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# Multifunctional probiotic and safety attributes *Heyndrickxia coagulans* isolated from stingless bee honey

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## Abstract

**Background** *Heyndrickxia coagulans*, recognized for its probiotic attributes and resilience as an endospore-forming bacterium, is increasingly studied for health supplement applications. This study aimed to evaluate the probiotic potential and safety of novel *H. coagulans* isolated from stingless bee honey, a new source for this bacterium, and to characterize their multifunctional probiotic properties.

**Results** We isolated two novel *H. coagulans*, TBRC-18260 and TBRC-18261, and conducted comprehensive in vitro analyses to assess their probiotic traits such as acid and bile salt tolerance, self-aggregation, and pathogen inhibition. Both isolates were also evaluated for safety through antibiotic susceptibility testing and hemolytic activity. Functional properties, including GABA production, antioxidant activity, were examined to establish their potential as probiotics. TBRC-18260 and TBRC-18261 exhibited core probiotic characteristics and showed excellent survivability under acidic conditions and in the presence of bile salts. They displayed strong antimicrobial activity against various pathogens and demonstrated significant GABA production and antioxidant capabilities. The safety assessments confirmed their non-hemolytic nature and susceptibility to a wide range of antibiotics.

**Conclusion** The novel *H. coagulans* isolates, TBRC-18260 and TBRC-18261, with their robust probiotic properties, antioxidant activities, and safety profiles, emerged as promising candidates for the development of functional foods and dietary supplements. This study enhances the biodiversity of available probiotics and supports the continuous search for novel strains with unique health-promoting characteristics.

## Highlights

- Novel *Heyndrickxia coagulans* isolates from stingless bee honey.
- Multifunctional probiotic attributes, including antioxidation and GABA production.
- Robust in various conditions: acid, bile salt tolerance, and high-temperature tolerance.
- Non-hemolytic and antibiotic-sensitive, confirming safety for supplementation.

**Keywords** *Heyndrickxia coagulans*, Multifunctional probiotic properties, Stingless bee honey, Thermotolerance, GABA production

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## Background

Due to the increasing demand for probiotic supplements in humans and livestock, the study of selected probiotic bacteria with appropriate properties from various sources has also received more attention. According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), which has revised the FAO/WHO definition, probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al. 2014). Numerous bacterial genera are recognized as probiotics, including *Lactobacillus*, a bacteria known for producing lactic acid. Another notable group is *Bacillus*, which is capable of forming endospores, making it more resilient to environmental pressures. *Bacillus* also produces a range of digestive enzymes that contribute to health benefits.

*Heyndrickxia coagulans* is a distinctive and promising probiotic, as it shares traits with both the *Lactobacillus* and *Bacillus* groups and was formerly classified under the Lactobacillaceae family. Its probiotic potential arises from its ability to produce lactic acid and its *Bacillus*-like capacity to form endospores (Narsing Rao et al. 2023). The formation of endospores allows the bacterium to survive harsh conditions in the gastrointestinal tract, while the lactic acid it produces inhibits pathogenic bacteria. Moreover, the U.S. Food and Drug Administration (FDA) recognizes *H. coagulans* as generally recognized as safe (GRAS) for consumption in both humans and animals (Cao et al. 2020; Liang et al. 2024).

The beneficial attributes of *H. coagulans* extend far beyond its lactic acid and endospore production. Numerous studies have elucidated its multifaceted probiotic properties. For instance, research based on a clinical trial, has shown that the spore-forming bacterium.

*H. coagulan*s plays a role in enhancing gut motility, providing evidence for its use as a supplement for adults with mild, occasional constipation, especially those with low fruit and vegetable intake (Kang et al. 2021). Clinical trials in non-alcoholic fatty liver disease (NAFLD) patients showed *H. coagulans* supplementation significantly reduced liver enzyme levels and improved metabolic parameters, including fasting blood sugar and insulin level, while also improving abdominal pain and bowel symptoms in irritable bowel syndrome patients (Abhari et al. 2020; Madempudi et al. 2019). These findings collectively highlight the diverse probiotic benefits of *H. coagulans*.

*H. coagulans* has been identified in various environments, ranging from fermented rice and soil (Sreenadh et al. 2022; Xu et al. 2023), to an assortment of fruits and vegetables like potatoes, pickles, corn, and tomatoes (Konuray Altun and Erginkaya 2021). The diversity of probiotic traits exhibited by *H. coagulans* across these sources suggests a significant influence of environmental conditions on its properties.

This research aims to uncover and evaluate the probiotic qualities of *H. coagulans* isolated from stingless bee honey, an unexplored source for *H. coagulans* presence. Stingless bees, known for their pollination activities, collect nectar from a wide range of flowers, each with its unique microbial community (Bueno et al. 2023; Damto et al. 2023; Nagamitsu and Inoue 2005). Stingless bees store honey in pots made of cerumen, a mixture of wax, and plant resins (Popova et al. 2021). This material may harbor its microbial community, which can interact with the honey's microbes that probably also influences microbial diversity. Additionally, the microbiome of stingless bees, comprising bacteria, fungi, and yeasts, plays a crucial role in honey production and preservation. These microbes can originate from the stingless bees' gut, body surfaces, and even the nest environment (Liu et al. 2023). During nectar collection and honey processing, these microbes can be incorporated into the honey, further enhancing its microbial diversity.

The investigation into stingless bee honey as a novel source of *H. coagulans* is motivated by the hypothesis that the diverse microbial communities found in stingless bee honey are influenced by a combination of factors, could impart distinct probiotic characteristics to the bacteria. While it is true that high-quality honey typically has a low number of microorganisms, stingless bee honey has been shown to contain a diverse array of beneficial microbes, including lactic acid bacteria (Kędzierska-Matysek et al. 2023; Xiong et al. 2023; Zamri et al. 2023). These beneficial microbes in stingless bee honey might enhance the probiotic properties and safety for human and livestock consumption.

## Methods

### Bacterial isolation

The two isolates of *H. coagulans* were isolated from 20 stingless bee honey samples of *Heterotrigona itama* sourced from the meliponists community enterprise in Pan Tae Subdistrict, Phatthalung Province, in March 2023. To begin the isolation process, 1 mL of fresh honey was mixed with 9 mL of sterile normal saline, and 0.1 mL was then plated onto Glucose Yeast Extract-*Bacillus coagulans* (BC) agar (GYE-BC) (Cat. No. M2102: HiMedia, India) and incubated at 37 °C for 24 h. Colonies that appeared on the GYE-BC agar were randomly selected and purified through re-streaking on GYE-BC agar. Subsequently, preliminary characterization of the isolates involved assessing cell morphology, Gram staining, and catalase enzyme activity after overnight incubation, prior to the onset of sporulation. These traits were observed when cultured on modified sporulation agar (prepared

from M1018 sporulation broth: HiMedia, India), supplemented with 1.5% agar (GRM026: HiMedia, India).

### Bacterial identification

The identification process utilized two principal methods. Initially, bacteria were identified based on being gram-positive, catalase-positive, and exhibiting endospore formation at the distal end of the cell. Bacteria meeting these criteria underwent further verification. Confirmatory tests included 16S rRNA gene sequencing, MALDI-TOF analysis. These methods were selected due to their higher accuracy and reliability compared to traditional biochemical tests.

### 16S rRNA gene amplification

Genomic DNA from pure *H. coagulans* samples was isolated following the guidelines provided with the GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Malaysia). The initial concentration and purity of the extracted DNA were assessed using a spectrophotometer (NanoDrop Lite, Thermo Scientific, USA).For PCR amplification, the universal primers 27 F (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTT GTTACGACTT-3′) (Lan et al. 2004) was used. Amplification was conducted in a 50 µL using the MultiGene™ Mini Personal Cycler (Labnet, USA). The PCR regimen was: 3 min at 95 °C, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, concluding with an extension at 72 °C for 5 min. The PCR products, approximately 1,525 bp, underwent agarose gel electrophoresis analysis and were purified with the GF-1 AmbiClean Kit (Gel and PCR from Vivantis Technologies, Malaysia). The purified PCR products were analyzed through DNA sequencing at 1st BASE DNA Sequencing Services, Singapore. The resulting sequences were then aligned using MEGA-X software. These sequences were cross-referenced via BLAST against sequences in the GenBank database to identify and confirm the specific genetic regions amplified in the PCR process. The purpose of aligning these sequences was to verify their accuracy, ensure they correspond to the target genes, and to compare them with known sequences in the database for further phylogenetic analysis.

The nucleotide sequences for these strains designated as TBRC-18260 and TBRC-18261, have been deposited in the GenBank database under accession numbers OR636959 and OR624758, respectively. Additionally, both strains have been deposited in the Thailand Bioresource Research Center (TBRC) culture collection under the accession numbers TBRC 18260 and TBRC 18261, respectively.

### Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF)

A fresh and pure bacterial colony (18–24 h) grown on GYE-BC agar was sent to the Office of Scientific Instrument and Testing, Prince of Songkla University, Thailand, for analysis via Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) using the MALDI Biotyper system (Bruker, Daltonik GmbH, Germany). Sample preparation and processing followed the Standard Operating Procedures outlined in the MALDI Biotyper manual (Bruker Daltonics 2012), including on-plate formic acid extraction to enhance protein ionization. Each sample was then subjected to automated data acquisition, with spectra analyzed against the Bruker Biotyper library for species identification. Quality control parameters were maintained according to the manual's recommendations.

### Carbohydrate utilization and biochemical profiling

The API 50 CHB and API 20 NE strips (bioMérieux, France) were used to assess the carbohydrate utilization patterns and biochemical profiles of the isolated *H. coagulans*. Pure bacterial colonies were suspended in sterile normal saline and adjusted to a turbidity of 2 McFarland, corresponding to a bacterial suspension with an approximate density of $6 \times 10^8$ CFU/mL, using a densitometer (DEN-1B McFarland: BIOSAN). The standardized suspensions were used to inoculate the API strips containing dehydrated substrates for various biochemical reactions. Following incubation at 37 °C for 24–48 h, the color changes in each well were interpreted by comparing them with the reference color chart provided by the manufacturer. Each color change corresponds to a positive or negative metabolic reaction, which was cross-referenced with the API system database to generate a metabolic profile of the isolates.

### Probiotics properties of *H. coagulans*

#### *Antibacterial activity*

The agar well diffusion method assessed the antibacterial activity of isolated *H. coagulans* against various pathogenic bacteria. *H. coagulans* cultures were grown on GYE broth, composed of 0.5% peptone, 0.05% yeast extract, 0.5% glucose, 0.05% di-potassium hydrogen phosphate, 0.03% magnesium sulfate, and 1 mL/L of a trace mineral solution (containing 0.01% sodium chloride, 0.018% iron (II) sulfate heptahydrate, 0.016% manganese (II) sulfate monohydrate, 0.0016% zinc sulfate heptahydrate, 0.0016% copper (II) sulfate pentahydrate, and 0.0016% cobalt (II) sulfate heptahydrate), and incubated at 37 °C for 24 h. The tested pathogens included *S.* Typhimurium (ESBL-producing: isolated strain, Accession: KT12325), from previous study (Lertworapreecha et al. 2016), *Staphylococcus aureus* (American Type

Culture Collection: ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), ESBL-producing *Klebsiella pneumoniae* (ATCC 700603), *Escherichia coli* (ATCC 25922), *Staphylococcus epidermidis* (Department of Medical Sciences, Thailand: DMST 5868), *Aeromonas veronii* (DMST 21255), *Aeromonas hydrophila* (DMST 2798), *Bacillus cereus* (DMST 6228), and *Streptococcus agalactiae* (DMST 11366). Each pathogen was cultured on trypticase soy agar (TSA) (HiMedia, India) and adjusted turbidity using densitometer equivalent to McFarland No. 0.5. Each test bacterium was spread onto Mueller-Hinton agar (MHA) (HiMedia, India), and a 6 mm Cork Borer was used to punch wells in the agar plate. After incubation at 37 °C for 24 h, *H. coagulans* cultures were centrifuged at 6,800 g for 10 min at 4 °C to pellet the bacterial cells. The supernatant was carefully decanted and filtered through a 0.22 µm pore-size filter to obtain a cell-free supernatant. This supernatant was used to assess the antimicrobial activity. The inhibition zones were measured after incubating at 37 °C for 16–18 h.

#### Acid and bile salt tolerance

Both *H. coagulans*, TBRC-18260 and TBRC-18261, were incubated at 37 °C, and the optical density at 600 nm (OD600) was measured using a spectrophotometer at the beginning (0 h) and after 3 h. This measurement was used as a proxy for bacterial growth and survival rates, as described in previous studies (Hyronimus et al. 2000).

*Lactobacillus plantarum* (B17) from previous research (Tuyarum et al. 2021), along with *Bacillus amyloliquefaciens* (TBRC-15434) and *B. subtilis* (TBRC-15435), demonstrated the ability to tolerate acidic and bile salt conditions. These strains were used as positive controls. Bacterial survival rates at both time points were assessed using the single plate-serial dilution spotting method (SP-SDS) (Thomas et al. 2015).

The resistance of the bacteria to acid and bile salt was measured using a method adapted from a previous study (Hyronimus et al. 2000). The turbidity of the *H. coagulans* suspension was adjusted using a densitometer to a McFarland standard of 0.5, corresponding to an approximate cell concentration of $10^8$ CFU/mL. To evaluate tolerance in an acidic environment, 0.5 mL of the bacterial suspension was added to 4.5 mL of GYE broth, which was adjusted to pH 3 using 1 M HCl. In contrast, bile salt resilience was tested by mixing 0.5 mL of the bacterial suspension with 4.5 mL of GYE broth containing 1% (w/v) bile salt (Cat. No. RM008: HiMedia, India).

Both bacterial mixtures were incubated at 37 °C, and the optical density at 600 nm (OD600) was measured using a spectrophotometer at the beginning (0 h) and after 3 h. This measurement was used as a proxy for bacterial growth and survival rates, as described in previous studies (Hyronimus et al. 2000). The bacterial survival rates at these two time points were determined through the single plate-serial dilution spotting technique (SP-SDS) (Thomas et al. 2015). The survival rate was calculated as follows:

$$Survival\ rate\ =\ [(N_1/N_0)]\times\ 100 \quad (1)$$

$N_0$ represents the initial bacterial concentration.
$N_1$ represents the count of the bacteria that tolerance.

#### Temperature tolerance of *H. coagulans*

Thermotolerance growth tests of *H. coagulans* were conducted at six different temperatures: 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C. An initial vegetative cell culture concentration was adjusted to achieve $10^5$ CFU/mL. From this culture, 0.1 mL aliquots were spread onto GYE-BC agar plates. These inoculated plates were then incubated at the specified temperatures for 24 h to assess the growth of *H. coagulans* at each temperature.

#### Screening of acid production

The acid formation screening method was adapted from a previous study (Ribeiro et al. 2021). *H. coagulans* were cultured in Modified GYE-PBS medium, consisting of GYE broth (as described previously) dissolved in 1X PBS buffer. The 1X PBS buffer was prepared with NaCl 137 mM, KCl 2.7 mM, $Na_2HPO_4$ 9.9 mM, $KH_2PO_4$ 1.8 mM and 0.001% (w/v) bromocresol purple as a pH indicator. The pH of the media was adjusted to 7.4 to ensure optimal bacterial growth conditions before sterilization by autoclaving at 121 °C for 15 min. The sterilized medium was then cooled to 50 °C. An exponentially growing culture of *H. coagulans* (18–24 h old) was introduced into the medium to achieve a final concentration of $10^6$ CFU/mL, followed by thorough mixing. The medium was aliquoted into 1.5 mL microcentrifuge tubes, with each tube receiving 200 µL. These tubes were stored overnight at 4 °C in a refrigerator. Acid production was monitored the next day by incubating the tubes at 37 °C for 8 h. If acid was produced, the medium would turn from purple to yellow.

#### Co-aggregation, auto-aggregation assay

Auto-aggregation and co-aggregation were derived from the protocol previous published (Dias et al. 2013). In brief, both isolates *H. coagulans* were grown in GYE broth at a temperature of 37 °C for 48 h. Post-culturing, they were rinsed twice using PBS (NaCl 137 mM, KCl 2.7 mM, $Na_2HPO_4$ 9.9 mM, $KH_2PO_4$ 1.8 mM, pH 7.4), and then their concentration was standardized to an OD600 of 0.6. A sample of 5 mL was vigorously vortexing for 2 min and then left to stand at 25 °C for 4 h. Subsequently, 100 µL of the top layer of the suspension was

gently moved to a 96-well plate already containing 100 µL of PBS. The decrease in light absorbance was measured at 600 nm. The percentage of auto-aggregation of each bacterial isolate was computed as given by:

$$1 - \left( \frac{\text{OD of the sampled layer}}{\text{OD of the complete bacterial mixture}} \right) \times 100 \quad (2)$$

OD of the sampled layer' refers to the optical density measurement of the top layer of the bacterial culture at the 4-hour mark.

OD of the complete bacterial mixture' represents the optical density measurement of the entire bacterial culture taken at the 0-hour mark.

The control for this experiment is the optical density measurement of the complete bacterial mixture at the 0-hour mark, which provides a baseline for comparison.

*Salmonella* Typhimurium, *S. aureus*, and *E. coli* were used for co-aggregation assessment. A 200 µL sample of each *H. coagulans* and pathogenic strain, standardized to an optical density at 600 nm ($OD_{600}$) of 0.6, was mixed (Konuray Altun and Erginkaya 2021). After a 4-hour standing period, the $OD_{600}$ was measured again. The co-aggregation percentage was calculated using the following formula:

$$\%\, co-aggregation = \left[ (A_{pat} + A_{pro}) - \frac{(A_{mix})}{A_{pat} + A_{pro}} \right] \times 100 \quad (3)$$

$A_{pat}$ and $A_{pro}$ represent absorbance of the controls.
$A_{mix}$ represents the absorbance of the mixed bacteria.

### Hydrophobicity assessment

The evaluation of hydrophobicity was adapted from a previous established method (Rahman et al. 2008). Briefly, *H. coagulans* was grown in GYE broth for 24 h. Following this, the bacterial pellet was collected using centrifugation (Hettich Universal 320 R: Germany; with Hettich swing-out rotor 1494) at a force of 5,031×g lasting 10 min and underwent two rounds of washing using PBS at a pH of 7.4. This bacterial pellet was then re-suspended and standardized to an $OD_{600}$ of 0.6 in PBS, referred to as $A_0$. Subsequently, a 3 mL sample of this was combined with 1 mL of xylene ($C_6 H_4 (CH_3)_2$), (SRJ; Sisco Research Laboratories Cat No. 54717: India). This mixture was vigorously mixed for 2 min using a vortex mixer and then allowed to equilibrate to room temperature (25 °C) for 15 min, ensuring the aqueous phase's separation. The drop in absorbance was then measured using spectrophotometry at 600 nm, termed $A_1$. The hydrophobicity percentage of each studied isolate was then determined using the following equation:

$$\%\, hydrophobicity = \left[ \left( \frac{A_0 - A_1}{A_0} \right) \right] \times 100 \quad (4)$$

$A_0$ represents the initial bacterial cell concentration in the aqueous phase.
$A_1$ represents the cell concentration in the aqueous phase after adsorption.

### Adhesion to Caco2 cells

Adhesion of bacteria to Caco2 cells was evaluated using a procedure based on previous method (Morita et al. 2002). Briefly, Caco-2 human colorectal adenocarcinoma cells (ATCC, Cat. No. HTB-37™, Lot No. 70064029) were cultured at a density of $1.2 \times 10^5$ cells/mL in 24-well plates using DMEM supplemented with 10% fetal bovine serum. Cultures were maintained at 37 °C in a $CO_2$ incubator, with medium changes on days 4, 8, 12, 16, and 18. Due to the cellular heterogeneity of Caco-2, the monolayer reached complete polarization and homogeneous differentiation between days 18 to 21 (Lea 2015).

The cultured cells were then rinsed twice with PBS (pH 7.4) and exposed to 1 mL of the 0.5 McFarland standards of *H. coagulans* in DMEM devoid of serum. After incubation at 37 °C in a $CO_2$ atmosphere for 90 min, the medium was discarded, and the cells were rinsed thrice with PBS. Subsequently, each well received 1 mL of 0.05% Triton X-100, followed by a 10 min incubation at ambient temperature. This was then diluted by a factor of ten using sterile saline and plated on GYE agar. After a 48 h incubation at 37 °C, *H. coagulans* colonies were enumerated. The adhesion rate was determined using the equation:

$$\%\, adhesion = \frac{Bacterial\ cells\ bound\ to\ Caco2\ cells}{total\ Caco2\ cell\ count} \times 100 \quad (5)$$

### Safety assessment

#### *Hemolytic activity*

*H. coagulans* was cultured on GYE agar and incubated at 37 °C for 24 h. Post-incubation, the bacterial isolates were streaked onto blood agar plates (Thermo Scientific™ Blood Agar Base, Cat. No. CM0055), supplemented with 5% v/v defibrinated sheep blood (Thermo Scientific™ Cat. No. R54016). These plates were then incubated at 37 °C for another 24 h. Following incubation, the reactions on the blood agar were analyzed. Based on the hemolytic pattern, the reactions were categorized as Beta (β) hemolysis, Alpha (α) hemolysis, or Gamma (γ) hemolysis.

### Antimicrobial susceptibility testing by disc diffusion

To assess the antimicrobial susceptibility of *H. coagulans*, it was cultured on GYE agar. After reaching the log phase of growth, the bacterial concentration was adjusted to approximately $10^8$ CFU/mL. A sterile cotton swab was

used to uniformly inoculate the adjusted bacterial culture onto GYE agar plates. Twelve antibiotic discs—ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), vancomycin (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), cephalothin (30 µg), streptomycin (10 µg), nalidixic acid (30 µg), and cotrimoxazole (25 µg), norfloxacin (10 µg), and tetracycline (30 µg) (Oxoid, UK)—were subsequently placed on the agar surface. The plates were incubated at 35 °C for 24 hours. After incubation, the diameter of the clear inhibition zones around each antibiotic disc was measured using a caliper by measuring the distance across the clear zone from one edge to the other. The results were then interpreted based on the size of the inhibition zones in accordance with the guidelines from the Clinical and Laboratory Standards Institute (CLSI) (Weinstein and Lewis 2020). *H. coagulans* ' susceptibility to each antibiotic was categorized as either resistant (R), moderately susceptible (MS), or susceptible (S).

#### *Minimal inhibitory concentration (MIC)*

The minimal inhibitory concentration (MIC) of *H. coagulans* was ascertained using the broth microdilution technique, adhering to the CLSI 2020 standards (Weinstein and Lewis 2020). Twelve antimicrobial agents, including ampicillin, chloramphenicol, erythromycin, vancomycin, ciprofloxacin, gentamycin, cephalothin, streptomycin, nalidixic acid, cotrimoxazole, norfloxacin and tetracycline, were subjected to serial two-fold dilutions. Concentrations ranged from 0.25 to 512 µg/mL, with an exception for cotrimoxazole, where the concentrations ranged from 3040 µg/mL for sulfamethoxazole and 160 µg/mL for trimethoprim to 0.742 µg/mL for sulfamethoxazole and 0.078 µg/mL for trimethoprim. These dilutions were prepared in Mueller Hinton broth (HiMedia, India). Within the microplates, each well contained a bacterial concentration of approximately $5 \times 10^5$ CFU/mL in a 200 µL volume. Following inoculation, plates were incubated at 35 °C for a period of 16 to 20 h. Subsequent analysis of susceptibility patterns was conducted with the WHONET 2023 software (Agarwal et al. 2009).

### Antioxidant activity

#### *Antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay*

*H. coagulans* was cultivated in GYE broth and incubated at 37 °C for 24 h. Following incubation, the culture was centrifuged at 6,800 g for 5 min. The supernatant was then subjected to antioxidant evaluation using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, following the method previously described (Xing et al. 2015). A standard ascorbic acid solution was prepared by dissolving 0.01 g of ascorbic acid in 50 ml of distilled water to yield a concentration of 200 mg/L. This solution was further diluted to concentrations ranging from 10 to 100 mg/L using distilled water. Separately, a DPPH solution was prepared at a concentration of 0.6 mmol by dissolving 0.0236 g of DPPH in 100 ml of methanol. For the assay, 100 µL of the sample was added to a 96-well plate, followed by 100 µL of the 0.6 mmol DPPH solution. The mixture was shaken for uniformity and then incubated in the dark for 30 min. Absorbance was measured using a microplate reader at wavelengths between 515 and 517 nanometers. The presence of antioxidants was indicated by a color shift from purple to yellow. Antioxidant activity was quantified as % DPPH free-radical inhibition using the following equation:

$$\%\,\text{DPPH free-radical inhibition} = [(A_{Sample} - A_{DPPH})/A_{Sample}] \times 100 \quad (7)$$

$A_{sample}$ represents the absorbance of the sample.
$A_{DPPH}$ denotes the absorbance of the DPPH solution without the inclusion of the sample.

#### Antioxidant activity by 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay

In the study, *H. coagulans* was cultured in GYE medium and allowed to grow at 37 °C for 24-hours. The pre-grown cultures were then subjected to centrifugation at 6,800 g for 5 min. The resulting supernatant was then evaluated for antioxidant presence using the 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay (Ilyasov et al. 2020).

For the ABTS assay, an ABTS solution with a concentration of 7 mM was prepared, combined with a 2.45 mM potassium persulfate solution at a 1:1 (v/v) ratio. This mixture was set aside to stand at ambient temperature for a full day. Before its application, the ABTS mixture was diluted with water to achieve an absorbance reading within $0.7 \pm 0.005$ when measured at 734 nm. Subsequently, 50 µL of the sample was combined with 100 µL of the diluted ABTS mixture. This solution was mixed thoroughly and kept away from light for 10 min. Absorbance readings were then taken using a microplate reader at the wavelength of 734 nm. Presence of antioxidants in the sample would manifest as a change in color from green to clear. The absorbance data was calculated in term of the antioxidant percentage with the following formula as % scavenging rate:

$$Scavenging\ rate\ (\%) = [(A_{Sample} - A_{ABTS})/A_{Sample}] \times 100 \quad (8)$$

$A_{sample}$ represents the absorbance of the sample.
$A_{ABTS}$ denotes the absorbance of the ABTS solution without the inclusion of the sample.

### Detection of gamma-aminobutyric acid (GABA) production by HPLC

To analyze the gamma-aminobutyric acid (GABA) content, High Performance Liquid Chromatography (HPLC) was performed. Firstly, *H. coagulans* was cultivated in GYE broth enriched with 5% monosodium glutamate (Cat No. TC561U: Himedia, India). The culture was then incubated at 37 °C for 24 h. Following incubation, the sample was centrifuged at 6,800 g for 5 min. The cell-free supernatant was then filtered using a 13 mm Nylon membrane syringe filter with a pore size of 0.22 µm (One PURE). The filtrate was analyzed using an Agilent 1260 HPLC system fitted with a Hypersil Gold C-18 column (250 × 4.6 mm I.D., 5 µm particle size; Thermo Scientific, Meadow, UK). The mobile phase composition was as follows: Solution A: 60% (100.02 mM sodium acetate, 3.59 mM triethylamine, and 12.49 mM acetic acid in 1000 mL of water), pH adjusted to 5.8. Solution B: 28% deionized water. Solution C: 12% acetonitrile. The mobile phase was pumped at a flow rate of 0.6 ml/min and maintained at a temperature of 26 °C. Detection was carried out using a UV detector set at 254 nm (Wan-Mohtar et al. 2020). Analysis of the concentration of GABA production was done by comparing with the standard curve of 4-aminobutanoic acid substance (Sigma-Aldrich).

### Statistical analysis

All the data were presented as the mean value ± SD. All data was analyzed statistically with GraphPad Prism version 9.5.1 (GraphPad Software, San Diego, CA). Analysis of variance (one-way ANOVA) performed by Dunnett's multiple comparisons test was used to compare more than two groups, and a paired t-test was used to compare the means of two groups. The P value less than 0.05 ($p < 0.05$) was considered statistically significant.

## Results

### *Heyndrickxia* isolation and identification and biochemical profiles

In the present study, Gram-positive and catalase-positive bacteria were isolated. Of these, only two isolates exhibited sporulation in their distal regions (Fig. 1A). Consequently, these isolates underwent detailed classification using 16S rRNA sequencing and MALDI-TOF analysis. The nucleotide sequences for these isolates, designated as TBRC-18260 and TBRC-18261, have been deposited in the GenBank database with accession numbers OR636959 and OR624758, respectively. Sequence comparison against type strains in GenBank revealed that TBRC-18260 and TBRC-18261 share 98.13% and 98.98% sequence identity with H. coagulans NR_041523 (Fig. 1B). Further validation through MALDI-TOF

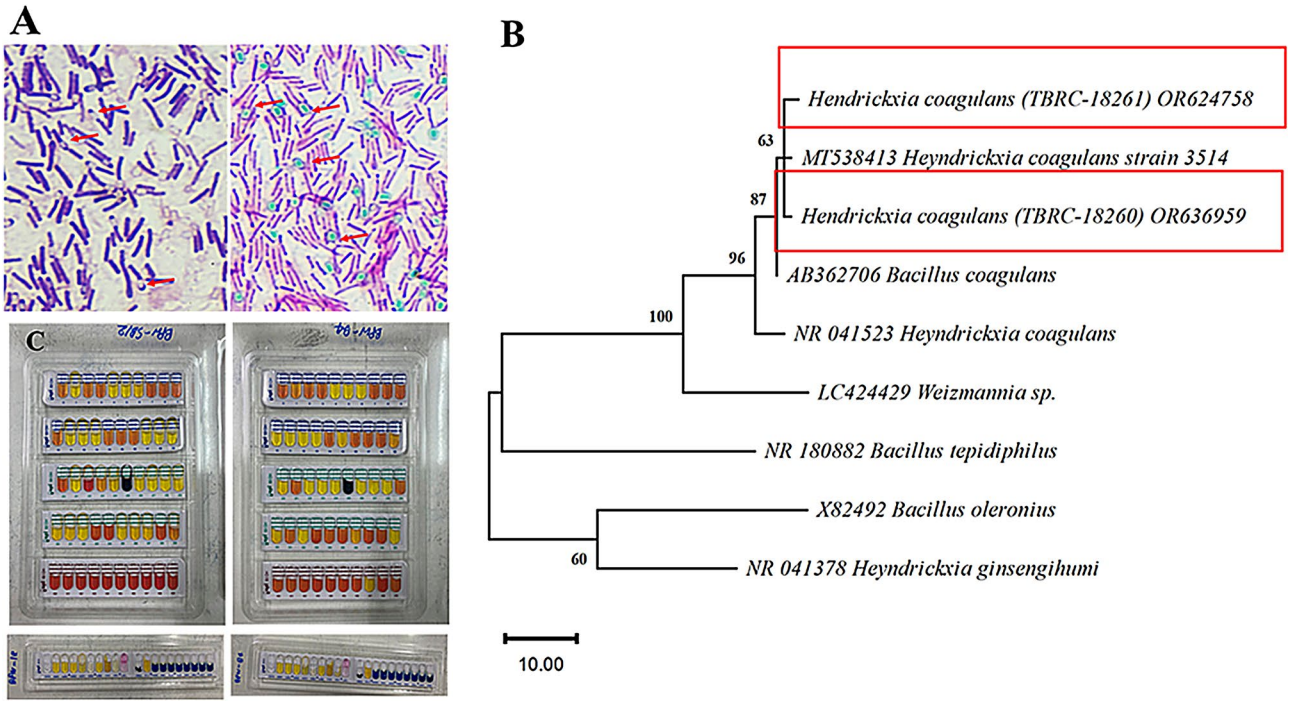


**Fig. 1** **A**) Microscopic Examination: The left panel depicts Gram-positive bacilli, and the right panel highlights endospore staining. Red arrows indicate the characteristic of spore production observed at the terminal ends of the bacterial cells. **B**) Phylogenetic Analysis: The tree, derived from 1,525 bp of the 16S rRNA gene sequence. The tree was constructed using the Maximum Likelihood method in MEGA-11 with 1,000 bootstrapping. **C**) Biochemical Characteristics: The top panel presents the distinct biochemical profiles of API-50 CHB and the bottom panel presents the biochemical profiles of API-20 NE of the two bacterial isolates

confirmed congruent genus and species classification. Biochemical profiling of both isolates using API 50 CHB and API 20 NE test strips also aligned with the characteristics of H. coagulans (Fig. 1C; Table 1). Additionally, both isolates have been successfully deposited at the Thailand Bioresource Research Center (TBRC).

In the present study, Gram-positive and catalase-positive bacteria were isolated. Of these, only two isolates exhibited sporulation in their distal regions (Fig. 1A). Consequently, these isolates underwent detailed classification using techniques such as 16S rRNA sequencing, MALDI-TOF and API-50 CHB/API-20 NE analyses. The nucleotide sequences for these isolates, designated as TBRC-18260 and TBRC-18261, have been deposited in the GenBank database with accession No. OR636959 and OR624758, respectively. Sequence comparison against type strains in GenBank revealed that TBRC-18260 and TBRC-18261 share 98.13% and 98.98% sequence identity with *H. coagulans* NR_041523 (Fig. 1B). Further validation through MALDI-TOF confirmed congruent genus and species classification, while API-50 CHB/API-20 NE analysis also shows biochemical profiles of both isolates of *H. coagulans* (Fig. 1C; Table 1). Additionally, both isolates have been successfully deposited at the *T*hailand *B*ioresource *R*esearch *C*enter (TBRC).

#### Antipathogens activity

Inhibitory effects against pathogenic bacteria are considered indicative of potential probiotic properties. In this study, the two *H. coagulans* isolates demonstrated varying degrees of inhibition against a range of pathogens. Both isolates exhibited antimicrobial activity against all tested pathogens. TBRC-18260 generally showed a stronger inhibitory effect than TBRC-18261, especially against *S.* Typhimurium (ST), *S. epidermidis* (SE), and *A. veronii* (AV), with inhibition zones measuring 15 mm, 24 mm, and 24 mm, respectively. The highest antimicrobial activity observed for TBRC-18,261 was against *A. veronii* (AV) with an inhibition zone of 25 mm. Both isolates exhibited similar levels of efficacy against *E. coli* (EC), *P. aeruginosa* (PA), and *E. faecalis* (EF), with inhibition zones ranging from 10 to 15 mm for TBRC-18261 and 12 to 15 mm for TBRC-18260 (Table 2).

#### Acid and bile salt tolerance

The study revealed that both isolates demonstrated a comparable ability to tolerate acidic conditions at pH 3.0 for a duration of 3 h. Specifically, TBRC18260 exhibited a slightly higher acid resistance with a survival rate of 70%, while isolate 18261 showed a survival rate of 66%. However, this difference was not statistically significant. In comparison to the control isolates, both isolates displayed greater acid resistance than *B. amyloliquefaciens*, although the survival rates were only slightly higher. Conversely, both isolates were less acid-resistant than *L. plantarum*. Again, no statistical significance was observed in these comparisons (Fig. 2A).

Regarding bile salt tolerance, both TBRC18260 and TBRC18261 displayed moderate tolerance to 1% bile salts after 3 h of incubation. TBRC18260 showed a survival rate of 65%, while TBRC18261 exhibited a survival rate of 60%. These rates were slightly higher than *B. amyloliquefaciens* but lower than *L. plantarum*. The differences in bile salt tolerance among the isolates were not statistically significant (Fig. 2A).

#### Acid production

Both isolates of *H. coagulans* were able to produce acid in the Modify GYE-PBS medium, as evidenced by the change in color of the bromocresol purple indicator from purple to yellow. The dark yellow color after the 12-hour incubation period suggests a strong acid production by both bacterial strains, indicating a significant drop in pH. This change happening in approximately 8 h aligns with the bacteria's characteristics of being potent acid producers (Fig. 2B).

Thermotolerance of *H. **coagulans***.

Temperature resistance assays of *H. coagulans* were conducted across a range of 40 °C to 60 °C. Both isolates demonstrated growth between 40 °C and 55 °C, with colony size and count comparable to the optimal control temperature of 37 °C, following the established approach of assessing thermotolerance through colony size and viability under elevated temperatures (Hurtado-Bautista et al. 2021). However, a noticeable reduction in both the size and number of colonies was observed upon incubation at 60 °C (Fig. 2C).

#### Co-aggregation, auto-aggregation

After 4 h of incubation, both isolates showed co-aggregation with the pathogenic strains. The co-aggregation abilities of TBRC-18260 and TBRC-18261 were at a similar level. Specifically, TBRC-18261 co-aggregated with *S.* Typhimurium, *S. aureus*, and *E. coli* at rates of 39.96%, 40.66%, and 40.41%, respectively. In comparison, TBRC-18260 co-aggregated with *S.* Typhimurium, *S. aureus*, and *E. coli* at rates of 36.91%, 37.30%, and 37.32%, respectively (Table 2).

Both *H. coagulans* isolates demonstrated the capability to auto-aggregate over time. For the BPW-4TBRC-18261 strain, auto-aggregation commenced at 4 h, showing 23.63% aggregation, which rose to 43.78% by the 8-hour mark. On the other hand, the TBRC-18260 strain began auto-aggregating at 2 h, with an initial rate of 17.16%, increasing to 50.94% after 8 h (Table 3).

**Table 1** Biochemical profile of the API-50 CH/API-20 NE kit (bioMérieux) of the two representatives *H. coagulans* isolates

| Test | Biochemical test API-50 CHB | TBRC 18261 | TBRC 18260 | Test | Biochemical test API-20 NE | TBRC 18261 | TBRC 18260 |
|---|---|---|---|---|---|---|---|
| 1 | Glycerol | + | + | 1 | ONPG: ß-galactosidase (Ortho NitroPhenyl-ßDGalactopyranosidase) | - | - |
| 2 | Erythritol | - | - | 2 | ADH: Arginine DiHydrolase | - | - |
| 3 | D-Arabinose | - | - | 3 | LDC: Lysine DeCarboxylase | - | - |
| 4 | L-Arabinose | + | + | 4 | ODC: Ornithine DeCarboxylase | - | - |
| 5 | Ribose | + | + | 5 | CIT: CITrate utilization | - | - |
| 6 | D- Xylose | + | - | 6 | H2S: H2S production | - | - |
| 7 | L-Xylose | - | - | 7 | URE: UREase | - | - |
| 8 | Adonithol | - | - | 8 | TDA: Tryptophane DeAminase | + | + |
| 9 | Methyl xyloside | - | - | 9 | IND: INDole production | - | - |
| 10 | Galactose | + | + | 10 | VP: acetoin production (Voges Proskauer) | + | + |
| 11 | D-Glucose | + | + | 11 | GE: GELatinase | - | - |
| 12 | D-Fructose | + | + | 12 | GLU: fermentation / oxidation (GLUcose) | - | - |
| 13 | D-mannose | + | - | 13 | MAN: fermentation / oxidation (MANnitol) | - | - |
| 14 | Sorbose | - | + | 14 | INO: fermentation / oxidation (INOsitol) | - | - |
| 15 | Rhamnose | + | - | 15 | SOR: fermentation / oxidation (SORbitol) | - | - |
| 16 | Dulcitol | - | - | 16 | RHA: fermentation / oxidation (RHAmnose) | - | - |
| 17 | Inositol | - | - | 17 | SAC: fermentation / oxidation (SACcharose) | - | - |
| 18 | Mannitol | - | - | 18 | MEL: fermentation / oxidation (MELibiose) | - | - |
| 19 | Sorbitol | + | + | 19 | AMY: fermentation / oxidation (AMYgdalin) | - | - |
| 20 | Methyl-D-mannoside | + | + | 20 | ARA.: fermentation / oxidation (ARAbinose) | - | - |
| 21 | Methyl-D-glucoside | - | + | 21 | $NO_2$: Reduction of nitrates to nitrites | - | - |
| 22 | N-acetyl-glucosamine | + | + | | | | |
| 23 | Amygdalin | + | + | | | | |
| 24 | Arbutin | + | + | | | | |
| 25 | Esculine | + | + | | | | |
| 26 | Salicin | + | + | | | | |
| 27 | Cellobiose | + | + | | | | |
| 28 | Maltose | + | + | | | | |
| 29 | Lactose | + | + | | | | |
| 30 | Melibiose | + | + | | | | |
| 31 | Sucrose | + | + | | | | |
| 32 | Trehalose | + | + | | | | |
| 33 | Inulin | - | - | | | | |
| 34 | Melizitose | - | - | | | | |
| 35 | D-raffinose | - | + | | | | |
| 36 | Starch | + | + | | | | |
| 37 | Glycogen | - | - | | | | |
| 38 | Xylitol | - | - | | | | |
| 39 | Gentibiose | + | + | | | | |
| 40 | Turanose | + | + | | | | |
| 41 | Lyxose | - | - | | | | |
| 42 | Tagatose | - | - | | | | |
| 43 | D-fucose | - | - | | | | |
| 44 | L-fucose | - | - | | | | |
| 45 | D-Arabitol | - | - | | | | |
| 46 | L-Arabitol | - | - | | | | |
| 47 | Gluconate | + | + | | | | |
| 48 | 2, Keto-gluconate | + | - | | | | |
| 49 | 5, keto-gluconate | + | - | | | | |

**Table 2** Antibacterial activity and adhesion properties of *H. coagulans*

| Isolates | Antimicrobial activity (Diameter of inhibition clear zone in mm.) | | | | | | | | Co-aggregation (%) | | | Auto-aggregation (%) | | Adhesion to Caco2 cells (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EC | ST | SA | PA | EF | SE | AV | AH | BC | KP | ST | SA | EC | 4 h | 8 h | |
| TBRC-18,261 | 11 | 11 | 10 | 15 | 15 | 20 | 25 | 18 | 17 | 12 | 39.96 ±0.45 | 40.66 ±0.45 | 40.41 ±0.25 | 23.63 ±0.24 | 43.78 ±0.24 | 32.15 |
| TBRC-18,260 | 11 | 15 | 14 | 15 | 15 | 24 | 24 | 17 | 15 | 10 | 36.91 ±0.47 | 37.30 ±0.32 | 37.32 ±0.23 | 17.16 ±0.69 | 50.94 ±0.33 | 7.13 |

Values are mean ± SD of three independent determinations ($n = 3$) of each sample. EC; *Escherichia coli* (ATCC 25922)
ST; *Salmonella* Typhimurium (ESBL strain; Accession: KT12325), SA; *Staphylococcus aureus* (MRSA strain, DMST 20637), PA; *Pseudomonas aeruginosa* (ATCC 27853), EF; *Enterococcus faecalis* ATCC 29,212, SE; *S. epidermidis* (DMST 5868), AV; *Aeromonas veronii* (DMST 21255), AH; *Aeromonas hydrophila* (DMST 2798), BC; *Bacillus cereus* (DMST 6228), KP; *Klebsiella pneumoniae* (ATCC 700603)

### Adhesion to caco-2 cell

Both *H. coagulans* isolates, demonstrate distinct adhesion characteristics. The data suggests that TBRC-18261 has a remarkably higher efficiency in adhering to Caco-2 cells compared to TBRC-18260. Specifically, the adhesion percentage for TBRC-18261 was calculated to be 32.15%, while for TBRC-18260, it was substantially lower at 7.13% (Tabel 2).

### Safety assessment

In the evaluation of the safety profile of *H. coagulans*, two distinct isolates were subjected to tests assessing their potential to lyse red blood cells and their susceptibility to a panel of 12 antimicrobial agents. Notably, neither of the isolates exhibited hemolytic activity (γ-hemolysis) and both demonstrated susceptibility to all the antimicrobial agents evaluated (Tables 3 and 4).

### Antioxidant activity

The antioxidant capabilities of two *H. coagulans* isolates were assessed using both DPPH and ABTS assays, comparing their performance against a 5 mg/mL ascorbic acid standard. Both isolates exhibited marked antioxidant activities that were on par with ascorbic acid. Specifically, the TBRC-18261 isolate demonstrated scavenging activity values of 78.36% and 68.79% for DPPH and ABTS respectively. Conversely, the TBRC-18260 isolate presented with 80.32% activity in the DPPH assay and 86.82% in the ABTS assay (Fig. 3).

### GABA production

GABA, a valuable compound, has been identified as a product of lactic acid bacteria. In the present investigation, the capacity of two *H. coagulans* isolates to synthesize GABA was evaluated. Results by HPLC technique indicated that both isolates were efficient GABA producers: TBRC-18261 generated 87.48 mg/mL of GABA and TBRC-18260 yielded 84.08 mg/mL of GABA (Fig. 4).

## Discussion

*Heyndrickxia coagulans*, formerly known as *Bacillus coagulans*, is a probiotic bacterium garnering significant interest for development as a dietary supplement for both humans.

and livestock (Cao et al. 2020; Zhou et al. 2020). *B. coagulans* was reclassified into the new genus *Weizmannia* due to phylogenetic distinction. *Bacillus coagulans* and related species form a distinct monophyletic clade that is separate from the Subtilis and Cereus clades, which are the core groups traditionally associated with the genus *Bacillus*. Moreover, the study identified specific conserved signature indels (CSIs) that are unique to the clade containing *B. coagulans*. These molecular signatures distinguish this group from other *Bacillus* species,

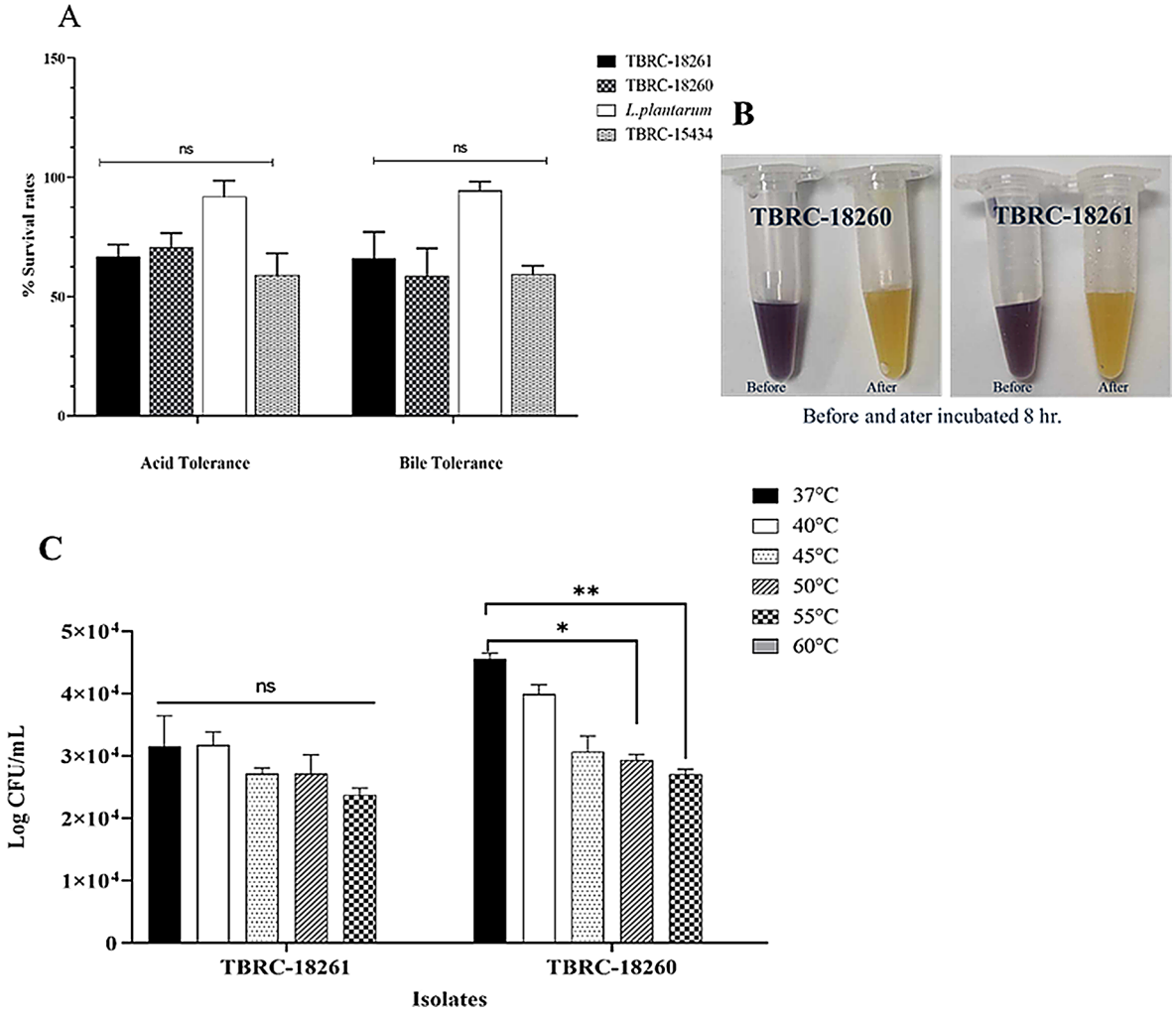


**Fig. 2** **A**) Acid and bile salt tolerance of both *H. coagulans* isolates compared to control *L. plantarum* B-17 and *B. amyloliquefaciens* (TBRC-15434). Bar graphs represent survival rates of two *H. coagulans* isolates and a control (*L. plantarum* and *B. amyloliquefaciens*) strain under acidic (pH 3.0) and 1% bile salt conditions over a 3-hours. Each bar denotes the mean ± SD from three independent experiments. **B**). Screening of acid production in Modified GYE-PBS medium. **C**). Temperature tolerance of both *H. coagulans* isolates to six different temperatures (37 °C, 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C)

supporting the need for a separate genus (Gupta et al. 2020).

However, recent advancements in phylogenomic analysis have prompted a further reclassification of *W. coagulans* into the genus *Heyndrickxia*. This reclassification is driven by more refined genomic data, which revealed that *B. coagulans* shares a closer evolutionary relationship with species in the *Heyndrickxia* genus. The refined analysis showed that the distinct clade containing *B. coagulans* aligns more closely with *Heyndrickxia* based on updated genetic markers and phylogenetic trees (Narsing Rao et al. 2023).

In this study, the isolated *H. coagulans* strains, TBRC-18260 and TBRC-18261, were identified using a comprehensive approach. Due to the high diversity and close similarity of strains within the *Bacillus* genus, classification based solely on 16S rRNA sequence analysis may not ensure precise identification, especially in genera with significant intra-species genomic variability (Celandroni et al. 2019). Therefore, multiple methods were employed, including the detection of spores at the terminal ends of the bacterial cells. Consistent results from 16S rRNA gene sequencing, MALDI-TOF analysis, and biochemical profiling with the API-50 CHB/API-20 NE systems provided accurate classification, confirming the isolates as *H. coagulans.*

Evaluating the inhibitory effect of probiotic strains on pathogenic bacteria is essential for assessing their probiotic capabilities. In our investigation, the two *H. coagulans* strains, TBRC-18260 and TBRC-18261, exhibited diverse inhibitory activities against several pathogens Notably, both isolates were equally effective in inhibiting pathogenic bacteria, particularly showing a significant ability to suppress *A. veronii* and *A. hydrophila*.

**Table 3** Antimicrobial susceptibility testing by agar disc diffusion and hemolytic activity

| Isolates | Antibiotics susceptibility by the disc diffusion agar method (zone dimeter in mm.) | | | | | | | | | | | Hemolytic activity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amp (10 µg) | Chl (30 µg) | Ery (15 µg) | Van (30 µg) | Cip (5 µg) | Gen (10 µg | Cep (30 µg) | Str (10 µg) | Na (30 µg) | Co-tri (25 µg) | Nor (10 µg) | Tet (30 µg) | |
| TBRC-18,261 | 28 (s) | 30 (s) | 30 (s) | 32 (s) | 28 (s) | 30 (s) | 35 (s) | 33 (s) | 23 (s) | 23 (s) | 29 (s) | 30 (s) | γ |
| TBRC-18,260 | 31 (s) | 31 (s) | 31 (s) | 32 (s) | 37 (s) | 25 (s) | 33 (s) | 26 (s) | 31 (s) | 33 (s) | 29 (s) | 39 (s) | γ |

Amp; ampicillin, Chl; chloramphenicol, Ery; erythromycin, Van; vancomycin, Cip; ciprofloxacin, Gen; gentamycin, Cep; cephalothin, Str; streptomycin, Na; nalidixic acid and Co-tri; cotrimoxazole, Nor; norfloxacin, Tet; tetracycline, S = susceptible

This aligns with prior studies that have demonstrated *H. coagulans* proficiency in combating pathogens such as *S.* Typhimurium, *S.* Enteritidis and *Clostridium perfringens* (Kawarizadeh et al. 2019; Xie et al. 2022). An intriguing finding from our study is the strain TBRC-18260's selective effectiveness against certain Gram-positive cocci, such as *S. aureus*, *E. faecalis*, and *S. epidermidis*, an aspect not previously reported. Furthermore, its antagonistic impacts on *A. veronii* and *A. hydrophila* underscore its promising potential for use in aquaculture.

The inhibitory effects of *H. coagulans* strains TBRC-18260 and TBRC-18261 against pathogenic bacteria are primarily attributed to organic acid production, which lowers the pH and creates an unfavorable environment for pathogen growth. This proposed mechanism aligns with findings from previous studies indicating that the acid production of *H. coagulans* contributes to its pathogen-inhibiting properties by reducing pH levels (Gao et al. 2023). Additionally, other studies have reported that *H. coagulans* may inhibit pathogenic bacteria through the secretion of various antimicrobial metabolites, including bacteriocins, hydrogen peroxide ($H_2O_2$), and antimicrobial peptides. These metabolites likely further enhance its antibacterial potential, suggesting that *H. coagulans* employs multiple strategies to suppress pathogen growth (A & Suresh, 2023; Styková et al. 2022; Su et al. 2025; Wang et al. 2023). However, it is important to note that our study did not directly confirm the presence of specific antimicrobial compounds in these strains, and further research, such as targeted metabolomic analyses, will be necessary to explore this possibility.

The ability of probiotics to withstand acidic and bile conditions is crucial for their survival in the gastrointestinal (GI) tract and subsequent colonization of the host's digestive system. Our study demonstrated that both *H. coagulans* strains, TBRC18260 and TBRC18261, exhibited significant tolerance to acidic conditions, with survival rates of 70% and 66%, respectively, at pH 3.0 after 3 h. Although TBRC18260 displayed slightly higher acid tolerance than TBRC18261, this difference was not statistically significant. Compared to control isolates, both strains demonstrated greater acid resistance than *B. amyloliquefaciens*, though they were slightly less resilient than *L. plantarum*, known for its high acid tolerance (Tuyarum et al. 2021).

For bile salt tolerance, both TBRC18260 and TBRC18261 showed moderate resilience to 1% bile salts (specifically cholic acid, RM008: Himedia, India), with survival rates of 65% and 60%, respectively, after 3 h of incubation. These rates were slightly higher than those for *B. amyloliquefaciens* and slightly lower than those for *L. plantarum*, though the differences were not statistically significant. This moderate tolerance to cholic acid aligns with previous studies indicating that *H. coagulans*

**Table 4** Antimicrobial susceptibility testing microbroth dilution technique

| Isolates | Antibiotics susceptibility by the microbroth dilution technique (MIC) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amp | Chl | Ery | Van | Cip | Gen | Cep | Str | Na | Co-tri | Nor | Tet |
| TBRC-18,261 | ≤ 0.25 | 2 | ≤ 0.25 | ≤ 0.25 | ≤ 0.25 | ≤ 0.25 | ≤ 0.25 | ≤ 0.25 | 4 | ≤ 1.48/0.156 | ≤ 0.25 | ≤ 0.25 |
| TBRC-18,260 | ≤ 0.25 | 8 | ≤ 0.25 | ≤ 0.25 | ≤ 0.25 | ≤ 0.25 | ≤ 0.25 | ≤ 0.25 | 4 | ≤ 1.48/0.156 | ≤ 0.25 | ≤ 0.25 |

Amp; ampicillin, Chl; chloramphenicol, Ery; erythromycin, Van; vancomycin, Cip; ciprofloxacin, Gen; gentamycin, Cep; cephalothin, Str; streptomycin, Na; nalidixic acid and Co-tri; cotrimoxazole, Nor; norfloxacin, Tet; tetracycline

Concentrations ranged from 0.25 to 512 µg/mL, with an exception for cotrimoxazole, which varied between 3040/160 to 0.742/0.078 µg/mL

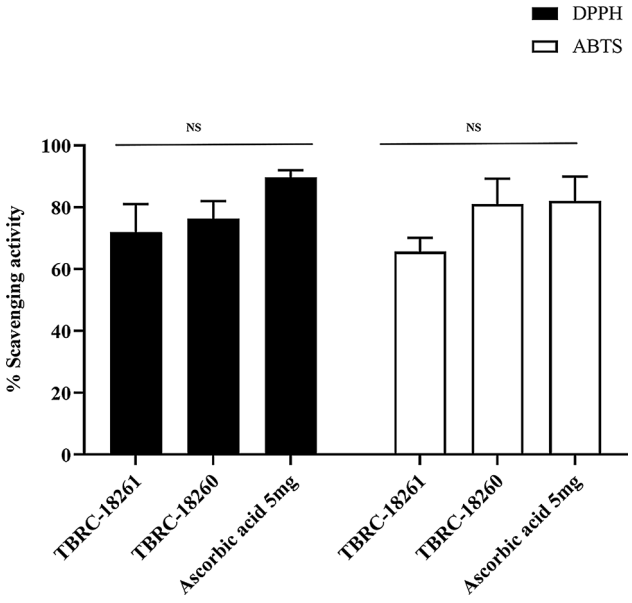


**Fig. 3** A) The graph represents the comparative antioxidant activity, measured as percentage scavenging activity, of two *H. coagulans* isolates (TBRC-18260 and TBRC-18261) using two different antioxidant assays: DPPH (solid bars) and ABTS (transparent bars). Additionally, ascorbic acid at a concentration of 5 mg is utilized as the standard antioxidant

generally exhibits bile resistance, with survival rates between 70% and 100% (Chaudhari et al. 2021). These findings underscore the importance of acid and bile salt tolerance in enabling the probiotic to endure bile exposure as it passes from the stomach to the intestine, where it can exert beneficial effect.

Strong acid and bile salt tolerance in probiotics is essential for industrial applications, as it ensures their viability during processing and storage in various food products. This resilience allows probiotics to survive in acidic or bile salt-rich environments found in fermented foods, beverages, and dairy products. Furthermore, it enhances product stability, supporting longer shelf life and maintaining probiotic efficacy throughout distribution.

Stingless bees often encounter fluctuating and harsh environmental conditions, such as varying pH levels in the nectar they collect and within their hive environments (Melia et al. 2022). The conservation of acid tolerance in bacteria associated with stingless bees, like *H. coagulans*, may reflect adaptation to these ecological pressures (Aguiar et al. 2023). The acid tolerance observed in *H. coagulans* may be crucial for the survival of stingless bees in deteriorating environments, where changes in nectar acidity or hive conditions could challenge the microbial communities, they rely on. This trait could play a significant role in maintaining the health and resilience of stingless bee populations as their natural habitats are increasingly compromised (Anderson et al. 2013; Vásquez et al. 2012).

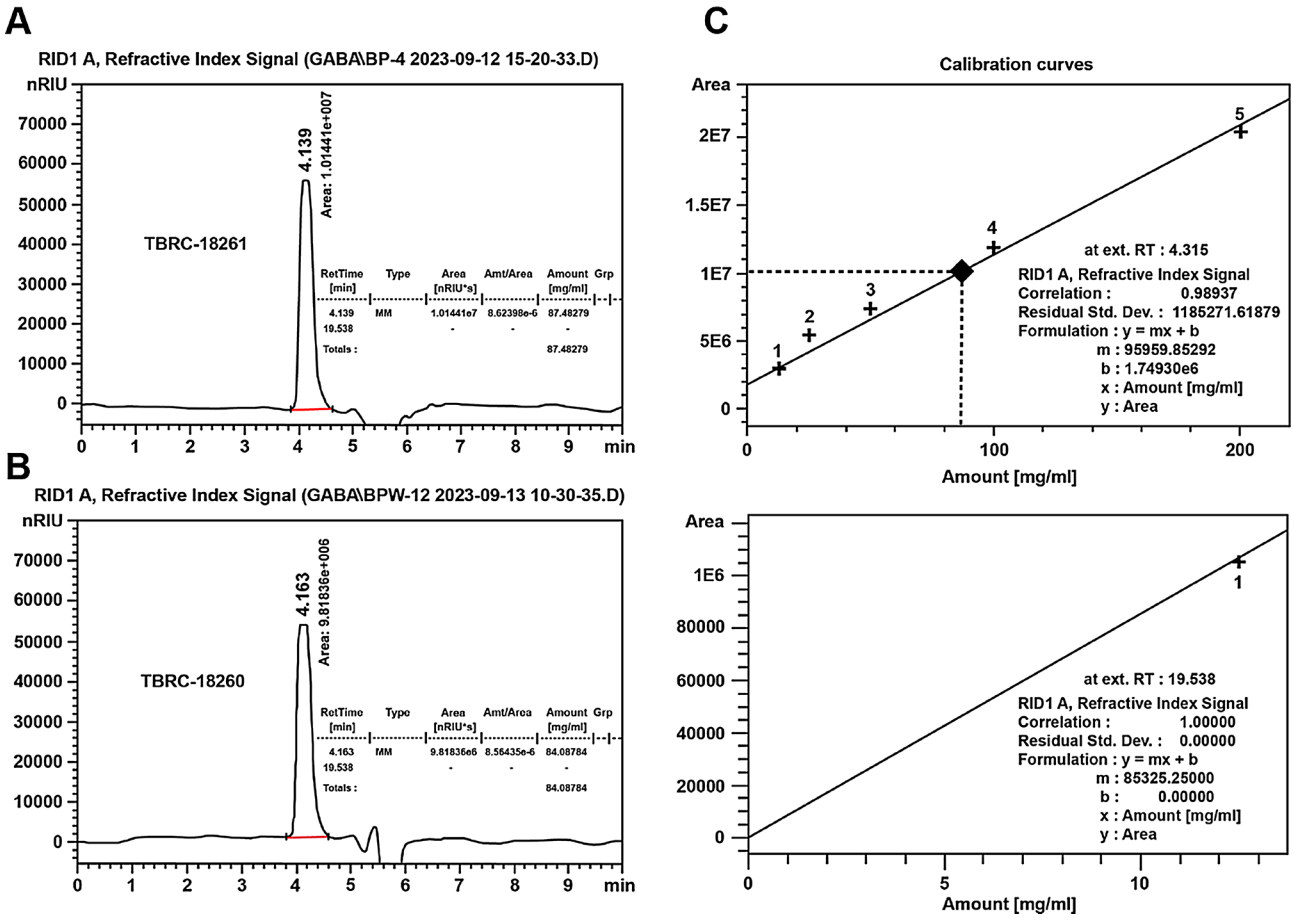


**Fig. 4** **A**) HPLC chromatogram for isolate TBRC-18261, showcasing the presence of GABA as indicated by the labeled peak. **B**) HPLC chromatogram for isolate TBRC-18260, similarly displaying the GABA peak. **C**) Calibration curves derived from pure 4-aminobutanoic acid standards (Sigma-Aldrich). The top panel is the calibration curve (with an expected retention time of 4.315 min) provides a strong linear correlation between GABA concentration and detected peak area. The bottom panel is the calibration curve, observed at an expected retention time of 19.538 min, reinforces this linear relationship

The safety evaluation of both TBRC18260 and TBRC18261 yielded encouraging results, highlighting their potential for further development and application. We found that neither isolate demonstrated resistance to any of the 12 drugs tested, with consistent findings observed in both disc diffusion and micro-broth dilution assays. Both isolates were obtained from flower honey, an environment that likely exerts lower selective pressure for antibiotic resistance compared to clinical or industrial settings. In addition, stingless bees may have limited exposure to synthetic antimicrobials in their natural environment. This could result in their gut microbiota, including *B. coagulans*, not developing resistance mechanisms against these compounds (Kwong and Moran 2016). Moreover, investigation of the microbiome of honey bees and their environment, demonstrating the intricate relationship between the bee host and its associated microbes. If stingless bees are sensitive to certain chemicals, this could disrupt their gut microbiome, potentially leading to the dominance of more susceptible bacterial strains (Raymann and Moran 2018a, b).

While the well diffusion assay offers a preliminary view of antimicrobial activity, further quantitative methods, such as minimum inhibitory concentration (MIC) tests, are needed to confirm and measure these effects and determine whether they are bactericidal or bacteriostatic. Additionally, relying solely on phenotypic assays may limit our understanding of antibiotic resistance. Although neither isolate showed resistance, they may harbor dormant antimicrobial resistance (AMR) genes that could activate under specific environmental conditions (Bengtsson-Palme et al. 2017; Poole 2012). Moreover, both isolates exhibited g-hemolysis characteristics, suggesting a lack of hemolytic activity. This indicates they may not possess certain virulence factors associated with hemolysis, it does not rule out the presence of other virulence traits, as virulence is a multi-faceted characteristic involving various factors. Non-virulence in bacteria can be linked to the absence of antimicrobial exposure, as selective pressures from antibiotics drive the development of resistance and virulence traits. Without these pressures, bacteria may prioritize growth over costly

virulence mechanisms, favoring non-virulent strains in low-stress environments. Thus, minimal exposure often results in bacteria that lack pathogenic traits (Beceiro et al. 2013; Martinez 2009). Future studies incorporating genomic analysis, such as whole-genome sequencing, would be valuable to identify any latent AMR, and virulence genes that could be activated in response to particular environmental pressures.

In addition to the general properties of probiotics We found a special feature that makes both isolates more interesting, namely their tolerance to high temperatures, antioxidant activity and GABA production. It was found that the vegetative form of both isolates can grow at temperatures of 60 °C. It was found that its ability to withstand high temperatures is superior to *B. coagulans* isolates from other reports that were found to be able to withstand heat in the range of 42–55 °C (Rhee et al. 2011; Tongpim" & Sakai, 2021). This indicates that both isolates are thermotolerant strains that will be of great benefit in the development of probiotic products that require heat treatment such as spray drying or the production of animal feed pellets.

Antioxidant activities in bacteria, particularly lactic acid bacteria, have increased attention due to their potential therapeutic applications and benefits in food preservation (Hu et al. 2023; Zapaśnik et al. 2022). In this study, the antioxidant capacities of two *H. coagulans*, TBRC-18261 and TBRC-18260, were thoroughly evaluated using two well-established radical scavenging assays: DPPH and ABTS. The antioxidant capabilities of the *H. coagulans*, TBRC-18261 and TBRC-18260 are notably significant, as they demonstrated scavenging solid activities and paralleled the antioxidant prowess of a 5 mg/mL ascorbic acid standard antioxidant reference (Sricharoen and Chanthai 2015).

Antioxidant Activiti between TBRC-18261 and TBRC-18260 was different especially in the ABTS assay. This variation may be attributed to distinct metabolic pathways or the presence of specific antioxidant molecules inherent to each isolate. However, it's essential to highlight that both isolates exhibited consistent scavenging activities above 65%, indicating their substantial antioxidant potential. When juxtaposed with prior research, the antioxidant capacities of different *H. coagulans* isolates vary significantly. A previous study in *B. coagulans* with high lactase-producing activity showed a high capacity for scavenging DPPH free radicals 35.0%, hydroxyl radicals 39.0%, and superoxide anion radicals 14.8%, and good reducing power 58.5 μmol/L ascorbic acid equivalent (Sui et al. 2020). Important antioxidant mechanisms reported in *B. coagulans* was that the *B. coagulans* boosts the concentration of proteins related to the Nrf2/Keap1 pathway (Nrf2, Keap1, heme oxygenase-1 (HO-1), along with enhancing the function of enzymes that combat oxidative stress (glutathione peroxidase (GSH-Px), catalase (CAT), superoxide dismutase (SOD). Furthermore, it reduced the levels of malondialdehyde (MDA) and diminished the expression of proinflammatory cytokines especially TNF-α (Gao et al. 2022).

While the production of GABA is well-documented in *Lactobacillus* strains, recent studies have increasingly explored its synthesis in *Bacillus* strains (Asun et al. 2022; Wang et al. 2019). However, reports specifically on GABA production by *H. coagulans* are relatively less common. The findings of this study, which document substantial GABA yields from *H. coagulans* isolates, present a significant addition to the existing body of literature. The levels of GABA produced by the TBRC-18261 and TBRC-18260 isolates notably position *H. coagulans* as a potent GABA producer among bacteria. The majority of research emphasizes the ability of lactobacilli, particularly *L. brevis*, to synthesize significant GABA quantities, with certain strains achieving concentrations of up to 176.04 g/L (Cha et al. 2023). Likewise, a few *Bacillus* strains, such as *B. subtilis*, have been documented to yield GABA levels around 12.5 g/L (Asun et al. 2022). Intriguingly, prior studies have not highlighted GABA production in *H. coagulans*. The findings from this investigation not only place *H. coagulans* on the GABA production but also suggest its superior synthesis capabilities compared to several *Lactobacillus* and *Bacillus* strains. These results underscore the potential of the examined *H. coagulans* isolates for probiotic applications.

In light of these results, it would be prudent to explore the potential of these probiotics in scenarios where controlled NO production is desirable, such as in the treatment of infections where an immune response needs to be modulated. Additionally, the differential effects observed between supernatant and pellet forms could be harnessed to tailor the therapeutic properties of these strains for specific applications.

## Conclusions

In conclusion, this research successfully isolated and characterized two novel *H. coagulans*, TBRC-18260 and TBRC-18261, from stingless bee honey, representing a significant advancement in the field of probiotics with potential applications in food science, human health, and animal husbandry. These strains demonstrate multifunctional probiotic properties, including resilience in various environmental conditions, high tolerance to acid and bile salts, and strong adhesion to epithelial cell lines. Their demonstrated antimicrobial activity against various pathogens further underscores their potential utility in food safety and human health. Of particular interest is the ability of these isolates to produce GABA, coupled with their antioxidant activities. The preliminary safety assessments, including hemolytic activity

and antimicrobial susceptibility, provide initial evidence of their potential for safe consumption; however, further studies are required to fully meet the WHO-FAO guidelines for probiotics.

The findings of this study support the use of natural sources for novel probiotics and highlight the need for further exploration in this field. Characterizing this novel H. coagulans strain opens opportunities for applications in human and animal health, providing a basis for future probiotic research and development.

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**Authors' contributions**
Benyapa Prakit & Rungravee Chaiyod: Methodology, Investigation, Formal analysis, Editing. Kittiya Khongkool: Investigation, Formal analysis, Writing - Original Draft. Wankuson Chanasit: Supervision, Writing - Review & Editing. Monthon Lertworapreecha: Funding acquisition, Conceptualization, Supervision, Visualization, Writing - Review & Editing. All authors contributed significantly to the discussion of the results, reviewed the manuscript, provided critical feedback, and approved the final version for publication.

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**Data availability**
All data generated or analyzed during this study are included in this published article.

## Declarations

**Ethics approval and consent to participate**
This work did not require ethical approval under the research governance guidelines operating during the research.

**Consent for publication**
All the authors have approved the manuscript that is enclosed.

**Competing interests**
The authors declare no competing interests.

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