditions such as high temperatures and dry environments during the manufacturing processes [3]. Therefore, spore-forming bacteria with their higher stress tolerance and better survival during storage have attracted great attention in the probiotic production.

Weizmannia coagulans (formerly known as *Bacillus coagulans)* was firstly isolated in 1915 by B.W. Hammer (1915) [4] from spoiled canned milk. By 2020, they are classified in the genus Weizmannia [5]. The bacterium occupied a broad range of environments, including many types of food (milk, potato, pickle, corn, and tomato), animal faeces (broiler, calf, and piglet), compost, and silage [6-9].

Similar to other spore-forming bacteria, spores of *W. coagulans* can survive in extreme conditions like high temperatures and dry storage [10]. It also has great viability in the gastrointestinal tract and good adhesion capacity to intestinal epithelial cells [9, 11]. In addition, the bacterium can produce some antibacterial substances, such as coagulin and lactosporin [12, 13], against some pathogenic bacteria like *Enterococcus* sp., *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus* [9, 14]. With these advantageous characteristics, *W. coagulans* is considered a potential probiotic candidate.

Several strains of *W. coagulans* have been identified as Generally Recognized as Safe (GRAS). The bacterium is also on the Qualified Presumption of Safety (QPS) list and authorized for human use [15]. Recently, an increasing number of studies have reported that probiotics derived from the original target host have evidently been revealed to be more efficacious than other sources (allochthonous) [16]. Therefore, exploiting native probiotic microorganisms should be considered. In Vietnam, the isolation of *W. coagulans* has only been reported from fermented cocoa beans [17] and Nam Dinh seawater [18]. Studies on *W. coagulans* isolated from other sources, and the characterization of bacterial probiotic properties, are limited. In this study, we aim to isolate, identify, and evaluate the probiotic properties of some indigenous *W. coagulans* strains.

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2. Materials and methods

2.1. Microorganisms and cell line

Pathogenic bacteria including *Aeromonas dhakensis* VTCC 70106, *Enterococcus faecalis* VTCC 70177, *Escherichia coli* VTCC 12272, *Listeria monocytogenes* VTCC 70147, *Staphylococcus aureus* VTCC 12275, and *Vibrio vulnificus* VTCC 70092 were obtained from the Vietnam Type Culture Collection (VTCC), Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Vietnam. These strains were stored at -70°C in nutrient broth (NB; Becton, Dickinson and Company, USA) supplemented with 20% glycerol (Fisher Scientific, UK) until use.

A human colon adenocarcinoma cell line (HT-29) was kindly provided by Dr. P.TT. Huong (Key Laboratory of Enzyme and Protein Technology, Vietnam National University, Hanoi).

2.2. Sampling and isolation of W. coagulans

In March 2021, 40 human and dairy cow samples were collected, including human breast milk (n=10), healthy vaginal secretion (n=10), and newborn faeces (n=10) at Hanoi Obstetrics and Gynaecology Hospital and raw cow milk (n=5) and cow faeces (n=5) at a dairy farm in Ba Vi, Hanoi. The milk samples were taken into sterile falcon tubes, the vaginal secretion samples were obtained by sterile swab sticks and the freshly voided faecal samples were collected in clean, dry, screw-top containers. All samples were transported immediately to the laboratory in a cool box and were processed the same day.

Human vaginal secretion and milk samples were spread directly onto nutrient agar (NA, Becton, Dickinson and Company, USA) plates. One gram of newborn or cow faeces was homogenized into 9 mL of sterile water. Then the diluted samples were heated at 80°C for 10 min before enrichment in the NB medium at 42°C for 24 h [6]. The enriched broths were diluted and plated out on the NA plates. The plates were incubated under aerobic and anaerobic conditions for 48-72 h. Then, suspected *W. coagulans* colonies with swollen sporangium were picked up and stored at -70°C as previously described. *W. coagulans* GBI-30, 6086 (BC30) obtained from a commercial probiotic Schiff was used as a reference strain in all experiments.

2.3. Identification of isolated strains

The identification of isolated strains was confirmed by 16S rRNA sequence analysis. Briefly, the PCR reaction was performed using universal primers of 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Successful PCR products were sequenced at First Base Laboratories (Malaysia). The 16S rRNA of *W. coagulans* strains were submitted to NCBI GenBank under accession numbers from OP592207 to OP592211. The 16S rRNA gene sequences were searched on the EzBioCloud database (https://www.ezbiocloud.net). Phylogenetic analysis was performed on MEGA X software using the maximum likelihood method in a Tamura 3-parameter model. Bootstrap analysis with 1000 re-samplings was assessed to confirm the consistency of the phylogram [5].

2.4. Safety test

Haemolysis activity was determined by streaking *W. coagulans* strains on the Columbia agar plates containing 5% (w/v) sheep blood (MELAB, Vietnam) [9]. After incubation at 37°C for 24 h, the strains formed a green zone, clear zone, and no clearance zone around the colony, which were classified as α-haemolysis, β-haemolysis, and γ-haemolysis, respectively. The antibiotic susceptibility of the bacterial strains was investigated by the Kirby-Bauer disk diffusion method [19]. The fresh bacterial cells (approximately 10^8 CFU/ml) of *W. coagulans*, prepared from overnight cultures, were spread on Mueller-Hinton (MH; Becton, Dickinson and Company, USA) agar plates. Antibiotic discs (Oxoid, USA) containing chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), neomycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), or vancomycin (30 µg) were placed on pre-inoculated plates. Plates were then incubated at 37°C for 20 h and the zone of inhibition diameter was measured.

2.5. Antibacterial activity

Well diffusion and agar plug diffusion methods were used to test the antibiotic production of the *W. coagulans* strains. For the first one, *W. coagulans* strains were shaken in trypticase soy broth (TSB, Becton, Dickinson and Company, USA) and de Man, Rogosa, and Sharpe (MRS, Becton, Dickinson and Company, USA) broth at 160 rpm. After shaking at 42°C overnight, the cell-free supernatants (CFSs) were collected by centrifugation at 8,000 rpm for 10 min and added into wells (d=5 mm) of the MH agar plates pre-inoculated with one of six pathogenic strains, namely, *E. faecalis* VTCC 70177, *L. monocytogenes* VTCC 70147, *E. coli* VTCC 12272, *S. aureus* VTCC 12275, *V. vulnificus* VTCC 70092, and *A. dhakensis* VTCC 70106. The plates were incubated at 37°C for 24 h and the clear diameter zone surrounding each well was measured (including the well) [14]. For the later one, *W. coagulans* strains were spread

on the trypticase soy agar (TSA, Becton, Dickinson and Company, USA) and MRS agar plates instead of the broths and the antibacterial activity was tested as described above.

2.6. Enzyme production of W. coagulans

The activity of amylase, cellulase, and protease was tested by spotting *W. coagulans* strains on NA plates supplemented with 0.2% soluble starch (Sigma-Aldrich, USA), 0.2% carboxymethylcellulose (Sigma-Aldrich, USA), and 0.1% casein (Sigma-Aldrich, USA), respectively [20]. The plates were incubated at 42°C for 48 h and stained with Gram's iodine solution (for amylase and cellulase) or Amido black solution (for protease) for visualization of the clear hydrolytic zones. The production of β-galactosidase was tested by streaking the bacteria on TSA plates containing 60 µl X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Thermo Fisher Scientific, Italy), which was diluted to a final concentration of 20 mg/ml in DMF (N, N- dimethylformamide; Wako, Japan) and 10 µl of IPTG (isopropyl-thio-β-D galactopyranoside; Thermo Fisher Scientific, Lithuania) solution as inducer [21]. β-galactosidase-producing strains appeared in blue colonies while non-producing enzyme strains appeared in white colonies.

2.7. Antioxidant activity

Antioxidant activities were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [22]. Briefly, a volume of 1.5 ml of each CFSs prepared from the TSB culture was added into tubes containing 1.5 ml of 0.2 mM DPPH (TCI chemical, Japan). A tube containing 1.5 ml of methanol and 1.5 ml of 0.2 mM DPPH was used as the blank. All tubes were incubated at 37°C for 30 min in the dark and the absorbance was measured at 517 nm using a UV-Vis spectrophotometer (DU® 730, Beckman Coulter, USA). The percentage of scavenged DPPH radical was calculated using the following formula:

$$\text{Scavenging effect (\%)} = \left(1 - \frac{A_{517}\ (\text{sample})}{A_{517}\ (\text{blank})}\right) \times 100$$

2.8. Adhesion capacity

HT-29 cell line was cultured at a density of approximately 2×10^5 cells per well in 24-well plates using Dulbecco's Modified Eagle Medium (DMEM; PAN-Biotech, Germany), which contained 1 g/l glucose, 10% (v/v) fetal bovine serum (Gibco, USA), and 1% (v/v) penicillin/streptomycin (Corning, USA) [11]. After overnight incubation at 37°C, the medium was removed, and the cell monolayer was washed once with phosphate buffered saline (PBS, pH 7.4). Subsequently, 900 µl of DMEM with 1 g/l glucose and 100 µl of vegetative cell suspension were added into the wells and incubated at 37°C for 2 h with 5% CO_2. The wells were washed twice with PBS (pH 7.4), added to 200 µl of 1% (v/v) triton X-100 (Merk, Germany), and incubated at 37°C for 5 min. The suspension in each well was diluted and then plated out on TSA plates to determine the number of adherent bacteria.

2.9. Stress tolerance to simulated digestion conditions

2.9.1. Preparation of cell and spore suspensions

Cell and spore suspensions were prepared as described by L.H. Duc, et al. (2003) [23]. Briefly, *W. coagulans* strains were cultured in TSB medium overnight. The culture broths were centrifuged at 8,000 rpm at 25°C for 10 min to collect the cells. The cell pellets were washed twice and resuspended in sterile PBS (pH 7.4) to obtain an optical density (OD) of 1.0 at 600 nm using a spectrophotometer (Ultrospec 10, Biochrom, USA). To produce spores, *W. coagulans* strains were cultured in a suitable broth after an appropriate time. The spore pellets were collected by centrifuging at 8,000 rpm for 10 min at 25°C and resuspended in sterile PBS (pH 7.4). The spore suspensions were heated at 80°C for 10 min and adjusted to obtain spore densities at log 7 CFU/ml.

2.9.2. Acid tolerance of the W. coagulans spores

The spore suspensions of *W. coagulans* were treated in PBS (pH 2.5) at 37°C for 2 h [24]. Then, the bacteria were counted, and the acid tolerance was determined using the following formula:

$$\text{Survival rate (\%)} = \frac{\log \text{CFU after treatment}}{\log \text{CFU before treatment}} \times 100$$

2.9.3. Survival of the W. coagulans strains in the simulated gastrointestinal tract

The spore suspensions of *W. coagulans* were incubated in both the simulated gastric juice (0.3% w/v pepsin (Sigma-Aldrich, USA), pH 2.5) for 2 h and the simulated small intestine fluid (0.3% (w/v) bile salts (Biobasic, Canada), 0.1% (w/v) pancreatin (Sigma-Aldrich, USA), pH 8.0) for 6 h [25]. The vegetative cell suspensions were only treated with the simulated small intestine fluid. Then, spores and vegetative cells were counted, and the survival rates were calculated using the following formula:

$$\text{Survival rate (\%)} = \frac{\log \text{CFU at } T_n}{\log \text{CFU at } T_0} \times 100$$

where T_n is the time after incubation with simulated gastric juice or small intestine fluid; T_0 is the initial time before incubation.

2.9.4. Effect of bile concentrations on the growth of vegetative W. coagulans cells

The TSB medium prepared with different concentrations of 0.1, 0.2, and 0.3% (w/v) bile salt were inoculated with the overnight cultures under anaerobic conditions at 37°C for 24 h. The growth of bacteria was monitored by measuring the OD_{600}. The TSB medium without bile salt was used as the positive control [24].

2.9.5. Germination in the simulated small intestine

The germination ability was conducted using the method of C.M. Theriot, et al. (2016) [26] with some modifications. Briefly, the spore suspensions were added to TSB medium supplemented with 0.1% w/v pancreatin (pH 8.0) and bile salt at concentrations of 0, 0.1, 0.2, and 0.3% (w/v). All tubes were incubated at 37°C for 24 h and the total bacteria were counted. The remaining spores were counted after a heating step at 80°C for 10 min.

2.10. Statistical analysis

Data from all experiments were expressed as mean ± standard error of the mean of triplicates. Differences between the two mean values were calculated by the student's *t*-test. The difference was considered to be statistically significant at $p<0.05$. All statistical analyses were performed using GraphPad Prism version 8.4.3.

3. Results and discussion

3.1. Isolation and identification of W. coagulans

From 40 samples, 150 bacterial colonies were collected. Of those, 22 gram-positive bacilli were recorded with 18 spore-forming strains. Ten strains were suspected as *W. coagulans* based on a slightly swollen sporangium of the cells [6]. The 16S rRNA sequences of were analysed and five strains were identified as *W. coagulans* with the DNA similarity range from 99.43 to 99.71%. All *W. coagulans* strains were isolated from cow faeces under both aerobic and anaerobic conditions. On the phylogenetic tree, five strains formed a clade with *W. coagulans* ATCC 7050^T (Fig. 1).

The presence of *Bacillus* in breast milk samples has been found in a few healthy women by analysing the metagenome [27, 28], although this genus has not been reported in vaginal fluid samples. Microbial diversity in breast milk could be influenced by geographic, genetic, dietary factors, and the lactation period [27]. Newborns are colonized by maternal microbiota via birth-associated exposure and breastfeeding [29] and the composition of these pioneer communities exhibits individual differences in early life depending on maternal microbiota. This could be explained by the absence of *W. coagulans* in newborn faeces in our study. However, a relatively small sample size in this study might limit the investigation. Involvement of larger human and livestock populations at different time points of life are further needed to confirm the ecology of *W. coagulans.*

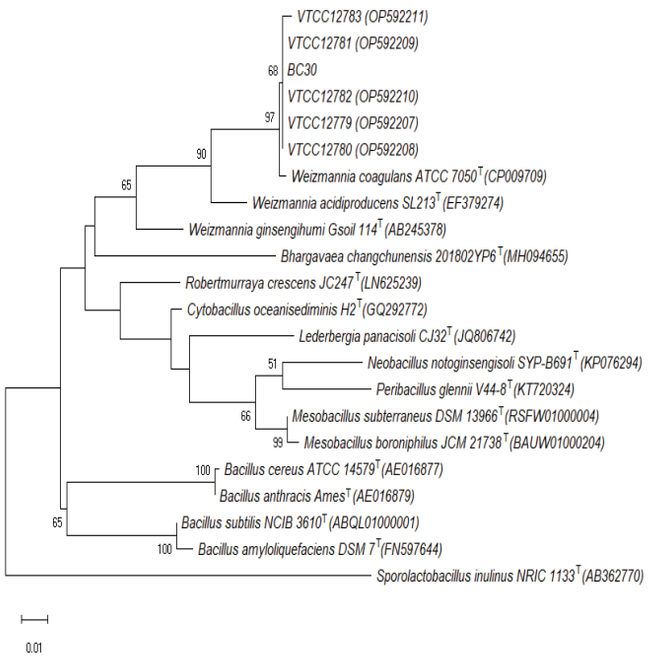

Fig. 1. Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequences showing the relationships between our *W. coagulans* strains and some representatives of the *Bacillaceae* family. Accession numbers are shown in parentheses. The numbers at the nodes indicate bootstrap values as percentages of 1,000 replicates. *Sporolactobacillus inulinus* was used as the outgroup taxon to root the tree.

3.2. Safety of the W. coagulans strains

All five strains were γ-haemolysis, which is a safety characteristic of probiotic [30]. The five strains were sensitive to seven tested antibiotics except for erythromycin and chloramphenicol in which strains VTCC 12779 and VTCC 12783 were moderate susceptibilities (Table 1). In 2012, the QPS program added the requirement of antimicrobial resistance for probiotic GRAS certification because of the horizontal gene transfer from helpful bacteria to harmful bacteria in the host's intestines [30]. Haemolysis and antibiotic sensitivity are two important criteria in evaluating the safety of probiotic bacteria. However, these two criteria are not sufficient to conclude whether a bacterial strain is safe to use. Therefore, other criteria such as production of D(-)-lactate, deconjugation of bile salts, and virulence factors (gelatinase, DNAse...) also need to be evaluated [1].

Table 1. Antibiotic resistance of the W. coagulans strains.

Antibiotic	Zone diameter (mm)					
	VTCC 12779	*VTCC12780*	*VTCC 12781*	*VTCC 12782*	*VTCC 12783*	*BC30*
Chloramphenicol (30 µg)	34.67±0.67 (S)	37.33±1.45 (S)	33.00±1.00 (S)	36.67±1.67 (S)	15.33±0.67 (M)	27.67±1.45 (S)
Erythromycin (15 µg)	15.33±0.67 (M)	32.00±0.00 (S)	34.67±1.76 (S)	31.33±0.67 (S)	18.67±0.67 (M)	33.33±1.76 (S)
Gentamicin (10 µg)	39.33±0.67 (S)	42.33±1.45 (S)	32.67±0.33 (S)	48.33±0.88 (S)	22.67±0.33 (S)	37.00±1.00 (S)
Neomycin (30 µg)	35.67±0.33 (S)	44.33±0.33 (S)	37.00±1.53 (S)	44.67±0.33 (S)	21.67±0.88 (S)	26.67±0.67 (S)
Streptomycin (10 µg)	31.33±0.33 (S)	34.33±1.20 (S)	33.33±0.67 (S)	35.00±1.53 (S)	24.33±1.20 (S)	30.00±1.15 (S)
Tetracycline (30 µg)	32.67±1.76 (S)	43.00±1.53 (S)	35.33±0.67 (S)	32.33±1.45 (S)	23.33±2.40 (S)	24.00±0.57 (S)
Vancomycin (30 µg)	30.67±0.67 (S)	32.67±1.20 (S)	32.67±0.33 (S)	33.67±0.67 (S)	36.00±0.00 (S)	32.00±0.00 (S)

Values are expressed as mean ± SEM (n=3). The inhibition zone diameters were measured, and the results were expressed as susceptibility (S), moderate susceptibility (M), and resistance (R).

3.3. Antibacterial activity

Using the MRS medium, *W. coagulans* strains showed antibacterial activity against the tested bacteria (Table 2). Strains VTCC 12779, VTCC 12781, and BC30 exhibited great antibacterial activity against *E. faecalis* and *L. monocytogenes* by the well diffusion method, while all *W. coagulans* strains were against *V. vulnificus* and *A. dhakensis* by the agar diffusion method. This characteristic of probiotic bacteria may suppress pathogenic bacterial growth and colonization in the gastrointestinal tract of hosts

Table 2. Antibacterial activity of the *W. coagulans* strains.

Bacteria	Media	Zone of inhibition (mm)					
		AD	*EF*	*EC*	*LM*	*SA*	*VV*
VTCC 12779	MRS broth	-	+	-	++	-	-
	MRS agar	+	-	-	-	-	+
VTCC 12780	MRS broth	-	-	-	-	-	-
	MRS agar	+	-	-	-	-	+
VTCC 12781	MRS broth	-	+	-	++	+	-
	MRS agar	+	-	-	-	-	+
VTCC 12782	MRS broth	-	-	-	-	-	-
	MRS agar	+	-	-	-	-	+
VTCC 12783	MRS broth	-	-	-	-	-	-
	MRS agar	+	-	-	-	-	+
BC30	MRS broth	-	+	-	+	-	-
	MRS agar	+	-	-	-	-	+

-: no inhibition zone; +: inhibition zone ≤10 mm; ++: inhibition zone >10 mm; AD: *A. dhakensis*; EF: *E. faecalis*; EC: *E. coli*; LM: *L. monocytogenes*; SA: *S. aureus*; VV: *V. vulnificus*.

[31]. A previous report on *W. coagulans* BDU3 also showed a wide spectrum of antimicrobial activity against pathogenic bacteria of *B. cereus*, *S. aureus*, *Enterococcus* sp., and *Micrococcus luteus* [14]. In the TSB medium, none of the *W. coagulans* strains showed antibacterial activity (data not shown). Variations in the culture medium often result in an alteration of antibiotic production, involving changes in both yields and the composition of the substances [32]. Therefore, the MRS medium might be a relevant culture medium for the production of antimicrobial substances by *W. coagulans*.

3.4. Enzyme production of W. coagulans

Amylase, cellulose, and protease activities ranged from 1.33 to 8.33 mm, 5.67 to 14.00 mm, and 4.00 to 12.00 mm, respectively (Table 3). VTCC 12783 showed the highest enzymatic activities. All strains were able to produce β-galactosidase. The results were consistent with previous reports. β-galactosidase was identified in extracellular protein extracts of *W. coagulans* MA-13 with a high expression level under culture containing 0.1% yeast extract and 0.1% lactose [33]. Moreover, *W. coagulans* MA-13 also produced soluble thermophilic cellulases in carboxymethyl-cellulose screening medium at 37°C and had an optimal operational activity at 50°C [34]. The production of extracellular, thermostable α-amylase by *W. coagulans* B49 was studied in solid state fermentation with a significant level of amylase produced in wheat bran [35]. The proteolytic activity of *W. coagulans* was also reported [36].

Table 3. Qualitative enzyme activity was measured by clear zone diameter.

Strain	Clear zone diameter ratio (mm)			β-galactosidase
	Amylase	*Cellulase*	*Protease*	
VTCC 12779	3.00±0.57	5.67±0.67	4.00±1.53	+
VTCC 12780	1.33±0.67	5.67±0.67	4.33±0.33	+
VTCC 12781	5.33±0.33	6.33±0.88	7.33±1.76	+
VTCC 12782	1.33±0.67	6.33±0.33	4.67±0.33	+
VTCC 12783	8.33±0.882	12.33±0.88	10.33±1.19	+
BC30	1.67±0.33	14.00±0.57	12.00±1.15	+
Mean ± SEM (n=3).				

3.5. Antioxidant activity

Oxidative stress can cause damage to lipids, proteins, and DNA when the levels of intracellular oxygen radicals are elevated. The scavenging DPPH rates of the *W. coagulans* strains ranged from 72 to 82% (Fig. 2). VTCC 12782 showed the strongest antioxidant activity

(82.97%), which was higher than BC30 (80.84%). The scavenging hydroxyl radical rates of our strains were higher than those of other species including *Pediococcus pentosaceus* SW01 (39.64%), *Lactobacillus pentosus* SW02 (34.31%), and *Lactobacillus plantarum* subsp. *plantarum* SW07 (28.63%) [22]. The antioxidant potential of *W. coagulans* was also reported through the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging ability [37].

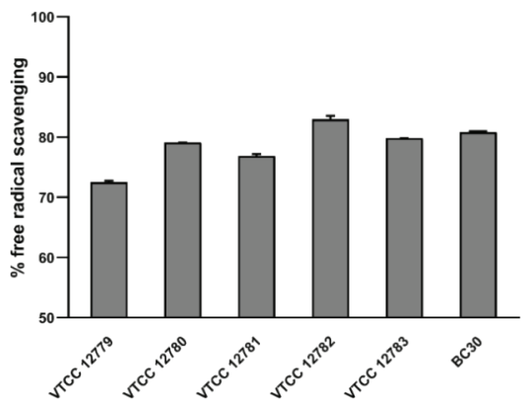

Fig. 2. Scavenging effect of the *W. coagulans* strains on DPPH free radical. Values are expressed as mean ± SEM (n=3).

3.6. Adhesion capacity

Three *W. coagulans* strains had remarkable adhesiveness toward intestine cells (Table 4). VTCC 12782 expressed the strongest adhesion (62.59%), followed by VTCC 12783 and VTCC 12779 with adhesion rates of 50.70 and 46%, respectively. This property ensures that probiotic bacteria colonize mucosal gut surfaces, interfere with pathogen binding, and interact with immune cells. *W. coagulans* isolated from calf faeces was able to adhere to INT407 cell monolayers [7]. *W. coagulans* CGMCC 9951 also exhibited strong adhesion to pig intestinal mucus with adhesion rates at 44.5, 48.9, 42.6 and 37.6% to the jejunum, ileum, transverse colon, and sigmoid colon, respectively [8].

Table 4. Adhesion of the *W. coagulans* strains to HT-29 cell line.

Adhesion period	VTCC 12779	VTCC 12780	VTCC 12781	VTCC 12782	VTCC 12783	BC30
Initial cell	6.99±0.08	6.71±0.13	7.13±0.05	7.03±0.08	7.16±0.14	7.07±0.13
Adhesion cell	3.20±0.17	ND	ND	4.40±0.22	3.63±0.19	ND
Adhesion ability (%)	46	-	-	62.59	50.70	-

Values are expressed as mean ± SEM (n=3). The means are presented as log-transformed values of CFU/ml of the bacterial strains. ND: not detected.

3.7. Stress tolerance to simulated digestion conditions

Spores of *W. coagulans* enter the body through the oral route and safely travel to the stomach. Then, the spores germinate in the duodenum and grow in the upper part of the small intestine. *W. coagulans* cells subsequently go down to the large intestine and sporulation begins in the lower part of the colon [2]. Therefore, we challenged vegetative cells with small intestine fluid, while spores were treated with acid, simulated gastric juice, and small intestine fluid.

3.7.1. Survival of vegetative W. coagulans cells in the simulated small intestine fluid

Based on the adherence test, the three strains VTCC 12779, VTCC 12782, and VTCC 12783 were selected. Survival rates of the vegetative cells of VTCC 12779, VTCC 12782, and VTCC 12783 in the simulated small intestine fluid were 94.71, 81.93 and 95.21%, respectively (Table 5). In more detail, after 6 h of incubation, the spore densities decreased from log 7.18 CFU/ml to log 6.80 CFU/ml (VTCC 12779), from log 7.14 CFU/ml to log 5.85 CFU/ml (VTCC 12782), and from log 7.31 CFU/ml to log 6.96 CFU/ml (VTCC 12783). These survival rates were considerably higher than those previously reported for *L. acidophilus* M92 (survival rate of 57%) and *B. subtilis* (survival rate of 0.0002% after the first hour of exposure) [23, 25].

Table 5. Survival of the *W. coagulans* vegetative cells in the simulated small intestine fluid.

Incubation period	VTCC 12779	VTCC 12782	VTCC 12783	BC30
Initial cell, 0 h	7.18±0.1	7.14±0.10	7.31±0.23	7.30±0.13
Small intestine fluid, 6 h	6.80±0.32	5.85±0.62	6.96±0.08	7.27±0.13
Survival rate (%)	94.71	81.93	95.21	99.58

Values are expressed as mean ± SEM (n=3). The means are presented as log-transformed values of CFU/ml of the bacterial strains.

3.7.2. Effect of bile salts on the growth of the vegetative W. coagulans cells

Resistance to bile salts is an important property of probiotic bacteria to survive and grow in the intestinal tract [24]. Three strains exhibited growth at a bile salt concentration of 0.1% (Fig. 3), whereas no growth was observed at higher concentrations. The data were consistent with previous studies, which showed *W. coagulans* strains were sensitive or weakly tolerant to a bile salt concentration of 0.3% [24, 38]. Because of higher growth in bile salt concentration of 0.1%, VTCC 12779 and VTCC 12782 were selected for further experiments.

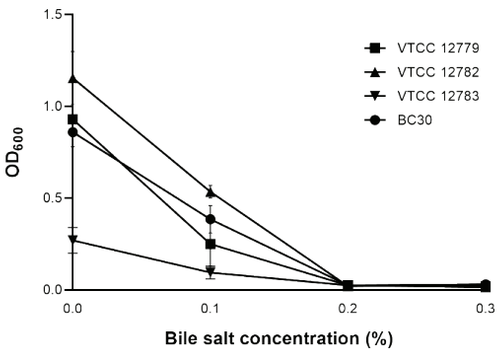

Fig. 3. Growth of the *W. coagulans* strains at different concentrations of bile salt. Values are expressed as mean ± SEM (n=3).

3.7.3. Acid tolerance of the W. coagulans spores

The survivability in gastric juice mainly depends on their tolerance to low pH. The tested *W. coagulans* spores showed survivability >90% at pH 2.5 (Table 6). After 2 h of incubation, the spore densities declined from log 7.69 CFU/ml to log 7.09 CFU/ml (VTCC 12779), from log 7.40 CFU/ml to log 7.14 CFU/ml (VTCC 12782), and from log 7.38 CFU/ml to log 6.81 CFU/ml (BC30). Spores of VTCC 12782 seemed to be more acid-tolerant than those of VTCC 12779 and BC30. Previous reports also showed high survival rates of spores of *W. coagulans* CGMCC (90.1%) and another *Bacillus* spp. (80%) [8, 24].

Table 6. Acid tolerance of the *W. coagulans* spores.

Incubation period	VTCC 12779	VTCC 12782	BC30
Initial spores, 0 h	7.69±0.00	7.40±0.04	7.38±0.15
pH 2.5, 2 h	7.09±0.12	7.14±0.05	6.81±0.23
Survival rate (%)	92.20	96.49	92.23

Values are expressed as mean ± SEM (n=3). The means are presented as log-transformed values of CFU/ml of the microbial strains.

3.7.4. Survival of the W. coagulans spores in the simulated gastrointestinal tract

Spores of VTCC 12779 were the most resistant to the simulated gastrointestinal tract, which decreased in viability from log 7.22 CFU/ml to log 7.17 CFU/ml (99.31% spore survival). The spore densities of VTCC 12782 decreased from log 7.59 CFU/ml to log 7.52 CFU/ml (99.08% spore survival) (Table 7). The survival rates of our *W. coagulans* strains were similar to that reported by T. Shinde, et al. (2019) [11] in which no significant decrease in *W. coagulans* spore count was detected after exposure to gastric conditions.

Table 7. Survival of the *W. coagulans* spores in the simulated gastric juice.

Incubation period	VTCC 12779	VTCC 12782	BC30
Initial spores, 0 h	7.22±0.00	7.59±0.04	7.37±0.02
Simulated gastric juice, 2 h	7.17±0.05	7.52±0.18	7.22±0.04
Survival rate (%)	99.31	99.08	97.96

Values are expressed as mean ± SEM (n=3). The means are presented as log-transformed values of CFU/ml of the microbial strains.

Spores of the tested strains also showed more than 99% survivability in small intestine fluid. After 6 h of incubation, the spore densities slightly increased from log 7.06 CFU/ml to log 7.07 CFU/ml (VTCC 12779) and from log 7.43 CFU/ml to log 7.77 CFU/ml (VTCC 12782) (Table 8). These results indicated that the spores of our strains had better survival ability in the simulated small intestine when compared to *B. subtilis* SC2362 (spore survival rate of 94%) [23]. For other species, *L. plantarum* and *L. brevis* strains exhibited survival rates in a range of 40 to 63% whereas *Pediococcus ethanolidurans* strains were sensitive to the simulated intestinal juice with no viability observed after 6 h of exposure [39].

Table 8. Survival of the *W. coagulans* spores in the simulated small intestine fluid.

Incubation period	VTCC 12779	VTCC 12782	BC30
Initial spores, 0 h	7.06±0.16	7.43±0.56	7.37±0.02
Small intestine fluid, 6 h	7.07±0.02	7.77±0.02	7.32±0.02
Survival rate (%)	100.14	104.58	99.32

Values are expressed as mean ± SEM (n=3). The means are presented as log-transformed values of CFU/ml of the microbial strains.

3.7.5. Germination of the W. coagulans spores in the simulated small intestine

The germination was tested based on the number of *W. coagulans* spores, which gradually decreased while the total number of bacteria increased (Fig. 4). Spores of VTCC 12782 reduced from 25 to 27% in all tubes containing different concentrations of bile salt while spores of VTCC 12779 reduced from 2 to 18%. Previous studies have reported the germination of *Bacillus* spp. spores, including *W. coagulans* in the gastrointestinal tract. According to L.H. Duc, et al. (2003) [23], the germination of *Bacillus* spores was expressed through the decrease of OD_{600nm} in AGK solution containing spores in the presence of bile salts. Using the *in vitro* model of the stomach and small intestine, the germinating percentage of *W. coagulans* BC30 spores was reported at 93%, which was higher than that of our strains. This might be due to the absence of germination triggers that were contained in the meal [40].

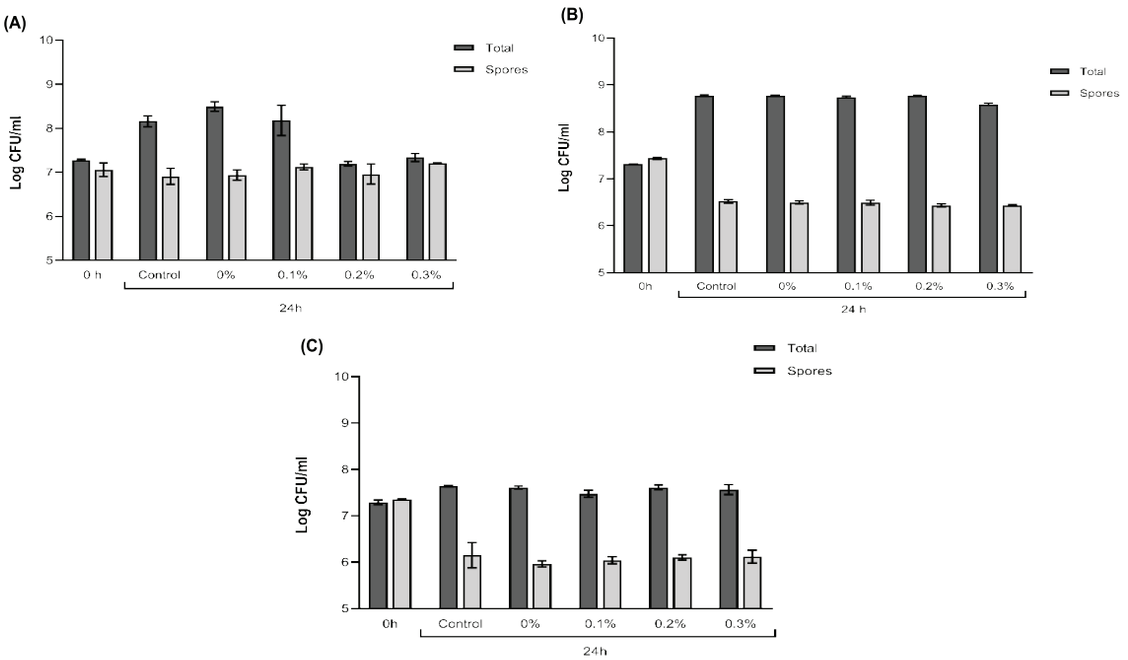

Fig. 4. Germination of the *W. coagulans* spores in the simulated small intestine. Spores of *W. coagulans* VTCC 12779 **(A)**, VTCC 12782 **(B)**, and BC30 **(C)** were treated in the simulated small intestinal condition and viabilities were assessed at after 24 h. Values are expressed as mean ± SEM (n=3).

4. Conclusions

In conclusion, from 40 human and cow samples, we isolated five *W. coagulans* strains from cow faeces. Not all strains showed haemolytic activity as well as resistance to tested antibiotics. Moreover, these strains were able to produce amylase, cellulase, protease, β-galactosidase, and antibacterial and antioxidant activity. The three strains VTCC 12779, VTCC 12782, and VTCC 12783 were able to adhere to HT-29 cells and survive in simulated intestinal conditions. VTCC 12779 and VTCC 12782 could grow in the presence of bile salt and had a high rate of spore survival in the gastrointestinal tract. The spores of VTCC 12782 exhibited the highest germination rate in the simulated small intestine. Therefore, strain VTCC 12782 is considered a potential candidate for probiotic production.

CRediT author statement

Thuy Duong Pham: Data collection, Experimental work, Writing; Thi Lan Anh Hoang: Study design, Reviewing and Editing; Thi Le Quyen Tran: Samples collection, Supporting data analysis; Thanh Trung Trinh: Supervision, Editing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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