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Research article

Characterization and Evaluation of Probiotic Potentials *In Vitro* of *Bacillus* Strains Isolated from Traditional Thai Natto

Vongsathorn Ngampuak[1]*, Natnicha Jumpadang[1], Thirayut Sombun[1] and Tantip Arigul[2]

[1]Department of Basic Science and Medical Science, Faculty of Allied Health Sciences, Pathumthani University, Pathum Thani, 12000, Thailand
[2]Department of Research, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, 10700, Thailand

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Abstract

Traditional Thai natto is one source of *Bacillus* spp. Due to their spore-forming ability, *Bacillus* spp. are considered promising probiotic candidates capable of surviving harsh environmental condition. This study aimed to genetically identify and characterize *Bacillus* strains isolated from traditional Thai natto and evaluate their probiotic potential, including survival ability under various acidic pH levels, bile salt concentrations, and electrolyte monogastric system model (EMSM). Antibacterial activity and safety assessments including hemolytic activity and antibiotic susceptibility were also evaluated. More than 10 colonies were found in the samples. Only 3 isolates were selected based on *Bacillus* morphology. The isolates displayed biochemical features consistent with *Bacillus* and were identified with 16S rRNA sequencing, showing high sequence similarity to: *B. subtilis* CMI 7 (99.86%), *B. subtilis* CMI 9 (99.72%), and *B. cereus* CMI 10 (98.91%). All isolates exhibited key probiotic traits and showed statistically significant differences in survival rates under acidic pH (over 62.35±4.50% at 4 h; and over 51.61±5.08% at 24 h), bile salt concentrations (over 93.60±2.42% at 4 h; over 82.23±3.90% at 8 h; and over 39.65±3.02% at 24 h), and EMSM conditions (over 73.08±3.18% at 4 h). Isolates CMI 7 and CMI 9 exhibited antagonistic activity against *S. aureus* ATCC 9144 and strain DMST 2933 and *E. coli* ATCC 25923, demonstrating non-hemolytic and antibiotic-sensitive features. In contrast, isolate CMI 10 lacked antagonistic activity, exhibited β-hemolysis, and showed resistance to several antibiotics, representing a significant safety concern. This study suggests that *B. subtilis* CMI 7 and CMI 9 are probiotic candidates with safety profiles. Further study should be conducted to explore other biological properties of these *Bacillus* spp.

Keywords: *Bacillus*; identification; natto; probiotics; fermented soybean

*Corresponding author: E-mail: Vongsathorn.n@ptu.ac.th
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1. Introduction

Thai fermented soybean, commonly known as traditional Thai natto, originates from Chiang Mai province in northern Thailand, a region with a long history of fermented soybean production. This unique delicacy undergoes a natural fermentation process primarily driven by bacteria, particularly *Bacillus* spp., which contribute to its characteristic sticky, stretchy texture and distinct aroma (Afzaal et al., 2022). Beyond its role in fermentation, Thai natto provides a nutrient-rich environment, including essential amino acids, vitamins, and short-chain fatty acids. Studies have highlighted the potential benefits of Thai natto, including improved digestion, modulation of gut microbiota, and antimicrobial properties that may support both human and animal health (Afzaal et al., 2022; Wang et al., 2023). Emphasis is placed on probiotic fermentation of edible products, specifically focusing on *Bacillus* spp. from Thai natto and their potential relevance as probiotics (Terlabie et al., 2006; Cao et al., 2019; Ngampuak et al., 2023).

Bacillus species are increasingly recognized for their probiotic potentials as they possess exceptional biological benefits such as antioxidant and antimicrobial activities, immune modulation, and enhancement of the nutritional and sensory properties of fermented foods (Lefevre et al., 2015; Shobharani et al., 2015). Probiotics are required to survive throughout the human digestive tract, which presents multiple challenges to survival, including exposure to gastric acid, digestive enzymes, and bile salts, which can significantly reduce bacterial viability (Golnari et al., 2024a). *Bacillus* species demonstrate a survival advantage over traditional and commercial probiotics, namely *Lactobacillus*, and *Bifidobacterium*, maintaining higher viability under acidic conditions and bile salt exposure, as reported in *in vitro* studies (Cutting, 2011; Zoldan et al., 2023).

Bacillus species have developed a range of adaptive mechanisms that allow them to endure and thrive in challenging environments. One of their most remarkable features is the ability to form endospores; highly durable, dormant structures that safeguard the bacteria during periods of environmental stress. This spore-forming capability provides exceptional resistance to extreme conditions, including highly acidic or alkaline pH, bile salt conditions, elevated temperatures, and enzymatic breakdown within the gastrointestinal tract (Golnari et al., 2024a,b). Consequently, *Bacillus* spp. can survive the entire passage through the digestive system, from ingestion to colonization in the intestines. *Bacillus* spp. contribute to gut microbiota balance, inhibit pathogens, and support immune modulation, making them strong candidates for therapeutic and nutritional applications (Daneshazari et al., 2023; Zoldan et al., 2023). This remarkable resilience of *Bacillus*, combined with their health-promoting properties, highlights their potential as next-generation probiotics, with promising applications not only in human health but also in animal nutrition and wellness. However, there has been limited exploration of the diverse *Bacillus* strains present in Thai fermented soybean, which may harbor unique strains with superior probiotic traits and the potential to serve as commercially viable spore-forming probiotics.

Consequently, this study aimed to isolate and characterize *Bacillus* strains from traditional Thai natto using biochemical assays and 16S rRNA gene sequencing. The isolates were evaluated *in vitro* for key probiotic potentials, including survival under acidic pH, bile salt, and electrolyte monogastric system model conditions (EMSM). Antibacterial activity, hemolysis, and antibiotic susceptibility were also assessed to determine their safety and efficacy as probiotic candidates.

2. Materials and Methods

2.1 Screening and isolation of bacteria

Five Thai Thua Nao Moe and Thua Nao Sa samples were collected from Chiang Mai province. Samples were homogenized in sterile 0.85% (w/v) NaCl solution (NSS) and serially diluted using a tenfold-dilution series. Aliquots were plated on nutrient agar plates (NA) (HiMedia, India) and incubated at 37ºC for 24-48 h. Colonies with typical *Bacillus* morphology—round, whitish, convex, sticky, and slime-producing—were selected for further study.

2.2 Biochemical characterization

Isolates were thoroughly evaluated for their key characteristics, including Gram-staining, spore-forming ability, and motility, following the method described by Lillie et al. (1977). The carbohydrate fermentation profile of isolates was assessed using standard biochemical tests, including the methyl red and Voges–Proskauer assays, the triple sugar iron (TSI) test, and specific carbohydrate fermentation assays includng glycerol, ribose, arabinose, xylose, mannose, mannitol, glucose, fructose, galactose, maltose, sucrose, lactose, and inulin (HiMedia, India; and Merck, Germany). The enzymatic production capacity of the isolates was further evaluated using standard biochemical tests such as catalase, cytochrome c oxidase, citrate utilization, urease, indole production, nitrate reductase activity, and hydrolytic assays such as skim milk casein and starch (HiMedia, India; and Merck, Germany). Results were compared with the reference strain *B. subtilis* DMST 5871, obtained from the Department of Medical Sciences, Thailand (DMST).

2.3 16S rRNA identification

The isolates were analyzed through 16S rRNA sequencing following genomic DNA extraction. The 16S rRNA gene was amplified using universal primers; 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'. The PCR product, approximate 1.5 kb, was confirmed by electrophoresis on a 1.2% (w/v) agarose gel. The 16S rRNA sequence was then analyzed for nucleotide identification using the BLAST program, accessing the National Center for Biotechnology Information (NCBI) databases. The Sanger sequencing data was initially converted from ab1 files to FASTA format using the online converter at https://sequenceconversion.bugaco.com. Low-quality bases were filtered out using SAMtools to ensure the integrity of the sequences. Consensus sequences were then generated from both the forward and reverse FASTA files using an in-house Python script. Sequence alignment was performed with MAFFT v7.526 to align the sequences accurately. A phylogenetic tree was constructed using IQ-TREE v2.4.0, applying maximum likelihood (ML) with 1000 bootstrap replicates to assess the robustness of the tree. Finally, the phylogenetic tree was visualized with FigTree v1.4.4 for interpretation and presentation.

2.4 Evaluation of probiotic potentials

In vitro probiotic potential was assessed based on survivability under acidic pH, bile salt concentrations (Oxgall from HiMedia, India), and electrolyte monogastric system model (EMSM) (chemicals from HiMedia, India). The EMSM consisted of simulated biological

fluids, including simulated salivary fluid (SSF) with salivary amylase (75 U/mL), simulated gastric fluid (SGF) with pepsin (2,000 U/mL), simulated intestinal fluid (SIF) with pancreatin (based on trypsin activity at 100 U/mL) and bile (10 mM). Rate of cell survivability was calculated using the Grosu-Tudorand and Zamfir (2012) equation. Prior to each experiment, isolates were incubated overnight in 20 mL nutrient broth (NB) (HiMedia, India) at 37ºC with 120 rpm. Cells were harvested by centrifugation (5,000 rpm, 10 min, 4ºC), washed twice in PBS (pH 7.2), and adjusted to 10^6 CFU/mL for assays. The experiments were performed in triplicates.

2.4.1 Tolerance to acidic pH

The cells were individually added to HCl-supplemented NB that had been adjusted to pH levels of 1, 2, 3, and 7. The mixtures were then incubated for 4 and 24 h, with the initial bacterial sample at 0 h serving as the control. Following that, 0.1 mL of the incubated experimental mixture was applied to NA by the spreading technique, then incubated overnight. After counting, the survival rate of each isolate was calculated as the percentage of bacterial colonies grown on NA, compared to the initial bacterial concentration, using the following equation 1:

$$\text{Survival rate under acidic conditions } (\%) = \left(\frac{log\ CFU\ N1}{log\ CFU\ N0}\right) x\ 100 \quad (1)$$

Where N1 is the viable count of isolates after incubation and N0 is the initial viable count.

2.4.2 Tolerance to bile salts

The cells were resuspended in sterile NB supplemented with bile salts at concentrations of 0.1% (w/v), 0.3% (w/v), and 0.5% (w/v). Samples were incubated, and measurements were taken at 4, 8, and 24 h, with the initial bacterial sample at 0 hour serving as the control. After bacterial counting, the survival rate of each isolate was calculated then assessed as described previously, using the following equation 2:

$$\text{Survival rate under bile salts conditions } (\%) = \left(\frac{log\ CFU\ N1}{log\ CFU\ N0}\right) x\ 100 \quad (2)$$

Where N1 is the viable count of isolates after incubation and N0 is the initial viable count.

2.4.3 Survival under electrolyte monogastric system model (EMSM)

The continuous simulation of EMSM in animals was conducted to assess the survival rate of the isolates. The EMSM solutions and protocol were prepared following the method described by Minekus et al. (2014), with minor modifications. The cells of the isolates were sequentially and continuously resuspended and incubated under the EMSM conditions. The isolates were counted on NA at each condition. They were first incubated in SSF for 2 min (at T2 min) at pH 7, followed by incubation in SGF for 2 h (at T2 h) at pH 3, and finally in SIF for an additional 2 h (at T4 h) at pH 7. The survival rate of each isolate was then calculated and assessed as previously described, using the following equation 3:

$$\text{Survival rate under EMSM } (\%) = \left(\frac{log\ CFU\ N1}{log\ CFU\ N0}\right) x\ 100 \quad (3)$$

Where N1 is the viable count of isolates after SGF incubation and N0 is the initial viable count.

2.5 Antimicrobial activity

Isolates were tested against *S. aureus* ATCC 9144 and strain DMST 2933, *E. coli* ATCC 25923, and *S. dysenteriae* DMST 2137 using the agar-well diffusion method, modified from Mulaw et al. (2019). The test bacteria were sourced from American Type Culture Collection (ATCC) and Department of Medical Sciences, Thailand (DMST). The test bacterial cells were adjusted to 0.5 McFarland Standard. Fifty microliters of overnight isolates culture were loaded into 7 mm wells on Mueller Hinton agar (MHA) (HiMedia, India) and incubated at 37ºC for 24 h. *B. subtilis* DMST 5871 served as the reference strain, NSS was used as the negative control, and Poly-Oph, a mixture of Neomycin sulfate (2 mg), Polymyxin B sulfate (5,000 units), and Gramicidin (0.025 mg), was used as the positive control. Zones of inhibition (ZOI) ≥1 mm were considered positive (Lleó et al., 1998). The experiment was conducted in triplicate, and the results were reported as the ZOI diameter±standard deviation.

2.6 Hemolytic activity

Isolates were cultured on 5% sheep blood agar (Becton Dickinson GmbH, Germany) and incubated at 37ºC for 24-48 h. Hemolytic activity was determined based on three distinct patterns: β (clear zone), α (greenish halo), or γ (no halo) as described by Reuben et al. (2019).

2.7 Antibiotic susceptibility

The antibiotic susceptibility was performed using disc diffusion method on MHA (Bauer et al., 1966). Antibiotics tested included Ampicillin (AMP, 10 mcg), Cefoxitin (CX, 30 mcg), Cefotaxime (CTX, 30 mcg), Amoxyclav (AMC, 30 mcg), Gentamycin (GEN, 10 mcg), and Ceftazidime (CAZ, 30 mcg) (HiMedia, India). Isolates were cultured on MHA at 37°C before antibiotic discs were placed on the inoculated plates. After 24 h of incubation, the zones of inhibition were measured. Susceptibility was interpreted according to the Clinical and Laboratory Standards Institute (2020) guidelines. For 30 mcg antibiotics, a clear zone ≥20 mm was Susceptible (S), 15-19 mm Intermediate (I), and ≤14 mm Resistant (R); for 10 mcg antibiotics, a clear zone ≥10 mm S, 7-9 mm I, and ≤6 mm R.

2.8 Statistical analysis

All the measurements were performed in triplicate and the results were expressed as mean standard deviation (SD). The significant difference among the various treated groups and control groups was analyzed using a two-sample *t*-test assuming unequal variances, performed in Microsoft Excel (version 16.78.3). Statistical significance was considered by *p* value. Asterisks indicated a significant difference *p <0.05, **p <0.01, and ***p <0.001.

3. Results and Discussion

3.1 Isolation and identification of bacteria

Three isolated colonies with sticky, slimy appearances were carefully chosen for further analysis. These colonies were stained to determine their Gram characteristics and assess their ability to produce endospores, as illustrated in Figure 1. The isolates exhibited irregular, flat, and morphologically circular colonies, characterized by a rough, opaque, and fuzzy white texture. Notably, isolates CMI 7 and CMI 9 shared similar colonial structures with periodic expansion, creating a distinctive pattern. In contrast, isolate CMI 10 displayed a more conventional colonial appearance, lacking the periodic growth seen in the others. Examination of their cell walls revealed that all isolates were distinctly Gram-positive, characterized by their strong retention of crystal violet stain. Furthermore, each isolate demonstrated a remarkable ability to produce spores, as evidenced by the retention of malachite green in their clearly defined, circular spore structures. These findings highlighted fundamental characteristics shared with *Bacillus* species, all widely recognized for their Gram-positive bacilli spore-producing capabilities (Li et al., 2023; Ngampuak et al., 2023; Kuang et al., 2024). Due to their ability to produce spores, *Bacillus* species manifest extraordinary resistance properties. The isolates can withstand extreme or unfavorable environments. Within the human gastrointestinal system, spores of isolates may show impressive durability. They survive the acidic conditions in stomach and resist the action of bile salts in the intestines, allowing them to pass through the system unharmed. When they reach the intestines and encounter favorable conditions, the spores can germinate, returning to their active and vegetative form, and may contribute positively to gut health (Elshaghabee et al., 2017).

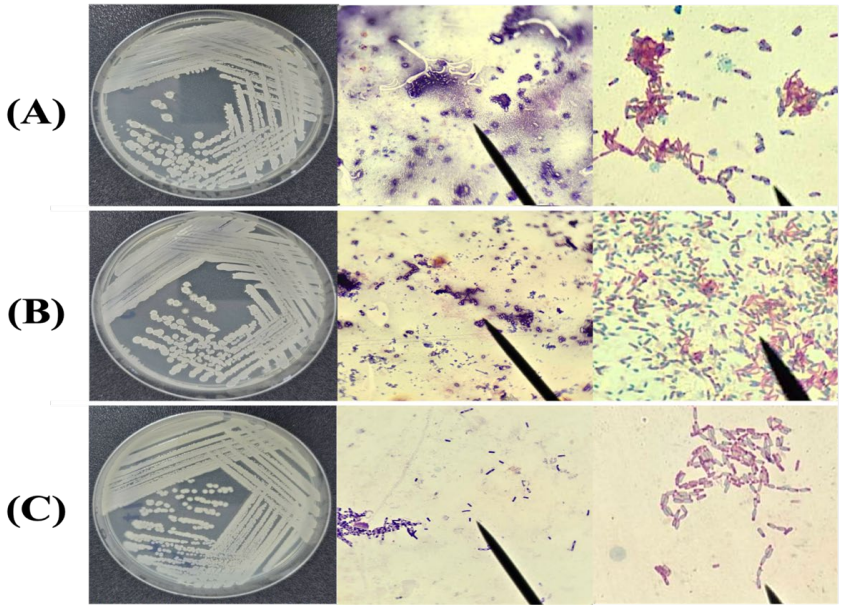

Figure 1. The appearance of isolated bacterial colonies on NA (left), Gram staining (middle), endospore staining (right) of (A) isolate CMI 7, (B) isolate CMI 9, and (C) isolate CMI 10. All bacterial staining was observed at 1,000× magnification.

B. subtilis DMST 5871 served as a reference species for comparison with the isolates, as shown in Table 1. It exhibited a comparable ability to produce enzymes such as catalase and cytochrome c oxidase. Additionally, all strains exhibited similar starch hydrolysis activity. In contrast, all strains showed negative results for indole production, indicating a lack of enzyme-producing ability in this aspect. Nitrate reductase exhibited positive results for isolates CMI 7 and CMI 9, together with the reference species, indicating the strong genetic relationship. Conversely, isolate CMI 10 differed from the reference species. Moreover, all isolates displayed skim milk hydrolysis pattern similar to that of the reference species, showing that they were able to produce proteases when exposed to protein substrate, but exhibited negative result for isolate CMI 7. Thereby, each strain revealed distinct characteristics in enzyme-producing ability of *Bacillus* strains.

The ability to ferment carbohydrates, produce mixed acids, and generate acetylmethyl carbinol (acetoin) was observed in the isolates and in *B. subtilis* DMST 5871. Among the isolates, only isolate CMI 7 and *B. subtilis* DMST 5871 could utilize glycerol (C3), the simplest building block of lipids, while the others could not. Focusing on C5 carbohydrates, ribose, arabinose, and xylose were utilized and fermented by the isolates, following the same pattern as *B. subtilis* DMST 5871. For C6 carbohydrates, fermentation occurred with mannose, mannitol, glucose, fructose, and galactose; however, *B. subtilis* DMST 5871 tested negative for mannitol fermentation. Similarly, oligosaccharides and starch were fermented by the isolates. Except that isolate CMI 9 tested negative for maltose, and isolate CMI 10 tested negative for lactose. In addition, TSI test was assessed the ability to utilize three carbohydrates—trace amounts of glucose, as well as larger quantities of lactose and sucrose—along with the production of H_2S. The results were consistent with previously observed carbohydrate fermentation patterns. Therefore, the biochemical character assessment confirmed that the isolates belonged to the genus *Bacillus*, as determined by comparison with *B. subtilis* DMST 5871. The *Bacillus* genus exhibits significant species diversity, with various studies highlighting notable biochemical differences among its members (Sawant et al., 2022; Mukhtar et al., 2022; Di et al., 2023). The genetic confirmation of the isolates was achieved through amplification of the 16S rRNA gene, which serves as a molecular fingerprint for bacterial identification. PCR amplification yielded an amplicon of approximately 1.5 kb, confirming the bacteria belonged to the genus *Bacillus,* as shown in Figure 2 and Table 2. Specifically, isolate CMI 7 showed a high similarity of 99.86% to *B. subtilis* strain DCK5, whereas isolate CMI 9 manifested a similarity of 99.86% to both *B. subtilis* strain G7 and *B. subtilis* strain MR3. On the other hand, isolate CMI 10 exhibited a similarity of 98.91% to *B. cereus* strain a35. Therefore, all isolates in this study belonged to the genus *Bacillus*. They clustered with previously reported *Bacillus* isolates and established type strains, which were consistent with and supported the earlier biochemical identification results.

3.2 Survival under acidic conditions

The ability of probiotic bacteria to survive and remain viable in acidic environments is a crucial factor in ensuring their therapeutic benefits. The stomach's pH typically ranges from 2.5 to 3.5, serving as a natural barrier that prevents bacterial entry into the intestinal tract (Lin, et al., 2007; Boke, et al., 2010). The isolated *Bacillus* CMI 7, CMI 9, and CMI 10 were exposed to acidic pH conditions. Compared with the control group at pH 7 and its initial cells (at 0 h), isolates demonstrated the ability to survive under acidic environments, as shown in Figure 3. The outstanding survival of isolate CMI 7 under acidic pH levels was

Table 1. Biochemical characterization of the isolates

Parameters	Isolates			
	CMI 7	**CMI 9**	**CMI 10**	***B. subtilis* DMST 5871**
Characteristics				
Gram	+	+	+	+
Endospore	+	+	+	+
Motility	+	+	+	+
Enzymatic production				
Catalase	+	+	+	+
Cytochrome c oxidase	+	+	+	+
Citrate	-	-	-	-
Urease	+	+	-	+
Indole	-	-	-	-
Nitrate reductase	+	+	-	+
Skim milk Hydrolysis	-	+	+	+
Starch Hydrolysis	+	+	+	+
Carbohydrate Utilization				
Methyl Red	+	+	+	-
Voges-Proskauer	+	+	+	d
Triple Sugar Iron	K/A	A/A	A/A	K/A
Glycerol ferment	+	-	-	+
Ribose ferment	+	+	+	+
Arabinose ferment	+	+	+	+
Xylose ferment	+	+	+	+
Mannose ferment	+	+	+	+ D
Mannitol ferment	+	+	+	-
Glucose ferment	+ D	+	+	+ D
Fructose ferment	+	+	+	+
Galactose ferment	+	+	+	+
Maltose ferment	+	-	+	+
Sucrose ferment	+	+	+	+
Lactose ferment	+	+	-	+
Inulin ferment	+	+	+	+

The symbols used for interpretation are as follows: **'+'** = positive (≥ 90%), **'-'** = negative (≥ 90%), **'**-D' = delayed reaction, and "d" = doubtful. Triple Sugar Iron (TSI) test results are interpreted as A= acid, and K= alkaline.

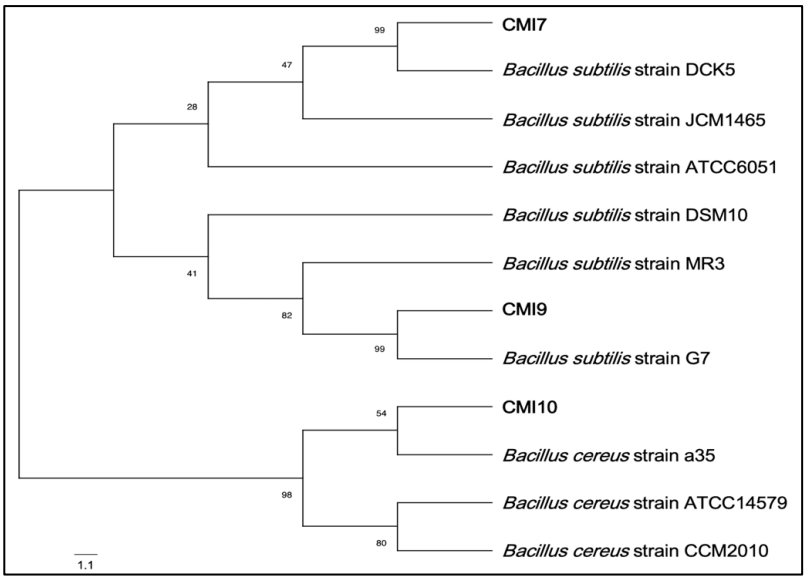

Figure 2. Phylogenetic tree illustrating the relationships among the isolated bacterial strains, *Bacillus*-related species, and *Bacillus* type strains, constructed using the maximum-likelihood method based on 16S rRNA sequences. The scale bar represents 1.1 nucleotide substitutions per site.

Table 2. The similarity of partial 16S ribosomal RNA gene sequence and accession numbers of the isolates

Isolates	Submission	16S ribosomal RNA Gene (% similarity)	Accession Number
CMI 7	SUB15268966	*Bacillus subtilis* strain DCK5 (99.86%)	PV544817
CMI 9	SUB15268966	*Bacillus subtilis* strain G7	PV544818
		Bacillus subtilis strain MR3 (99.72%)	
CMI 10	SUB15268966	*Bacillus cereus* strain a35 (98.91%)	PV544819

statistically significant differences over time (p<0.05 and p<0.01). It displayed a survival rate of 81.26±2.21 % after 4 h and 73.77±5.88% after 24 h at pH 1. At pH 2, its survival rate was 84.61±3.96% after 4 h and 75.75±4.91% after 24 h. At pH 3, it manifested a survival rate of 86.94±3.90% after 4 h and 79.02±3.69% after 24 h. The next highest survival rate was observed in isolate CMI 10, which showed statistically significant differences over time at pH 1 and pH 2 conditions. It showed a survival rate of 62.35±4.50% at pH 1 after 4 h and 59.15±3.47% after 24 h (p<0.001). At pH 2, its survival rate was 93.98±3.45% after 4 h and 83.26±2.69% after 24 h (p<0.01). At pH 3, it demonstrated 98.36±1.21% survival after 4 h and 93.82±3.22% after 24 h.

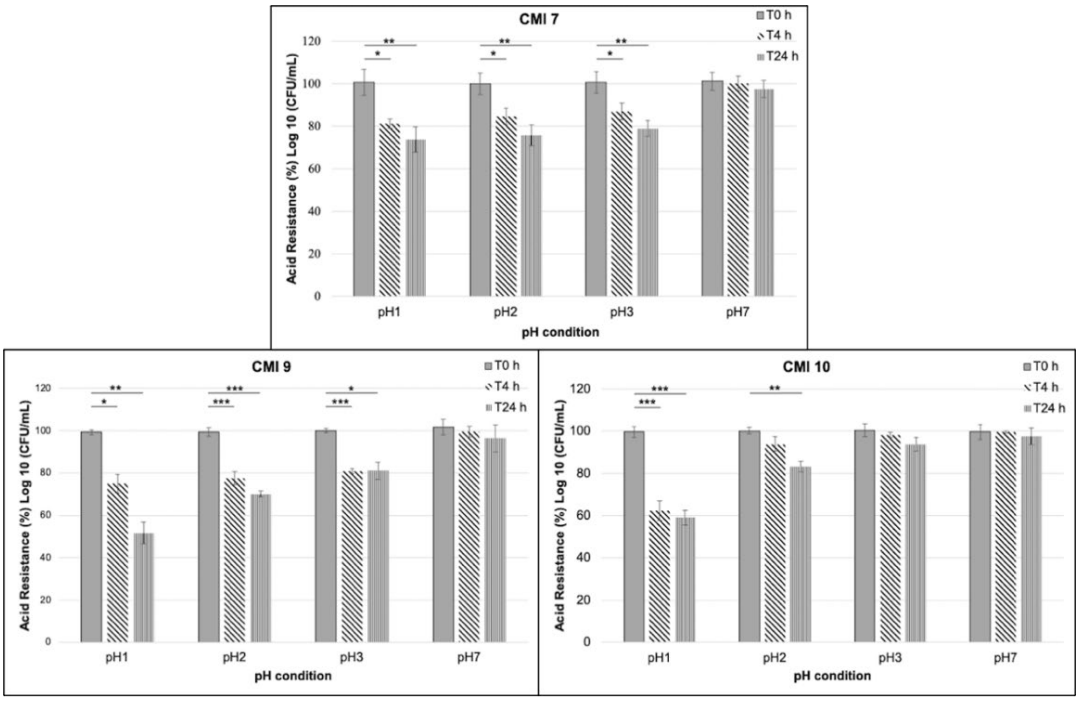

Figure 3. The percentage survival rates of isolates CMI 7, CMI 9, and CMI 10 under various acidic conditions (pH 1, 2, 3, and 7) were assessed at T0, T4, and T24 h. Bacterial counts were recorded as $\log_{10}$ CFU/mL to determine their survival rates.

Lastly, the survival of isolate CMI 9 illustrated statistically significant differences over time. At pH 1, its survival rate was 74.89±4.29% after 4 h and 51.61±5.08% after 24 h (p<0.05 and p<0.001). At pH 2, the survival rate displayed was 77.54±3.01% after 4 h and 70.07±1.31% after 24 h (p<0.001). At pH 3, its survival rate was 80.82±1.25% after 4 h and 81.02±3.96% after 24 h (p<0.001 and p<0.05).

Bacillus subtilis CMI 7 and CMI 9 displayed survival rates exceeding 70% even after 24 h at pH 1. This high level of survival was notable, as exposure to gastric acid is a critical bottleneck for probiotics, and many *Lactobacillus* and *Bifidobacterium* strains are reported to lose viability rapidly below acidic pH 3 (Jin et al., 2012; Fang et al., 2015; Liu et al., 2020). Andriani et al. (2017) also reported favorable survival rates of *B. subtilis* and *B. licheniformis* in acidic conditions at pH 2 after 6 h. Ngampuak et al. (2023) consistently observed survival rates of *B. subtilis* strain VN2, *B. aureus* strain VN3, *B. subtilis* strain VN5, and *B. licheniformis* strain VN7 in acidic conditions at pH 1-6 over 24 h. Interestingly, while isolate CMI 10 exhibited excellent survival at pH 2 and 3, its performance at pH 1 was significantly lower than that of isolate CMI 7, and its identification as *B. cereus* raises safety concerns that preclude its consideration as a probiotic despite its acid resilience.

Therefore, *B. subtilis* CMI 7 and CMI 9 present remarkable acid tolerance, underscoring their potential to withstand gastric passage and maintain probiotic functionality in the human gut.

3.3 Survival under bile salts conditions

Bile salts act as biological emulsifiers, breaking down lipids to facilitate fat digestion (Begley et al., 2006). Although the composition of Oxgall solution differs from that of human bile juice, it is widely used to evaluate the viability of potential probiotic bacterial strains (Begley et al., 2006; Gomes et al., 2012; Tulini et al., 2013). The isolated *Bacillus* CMI 7, CMI 9, and CMI 10 were exposed to different bile salts concentrations to assess their survival rate. Compared to the control group (without bile salts) and its initial cells, these isolates demonstrated significant survival rates under different bile salts environments, as shown in Figure 4.

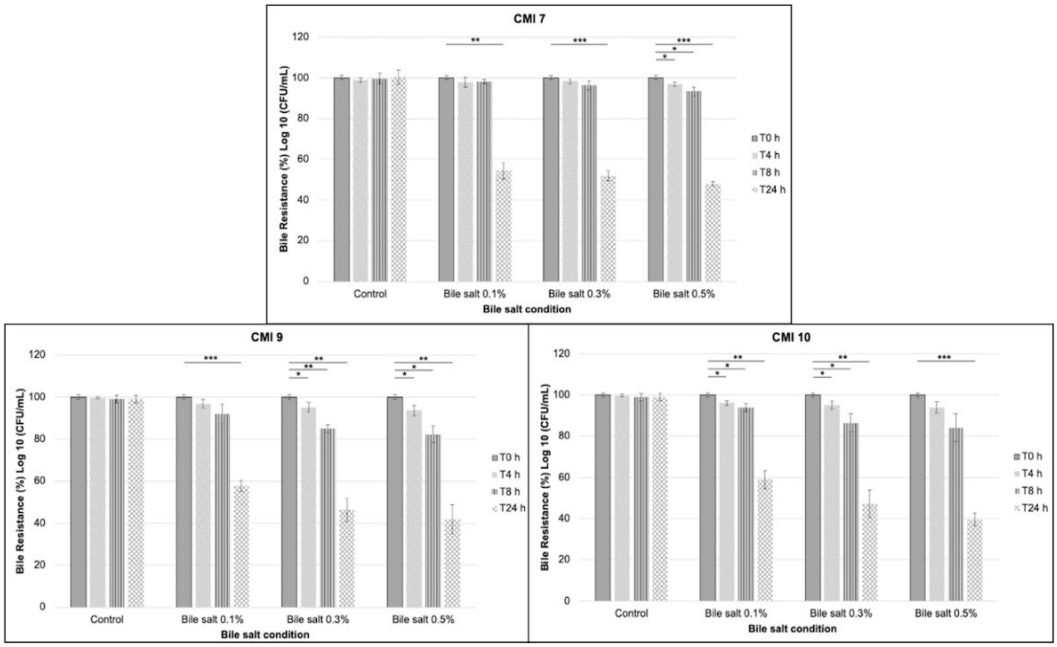

Figure 4. The percentage survival rates of isolates CMI 7, CMI 9, and CMI 10 under bile salts conditions (0.1% (w/v), 0.3% (w/v), and 0.5% (w/v) at T0, T4, T8, and T24 h. Bacterial counts were recorded as $\log_{10}$ CFU/mL to determine their survival rates.

The survival of isolate CMI 7 under Oxgall concentrations showed statistically significant differences over time. At 0.1% Oxgall, a survival rate displayed 97.78±2.41% after 4 h, 98.00±1.00% after 8 h, and 54.30±4.00% after 24 h (p<0.01). At 0.3% Oxgall, its survival rate was 98.29±1.12% after 4 h, 96.45±2.15% after 8 h, and 51.89±2.46% after 24 h (p<0.001). At 0.5% Oxgall, a survival rate exhibited 97.01±1.02% after 4 h (p<0.05), 93.36±2.09% after 8 h (p<0.05), and 48.06±1.10% after 24 h (p<0.001). The survival rate of isolate CMI 9 under Oxgall concentrations showed statistically significant differences over time. At 0.1% Oxgall, its survival rate was 96.69±2.12% after 4 h, 91.97±4.64% after 8 h, and 57.73±2.61% after 24 h (p<0.001). At 0.3% Oxgall, its survival rate was 95.18±2.27% after 4 h (p<0.05), 84.89±1.84% after 8 h (p<0.01), and 46.28±5.62% after 24 h (p<0.001). At 0.5% Oxgall, a survival rate was 93.60±2.42% after 4 h (p<0.05), 82.23±3.90% after 8 h (p<0.05), and 41.49±6.81% after 24 h (p<0.01). Lastly, the survival rate of isolate CMI 10 under Oxgall concentrations showed statistically significant

differences over time. At 0.1% Oxgall, a survival rate was 96.13±1.20% after 4 h (p<0.05), 93.76±1.97% after 8 h (p<0.05), and 58.88±4.38% after 24 h (p<0.01). At 0.3% Oxgall, a survival rate exhibited 94.95±1.92% after 4 h (p<0.05), 86.45±4.36% after 8 h (p<0.05), and 47.15±6.77% after 24 h (p<0.01). At 0.5% Oxgall, a survival rate of 93.88±2.81% after 4 h, 84.10±6.71% after 8 h, and 39.65±3.02% after 24 h (p<0.001) were observed.

The results of this study revealed an inverse relationship between bile salts concentration and the survival rate of isolates. Both *B. subtilis* CMI 7 and CMI 9 maintained exceptionally high viability (>90%) during the initial 4-8 h at bile concentrations ranging from 0.1% to 0.5% Oxgall, before showing a gradual decline at 24 h, where survival still remained above 40-55%. The bile tolerance observed here aligns with previous reports on *Bacillus* spp. (Daneshazari et al., 2023; Nakharuthai et al., 2023; Ngampuak et al., 2023). These findings are particularly significant when compared with *Lactobacillus* and *Bifidobacterium* strains, which generally show markedly reduced survival above 0.3% bile (Begley et al., 2005; Ruiz et al., 2013). A similar trend was observed for *B. cereus* CMI 10, although it raises critical safety concerns that may outweigh its apparent tolerance.

These results underscore the remarkable resilience of the *B. subtilis* CMI 7 and CMI 9 to higher bile salts concentrations, thereby underscoring their relevance as resilient probiotic candidates.

3.4 Survival under electrolyte monogastric system model

It is essential to evaluate potential probiotics for their ability to survive under the continuous digestion conditions simulated by the EMSM. This evaluation was framed by parameters including oral, gastric, and small intestinal digestion, with their relevance of the results discussed in relation to available *in vivo* data (Minekus et al., 2014). Isolates were subjected to the EMSM to assess their survival relative to initial cell counts. These strains showed statistically significant differences of the survival rate under EMSM environments, as shown in Figure 5.

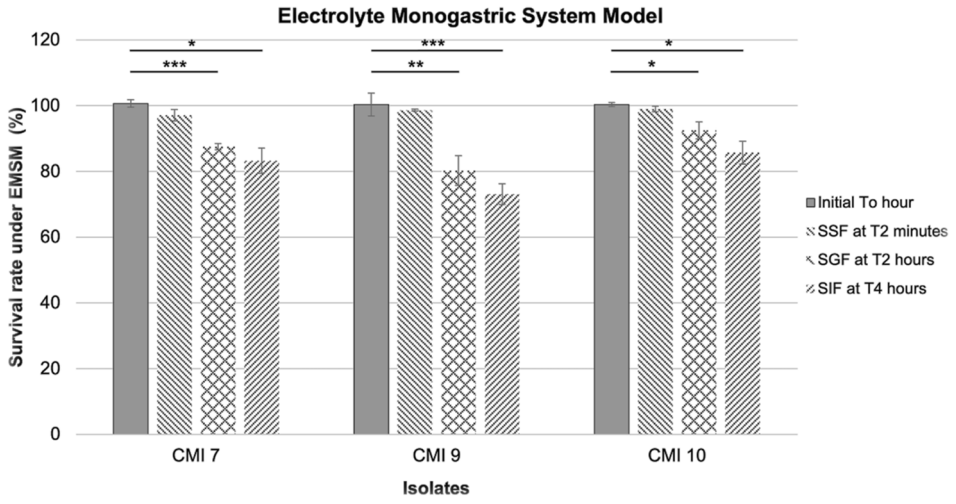

Figure 5. The percentage survival rates of isolates CMI 7, CMI 9, and CMI 10 under EMSM conditions were assessed at continuous time points: SSF at T2 mins, SGF at T2 hours, SIF at T4 h. Bacterial counts were recorded as $\log_{10}$ CFU/mL to determine their survival rates.

The isolates demonstrated strong tolerance to continuous exposure under SSF, SGF, and SIF conditions. Isolate CMI 10 exhibited the highest survival rate; 99.03±058% in SSF, 92.48±2.59% in SGF (p<0.05), and 85.72±3.49% in SIF (p<0.05). Isolate CMI 9 ranked next, the survival rate was 98.62±0.30% in SSF, 80.23±4.54% in SGF (p<0.01), and 73.08±3.18% in SIF (p<0.001). Lastly, the survival rate of isolate CMI 7 was 97.11±1.75% in SSF, 87.55±0.96% in SGF (p<0.001), and 83.25±3.81% in SIF (p<0.05).

This study clearly demonstrated that the isolates exhibited exceptional survival under these conditions, with survival rates exceeding 73.08±3.18% after 4 h. These results are consistent with the findings of Barbosa et al. (2005), who observed that spore-forming bacteria such as *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. clausii*, *B. megaterium*, *B. firmus*, and *B. cereus* exhibited tolerance to bile salts and simulated gastrointestinal tract conditions *in vitro*. The ability of spore-forming *Bacillus* strains to withstand stress and harsh environments demonstrates their potential resilience in challenging conditions (Leñini et al., 2023; Golnari et al., 2024b). This evidence is further supported by Zoldan et al. (2023), who observed that probiotic *B. subtilis* maintained the highest survival throughout all phases of an *in vitro* gastrointestinal simulation, with intestinal-phase counts exceeding 8.0 log CFU/g, significantly higher than those of *Lactiplantibacillus plantarum* and *L. casei*. In comparison, many *Lactobacillus* and *Bifidobacterium* strains exhibit sharply reduced survival under gastric acidity (pH <3) and bile concentrations above 0.3% (Corcoran et al., 2005; Andriantsoanirina et al., 2013). *Bacillus subtilis* CMI 7 and CMI 9 demonstrated superior resilience, maintaining survival above 80% under acidic conditions and up to 99% under bile salt exposure, a tolerance largely attributed to their robust spore-forming ability (Ruiz et al., 2013). Although *B. cereus* CMI 10 showed high survival, its safety concerns preclude its use as a probiotic.

This remarkable resilience observed under EMSM for *B. subtilis* CMI 7 and CMI 9 highlights their exceptional ability to withstand the harsh conditions of the digestive system, indicating their potential to survive ingestion in both humans and animals. These findings point to their promise as effective probiotics, capable of persisting throughout the gastrointestinal tract and exerting beneficial effects on the host.

3.5 Antimicrobial activity

Evaluating antimicrobial activity is important in probiotic property, as it indicates a strain's ability to suppress harmful pathogens and contribute to host protection (Fijan, 2023). Antimicrobial compounds are produced by some *Bacillus* strains for protecting themselves from competing microorganisms in their environment. These compounds include bacteriocins and secondary metabolites (Tran et al., 2023). The results of antimicrobial activities of isolates CMI 7, CMI 9, and CMI 10 are presented in Figure 6.

For *S. aureus* ATCC 9144 and strain DMST 2933, isolate CMI 9 showed a clear zone of 2.94±0.27 mm, while the reference displayed a larger clear zone of 4.19±0.77 mm, indicating positive antagonism. In contrast, isolate CMI 7 exhibited a slight clear zone of 0.65±0.05 mm. For *E. coli* ATCC 25923, isolates CMI 7 and CMI 9 showed minor inhibition with clear zones of 1±0.1 mm and 1.55±0.05 mm, respectively, indicating weak positive antagonism. In contrast, the reference produced a clear inhibition zone against *S. dysenteriae* DMST 2137 at 3.81±1.25 mm.

The antagonistic effect of *Bacillus* strains is largely attributed to their ability to produce a variety of antimicrobial metabolites. Among these, cyclic lipopeptides, including surfactin, iturin, and fengycin, have been reported as a key of this effect (Ongena & Jacques, 2008; Jasim et al., 2016). In addition, cyclic dipeptides, namely maculosin have

also demonstrated inhibitory effects against pathogens (Tran et al., 2023). Englerová et al. (2021) reported that *B. amyloliquefaciens* produces surfactin, fengycin, and iturin, which down-regulate genes expression in pathogenic bacteria, thereby preventing biofilm-related infections. Focusing on Gram-positive pathogenic *S. aureus*, isolate CMI 9 showed notable inhibition greater than isolate CMI 7, suggesting secretion of lipopeptides or bacteriocin-like compounds effective against Gram-positive pathogens (Stein, 2005; Ongena & Jacques, 2008). In the case of enteropathogenic *E. coli*, isolates CMI 9 and CMI 7 exhibited mild inhibition. These findings are consistent with previous reports showing that Gram-negative bacteria generally display lower susceptibility to bacteriocins and lipopeptides, largely because their outer membrane acts as an effective barrier (Gillor et al., 2008). Therefore, these results suggest that *B. subtilis* CMI 7 and CMI 9 in this study may have the ability to produce antimicrobial compounds that inhibit the growth of *S. aureus*.

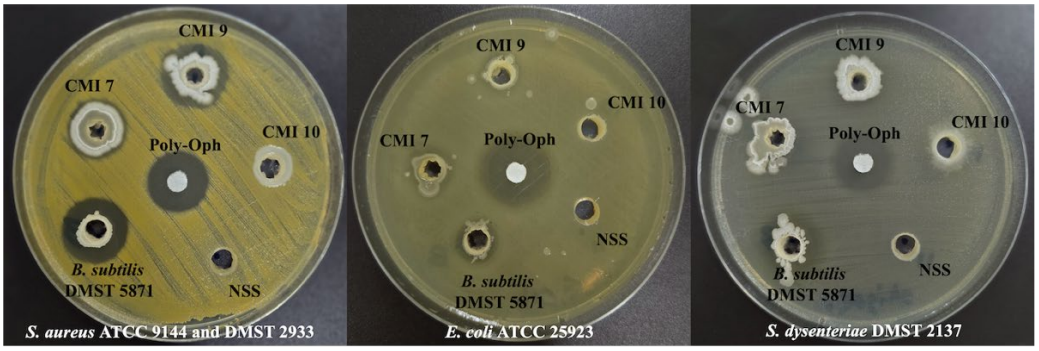

Figure 6. The antimicrobial activity of isolates CMI 7, CMI 9, and CMI 10 was tested against *S. aureus* ATCC 9144 and strain DMST 2933, *E. coli* ATCC 25923, and *S. dysenteriae* DMST 2137. *Bacillus subtilis* DMST 5871 served as the reference strain, NSS was used as the negative control, and Poly-Oph, a mixture of Neomycin sulfate (2 mg), Polymyxin B sulfate (5,000 units), and Gramicidin (0.025 mg), was the positive control.

3.6 Hemolysis evaluation

A crucial safety parameter when evaluating probiotic bacteria is their ability to lyse red blood cells (Shivani & Sathiavelu, 2024). Assessing hemolysis helps distinguish safe probiotic candidates from potentially pathogenic strains (Amoah et al., 2021). In this study, isolates were incubated with red blood cells at 37°C, as illustrated in Figure 7.

After 24 h, isolates CMI 7 and CMI 9 exhibited γ-hemolysis, while isolate CMI 10 displayed slight β-hemolysis. Meanwhile, the reference strain showed mild α-hemolysis. After 48 h, isolates CMI 7 and CMI 9 exhibited minimal α-hemolysis, while isolate CMI 10 showed strong β-hemolysis with creamy-greenish enlarged colonies. The reference strain also displayed mild α-hemolysis.

The hemolytic patterns observed in this study indicate strain-specific differences in the interaction of *Bacillus* isolates with red blood cells, aligning with previous studies. For instance, certain strains of *B. subtilis* and *B. tequilensis* primarily showed γ-hemolysis, whereas other strains of *B. subtilis, B. velezensis*, and *B. licheniformis* likely exhibited α- or β-hemolysis under comparable conditions (Amoah et al., 2021; Daneshazari et al., 2023; Ngampuak et al., 2023). Such variability likely reflected species-specific differences in the

production of hemolysins or other extracellular enzymes that interact with erythrocyte membranes. Notably, the predominance of γ-hemolysis and the minimal α-hemolytic activity observed in isolates CMI 7 and CMI 9 suggested that these isolates posed a low risk of cytotoxicity toward red blood cells. This feature is an important safety criterion for probiotic candidates as it indicates that the strains are unlikely to cause hemolytic damage within the gastrointestinal tract (Cutting, 2011). Unfortunately, isolate CMI 10 exhibited β-hemolysis. This finding aligns with the known physiology of *B. cereus*, a species well known for producing hemolysins such as cereolysin O and hemolysin BL (HBL), both of which contribute to cytotoxic effects and are closely linked to its virulence profile (Senesi & Ghelardi, 2010; Tuipulotu et al., 2021). From a food safety standpoint, finding a β-hemolytic isolate CMI 10 in traditional Thai natto underscores the importance of proper hygiene and controlled fermentation practices to ensure food safety.

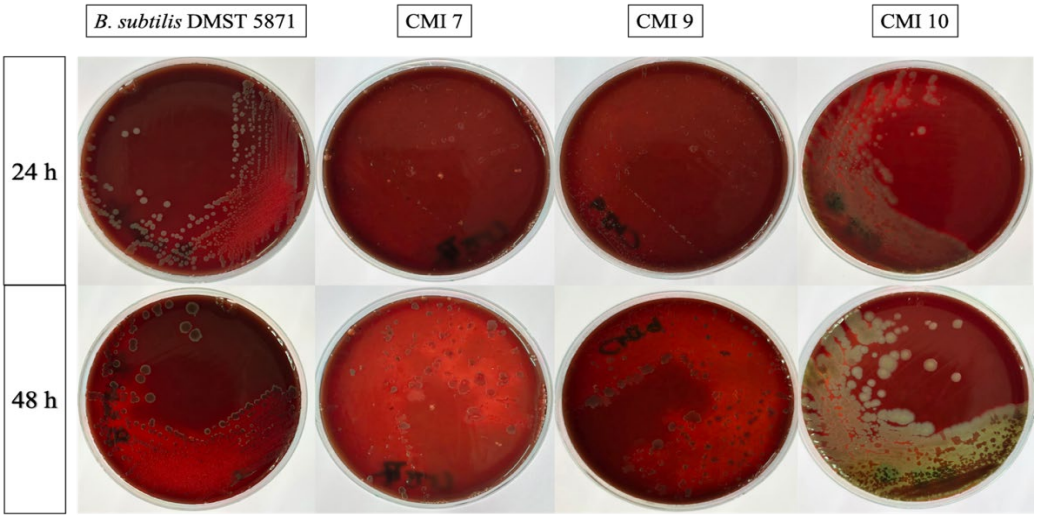

Figure 7. Hemolytic activity of isolates CMI 7, CMI 9, and CMI 10 was determined by incubation on blood agar at 37ºC for 24 and 48 h, with *B. subtilis* DMST 5871 serving as a reference.

Overall, the hemolytic profiles reinforce the potential of *B. subtilis* CMI 7 and CMI 9 as safe *Bacillus* probiotics, capable of supporting gut health without compromising erythrocyte integrity.

3.7 Antibiotic susceptibility

A key safety consideration is the antibiotic susceptibility of the isolated *Bacillus* strains, particularly if they need to be eliminated from the gastrointestinal tract (GIT). In this study, the isolates were tested with antibiotics from two groups: (i) cell wall synthesis inhibitors (Ampicillin, Cefotaxime, and Cefoxitin) and (ii) protein synthesis inhibitors (Gentamicin), as shown in Table 3 and Figure 8.

Both isolates CMI 7 and CMI 9 were susceptible to all antibiotics, including cell wall synthesis inhibitors (Ampicillin, Cefotaxime, Cefoxitin) and the protein synthesis inhibitor Gentamicin. These findings align with the expected intrinsic susceptibility of *B. subtilis* to β-lactams and aminoglycosides, in agreement with the reports of Horikawa and Ogawara

(1980) and Adimpong et al. (2012). This broad susceptibility was consistent with previous reports showing that many *B. subtilis* strains naturally remain sensitive to common antibiotics, reflecting a non-resistant phenotype (Daneshazari et al., 2023; Golnari et al., 2024a,b). On the other hand, several studies have shown that *B. subtilis* can naturally possess intrinsic resistance and acquired resistance traits. Butcher and Helmann (2006) demonstrated that sigma(W)-dependent operons in *B. subtilis* conferred intrinsic resistance and played a key role in enabling it to withstand antibiotic-induced killing. In addition, Tojo et al. (2015) reported that *B. subtilis* strain 168 can acquire resistance to drugs such as streptomycin, through ribosomal mutations, and rifampin, through RNA polymerase mutations, involving specific drug-resistance genes (e.g., rpsL, rpoB, and mthA). Consequently, *B. subtilis* CMI 7 and CMI 9 are sensitive to antibiotics, a characteristic considered safe and indicative of probiotic potential, thereby supporting their suitability for use as probiotics.

Table 3. Antibiotic susceptibility profile of the isolated *Bacillus* strains

	Diameter of Inhibition Zone				
Isolates	**Ampicillin (AMP) 10 mcg**	**Cefotaxime (CX) 30 mcg**	**Cefoxitin (CTX) 30 mcg**	**Amoxyclav (AMC) 30 mcg**	**Gentamycin (GEN) 10 mcg**
CMI 7	S (29.02±0.68)	N/M	N/M	S (30.24±0.39)	S (12.84±0.48)
CMI 9	S (23.91±0.1)	S (31.07±0.72)	S (38.14±0.05)	S (29.77±0.38)	S (16.71±0.35)
CMI 10	R	R (10.67±0.06)	R (11.56±0.24)	R (10.45±0.84)	S (22.57±0.49)

The results were categorized as follows: S = susceptible, I = intermediate, R = resistant, and N/M = not measurable. The diameter of the inhibition zone (measured in mm) was recorded for each antibiotic and presented as the mean±standard deviation (SD) from three independent experiments performed in triplicate.

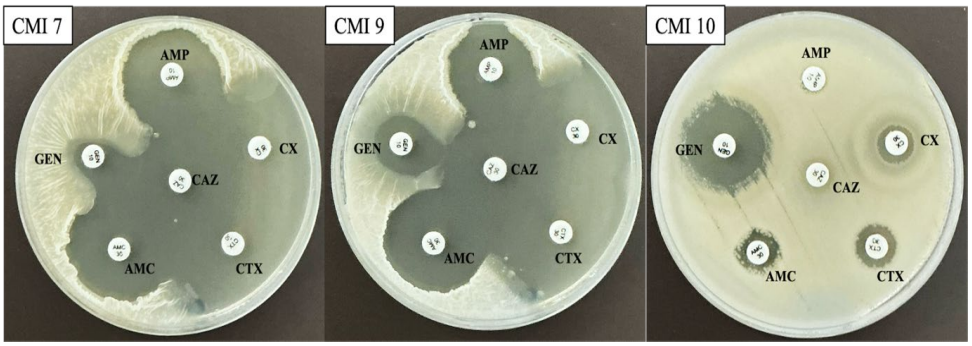

Figure 8. Antibiotic susceptibility of isolates CMI 7, CMI 9, and CMI 10 was evaluated using the following antibiotics: Ampicillin (AMP), Cefoxitin (CX), Cefotaxime (CTX), Amoxyclav (AMC), Gentamycin (GEN), and Ceftazidime (CAZ).

In contrast, isolate CMI 10 exhibited selective resistance, being susceptible to Gentamicin but resistant to Ampicillin, Cefoxitin, and Cefotaxime. A phenotype commonly reported among *B. cereus* due to the presence of chromosomally encoded resistance determinants such as β-lactamases, as well as efflux pumps and ribosomal protection proteins (Fiedler et al., 2019; Bottone, 2010). This pattern was consistent with observations in other *Bacillus* strains, where resistance to β-lactam antibiotics was linked to the presence of antibiotic-resistance genes on plasmids or chromosomal loci. It indicates the presence of acquired resistance mechanisms. These findings aligned with those of Jin et al. (2024), who reported plasmid-associated antibiotic resistance genes in *Bacillus* strains. In their study, *B. cereus* strain J-7-A carried plasmids encoding multiple resistance genes, including those for chloramphenicol, erythromycin, and gentamicin. Importantly, the presence of these plasmids corresponded with the observed resistance profiles, underscoring the role of horizontal gene transfer in spreading antibiotic resistance among *Bacillus* species.

Probiotic strains must be sensitive to clinically important antibiotics to avoid contributing to the spread of antimicrobial resistance. Based on these results, the antibiotic susceptibility patterns of isolates CMI 7 and CMI 9 qualify them as suitable candidates for selection as probiotic *Bacillus* strains. In contrast, the resistance profile of CMI 10 conflicts with established safety criteria for probiotic assessment.

4. Conclusions

Three isolated strains from traditional Thai natto were biochemically and genetically identified as spore-forming *B. subtilis* CMI 7, *B. subtilis* CMI9, and *B. cereus* CMI10. Among them, *B. subtilis* CMI 7 and CMI 9 demonstrated clear probiotic potential *in vitro*, showing high survival rates under various pH conditions, different bile salt concentrations, and simulated gastrointestinal stress (EMSM). They also showed antagonistic activity against pathogenic *S. aureus* ATCC 9144 and strain DMST 2933 and *E. coli* ATCC 25923, alongside favorable safety profiles, including the absence of hemolytic activity and antibiotic resistance. For these reasons, this study highlights the potential of spore-forming *B. subtilis* CMI 7 and CMI9 as safe probiotic candidates, offering advantages beyond commercial strains due to their robust resistance to harsh gastrointestinal conditions. Further study should include whole-genome sequencing to identify functional genes, *in vivo* studies to evaluate gut colonization and host interactions, metabolite profiling to characterize bioactive compounds, and evaluations of their applicability in food industry to support practical probiotic use.

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6. Authors' Contributions

Vongsathorn Ngampuak designed research; coordinated research; and wrote the paper; **Vongsathorn Ngampuak** and **Natnicha Jumpadang** performed research; **Vongsathorn Ngampuak** and **Thirayut Sombun** contributed new reagents/analytic tools; **Vongsathorn Ngampuak** and **Tantip Arigul** analyzed genetic data.

ORCID
Vongsathorn Ngampuak https://orcid.org/0009-0003-5184-4676
Natnicha Jumpadang https://orcid.org/0009-0009-8241-8526
Thirayut Sombun https://orcid.org/0009-0005-8137-3355
Tantip Arigul https://orcid.org/0009-0006-2852-3593

7. Conflicts of Interest

All authors declare there is no conflict of interest regarding this publication.

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