Isolation, Characterization, and Assessment of Probiotic Properties of *Bacillus clausii* Isolated from Children's Stools in a Northern Province of Vietnam

Nguyen Quynh Anh Ngo[1*], Xuan Thanh Dam[2], Tiep Khac Nguyen[2], Chien Ngoc Nguyen[3*], Nhi Dinh Bui[4*]

[1]Center for Pharmaceutical Research and Business, Hai Duong Central College of Pharmacy, 324 Nguyen Luong Bang, Hai Duong City, Vietnam.
[2]Faculty of Biotechnology, Hanoi University of Pharmacy, 13-15 Le Thanh Tong, Ha Noi City, Vietnam.
[3]National Institute of Pharmaceutical Technology, Hanoi University of Pharmacy, 13-15 Le Thanh Tong, Ha Noi City, Vietnam.
[4]Viet Tri University of Industry, Viet Tri City, Vietnam

ABSTRACT

Bacillus clausii is a widely utilized human probiotic in various commercial products; however, there has been limited research on the isolation from diverse sources and evaluation of probiotic characteristics of *Bacillus clausii*. For the first time in this study, *Bacillus clausii* strains were isolated and evaluated from stool samples obtained from healthy volunteer children in a northern province of Vietnam. The inherent biological properties of the isolated *Bacillus clausii* strains were specifically examined to explore their potential application as probiotics. Thirteen colonies underwent screening through morphological and biochemical analyses, along with protein Maldi Tof MS. Among these isolates, *Bacillus* M23 and M31 were identified. In the preliminary safety screening, both strains exhibited negative hemolytic activity. Additionally, *in vitro* characteristics, such as spore formation, resistance to acid and bile salts, resistance to pathogenic microorganisms, assessment of extracellular enzyme production, and antibiotic sensitivity testing were determined for these strains, falling within the observed range for other probiotic strains. The 16S rRNA gene sequencing revealed that *Bacillus* M31 shared 97% similarity with *Bacillus clausii* DSM 8716 in the Genbank database. These findings suggest that the *Bacillus clausii* M31 shows promise as a probiotic candidate, although further extensive *in vitro/vivo* studies are necessary to validate its efficacy and safety.

Keywords: *Bacillus clausii*, probiotic, children's stools.

1 INTRODUCTION

Probiotics are defined as live microorganisms that, when administered in sufficient quantities, can impart beneficial health effects [1]. The recognition of their health benefits, such as inhibiting intestinal pathogens, promoting the growth of healthy microflora in the gastrointestinal tract, reducing cholesterol levels, controlling diarrhea, enhancing immune responses, and exhibiting anti-mutagenic and anti-carcinogenic activities, has led to a growing market for probiotic food supplements [2, 3].

While the two main genera, *Lactobacillus* and *Bifidobacteria*, are commonly represented in the market as conventional probiotics primarily isolated from sources like the gastrointestinal tract, feces, milk, and fermented foods, various species from *Streptococcus*, *Propionibacterium*, *Bacillus*, *Enterococcus*, and *Saccharomyces* from different sources are also claimed to have probiotic properties [3, 4].

Corresponding authors:
Nguyen Quynh Anh Ngo: qanhk63hup@gmail.com
Chien Ngoc Nguyen: nhibd@vui.edu.vn
Nhi Dinh Bui: chiennn@hup.edu.vn
Received: 28/02/2024 Accepted: 18/05/2024.
DOI: https://doi.org/10.35516/jjps.v17i4.2444

It is noteworthy that many accessible probiotic strains belong to lactic acid bacteria (LAB), a group of non-sporulating bacteria. However, it is crucial to recognize that spore-forming bacteria, such as *Bacillus* species, have gained significant attention due to their unique properties when compared to vegetative cells [1, 5].

Probiotic strains must meet essential standards and endure various stages of manufacturing, storage, transportation, and application [6]. Spore-forming bacteria, like *Bacillus* species, exhibit remarkable resistance to heat, UV irradiation, pH conditions, desiccation, and solvents. This resistance provides them with the capability for long-term storage at low or room temperature, increased stability in heat processing, and enhanced acid tolerance-important traits that address challenges associated with *Bacillus* usage as probiotics [4, 7, 8].

This resilience opens up the possibility of incorporating spore-forming bacteria into food products, making them potentially dominant microorganisms in pasteurized milk-based products [8]. While numerous *Bacillus* strains with probiotic potential have been studied in various *in vitro* and *in vivo* experiments, some, including *B. subtilis*, *B. polyfermenticus*, *B. clausii*, *B. coagulans*, *B. licheniformis*, and *B. pumillus*, have received approval for commercial use as dietary supplements or growth promoters in aquaculture and animals. Extensive research has focused on isolating *Bacillus* strains from diverse sources for the development to develop products [9, 10]. *Bacillus* species, especially *B. subtilis*, are prevalent in soil but have been identified in water, air, human and animal gut, vegetables, fermented foods, raw and pasteurized milk, and dairy products [3]. Consequently, their ubiquitous presence in different environments allows them to easily find their way into food products, often being part of milk microflora [4].

Recent research highlights the extensive use of these bacteria for generating metabolites, including amino acids, antibiotics, bacteriocins, surfactants, and bioactive peptides. Moreover, certain *Bacillus* species, such as *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis*, and *B. pumilus*, have gained attention for their potential as probiotics or feed additives, as evidenced by increasing proposals for their utilization in these roles [11, 12].

Bacillus clausii has been available in the market for more than 55 years and is distinguished by the presence of four probiotic strains [10]. Its resilience to various physicochemical conditions, including heat, antibiotics, and gastric pH, is attributed to the presence of spores [12, 13]. The notable resistance of *B. clausii* to a broad spectrum of antibiotics ensures that its effectiveness remains unaffected even when administered alongside antibiotic therapy [13]. Additionally, *B. clausii* stands out due to its rapid growth in both aerobic and anaerobic environments [10]. *B. clausii* spores can thrive in young shoots, undergo growth and proliferation, and subsequently regenerate spores [2, 3]. These spores can be present in various environments, including soil, straw, mud, and are also identified in the stems of insects, animals, and humans [3, 4]. Numerous studies indicate that *Bacilli* can be readily obtained from the human gastrointestinal tract through biopsy and the isolation of stool samples [3, 4]. This study focuses on isolating potential *Bacillus clausii* strains from the human digestive tract - finding a safe strain that has good acid and antibiotic resistance and can be used as a probiotic. This study focuses on isolating potential *Bacillus clausii* strains from the human digestive tract - finding a safe strain that has good acid and antibiotic resistance and can be used as a probiotic. Hence, the objective of this study was to explore the probiotic properties of the *B. clausii* M31 strain and assess its safety, with the aim of to utilize it as a raw material for the production of probiotic products.

2 MATERIALS AND METHODS

2.1 Materials

Bacillus clausii strains were isolated from stool samples of healthy children in Hai Duong Province, a northern province of Vietnam. Pathogenic bacterial strains, including *Escherichia coli* ATCC 8739,

Staphylococcus aureus ATCC 6538, *Salmonella typhimurium* ATCC 13311, and *Candida albicans* ATCC 10231, utilized in the study were provided by the National Institute of Drug Quanlity Control.

Reference Strain: *Bacillus clausii* 088AE (sourced from commercial preparations are known as SEBclausii, BioSEB CII).

All other chemicals were of reagent grade and were used without further purification such as Nutrient Broth (*NB, Himedia, India*); Luria-broth agar (*LB, Himedia, India*); Glycerol, MRS agar, MRS broth, Bile Salt; 5N hydrochloric acid (*Merck, Germany*); Tryptic Soy Agar (*TSA, Himedia, India*); Mueller-Hinton (*Himedia, India*); Sheep blood (*MELAB, Vietnam*); Brain Heart Infusion (BHI) broth (Italy).

2.2 Research Methods

2.2.1 Isolation of Probiotic Bacteria

Multiple stool samples were gathered from healthy children who had refrained from consuming probiotic products for a consecutive two-week period. Following the methodology detailed by Lee *et al*., 2019 [9], 1 g of each sample underwent homogenization in 10 mL of Nutrient Broth (NB, Himedia, India) and was subsequently heated at 80 °C for 10 min to eliminate vegetative cells. A ten-fold serial dilution of the supernatant was spread-plated onto Luria-broth agar (LB, Himedia, India), and the LB plates were incubated at 37 °C for 24 h. Individual colonies were streaked across the plate to obtain pure isolated colonies. Subsequently, bacterial isolates underwent observation for colony shape, cell morphology, and Gram stain. Distinct strains were stored at 4 °C for future use to examine probiotic properties, while the isolated Gram-positive strains were preserved in 20 % glycerol at 4 °C.

2.2.2 Biochemical identification of *Bacillus* strains using protein *MALDI TOF MS*

The isolates were tested in duplicate. Following the methods outlined by Starostin *et al.*, 2015 [14], a colony was directly spotted on the MALDI plate with 1 µL of 70 % formic acid added to each spread sample and allowed to dry. Subsequently, 1 µL of the HCCA Matrix solution (composed of 47.5 % water, 50 % acetonitrile, and 2.5 % trifluoroacetic acid) was applied to each position containing a sample. The plate was then inserted into the MALDI Biotyper and the Flex Control software (*Bruker, Germany*) was initiated. The mass spectra were acquired within 10 min. The spectra were imported into the integrated Bruker Bacterial Test Standard (*BTS, Bruker Daltonik GmbH, Bremen, Germany*). The obtained mass spectra were subsequently compared with those of known bacterial strains from commercial libraries provided by Bruker, which presently encompasses around 9000 reference bacterial proteins [15].

2.2.3 Evaluating the Ability to Form Spores

In adverse environmental conditions, *Bacillus* strains demonstrate the capability to undergo spore formation [3]. The bacterial strains were cultivated in liquid NB at a temperature of 37 ℃, with a shaking speed set at 120 rpm, over a duration for 24 h. Subsequently, the bacterial cultures underwent heat treatment at varying temperatures for a duration of 15 min, specifically at 40 ℃, 50 ℃, 60 ℃, 70 ℃, and 80 ℃. Following this treatment, the samples were appropriately diluted, and the bacterial suspension was evenly spread onto agar plates containing LB agar. The subsequent step involved the observation and quantification of the colonies proliferating on the agar plates, allowing for the calculation of bacterial density.

2.2.4 Assessment of Bile Salt Tolerance

To evaluate bile salt tolerance, *Bacillus* cultures from overnight incubation were re-suspended in sterile Phosphate-Buffered Saline (PBS) pH 7.2 (*Merck, Germany*). The suspension was then adjusted to reach a concentration of 10^8 CFU/mL. This adjusted culture was introduced into fresh MRS broth containing 0.3 % (w/v) Bile Salt and incubated for 6 h. Cell viability was determined at 0, 3, and 6 h of incubation through serial dilution and plating onto MRS agar (Jose *et al.*, 2015) [17].

2.2.5 Test for Survivability in Acid Tolerance

For the determination of acid tolerance, various pH

levels were established using a 5N hydrochloric acid solution, namely pH 1.0, 2.0, 3.0, and 4.0, in PBS. The isolates underwent incubation in NB for 18 h at 37 °C. Subsequently, the cell pellet was collected, washed in PBS, and resuspended in solutions with pH values of 2 and 4. The incubation period lasted 4 h at 37 °C. To assess survivability, plate counts on nutrient agar were conducted at 0, 1, 2, and 4 h [18]. The survival rate was determined using the following equation:

Survival rate (%)= ($N0$/$N1$)×100

$N1$ and $N0$ represent the logarithmic colony-forming units per milliliter (log cfu/mL) count of the selected species after and before treatment.

2.2.6 Assessment of Resistance to Pathogenic Microorganisms

The inhibitory effect of *Bacillus* isolates against certain pathogens was initially determined using the agar well diffusion technique [5]. *Bacillus* isolates were cultured in MRS broth at 37 °C overnight, while the target pathogens were pre-cultured under the same conditions in Brain Heart Infusion (BHI) broth (*Panreac, Italy*). Exactly 200 µL of the test pathogens (10^7 CFU/mL) were then spread onto the surface of Mueller Hinton Agar plates (*Himedia, India*).

Wells were punctured into the inoculated plates and filled with 100 µL of cell-free supernatant obtained by centrifugation of *Bacillus* cultures at 6000 rpm for 10 min (*Hettich Eba, Germany*). The plates were incubated at 37 °C for 24 h, and the antagonistic activity of *Bacillus* was assessed by measuring the formation of inhibition zones (mm) around the wells. This technique was performed in triplicate for each *Bacillus* isolate, and the mean result was recorded. Refining the methodology outlined by Amoah K *et al*., 2019 [19], the determination of resistance to deleterious bacterial and fungal strains involves the agar perforation method using the following test microorganisms: *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *S. typhimurium* ATCC 13311, and *C. albicans* ATCC 10231. The observation of the agar plate was conducted after 24 - 48 h, and any inhibition rings around the agar hole were measured for diameter (D - d, mm). In this context, D represents the diameter of the inhibition ring in millimeters, and d signifies the diameter of the agar hole in millimeters [6, 18].

2.2.7 Assessing the Ability to Produce Extracellular Enzymes

The *Bacillus* strains under scrutiny were subjected to assessments for extracellular amylase, protease, and cellulase activities on plate starch-agar medium (LB give more 1% starch, *Everest-India)*, casein-agar (LB give more 1% casein, *GNC-India*), and carboxymethylcellulose (CMC)-agar media plates (LB give more 1% casein, *Wealthy - China*) , respectively. These evaluations followed the methodologies outlined by Amoozegar *et al*., 2003, and Niranjana *et al*., 2020 [21, 22]. In the screening process, each *Bacillus* strain's colony was positioned at the center of the corresponding selective medium agar plate. After the incubation period, a thorough assessment of all plates took place, with measurements taken for the diameters of the clearance zones, excluding the bacterial colony's diameter. The relative enzyme activity (REA) was calculated using the formula: REA = (D/ d) following the methodology specified by Latorre *et al*., 2016 [23]. Based on the outcomes of the REA test, organisms were classified as excellent (REA > 5.0), good (REA > 2.0 – 5.0), or weak (REA < 2.0).

D: the diameter of the zone of clearance

d: the diameter of the bacterial colony in millimeters.

2.2.8 Antibiotic Sensitivity Test

The antibiotic susceptibility of the selected *Bacillus* strains was assessed using the Kirby-Bauer disk diffusion technique, which adheres to the Clinical and Laboratory guidelines Institute 2021 (CLSI) performance guidelines for antimicrobial susceptibility testing. Several antibiotics (*Oxoid, United Kingdom*) were tested, including ampicillin (10 µg), streptomycin (10 µg), erythromycin (15 µg), tetracycline (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), trimethoprim

(1.25 µg)/sulfamethoxazole (23.75 µg). Bacterial strains were cultured on Tryptic Soy Agar (Himedia, India) plates for 24 h at 37 °C. Following this, the colonies of each strain were suspended in a 0.9 % NaCl solution to prepare a concentration of approximately 1×10^8 CFU/mL. This suspension was then applied to Mueller-Hinton agar (Himedia, India) agar plates using a swab. Antibiotic discs were positioned on the seeded plates, and the zones of growth inhibition were measured after 18 h of incubation at 37 °C following methods by Spears *et al*., 2021 [24].

2.2.9 Hemolytic activity

The hemolytic activity of *Bacillus* strains was determined by using Columbia agar containing 5 % (w/v) sheep blood (*MELAB, Vietnam*). After 24 h of incubation at 37 °C, the hemolytic activity of strains was evaluated and classified based on the lysis of red blood cells in the medium around the colonies. The formation of clean and green areas on the plates was judged as hemolysis. No surrounding area should be identified as non-hemolytic activity by Thi *et al*., 2022 [25]. Alpha hemolysis (α), beta hemolysis (β), and gamma-hemolysis (γ) appeared as green-hued, clear, and no clear zones around the colonies, respectively. Strains with γ-hemolysis are considered safe.

2.2.10 Molecular identification by 16S rRNA sequencing

Separate colonies of selected *Bacillus* strains were used for DNA extraction using the DNA Blood and Tissue DNA Extraction Kit according to the manufacturer's instructions (Qiagen). The purified DNA is used as a template for PCR to amplify the gene segment encoding 16S rRNA with specific primer pairs. The DNA sequences were then analyzed using Illumina MiSeq software (*Illumina, Inc., USA*) and compared with data available in NCBI's gene bank (GenBank), using the BLAST program to find the strain with the closest sequence with a similar ratio copper. The amplification of the 16S ribosomal RNA (rRNA) gene was carried out utilizing the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAAGGAGGTGATCCAGCC-3'). PCR was employed to amplify the DNA products, and the process followed these conditions: an initial denaturation at 95°C for 3 min, succeeded by 35 cycles, each comprising 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min. The amplification concluded with a final extension step at 72°C for 5 min [26].

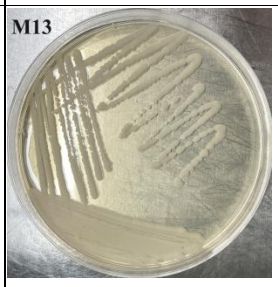
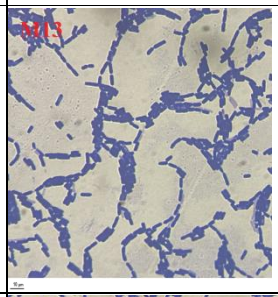
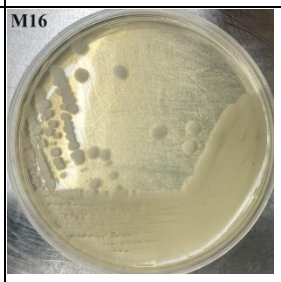
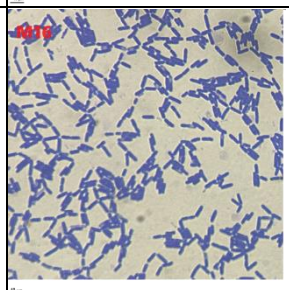
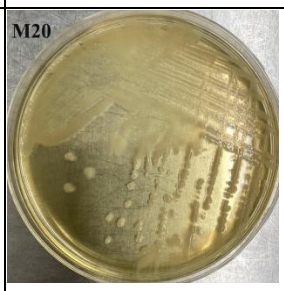
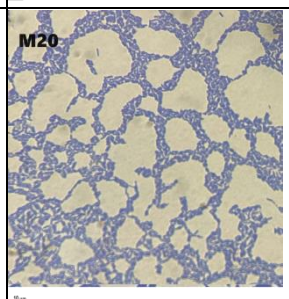
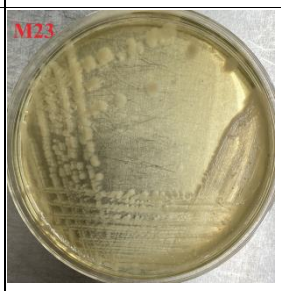
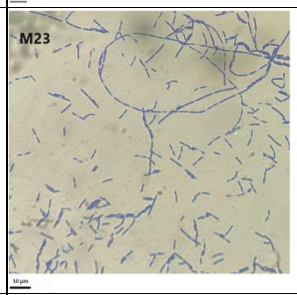
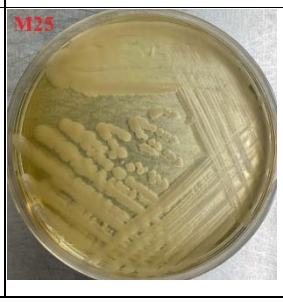
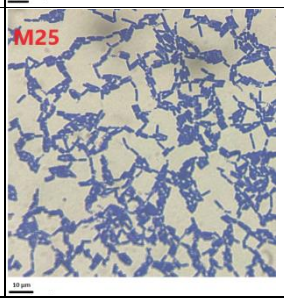
Morphological and biochemical tests such as Gram-positive, catalase and oxidase-positive, motility, API CH50 and cell cell-shaped tests were implemented following the mentioned methods before [9, 11]

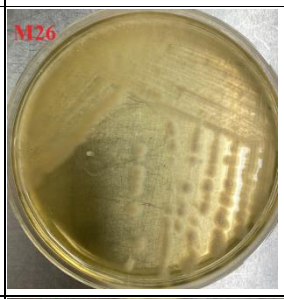
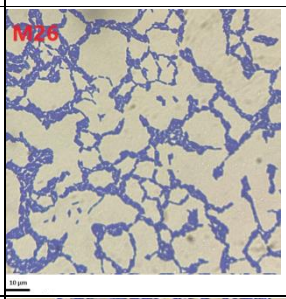
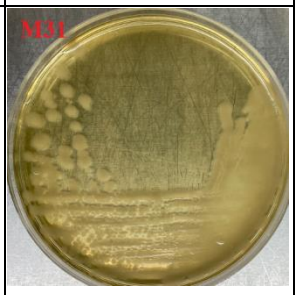
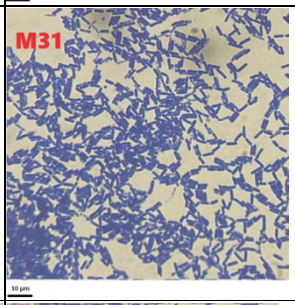
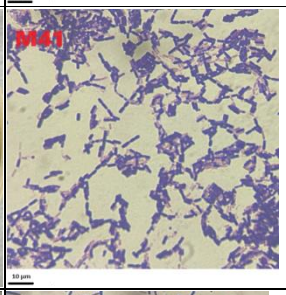
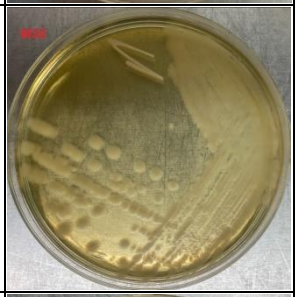
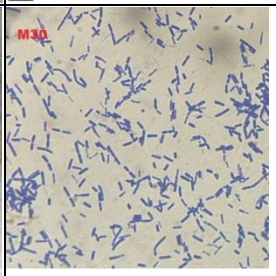
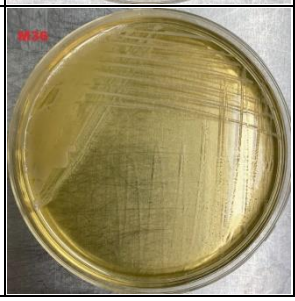
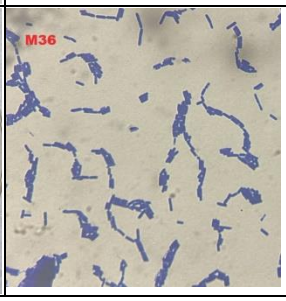
3 RESULTS AND DISCUSSION

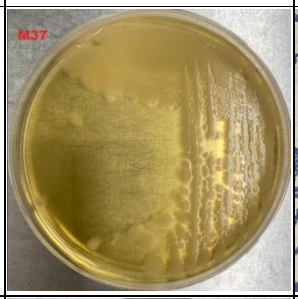
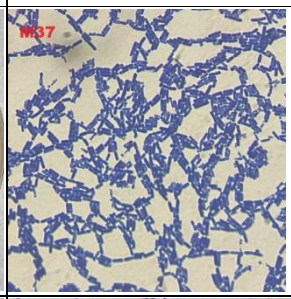
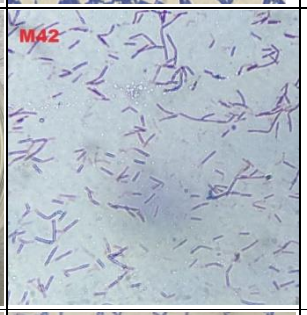
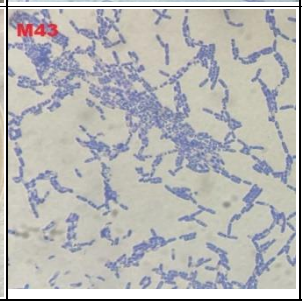
3.1 Isolation of *Bacillus*

The guidelines for identifying and choosing the most potential *Bacillus* probiotic strains for probiotic use are illustrated in *Figure 1* below. A total of 13 strains were extracted from fecal samples of healthy children, employing specific criteria such as their characteristic morphological features (small pinpointed and creamy white colonies). These strains were identified by performing morphological and biochemical tests such as Gram, catalase and oxidase-positive, motility, and coccus and rod-shaped tests [4, 12]. Initially, 13 different characteristic colonies were isolated from feces's children samples and predominantly identified using morphological and biochemical assays (*Fig. 1, Table 1*)

Table 1. Characteristics of isolated *Bacillus* strains

Strain code	(a)	(b)	(c)	(d)	(e)	(g)	*Colony image*	*Gram staining image*	API CH50
M13	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus cereus***
M16	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus cereus***
M20	(+)	(+)	(+)	(+)	(+)	(+)			***Bacillus pumilus***
M23	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus clausii***
M25	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus cereus***

Strain code	(a)	(b)	(c)	(d)	(e)	(g)	*Colony image*	*Gram staining image*	API CH50
M26	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus subtilis***
M31	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus clausii***
M41	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus amyloliquefaciens***
M30	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus cereus***
M36	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus subtilis***

Strain code	(a)	(b)	(c)	(d)	(e)	(g)	*Colony image*	*Gram staining image*	API CH50
M37	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus flexus***
M42	(+)	(+)	(+)	(+)	(+)	γ			***Bacillus licheniformis***
M43	(+)	(+)	(+)	(+)	(+)	(+)			***Bacillus pumilus***

** (a) Gram staining; (b) Catalase; (c) Motility; (d) Glucose; (e) VP test; (f) Hemolytic activity*

Results of Gram staining and microscopic observation show that the bacteria were in the form of short rods, with round ends, standing alone, sometimes joined together to form long filaments (*Fig. 1*). These bacterial strains are all Gram Gram-positive. Through the morphological characteristics of colonies, cells, the results of physiological and chemical characteristics of the strains and the API CH50 test, we can preliminarily conclude that these strains have many characteristics that overlap with *Bacillus* according to Bergey's Classification of Bacteria and Elshaghabee *et al*, 2017 [7]. *Bacillus spp.* has been considered one of the most prominent probiotics due to its better properties compared to other probiotics. The isolation of *Bacillus* bacteria in human feces samples in this study also confirmed results from previous studies suggesting that probiotics that can naturally be isolated from the human gut are likely to have the ability to survive passage through the gut [30]. *Bacillus clausii* and *Bacillus licheniformis* have been isolated from healthy human adult feces, indicating their ability to survive passage through the gastrointestinal tract [31].

3.2 Results of *Bacillus* identification using MALDI TOF MS protein mass spectrometry technology

Mass spectra obtained from each tested species were compared to each other. For each species, a master spectrum (MSP) was generated and included in the reference library. In the program created by MSP using

Flex software from Bruker Daltonik, Bremen, Germany, the *Bacillus* strain protein mass spectra are placed on a separate branch from the branch containing most of the protein mass spectra of *Bacillus spp.* species, and the results are returned. The obtained identification results using the MALDI TOF protein mass spectrometry method in *Table 2* show that the strains identified are *Bacillus* spp. Among them, M23 and M31, identified as *Bacillus clausii*, are the strains being sought. The remaining strains are identified as *Bacillus subtilis*, *Bacillus cereus* etc. The experiments were repeated three times (n=3) for a high confidence level (confidence level, also known as the threshold criterion, set at 2.0 by Bruker for safe genus identification, determined to the species level). These identification results are likely to be reliable and consistent with previous research results of authors such as Starostin *et al.*, 2015 and Manzulli *et al.*, 2021, which has have demonstrated a high degree of reproducibility [14, 32].

Table 2. Results of strain identification test by MALDI TOF MS

Strain	**Detected species**	***Score****
M13	*Bacillus cereus*	2.23
M16	*Bacillus cereus*	2.15
M20	*Bacillus pumilus*	2.13
M23	*Bacillus clausii*	2.26
M25	*Bacillus cereus*	2.23
M26	*Bacillus subtilis*	2.15
M30	*Bacillus cereus*	2.13
M31	*Bacillus clausii*	2.25
M36	*Bacillus subtilis*	2.02
M37	*Bacillus flexus*	2.01
M41	*Bacillus amyloliquefaciens*	2.11
M42	*Bacillus licheniformis*	2.13
M43	*Bacillus pumilus*	2.04

** The experimental results were repeated n=3; high level of confidence (confidence level, also known as threshold criterion > 2.0, defined by Bruker as "safe genus identification, determined to the species level")*

The characteristic spectrum called PMF (Peptide Mass Signature) is generated for the analytes in the sample. Bacterial identification is performed by comparing the PMF of the unknown organism with the PMF present in the database or by comparing multiple biomarkers of the unknown organism with the proteome database. The MALDI-TOF method is often used for simple protein samples, and is quite pure. The advantage is that it can sequence amino acids and peptide fragments and accurately measure mass, but it is not suitable for analyzing complex protein mixtures. DNA has been extracted to be pure and from there, analyzed to the species level with highly reliable score results (using the MALDI TOF protein mass spectrometry method). This is also the basis for performing further experiments.

3.3 Evaluating the ability to form spores

After time from cell stimulation at increasing temperatures, the bacterial strains have an increased ability to produce spores and have relatively high spore density [30]. At 80°C after 15 min, strains M23 and M31 have high spore formation ability.

Table 3. Results of evaluatingAssessing the ability of bacterial strains to produce spores. the spore production ability of bacterial strains

Strain code	Rate of spore formation (log CFU/mL)				
	40°C	**50°C**	**60°C**	**70°C**	**80°C**
Bacillus M23	0.0075 ± 0.001	1.35 ± 0.21	5.19 ± 0.23	7.40 ± 0.22	9.64 ± 0.28
Bacillus M31	0.0264 ± 0.001	1.62 ± 0.17	5.21 ± 0.12	7.64 ± 0.16	10.54 ± 0.36
Bacillus clausii 088AE	0.0318 ± 0.001	1.71 ± 0.34	5.15 ± 0.24	7.31 ± 0.23	10.04 ± 0.16

Note: Cultures started with spores (10^8 *CFU/mL), in nutrient broth, at 37°*C.

Mean values with SE from three biophotometer experiments

A number of other studies also confirmed that *Bacillus spp*. spores are formed, but the specific quantity is not mentioned [30, 31]. In this study, we found that the number of spores of *Bacillus* M23, M31 was relatively high after 15 min of heating and gradually increased as the temperature increased. The ability to sporulate is a crucial characteristic of *B. clausii* to sustain effects on the digestive tract, as vegetative cells are lost when the pH drops below 2. In this study, the spore production rate of M23 and M31 was approximately 10^{10} CFU/mL when the medium was stimulated at high temperatures. However, the spore formation rate of M23 is weaker than that of M31. M31 is nearly equivalent to the reference *B. clausii* 088AE. And M31 at 80°C reached the highest number of about 10^{10} CFU/mL, with high potential in probiotic products.

3.4 Survivability in Simulated in Bile Salts

Bacillus M23 and M31 were tested for their ability to grow in 0.3% bile salts for varying times, an important taxonomic characteristic of the species. The bile salt tolerance of all tested bacteria was confirmed and there were minor differences between strains.

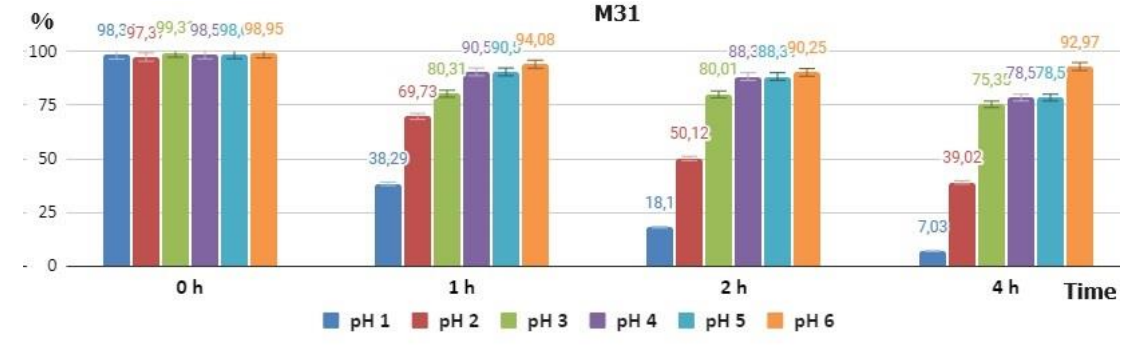
Table 4. Viability of the *Bacillus* isolated strains (log10 CFU/mL) after 0, 3 h, and 6 h of incubation in 0.3% bile salt

Strain code	Bile salt		
	0 h	**3 h**	**6 h**
Bacillus M23	8.96 ± 0.06	8.97 ± 0.03	9.32 ± 0.09
Bacillus M31	8.92 ± 0.05	8.93 ± 0.05	9.65 ± 0.05
B. clausii 088AE	8.95 ± 0.05	8.96 ± 0.05	9.75 ± 0.05

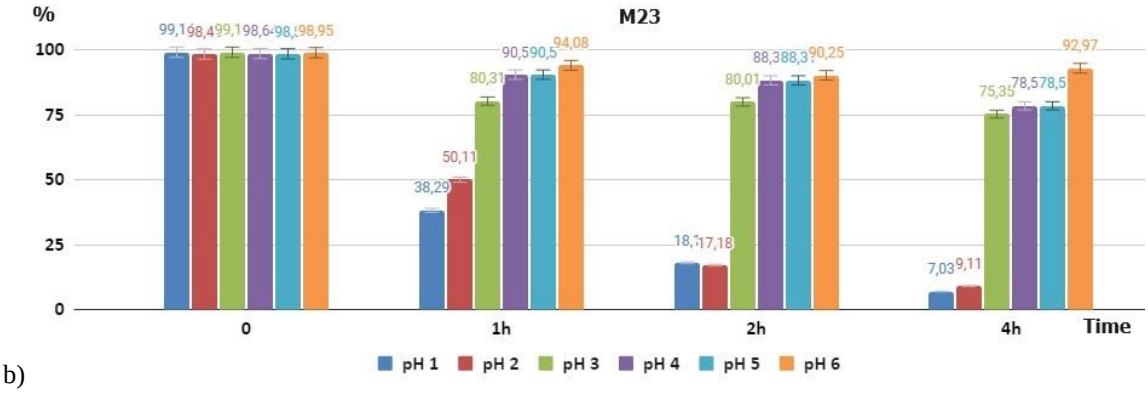
The survivability and growth of potential probiotic strains in the gastrointestinal tract (GIT) depend significantly on their capability to endure and resist intestinal bile salts. Therefore, the ability to tolerate bile salts is a crucial criterion for selecting probiotics [15]. Many studies have assessed the bile salt tolerance of potential probiotics, commonly using an average level of 0.3 % bile salt in their evaluations [16]. In this study, two *Bacillus* isolates, namely M23 and M31, exhibited robust tolerance to 0.3 % bile salt after a 6 h exposure. There was no significant difference ($P > 0.05$) in the viability of *Bacillus* strains between 3 h and 6 h of incubation. Additionally, the range of viability for *Bacillus* strains in the bile salts environment, measured in log10 CFU/mL, after a 6 h incubation, varied from 9.32 ± 0.09 to 9.65 ± 0.05. This was comparable to the recorded range of 8.95 ± 0.05 to 9.75 ± 0.05 (*Table 4*).

3.5 Results of acid tolerance survey

Probiotic bacterial strains exhibit a critical attribute of resilience to low acidity, a prevalent condition in the upper gastrointestinal tract [12, 15]. Consequently, from the isolated bacterial strains, their capacity to endure varied pH conditions (ranging from pH 1 to 7) in nutritional environments was investigated at different time points, specifically 0 h, 1 h, 2 h and 4 h. The findings indicate that the two identified *Bacillus* strains demonstrated noteworthy tolerance to low pH levels (pH 1, 2, 3, 4, 5, and pH 6), along with 0.3 % bile salts, as assessed. Both strains exhibited survival under acidic conditions, signifying a high level of tolerance in these isolates. Additionally, both strains displayed resistance to bile salts, as detailed in *Fig 3*.



a)



b)

Fig 2. Survival of *Bacillus* strains M23 and M31 in culture environments with different pH levels

a) Survival of Bacillus M31 in different pH conditions

b) Survival of Bacillus M23 in different pH conditions

Note: Cultures started with spores (10^8 *CFU/mL), in nutrient broth different pH, at 37*°C.

Mean values with SE from three biophotometer experiments

At pH 1, both strains decreased in number rapidly, proving that *Bacillus* cannot tolerate pH 1 environment. After only 1 hour, the number decreased by less than 40%. At pH 2, *Bacillus* M31 after 1 h survival 70%, after 4 h remains 39%. However, *Bacillus* M23 survival sharply to 40% after just 1 h, proving that the digestive tract's survival ability to withstand harsh conditions is not good. Next, at pH 3, two strains M23 and M31 both survived about 80% after 4 h. Similar to pH 4 and pH 5, survival is more than 80% after 4 h. With pH 6, the survival rate is higher than

about 90%. Among the two strains of *Bacillus clausii*, which have undergone preliminary identification via biochemistry and MALDI TOF, it has been observed that they exhibit growth capability at levels below pH 3. Notably, M31 demonstrates resilience and pronounced resistance at acidic pH levels. Prior investigations conducted by Sorokulova *et al*. (2013) and Amoah *et al*. (2021) have elucidated that probiotic bacterial strains within the *Bacillus* genus can endure acidic conditions ranging from pH 2 to pH 3 for a duration of 2 h [4, 19]. Moreover, Piggo *et al*. (2008) documented the capacity of *Bacillus* genus bacterial strains to endure pH 3 for 120 min [33]. In our study, bacterial strains M23 and M31 exhibit the ability to withstand pH 2 for an equivalent duration of 2 h. Notably, M31 demonstrates a heightened advantage, displaying the highest bacterial survival density when subjected to acidic pH conditions and survival in pH 1/1h around 30%; in pH 2/ 2h around 70% was better survival ability than others studies. This result also shows that it is necessary to protect isolated *Bacillus* strains at lower pH to achieve better effects on the digestive tract.

3.6 Evaluating antibacterial ability

Bacillus strains M23 and M31 were assessed for their antibacterial activity using the agar well diffusion method. The capability to resist pathogenic microorganisms is a significant classification characteristic of probiotics. The antibacterial effectiveness of all tested bacteria was verified. There were minor distinctions between the strains, as illustrated in *Table 5*.

Table 5. Antibacterial activity of *Bacillus* M23, M31 strains using well diffusion method

Strain code	Inhibition zone (mm)		
	Bacillus **M23**	*Bacillus* **M31**	*B. clausii* 088AE
***S. aureus* ATCC 6538**	8.26 ± 0.21	13.50 ± 0.41	12.43 ± 0.24
***E. coli* ATCC 8739**	10.17 ± 0.25	12.58 ± 0.40	12.38 ± 0.23
***S. typhimurium* ATCC 3311**	9.33 ± 0.61	9.52 ± 0.81	10.70 ± 0.60
***C. albicans* ATCC 10231**	11.24 ± 0.13	12.93 ± 0.47	12.67 ± 0.22

The assessment of antibacterial efficacy for the isolated *Bacillus* M23 and M31 strains involved the measurement of the growth inhibition zone diameter against various pathogenic bacteria within an agar plate environment supplemented with probiotics. The findings presented in *Table 5* indicate that *Bacillus* M31 exhibits superior effectiveness in restraining the proliferation of both Gram-positive and Gram-negative pathogenic bacteria with *Bacillus* M31 and *B. clausii* 088AE. Additionally, it demonstrates notable inhibitory capabilities against fungal growth [7, 15, 35]. Inhibition zones of *Bacillus* M31 were larger than *Bacillus* M23 and similarly *B. clausii* 088AE with *E. coli* ATCC 8739; and larger than *B. clausii* 088AE with *S. aureus* ATCC 6538. This result is similar to the results of evaluating Bacillus strains isolated from other sources such as poultry feces, pig feces, fish or other human gastrointestinal tract [5, 8, 19, 21]. Furthermore, in this study, *Bacillus* strains M23 and M31 showed larger antibiotic inhibition zone larger. Furthermore, in this study, *Bacillus* strains M23 and M31 showed antibacterial inhibition zone larger than related isolated *Bacillus* from other sources (12.93mm with *C. albicans* ATCC 10231)

3.7 Assessing the ability to produce extracellular enzymes

The enzyme production ability was assessed by calculating the ratio of the hydrolysis circle diameter (D) to the colony diameter (d). Investigation of the ability to produce extracellular enzymes using the agar well method was performed with bacterial *Bacillus* M23 and M31.

Table 6. Relative enzyme activity values produced by selected *Bacillus* M23 and M31 *Relative enzyme activity (REA)*

Strain	Ring diameter (mm)	
	Amylase	***Protease***
Bacillus **M23**	1.50 ± 0.21	3.00 ± 0.14
Bacillus **M31**	1.57 ± 0.13	2.60 ± 0.15
B. clausii 088AE	2.00 ± 0.11	2.75 ± 0.17

Bacillus M23 and M31 are both capable of producing amylase and protease enzymes. The amylase production ability of the strains is lower than the protease production ability, showing that the diameter of the starch substrate degrading circle (1.5 - 2.0 mm) is higher than the casein degrading ring diameter (2.6 - 3.0 mm). This result is also consistent with some previous studies, therefore *B. clausii* strain has the ability to produce extracellular enzymes to degrade substrates [21, 34]**.**

3.9 Antibiotic sensitivity testing

Bacillus M23 and M31 were tested for antibiotic sensitivity. Determining whether probiotics are antibiotic resistant or not is an important classification characteristic. The information of *Table 7* illustrated the antibiotic resistance of all tested bacteria was recorded and the differences between M23 and M31.

Table 7. Antibiotic sensitivity profiles of *Bacillus* strains

Antibacteria	Strain	
	Bacillus **M23**	*Bacillus* **M31**
Ampicillin 10 µg	26.08±1.10* (S)**	27.16±1.45 (S)
Streptomycin 10 µg	18.02±0.71 (S)	30.27±1.61 (S)
Erythromycin 15 µg	21.02±0.60 (S)	24.17±1.18 (S)
Tetracycline 30 µg	26.10±1.08 (S)	34.00±1.20 (S)
Chloramphenicol 30 µg	23.05±1.41 (S)	26.03±1.11 (S)
Ciprofloxacin 5 µg	20.27±1.14 (S)	25.67±1.12 (S)
Gentamycin 10 µg	26.15±1.20 (S)	28.01±1.09 (S)
Trimethoprim 1.25 µg/ Sulfamethoxazole 23.75 µg	17.36±0.80 (S)	20.24±1.21 (S)

** Mean±SD expressing data of inhibition diameter (mm) in three replications.*

*** Zone Diameter Breakpoints, nearest whole (mm): Sensitive (S), Resistance (R)*

The antibiotics utilized in this investigation were evaluated in studies by Guo *et al*., 2016 [38]. The antibiotic resistance and sensitivity levels of isolated *Bacillus* strains were assessed using the CLSI 2020 standard values and the specific S and R indices were different for each type of antibiotic. *Table 7* results are also consistent with earlier research showing that *Bacillus* M23 and M31 isolates are particularly sensitive (S) to numerous antibiotics [40]. Furthermore, the recovered *Bacillus* strain was sensitive to Ciprofloxacin, Chloramphenicol, Streptomycin, and Tetracycline, which was consistent with the findings of other studies such as Abbrescia *et al*., 2018, Guo et al., 2016, Cutting M., 2020 [38, 39, 40].

3.8 Evaluation of hemolytic activity

In accordance with FAO Animal Production and Health [29], it is advisable for microbial strains intended for use as probiotics to be safe within the host. Choosing and utilizing strains that lack haemolytic activity as probiotics highlights their non-virulent characteristics. The results of testing hemolytic activity with isolated strains are shown in *Table 1*. Among them, two hemolytic strains M20 and M43 were eliminated from subsequent

experiments. Out of the examined *Bacillus* strains for haemolytic activity, demonstrated non-haemolytic behavior, making them suitable for further evaluation due to their safety as probiotics. This aligns with prior findings that indicate a significant portion of bacterial strains had non-haemolytic properties [18, 28]. Evaluation of potential *Bacillus clausii* strains including *Bacillus* M23 and M31 did not show hemolytic activity, the results of culture on agar plates are shown in *Fig 4* below.

a)*Bacillus clausii* 088AE **b)*Bacillus* M31** **c)*Bacillus* M23**

Fig 4. Hemolytic activity of *Bacillus* strains M23, M31

Detection of hemolytic activity is regarded as a crucial indicator of virulence and serves as a fundamental safety assessment when pre-screening a strain for potential investigation as a probiotic or feed additive. The European Food Safety Authority (EFSA) strongly advises the assessment of hemolytic activity to ensure that a bacterial strain, even if it holds Generally Recognized As Safe (GRAS) or Qualified Presumption of Safety (QPS) status, is devoid of toxigenic potential (Yasmin *et al.*, 2020) [41]. Strains displaying hemolytic activity may be deemed unsuitable for applications in human or animal health until the impact of this virulence factor is either mitigated, altered, or confirmed as non-harmful to the eukaryotic host. In the present study, *Bacillus* M23 and M31 were examined, revealing no transparent or greenish zones around their colonies on blood agar plates. This observation contrasts with the henomenon noted in control strains of *Bacillus clausii* 088AE.

3.9 Results of gene sequencing using the 16S rRNA method

The 16S rRNA gene sequence region of the bacterial strains was amplified by PCR and sequenced. With the primer pair used, it was shown that this gene region was amplified in all bacterial strains. The size of the amplified fragment is about 350-400 bp.

The 16S rRNA gene sequence of the bacterial strains was used to build a phylogenetic tree, combining the 16S rRNA gene sequence region with reference bacterial strains in Genbank (*Fig. 5*). Analysis results from the phylogenetic tree of strain M31 are in the same branch as strain *Bacillus clausii* DSM 8716 with a bootstrap ratio of 97% in Genbank database under accession number CP019905.1, with a high level of confidence. This is also consistent with the studies of Simon *et al.*, 2011 and Huynh A *et al.*, 2009 when demonstrating that Bacillus bacterial strains are present in the human intestinal tract [5, 15, 17].

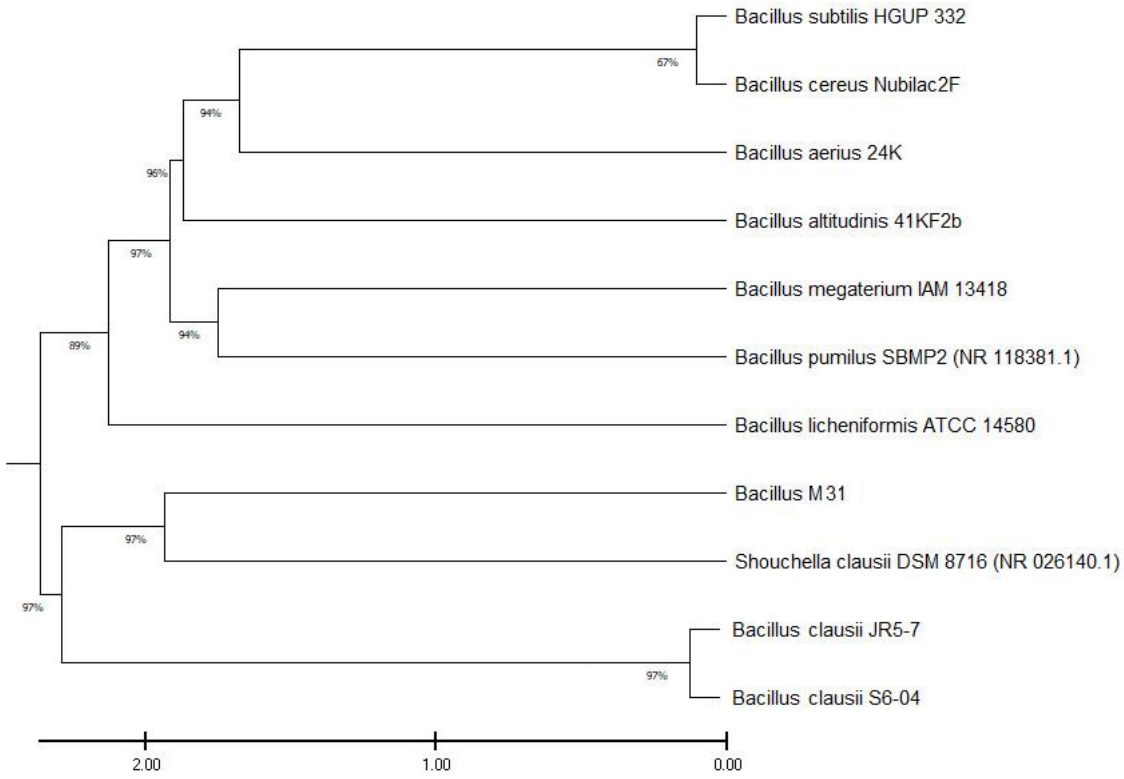


Fig 5. Phylogenetic tree of Bacillus M31 strain

4 DISCUSSION

The selective examination of *Bacillus* strains isolated from the stools of healthy children in this study highlights their distinct probiotic properties, particularly in the case of *Bacillus clausii*, showcasing their ability to thrive and flourish within the human gastrointestinal tract [1, 2]. The *Bacillus clausii* M31 strain, specifically isolated in this study, demonstrates proficiency in spore formation, resilience in challenging environments like low pH and bile salts, and resilience in the digestive tract. This strain proves beneficial in the digestion and hydrolysis of carbohydrates and amino acids, making it a viable candidate for probiotic applications in the food industry, as previously documented in other research studies [9, 20]. Thus, based on the most recent studies and available reports, there is a noticeable absence of data regarding the isolation of *Bacillus* probiotics from children's stools. Consequently, this study aimed to fill this gap by preparing children's stool samples for the isolation of *Bacillus* strains with potential probiotic properties as a novel source. The isolates, identified through biochemical and molecular testing, were associated with *B. clausii* and subsequently utilized for probiotic assessment.

It is crucial to emphasize that the probiotic attributes are inherently specific to each strain, contingent upon both the source of isolation and the intended target. Therefore, evaluating *in vitro/vivo* probiotic properties becomes a pivotal step in considering a microorganism as a probiotic, a point consistently highlighted in various studies [42–44]. Hemolytic activity, often considered detrimental to host cells and tissues [45], necessitates screening bacteria for these products to ensure the safety of an isolate [43]. Fortunately, neither of the two strains in this study exhibited hemolytic activity, aligning with similar

observations reported for probiotic candidates such as *B. clausii* UBBC07 and *Bacillus* BS 3 and BS 31 [4]. Comparable results were also reported by Keubutornye *et al*., 2017 [34], and Naeem *et al*.'s investigation on potential probiotics of *Bacillus* strains showed no hemolysis for their isolates [45].

The evaluation of antibiotic susceptibility is a crucial aspect to guarantee that bacterial strains are not resistant to antibiotics. Understanding the transfer of antibiotic resistance determinants is equally essential for investigating safe probiotics [27]. Antibiotic resistance modeling for *Bacillus* M23 and M31 demonstrated susceptibility, confirming their non-resistance to antibiotics [28]. This aligns with previous studies revealing the sensitivity of *Bacillus* strains to antibiotics [26, 29]. The antimicrobial capability is an undeniably crucial attribute of potentially probiotic bacterial strains. We demonstrated that *B. clausii* M31 has the capacity can impede the growth of opportunistic pathogens, namely *E. coli, S. aureus,* and *P. aeruginosa*, during co-cultivation in a liquid medium. Probiotic strains of *B. clausii* are recognized for their production of substances with antimicrobial properties, with some of these substances having been identified and characterized. Notably, *B. clausii* has been observed to generate antimicrobial substances effective against both Gram-negative and Gram-positive species during whey fermentation [42], underscoring the significance of the starting substrates in the synthesis of antimicrobials. Another noteworthy instance of indirect antimicrobial activity was reported by Ripert G *et al*., 2016 [46].

Given that acid and bile salts in the stomach and intestine represent the initial biological barriers that probiotic strains must overcome after ingestion, acid, and gastric juice tolerance, as well as bile resistance, are paramount factors for the viability and growth of probiotic strains during their journey through the gastrointestinal tract [44]. Both isolates in this study exhibited tolerance to acidic pH and artificial gastric juice conditions, and both strains demonstrated resistance to bile salts. These findings are in line with previous results regarding *Bacillus* strains with probiotic potential [48, 49]. The spores of *Bacillus clausii* M31 exhibited remarkable resilience against simulated gastric and small intestinal environments, suggesting their potential to endure the passage through the upper gastrointestinal tract. Earlier studies have shown that spore formulations of widely recognized *B. clausii* probiotic strains can withstand pH 2 and up to 0.3% bile salts. Furthermore, these spores can germinate and multiply in conditions mimicking the human intestinal environment [50, 51]. The sporulation rate results of M31 show good sporulation ability, which can be further improved through optimization of culture conditions, such as aeration and cell density, in industrial bioreactors. In addition, our findings confirm that the spores of *B. clausii* M31 can endure simulated gastric and small intestinal conditions, too.

Traditional methods for microbial identification, such as biochemical tests and DNA sequencing, are known for their time-consuming and labor-intensive nature. In contrast, the recently employed MALDI TOF mass spectrometry method proves to be simple and rapid, aiding in narrowing down the scope of strain evaluation to save time and costs. However, the effective application of mass spectrometry necessitates a comprehensive reference database and specific software for spectral comparison. Despite this, the identification of isolates through morphological characteristics, biochemical testing, or MALDI TOF still requires further confirmation through tests involving 16S rRNA gene sequencing [21, 23]. Classification of bacterial strains on the phylogenetic tree based on the bootstrap index corresponding to each branch. The higher the bootstrap index, the greater the confidence level that the bacterial strain to be identified is similar to the bacterial strain on the same branch. According to Hillis and Bull (1993), the confidence level of phylogenetic analysis based on the bootstrap index is conventionally defined as: bootstrap index < 65: low confidence level; 65

≤ bootstrap index < 85: average confidence level; Bootstrap index ≥ 85: high confidence level [22].

In conclusion, *B. clausii* M31 emerges as a promising candidate for probiotic applications. This designation is grounded in a comprehensive analysis of test results, which consistently demonstrated the strains' favorable probiotic potential. Nevertheless, for a definitive determination regarding their suitability as probiotic strains, further *in vitro* and *in vivo* assessments are essential. These evaluations should encompass factors such as enzymatic activity, co-aggregation, antimicrobial activity, biofilm formation, cholesterol reduction, and animal models, paving the way for a more conclusive decision on their application as probiotics.

5 CONCLUSION

Out of the thirteen isolated *Bacillus* strains examined in this study, two were identified as *Bacillus clausii*. Subsequently, the identification of *Bacillus clausii* M31 was refined based on an assessment of both the pharmaceutical product type and its corresponding activity, establishing it as the most viable candidate. Strain M31 exhibited a nucleotide sequence closely resembling *Bacillus clausii* DSM 8716, displaying 97% sequence similarity. The nucleotide sequence for *Bacillus clausii* M31 has been documented in the NCBI GenBank. *B. clausii* M31 is characterized as a Gram-positive bacillus with spore-forming capabilities. It demonstrates resilience under acidic conditions (pH 3) for a duration of 3 h, resistance to bile salt environments, resistance to pathogenic bacteria, susceptibility to antibiotics, and proficiency in amylase and protease production. Notably, *Bacillus clausii* M31 does not induce hemolysis. Finally, it can be concluded that *B. clausii* M31 could be notable as probiotic candidates.

ACKNOWLEDGMENTS

The authors would like to thank Hanoi University of Pharmacy and Haiduong Central College of Pharmacy for facilitating this study. The several writers whose work was included intoin this review are also acknowledged by the authors.

Credit authorship contribution statement

Nguyen Quynh Anh Ngo: writing of the original draft, conceptualization, methodology, writing - review & editing, supervision; Chien Ngoc Nguyen : methodology, data curation, formal analysis, writing - review & editing, and project administration; Xuan Thanh Dam: methodology, formal analysis, and project administration.

Declarations

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.

REFERENCES

1. Lefevre M., et al. Safety assessment of *Bacillus subtilis* CU1 for use as a probiotic in humans. *Regul. Toxicol. Pharmacol.* 2017; 83:54–65. https://doi.org/10.1016/j.yrtph.2016.11.010
2. Kim B. J., Hong J. H., Jeong Y. S., and Jung H. K. Evaluation of two *Bacillus subtilis* strains isolated from Korean fermented food as probiotics against loperamide-induced constipation in mice. *J. Korean. Soc. Appl. Biol. Chem.* 2014; 57(6):797–806. https://doi.org/10.1007/s13765-014-4106-0
3. Hameed A., et al. Isolation and characterization of a cholesterol-lowering bacteria from *Bubalus bubalis* raw milk. *Fermentation.* 2022; 8:163. https://doi.org/10.3390/fermentation8040163.
4. Sorokulova I. Modern status and perspectives of *Bacillus* bacteria as probiotics. *J. Prob. Health.* 2013; 1:e106. https://doi.org/10.4172/2329-8901.1000e106.
5. Naeem M., et al. Screening of cattle gut-associated *Bacillus* strains for their potential use as animal probiotics. *Indian J. Anim. Res.* 2018.

https://doi.org/10.18805/ijar.B-948.

6. Barakat M., Al-Najjar M. A., Abdulrazzaq S., Talib W. H. and Athamneh T. The immunostimulatory effect of probiotic conditioned medium on RAW264.7 murine macrophages. *Jordan Journal of Pharmaceutical Sciences.* 2023; 16(2):462. https://doi.org/10.35516/jjps.v16i2.1501
7. Elshaghabee F. M. F., Rokana N., Gulhane R. D., Sharma C. and Panwar H. *Bacillus* as potential probiotics: Status, concerns, and future perspectives. *Front. Microbiol.* 2017; 8:1490. https://doi.org/10.3389/fmicb.2017.01490.
8. Permpoonpattana P., Hong H. A., Khaneja R. and Cutting S. M. Evaluation of *Bacillus subtilis* strains as probiotics and their potential as a food ingredient. *Benef. Microbes.* 2017; 3(2):127–135. https://doi.org/10.3920/BM2012.0002
9. Lee N. K., Kim W. S. and Paik H. D. *Bacillus* strains as human probiotics: Characterization, safety, microbiome, and probiotic carrier. *Food. Sci. Biotechnol.* 2019; 28:1297–1305. https://doi.org/10.1007/s10068-019-00691-9
10. Urdaci M. C., Bressollier P. and Pinchuk I. *Bacillus clausii* probiotic strains: Antimicrobial and immunomodulatory activities. *J. Clin. Gastroenterol.* 2004; 38(6):86. https://doi.org/10.1097/01.mcg.0000128925.06662.69
11. Dilshad R. and Batool R. Antibacterial and antioxidant potential of *Ziziphus jujube*, *Fagonia arabica*, *Mallotus phillipensis*, and *Hemidesmus indicus*. *Jordan Journal of Pharmaceutical Sciences.* 2022; 15(3):413–427. https://doi.org/10.35516/jjps.v15i3.417
12. Ocaña A. M. Review of *Bacillus clausii* and its use in clinical practice. *In Focus.* 2007. https://doi.org/10.1186/s40794-019-0089-5
13. Odo K. E., Agbo M. O. and Osadebe P. O. Extract and fractions from soil bacteria (*Streptomyces canus* ATCC 12647) possess antimicrobial and antioxidative potential *in vitro*. *Jordan Journal of Pharmaceutical Sciences.* 2022; 15(3):405–412. https://doi.org/10.35516/jjps.v15i3.416
14. Starostin K. V., et al. Identification of *Bacillus* strains by MALDI TOF MS using a geometric approach. *Sci. Rep.* 2015; 5:16989. doi: 10.1038/srep16989 https://doi.org/10.1038/srep16989
15. Vecchione A., Celandroni F., Mazzantini D., Senesi S., Lupetti A. and Ghelardi E. Compositional quality and potential gastrointestinal behavior of probiotic products commercialized in Italy. *Frontiers in Medicine.* 2018; 5. https://doi.org/10.3389/fmed.2018.00059
16. Suva M., Sureja V. P. and Kheni D. B. Novel insight on probiotic *Bacillus subtilis*: Mechanism of action and clinical applications. *J. Curr. Res. Sci. Med.* 2016; 2:65–72. https://www.researchgate.net/publication/312317617
17. Jose N. M., Bunt C. R. and Hussain A. M. Comparison of microbiological and probiotic characteristics of lactobacilli isolates from dairy food products and animal rumen contents. *Microorganisms.* 2015; 3:198–212. https://doi.org/10.3390/microorganisms3020198
18. Corcoran B. M., Stanton C., Fitzgerald G. F., and Ross R. P. Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Appl Environ Microbiol*. 2005; 71(6):3060–3067. https://doi.org/10.1128/AEM.71.6.3060-3067.2005
19. Amoah K., Huang Q. C., Tan B. P., Zhang S., Chi S. Y. and Yang Q. H. Dietary supplementation of probiotic *Bacillus coagulans* ATCC 7050 improves growth performance, intestinal morphology, microflora, immune response, and disease confrontation of Pacific white shrimp (*Litopenaeus vannamei*). *Fish Shellfish Immunol.* 2019; 87:796–808. https://doi.org/10.1016/j.fsi.2019.02.029
20. Giua C., Romano F., Keber E., et al. A prospective real-world study of *Bacillus clausii* evaluating use, treatment habits, and patient satisfaction in Italian community pharmacies: The PEGASO Study. *Drugs - Real World Outcomes.* 2023. https://doi.org/10.1007/s40801-023-00402-1

21. Amoozegar M., Malekzadeh F. and Malik K. A. Production of amylase by a newly isolated moderate halophile, *Halobacillus* sp. strain MA-2. *J. Microbiol. Methods.* 2003; 52:353–359. https://doi.org/10.1016/S0167-7012(02)00191-4
22. Niranjana J. and Bavithra P. S. I. A comparative study on screening methods for the detection of protease activity-containing bacteria. *Int. J. Sci. Res.* 2020; 9:169–171. https://doi.org/10.21275/ART20203705
23. Latorre J. D., Hernandez-Velasco X., Wolfenden R. E., Vicente J. L., Wolfenden A. D., Menconi A., Bielke L. R., Hargis B. M. and Tellez G. Evaluation and selection of *Bacillus* species based on enzyme production, antimicrobial activity, and biofilm synthesis as direct-fed microbial candidates for poultry. *Front. Vet. Sci.* 2016; 3:95. https://doi.org/10.3389/fvets.2016.00095
24. Spears J. L., Kramer R., Nikiforov A. I., Rihner M. O. and Lambert E. A. Safety assessment of *Bacillus subtilis* MB40 for use in foods and dietary supplements. *Nutrients.* 2021; 13:733. https://doi.org/10.3390/nu13030733
25. Thi Lan Anh H., Thi Thanh Hue L., Hai Linh B. N., Tuan Dung N. H., Duong Minh D., Thi Le Quyen T. and Trung T. T. *In vitro* safety evaluation of *Bacillus subtilis* species complex isolated from Vietnam and their additional beneficial properties. *Vietnam Journal of Biotechnology.* 2022; 20(4):727–740. https://doi.org/10.15625/1811-4989/16917
26. Mohkam M., et al. Multifaceted toxin profile of *Bacillus* probiotic in newly isolated *Bacillus* spp. from soil rhizosphere. *Biologia.* 2020; 75:309–315. https://doi.org/10.2478/s11756-019-00357-1
27. Hoyles L., Honda H., Logan N. A., Halket G., La Ragione R. M., and McCartney A. L. Recognition of greater diversity of *Bacillus* species and related bacteria in human faeces. *Res. Microbiol.* 2012; 163:3–13. https://doi.org/10.1016/j.resmic.2011.10.004
28. Lopetuso L. R., Scaldaferri F., Franceschi F., and Gasbarrini A. *Bacillus clausii* and gut homeostasis: State of the art and future perspectives. *Expert Rev. Gastroenterol. Hepatol.* 2016; 10:943–948. https://doi.org/10.1080/17474124.2016.1200465
29. Bajagai Y. S., Klieve A. V., Dart P. J. and Bryden W. L. (Eds). *Probiotics in Animal Nutrition – Production, Impact and Regulation.* Rome: FAO Animal Production and Health; 2016. 179.
30. Fakhry S., Sorrentini I., Ricca E., De Felice M. and Baccigalupi L. Characterization of spore-forming bacilli isolated from the human gastrointestinal tract. *Journal of Applied Microbiology*, 2008; 105(6):2178–2186. https://doi.org/10.1111/j.1365-2672.2008.03934.x
31. Cenci G., Trotta F., and Caldini G. Tolerance to challenges mimicking gastrointestinal transit by spores and vegetative cells of Bacillus clausii. *Journal of Applied Microbiology*, 2006; 101(6):1208–1215. https://doi.org/10.1111/j.1365-2672.2006.03042.x
32. Manzulli V., Rondinone V., Buchicchio A., Serrecchia L., Cipolletta D., Fasanella A., Parisi A., Difato L., Iatarola M., et al. Discrimination of Bacillus cereus group members by MALDI-TOF mass spectrometry. *Microorganisms.* 2021; 9(6):1202. https://doi.org/10.3390/microorganisms9061202
33. Piggot P. J. Bacillus subtilis, Editor(s): Moselio Schaechter, Encyclopedia of Microbiology (Third Edition), Academic Press; 2009:45–56. https://doi.org/10.1006/rwgn.2001.0099
34. Kuebutornye F. K., et al. *In vitro* assessment of the probiotic characteristics of three *Bacillus* species from the gut of Nile tilapia, *Oreochromis niloticus*. *Probiot. Antimicro. Prot.* 2017; 12:412–424. https://doi.org/10.1007/s12602-019-09562-5
35. Khan F. F., Sohail A., Ghazanfar S., Ahmad A., Riaz A., Abbasi K. S., Ibrahim M. S., Uzair M., and Arshad M. Recent Innovations in Non-dairy Prebiotics and Probiotics: Physiological Potential, Applications, and Characterization. *Probiotics Antimicrob Proteins*. 2023. https://doi.org/10.1007/s12602-022-09983-9
36. Manhar A. K., et al. Cellulolytic potential of probiotic *Bacillus subtilis* AMS6 isolated from traditional fermented soybean (Churpi): An in vitro study with regards to application as an animal feed additive. *Microbiol. Res.* 2016; 186–187:62–70. https://doi.org/10.1016/j.micres.2016.03.004

37. Tirabunyanon M., and Tongwittaya N. Protection activity of a novel probiotic strain of Bacillus subtilis against Salmonella Enteritidis infection. *Res. Vet. Sci.* 2012; 93(1):74–81. https://doi.org/10.1016/j.rvsc.2011.08.008
38. Guo X., Chen D-D., Peng K-S., Cui Z-W., Zhang X-J., Li S., et al. Identification and characterization of *Bacillus subtilis* from grass carp (Ctenopharyngodon idellus) for use as probiotic additives in aquatic feed. *Fish Shellfish Immunol.* 2016; 52:74–84. https://doi.org/10.1016/j.fsi.2016.03.017
39. Cutting S. M. *Bacillus* probiotics. *Food Microbiol.* 2011; 28:214–220. https://doi.org/10.1016/j.fm.2010.03.007
40. Yasmin I., Saeed M., Khan W. A., Khaliq A., Chughtai M. F. J., Iqbal R., et al. *In vitro* probiotic potential and safety evaluation (hemolytic, cytotoxic activity) of *Bifidobacterium* strains isolated from raw camel milk. *Microorganisms.* 2020; 8:354. https://doi.org/10.3390/microorganisms8030354
41. Daneshazari R., Rabbani Khorasgani M., Hosseini-Abari A. and Kim J. H. *Bacillus subtilis* isolates from camel milk as probiotic candidates. *Sci. Rep.* 2023; 13(1):3387. https://doi.org/10.1038/s41598-023-30507-0
42. Fijan S. Microorganisms with claimed probiotic properties: An overview of recent literature. *Int. J. Environ. Res. Public Health.* 2014; 11:4745–4767. https://doi.org/10.3390/ijerph110504745
43. Ahire J. J., Kashikar M. S., and Madempudi R. S. Comparative accounts of probiotic properties of spore and vegetative cells of Bacillus clausii UBBC07 and in silico analysis of probiotic function. 3 Biotech. 2021; 11(3):116. https://doi.org/10.1007/s13205-021-02668-0
44. Shakira G., et al. (2018) Effect of indigenously isolated Saccharomyces cerevisiae probiotics on milk production, nutrient digestibility, blood chemistry and fecal microbiota in lactating dairy cows. *J. Anim. Plant Sci.* 2018; 28(2):407–420. https://api.semanticscholar.org/CorpusID:184482037. Accessed 22 Dec 2023.
45. Naeem M., et al. Screening of cattle gut associated Bacillus strains for their potential use as animal probiotic. *Indian J. Anim. Res.* 2018. https://doi.org/10.18805/ijar.B-948
46. Ripert G., Racedo S. M., Elie A. M., Jacquot C., Bressollier P. and Urdaci M. C. Secreted Compounds of the Probiotic Bacillus clausii Strain O/C Inhibit the Cytotoxic Effects Induced by Clostridium difficile and Bacillus cereus Toxins. *Antimicrob Agents Chemother*. 2016. https://doi.org/10.1128/AAC.02815-15
47. Nair A. S., and Dubhashi A. V. In-vitro transit tolerance of probiotic *Bacillus* species in human gastrointestinal tract. *Int. J. Sci. Res.* 2015; 5(6):1899–1902. https://doi.org/10.21275/v5i6.nov164343
48. Talebi S., Makhdoumi A., Bahreini M., Matin M. M., and Moradi H. S. Three novel Bacillus strains from a traditional lactofermented pickle as potential probiotics. *J. Appl. Microbiol.* 2018; 125(3):888–896. https://doi.org/10.1111/jam.13901
49. Araya M., Morelli L., Reid G., Sanders M. E. and Stanton C. *Joint FAO/WHO Working Group Report on Guidelines for the Evaluation of Probiotics in Food London*, Ontario. 2002. http://ftp.fao.org/es/esn/food/wgreport2.pdf.
50. Barbosa T. M., Serra C. R., La Ragione R. M., Woodward M. J., and Henriques A. O. Screening for Bacillus isolates in the broiler gastrointestinal tract. *Appl. Environ. Microbiol.* 2005; 71(2):968–978. https://doi.org/10.1128/AEM.71.2.968-978.2005
51. Lippolis R., Siciliano R., Mazzeo M., Abbrescia A., Gnoni A., Sardanelli A., and Papa S. Comparative secretome analysis of four isogenic Bacillus clausii probiotic strains. *Proteome Science.* 2013; 11(1):28. https://doi.org/10.1186/1477-5956-11-28

عزل وتوصيف وتقييم خصائص البروبيوتيك لبكتيريا *Bacillus clausii* المعزولة من براز الأطفال في إحدى المقاطعات الشمالية في فيتنام

نجوين كوينه آنه نجو[1*] ، شوان ثانه دام[2]، تيب خاك نجوين[2]، شين نجوك نجوين[3*] ، نهي دينه بوي[4*]

[1]مركز البحوث والأعمال الصيدلانية، كلية هاي دونج المركزية للصيدلة، 324 شارع نجوين لونج بانج، مدينة هاي دونج، فيتنام.

[2]كلية التكنولوجيا الحيوية، جامعة هانوي للصيدلة، 15-13 شارع لي ثانه تونج، مدينة هانوي، فيتنام.

[3]المعهد الوطني للتكنولوجيا الصيدلانية، جامعة هانوي للصيدلة، 15-13 شارع لي ثانه تونج، مدينة هانوي، فيتنام.

[4]جامعة فييت تري للصناعة، مدينة فييت تري، فيتنام

ملخص

Bacillus clausii هو بروبيوتيك بشري يستخدم على نطاق واسع في العديد من المنتجات التجارية؛ ومع ذلك، كان هناك بحث محدود حول عزل *Bacillus clausii* من مصادر متنوعة وتقييم خصائص البروبيوتيك .لأول مرة في هذه الدراسة، تم عزل سلالات *Bacillus clausii* وتقييمها من عينات البراز التي تم الحصول عليها من أطفال متطوعين أصحاء في مقاطعة شمال فيتنام .تم فحص الخصائص البيولوجية المتأصلة لسلالات *Bacillus clausii* المعزولة على وجه التحديد لاستكشاف تطبيقها المحتمل كبروبيوتيك .خضعت ثلاث عشرة مستعمرة للفحص من خلال التحليلات المورفولوجية والكيميائية الحيوية، جنبًا إلى جنب مع بروتين Maldi Tof MS. من بين هذه العزلات، تم تحديد *Bacillus* M23 و .M31 في فحص السلامة الأولي، أظهرت كلتا السلالات نشاطًا انحلاليًا سلبيًا .بالإضافة إلى ذلك، تم تحديد خصائص المختبر، مثل تكوين الجراثيم، ومقاومة الأحماض وأملاح الصفراء، ومقاومة الكائنات الحية الدقيقة المسببة للأمراض، وتقييم إنتاج الإنزيمات خارج الخلية، واختبار حساسية المضادات الحيوية لهذه السلالات، والتي تقع ضمن النطاق المرصود لسلالات البروبيوتيك الأخرى .كشف تسلسل جين 16S rRNA أن *Bacillus* M31 يشترك في تشابه بنسبة97 ٪ مع *Bacillus clausii* DSM 8716في قاعدة بيانات Genbank. تشير هذه النتائج إلى أن *Bacillus clausii* M31 يظهر وعدًا كمرشح بروبيوتيك، على الرغم من ضرورة إجراء المزيد من الدراسات المكثفة في المختبر / الجسم الحي للتحقق من فعاليته وسلامته.

الكلمات الدالة: العزل، التوصيف، *Bacillus clausii*، البروبيوتيك، براز الأطفال.

* ***المؤلف المراسل:***

نجوين كوينه آنه نجو qanhk63hup@gmail.com

شين نجوك نجوين: nhibd@vui.edu.vn

نهي دينه بوي: chiennn@hup.edu.vn

تاريخ استلام البحث 2024/02/28 وتاريخ قبوله للنشر 2024/05/18.