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Research article

Biological properties and potential probiotic bacteria isolated from honey collected in Chiang Mai, Thailand

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Abstract

Importance of the work: Exploring the physicochemical and biological properties of probiotic bacteria provides insights into the quality of honey from Chiang Mai, one of Thailand's important honey-producing sites.

Objectives: To explore the quality and biological potential of local raw honey produced in Chiang Mai province.

Materials & Methods: The quality and potential were investigated of four non-processed stingless bee honey (SH) and four honeybee honey (HH) samples locally collected from several districts in Chiang Mai province, Thailand. Assessment consisted of high-performance liquid chromatography and dinitrosalicylic acid method for sugar quantitation, Bradford's protein assay for soluble protein content, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid antioxidant assay and disc diffusion assay for antibacterial properties. Additionally, the bacteria from natural honey were isolated, characterized and evaluated for their probiotic potential.

Results: Chiang Mai's local honey had potent bioactivities. Though the physicochemical properties of the examined honey varied among samples, the glucose-to-fructose ratio was significantly higher in HH compared to SH. Both the SH and HH samples were antioxidative and antibacterial. Aside from the properties of the honey samples themselves, the natural bacteria from honey were isolated and characterized for potential utilization. Some of the honey bacterial isolates had promising properties as probiotics.

Main finding: Chiang Mai's local, non-processed honey types varied in quality and biological activity, as well as serving as a reservoir of environmental bacteria in the *Bacillus/Priestia* genera, some of which can be further utilized in food applications.

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Introduction

Honey has long been recognized as a natural healthcare and longevity-promoting product (Kumar et al., 2010). Traditional Thai medicine and treatment regimens have regarded honey as one of the common ingredients, providing sweetness, binding and healing properties (Chotchoungchatchai et al., 2020). Honey has been documented as a part of ethnobotanical medical treatment for cognitive impairment in the elderly of northern Thailand (Offringa, 2015). Honey combinations with various herbs, including Gotu kola, garlic, noni and Thai pepper, constitute recipes for age-delaying and immune-boosting effects (Matichon Public Company Ltd., 2019).

Honey is closely related to the lives of Chiang Mai's native population, as many households cultivate *Dimocarpus longan*, longan, known in Thai as 'lam-yai'), one of the most commonly produced fruits in northern Thailand (Narjes and Lippert, 2016). Longan honey (known in Thai as 'dok lam-yai') has become an iconic product from northern Thailand, as the longan growers gain secondary income from beekeepers utilizing their orchards, while some attempt to raise bees themselves, with Chiang Mai producing almost 300,000 t of longans from over nearly 73,000 ha in 12 districts in 2022 (National News Bureau of Thailand, 2022). In addition, Chiang Mai serves as one of the largest homes of bee farms and beekeepers in Thailand, with about 150 beekeepers and nearly 25,000 hives of honeybees (Agricultural Technology Promotion Center, 2020).

The biological and therapeutic properties of honey have been demonstrated in numerous studies. For example, antibacterial activity (Packer et al., 2012; Hammond and Donkor, 2013; Matzen et al., 2018) and antioxidant properties through *in vitro* assays (Hegazi et al., 2009). Similarly, it was shown that stingless bee honey, along with propolis and bee bread, had antioxidant capacities (Ahmad et al., 2019a). Wound-healing (Saikaly and Khachemoune, 2017) and probable immunomodulatory effects (Majtan, 2014) of honey have been reviewed. The anticancer property of honey was shown using oral squamous cell carcinoma and osteosarcoma cell lines (Ghashm et al., 2010). Ahmad et al. (2019b) showed that honey from the Malaysian stingless bee (*Heterotrigona itama*) was cytotoxic against malignant glioma cells.

While honeybee honey is the most common in the market, stingless bee honey has emerged as an alternative, more nutritious and more expensive food ingredient (Ávila et al., 2018). The average price of stingless bee honey (SH) can be 5–10 times higher than that of honey bee honey (HH), with beekeepers selling SH *honey* for well over $30/kg in Thailand. Due to the attractive commercial incentives, food fraud practices have occurred, such as adulterating additives, supplemental feeding of honey bees and illegal importation (Strayer et al., 2014; Moore et al., 2012).

HH regulations are diligently practiced. For example, in addition to the physical properties (such as color, odor and moisture), National Committee on Agricultural Commodity and Food Standards (2013) requires the sucrose content to not exceed 5%, while the combined glucose and fructose content must exceed 60% of honey and microbial contamination must be considered (Codex Alimentarius Commission, 2019). In contrast, a worldwide standard characterization for stingless bee products is not yet available and any regulation is only vaguely defined in Thailand.

Consequently, the current study explored the quality and potential of local honey produced in Chiang Mai. SH samples and HH samples from several districts in Chiang Mai were examined. Specifically, these honey samples were raw and not subjected to quality control, being purchased from local markets or at farm sites (for rituals and household consumption). The amount of total reducing sugars and individual sugars, including glucose, fructose and sucrose, were quantified. The antioxidant and antibacterial properties of the samples were assayed. In addition, native bacteria from honey samples were isolated and further characterized molecularly and biochemically. In collaboration with the Agricultural Technology Promotion Center (Economic Insects), Chiang Mai, Thailand, this study aimed to document information regarding Chiang Mai's raw honey properties and their bacterial isolates. The resultant data should be useful in promoting opportunities to utilize Chiang Mai's honey as a bioactive reservoir for potential probiotics.

Materials and Methods

Honey samples

The honey samples were sourced in Chiang Mai, Thailand (Fig. 1). The samples comprised SH (SH1–SH4) and HH (HH5–HH8), as shown in Table 1. The SH samples were harvested from honey pots (separate from pollen pots), crushed with a spoon and strained on a cheesecloth on top of a strainer. On the other hand, the HH from the honeybee *Apis cerana* was harvested from the honeycomb, with honey dripping from the combs being strained to remove large particles before being transported to the Agricultural Technology Promotion Center (ATPC; Economic Insects), Chiang Mai, Thailand. The honey samples were aliquoted and subject to analyses (moisture content, water activity, pH, sugar content) and total soluble solids (TSS) measurement.

Table 1 Detail of honey samples examined in this study

Group	Sample name	Bee strains	Source (District in Chiang Mai)	Month collected (2021)	Associated plants (food sources)
Stingless bee honey	SH1	*Lepidotrigona* sp.	Samoeng	June	Wild flower(s)
	SH2	*Tetragonula* sp.	San Pa Tong	July	Wild flower(s)
	SH3	*Tetragonula* sp.	San Kamphaeng	July	'*Lam-yai*' (longan)
	SH4	*Tetragonula* sp.	San Pa Tong	July	Wild flower(s)
Honey bee honey	HH5	*Apis cerana*	Samoeng	May	Wild flower(s)
	HH6	*Apis cerana*	Chai Prakan	May	Wild flower(s)
	HH7	*Apis cerana*	San Kamphaeng	May	'*Lam-yai*' (longan)
	HH8	*Apis cerana*	Mae On	May	Wild flower(s)

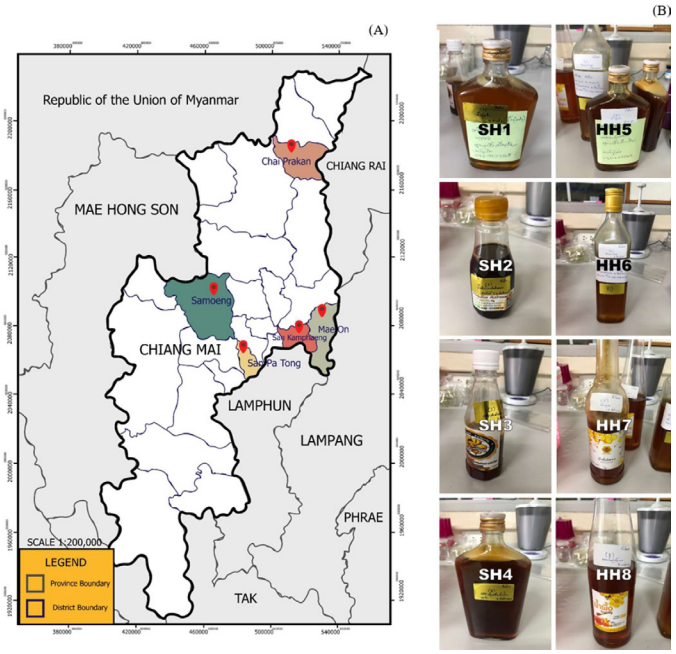

Fig. 1 Map of honey collection sites obtained from several districts: (A) in Chiang Mai province, Thailand, indicating geographical range of honey collection sites (Table 1); (B) honey samples examined, where SH = stingless bee honey; HH = honeybee honey

Chemical property analysis

Sugar contents were determined using two methods. First, the reducing sugars were determined using a colorimetric assay with 3,5-dinitrosalicylic acid (the DNS method), according to Wood et al. (2012). The DNS reagent was prepared in a 1,000 mL beaker by dissolving 10 g of 3,5-dinitrosalicyclic acid in 250 mL of H_2O. This step was followed by slow, continuous stirring with the addition of a 200 mL solution of dissolved 16 g NaOH. The mixture was incubated at 50°C with stirring to obtain a clear solution. Then, 403 g of potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6 \cdot 4H_2O$) was gradually added. The mixture was filtered before the final volume was adjusted to 1 L with water. The resultant DNS reagent was stored in the dark at below 20°C. Once the reagent was ready, 0.5 mL of each diluted honey was mixed with 0.5 mL of the DNS reagent. The resultant mixtures were heated for 5 min in boiling water. Once they had cooled, 4 mL water was added to each test tube, after which, the absorbance of each mixture was measured at 545 nm using a spectrophotometer. Glucose was used as a standard compound for standard curve-based quantitation.

Second, the glucose, fructose and sucrose contents were determined using a high-performance liquid chromatography (HPLC) system with a refractive index detector (HPLC-RID; Agilent 1260 Infinity; USA), as described by Ghramh et al. (2020).

The soluble protein content was determined using Bradford's protein assay (Bradford, 1976; Villacrés-Granda et al., 2021). In brief, 150 µL of the diluted sample was mixed with 850 µL of Bradford's reagent (Himedia®; India). The absorbance of the resultant mixture was measured at 595 nm and bovine serum albumin (BSA) in the range 5–100 µg/mL was used to generate a standard curve for quantification ($y = 9.1801x + 0.0228$, with a coefficient of determination (R^2) of 0.9949) in molecular-grade water (Merck; USA). The protein content of each honey sample was expressed as micrograms of BSA per milliliter of honey (µg BSA/mL honey).

The amount of total soluble solids (°Brix) was determined using an Abbe refractometer (NAR-1T; ATAGO; Japan) at room temperature, while the TSS content was calculated as a percentage using TS = 100 – percentage moisture content (Kamal et al., 2019).

2,2′-Azinobis-3- ethylbenzothiazoline-6-sulfonate assay for the antioxidant properties

The 2,2′-azinobis-3- ethylbenzothiazoline-6-sulfonate (ABTS) free radical-scavenging activity of each honey sample was determined using a decolorization assay, as described

by Re et al. (1999) with some modifications. In brief, 7 mM ABTS radical ($ABTS^{\bullet+}$) solution was prepared with 2.45 mM potassium persulfate (oxidizing agent) and incubated for 16 hr in the dark at room temperature. The honey samples were prepared with dilution factors of 0, 1:3, 1:5, 1:10, 1:15 and 1:20. For each solution, five replicates of the test were performed by mixing a 10 µL honey sample with 990 µL $ABTS^{\bullet+}$ solution. The mixtures were kept in the dark at room temperature for 1 hr before the absorbance was measured at 734 nm (A_{734}). Methanol (10 µL) was used as a control. The antioxidant activity was expressed as the percentage inhibition calculated using Equation 1:

$$\% \text{ Inhibition} = [(\text{Control } A_{734} - \text{Sample } A_{734})/\text{Control } A_{734}] \times 100 \quad (1)$$

where A734 is the absorbance measured at 734 nm for both the control and sample.

Anti-radical power was estimated as the half maximal inhibitory concentration (IC_{50}) extrapolated from the % inhibition of the honey samples for the different honey-to-sterile water dilution factors (1:2, 1:5, 1:8 and 1:10). Ascorbic acid (VitC) in ethanol was used as a standard antioxidant agent, with the results also reported in VitC equivalent antioxidant capacity. The IC_{50} values of antioxidant activity were extrapolated from the ABTS assay plot and were defined as the honey concentration (%) needed to decrease the initial ABTS concentration by 50% (Rivero-Cruz et al., 2020).

Antibacterial property evaluation

Initially, disc diffusion assay was attempted; however, there were viscosity and contamination problems. Consequently instead, an agar well diffusion assay was applied using a 100 µL honey sample per well. The antibacterial properties were assessed of honey samples against *Escherichia coli NCTC 9001 (ATCC® 11775™)*, *Staphylococcus epidermidis ATCC® 12228™*, and *Staphylococcus aureus NCTC 8325* on approximately 5 mm thick Mueller-Hinton (MH) agar (2 g/L beef extract, 17.5 g/L acid hydrolysate of casein, 1.5 g/L starch, 15 g/L bacteriological agar) punched with a 6 mm diameter sterile cork borer. The tested bacterial cultures were grown to the exponential phase and diluted to an optical density at 600 nm (OD_{600}) of approximately 0.45 and spread (100 µL) onto MH agar using sterile cotton swabs. After 20 hours, the clear zone diameters were measured. The experiment was carried out in biological triplicates, each with technical duplicates. Ampicillin (50 µg/mL) was used as a reference antibiotic agent.

Bacterial isolation and 16S rRNA characterization

Isolation of strains from honey

Isolation of the bacteria from honey involved spreading diluted honey samples onto de Man, Rogosa and Sharpe agar (MRS) and Luria-Bertani (LB) agar plates and incubating them aerobically or sub-aerobically (in an anaerobic jar with GasPak™; Becton, Dickinson and Company; USA) at 37°C. Colonies with different morphologies were subject to DNA extraction (QIAamp DNA kit; QIAGEN; USA) and subsequent 16S rRNA gene amplification using the universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), according to Heuer et al. (1997). In this experiment, all 21-one isolates (Table S1) with different observable colony morphologies were analyzed. Then, 15 strains amplifiable with the universal primers targeting the 16S rRNA gene were stored as -80°C stocks. Further analyses through sequencing (targeted) were performed on the strains that survived a freeze-thaw cycle (Table 4) using the published primers 27F, targeting the 16S rRNA gene, and AntiR-F (5'-ACACACGTGCTACAATG-3') and 23S-23-38-R (5'-TGCCAAGGCATCCACC-3') targeting the 16S-23S internal transcribed spacer (ITS) region (Osorio et al., 2005).

Sequencing and data analysis

Polymerase chain reaction (PCR) samples were purified (QIAquick PCR purification kit; QIAGEN; USA) and submitted for Sanger sequencing (ATGC Co., Ltd; Thailand). The obtained FASTA files were trimmed using the BioEdit software (Hall et al., 2011) and subjected to nucleotide BLAST (based on the NCBI database at https://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification. The sequence of the 23S-ITS region was used for phylogenetic tree construction using the CLUSTALW software (https://www.genome.jp/tools-bin/clustalw). Alignment and phylogenetic reconstructions were performed using the function "build" in the ETE3 3.1.2 software (Huerta-Cepas et al., 2016).

Screening for probiotic potential

Acid and bile tolerance

The acid and bile tolerance capacities of the bacterial isolates were assessed. The strains (characterized as *Bacillus*

spp.) were grown overnight in LB broth. The cultures were inoculated in fresh media and the exponential-phase cells were harvested and washed twice with PBS (pH 7.2). The washed cells were resuspended in PBS pH 3.5 and PBS pH 2.0 to assess acid tolerance. At 0 hr, 2 hr and 4 hr of 37°C incubation, the bacterial survival was estimated using a drop plate method. Aliquots of the samples treated for 2 hr were pH-adjusted to 6.8 using 1*N* NaOH. The bile salt was added to achieve the final concentration of 0.2% (weight per volume). The cell survival was estimated following 0 hr, 3 hr and 6 hr of incubation at 37°C.

The percentage survival rate was calculated using Equation 2:

$$\text{Survival rate (\%)} = N_t / N_0 \times 100 \quad (2)$$

where N_t is the total viable count at time t and N_0 is the total viable count at time 0.

Simulated gastric fluid and simulated pancreatic fluid tests

The ability of the bacterial isolates to tolerate gastric fluid and pancreatic fluid was tested using simulated gastric fluid (SGF) and simulated pancreatic fluid (SPF). The solutions were prepared as follows: SGF (0.4 g/L glucose, 3.0 g/L yeast extract, 1.0 g/L peptone, 4.0 g/L porcine mucin, , 0.5 g/L cysteine, 0.08 g/L NaCl, 0.4 g/L $NaHCO_3$, 0.04 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 0.008 g/L $CaCl_2$• $2H_2O$, 0.008 g/L $MgSO_4$• $7H_2O$, 1.0 g/L xylan, 3.0 g/L soluble starch, 2.0 USP/L pectin, 0.1% (v/v) Tween-80, autoclaved and the pH adjusted to 2.0 using HCl, 3.0 g/L pepsin) and SPF (adjusted pH to 8.0–8.3 with NaOH; 0.3% (weight per weight, w/w) porcine bile extracts, 0.1% (w/w) pancreatin), according to Paramera et al. (2011).

Autoaggregation assay

The autoaggregation property of the strains was carried out as described by Del Re et al. (2000) with some modifications. In brief, the bacterial liquid cultures were subjected to centrifugation at 5000×*g* for 15 min to harvest the cells. The cell pellets were washed twice and resuspended in PBS. The OD_{600} was initially measured and adjusted accordingly for all cultures; then, 4 mL of each culture were set for room-temperature incubation. At times t = 0 hr, 1 hr, 2 hr and 3 hr of incubation, 0.1 mL of the upper suspension was removed and mixed with 0.9 mL PBS and the OD_{600} was measured. The autoaggregation percentage was calculated using Equation 3:

$$\text{Autoaggregation percentage} = (1 - (A_t / A_0)) \times 100 \quad (3)$$

where A_0 is the absorbance at t = 0 and A_t is the absorbance at t = 1 hr, 2 hr and 3 hr.

Cell surface hydrophobicity evaluation

Microbial adhesion to solvents (MATS) was evaluated using the method of Bellon-Fontaine et al. (1996). Bacterial cultures were prepared as described for the autoaggregation assay, except they were resuspended in 100 mM KNO_3 (pH 6.2). The absorbance at 600 nm was measured as A_0. A sample (1 mL) of xylene (PanReac™; Spain) was added to 3 mL of the cell suspension, incubated for 10 min at room temperature (RT) and vortexed for 2 min to allow thorough mixing. The mixture was further incubated at RT for 20 min before removing the aqueous phase to measure absorbance at 600 nm (A_a). The MATS percentage was expressed as $1 - (A_a / A_0) \times 100$ (similar to the autoaggregation percentage calculation).

Spore formation

As the bacteria isolated from honey were mostly *Bacillus*, their ability was assessed to form heat-resistant spores. The bacterial isolates were grown overnight in LB and inoculated into sporulation medium (1.04 g/L glucose, 0.59 g/L $MgSO_4$·7H2O, 6.0 g/L KH_2PO_4, 5.0 g/L yeast extract, 3.0 g/L peptone, 0.01 g/L NaCl, trace elements) as described by Posada-Uribe et al. (2015). The cultures were incubated with agitation (120 revolutions per minute) at 37°C for 48 hr before being aliquoted into two portions in 1.5-mL microcentrifuge tubes. The aliquots were either incubated at room temperature or at 80°C for 20 min before being serially diluted and plated onto LB agar.

Hemolytic activity

The bacterial strains isolated from mixed honey were streaked onto Columbia agar containing 5% (v/v) sheep's blood (Oxoid; UK) and incubated at 37°C for approximately 20 hr.

Antibiotic susceptibility

The antibiotic susceptibility test was carried out using the disk diffusion method according to the Clinical and Laboratory Standard Institute Standards for Antimicrobial Susceptibility Testing (Humphries et al., 2021). In brief, the bacterial cultures were grown overnight on nutrient agar plates and inoculated into fresh nutrient broth. The liquid cultures in the exponential phase were diluted to an approximate cell density of 1.5×10^8 colony forming units (CFU)/mL using 0.5 McFarland standard (0.05 mL of 1.175% $BaCl_2$•$2H_2O$ mixed with 9.95 mL of 1% H_2SO_4), according to Wikler (2006). The diluents were spread onto nutrient agar plates. Antibiotic discs (Himedia®; India),

loaded with ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), rifampicin (5 µg), streptomycin (10 µg), tetracycline (30 µg) or vancomycin (30 µg), were deposited onto the agar. The cultures were incubated at 37°C for 24 hr before measuring the diameter of the inhibition zone.

Statistical analysis

The data were reported as mean ± SD values and analyzed using analysis of variance followed by Duncan's multiple range test using the IBM® SPSS® statistics v.28.0.0.0(190) software package (IBM Corp.; USA).

Results

Physicochemical properties of honey samples collected from Chiang Mai, Thailand

Some of the physical properties of the honey samples were measured and recorded at the collection sites by the ATPC (Economic Insects), Chiang Mai. For example, typically, beekeepers would aim to maintain the moisture content in the range 17–21%. However, some honey samples evaluated in the current study did not meet the standard according to (Codex Alimentarius Commission, 2019). A higher moisture content was observed in SH, in the range 19.0–25.5%, which is generally more hydrated compared to HH (18.5–21.0%, Table 2). Nonetheless, the moisture content, total solids (TS) and total soluble solids (TSS) measured in °Brix were not significantly different between the SH and HH samples. Notably, while the reducing sugar content was somewhat comparable among all honey samples, the individual sugars, especially fructose, were outstanding among the SH samples. For example, the fructose concentration in SH4 was as high as 43.99 ± 4.37%, which was at the higher end of the range in Bobiş et al. (2018). The fructose-to-glucose ratios were also significantly higher in SH compared to the HH samples (Table 2). The sucrose level was generally low in all samples except SH3 (5.24 ± 0.27 g/100 g honey), which exceeded the maximum allowable value for sucrose (5%) in finished honey based on honey legislation (Mesele, 2021). Though SH was commonly acclaimed for its potency and nutrition, the amount of soluble protein content was similar to some HH samples. Table 2 details the chemical properties of honey samples evaluated in this study.

Table 2 Physicochemical properties of honey samples in this study

Property tested	Honey sample								*p*-value[1]	*p*-value[2]
	SH1	SH2	SH3	SH4	HH5	HH6	HH7	HH8		
Moisture content (%)*	25.5±0.12^{A}	25.0±0.12^{A}	20.5±0.12^{A}	19.0±0.11^{A}	19.5±0.11^{A}	18.5±0.10^{A}	21.0± 0.12^{A}	20.5± 0.10^{A}	NC	0.176
Total solids (%)	74.5^{A}	75.0^{A}	79.5^{A}	81.0^{A}	80.5^{A}	81.5^{A}	79.0^{A}	79.5^{A}	NC	0.176
°Brix*	72.5^{A}	73.0^{A}	79.0^{A}	79.5^{A}	79.0^{A}	80.0^{A}	77.5^{A}	78.0^{A}	NC	0.229
A_w	0.704±0.030^{aA}	0.697±0.030^{baA}	0.631±0.030^{caA}	0.603±0.030^{daA}	0.615±0.030^{faA}	0.591±0.030^{baA}	0.643±0.030^{eaA}	0.635±0.030^{daA}	< 0.0001	0.217
pH	3.075±0.106^{bA}	3.100±0.14^{bA}	3.975±0.035^{aA}	3.940±0.085^{aA}	4.005±0.007^{aA}	3.955±0.064^{aA}	3.870±0.184^{aA}	3.115±0.163^{bA}	< 0.0001	0.344
Total reducing sugar (g/100 g)	68.38±5.76^{bcB}	60.71±4.28^{cB}	50.96±3.14^{dB}	63.85±2.88^{cB}	76.07±7.46^{bcA}	61.6±5.46^{cA}	88.11±5.74^{aA}	67.84 ± 0.91^{cA}	< 0.0001	0.001
Glucose (g/100 g)	36.38±1.82^{dB}	24.77±1.24^{eB}	8.34±0.42^{gB}	18.31±0.92^{fB}	66.41±3.32^{aA}	38.6±1.93^{cdA}	69.46 ± 3.47^{aA}	57.03 ± 2.85^{bA}	< 0.0001	< 0.0001
Fructose (g/100 g)	26.36±2.43^{cA}	24.68±1.99^{dA}	34.57±2.74^{bA}	43.99±4.37^{aA}	6.82±2.07^{efB}	8.34±0.93^{eB}	6.61±1.08^{fB}	3.85 ± 1.05^{gB}	< 0.0001	0.001
Fructose-to-glucose ratio	0.742±0.055^{cdA}	1.042±0.146^{cA}	3.954±0.907^{aA}	2.389±0.169^{bA}	0.120±0.018^{eB}	0.235±0.024^{deB}	0.091±0.013^{eB}	0.070±0.001^{eB}	< 0.0001	< 0.0001
Sucrose (g/100 g)	2.65±0.13^{cA}	2.98±0.15^{bA}	5.24±0.27^{aA}	ND	0.24±0.02^{eB}	0.74±0.03^{dB}	ND	ND	< 0.0001	0.0003
Soluble protein content (µg BSA/mL)	1106.306 ± 20.668^{bcA}	1860.360±224.535^{abcA}	2514.414±410.016^{aA}	2226.126±268.223^{abA}	1289.640±95.856^{bcB}	1760.360±112.112^{abcB}	1850.901±214.748^{abcB}	1252.703±106.78^{bcB}	0.076	0.041

SH = stingless bee honey; HH = honey bee honey; ND = not determined; * = values measured at collection sites by the Agricultural Technology Promotion Center (ATPC, Economic Insects), Chiang Mai, Thailand; A_w = water value; BSA = bovine serum albumin; NC = not calculated.

Duncan's Multiple Range Test (DMRT) was performed in two ways: [1] eight individual samples were compared with each other, and [2] four SH samples were compared with four HH samples. Mean ± SD values in a column superscripted with different lowercase letters are significantly ($p < 0.05$) different when comparing individual honey samples[1]. The different uppercase letter superscription denotes significant different among means of SH vs. HH samples[2].

Both stingless bee honey and honeybee honey were highly bioactive

Honey has been reported to have antioxidant properties (Mohamed et al., 2010; Al-Hatamleh et al., 2020). Such properties, in part, stem from the vitamin, phenolics and flavonoid components of honey (Mohamed et al., 2010; Chua et al., 2013). The current honey samples exhibited antioxidant and antibacterial properties. The ABTS radical scavenging assay was used to evaluate the honey's antioxidant capacity. There was a significant difference between the antioxidant capacity of the SH and HH samples ($p = 0.008$; analysis of variance with Duncan's multiple range test). Nevertheless, some significant differences were also found among individual honey samples (Table 3).

In addition, honey has been reported to have antibiotic properties (Hammond and Donkor, 2013; Israili, 2014; Matzen et al., 2018). The current study tested honey samples for their antibacterial properties against pathogens (*Escherichia coli*, *Staphylococcus aureus* and *S. epidermidis*) based on agar well diffusion assay. All these honey samples had inhibitory effects against the tested bacteria. Generally, SH had higher antibacterial activity compared to HH. In particular, SH1 and SH2 were highly potent based on their clear zone diameters (agar well diffusion assay; Table 3).

Honey was a reservoir for environmental bacteria

The diluted honey samples were swabbed onto MRS and LB plates and incubated at 30°C aerobically or anaerobically (BD GasPak™ system; Becton, Dickinson and Co.; USA). The distinct colonies were subjected to further isolation and DNA extraction followed by PCR amplification (16S rRNA gene). The 16S rRNA gene (partial) sequence revealed all isolates as *Bacillus*/*Priestia* species (Table S1).

The fifteen honey bacterial isolates (Table S1) were stored in 12.5% (v/v) glycerol at -80°C and were further characterized based on their survival when subjected to a freeze-thaw cycle using the 16S-23S ITS-targeting primers. However, only seven isolates remained after several freeze-thaw cycles (Table 4). Cody et al. (2008) reported that skim milk Carvalho et al. (2004) that non-glucose carbon sources, such as fructose, lactose, and mannose, could enhance the preservation of bacterial stocks. In addition, Boisvert and Boisvert (2001) demonstrated that *B. thuringiensis* reduced by almost one-half efficacy against *Aedes triseriatus* after 1 wk of freezing. These findings suggested that the type of bacterial storage plays an important role in later applications. Notably, the seven strains surviving freezing were mainly *Bacillus* spp. Bacilli were found to be associated with plants and soils surrounding the habitats of the honey bees habitats (Cano et al., 1994; Nicholson, 2002). Fig. 2 shows that the honey isolates were different from the laboratory *B. subtilis* strains, including the domesticated *B. subtilis* 168 and the wild-type strain *B. subtilis* NCIB3610 (Zeigler et al., 2008). The honey of the stingless bee (*Heterotrigona itama*) collected across Malaysia reportedly contained *Bacillus* spp. (Amin et al., 2020). Notably, *Bacillus* spp. with potential antifungal peptides have also been isolated from honey (Zhao et al., 2013). Therefore, additional characterization of the honey isolates in this study is of interest.

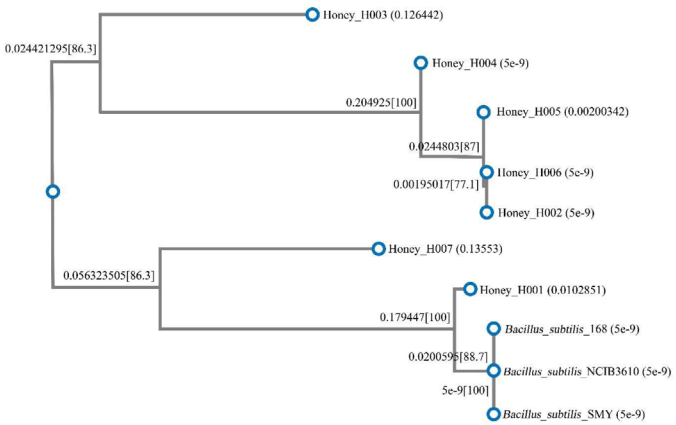

Fig. 2 Phylogenetic tree of honey bacterial isolates generated using 16S-23S internal transcribed spacer region of ribosomal DNA extracted from isolates, using CLUSTALW to align multiple sequences and subsequently produce phylogenetic generation (ETE3 pipeline)

Table 3 Antioxidant capacity and antibacterial (based on agar well diffusion method) properties of honey samples

Sample	Inhibition (%)	Ascorbic acid equivalent concentration (mM)	IC_{50} (%)	Diameter of inhibition zones against pathogenic bacteria (mm)		
				S. aureus	*E. coli*	*S. epidermidis*
SH1	86.54±27.03^{bcd}	2.81±0.86^{cd}	76.79±1.69^{a}	35.81^{b}	29.28^{a}	24.11^{a}
SH2	127.02± 32.06^{b}	4.04±1.02^{bc}	58.12±1.98^{a}	40.68^{a}	28.47^{a}	21.16^{b}
SH3	105±39.15^{bc}	3.55±1.36^{cd}	50.52±0.15^{a}	33.13^{b}	15.74^{d}	15.09^{d}
SH4	177.04±68.08^{a}	6.07±2.52^{a}	37.72±0.28^{a}	15.45^{d}	14.94^{d}	12.32^{e}
HH5	180.84±156.4^{a}	5.86±2.32^{ab}	48.48±0.59^{a}	32.54^{b}	19.43^{c}	18.96^{c}
HH6	48.1±25.38^{d}	1.62±0.68^{d}	268.97±25.51^{c}	16.61^{d}	15.80^{d}	15.89^{d}
HH7	56.67±15.34^{d}	1.81±0.42^{d}	128.13±5.84^{b}	0.00^{e}	0.00^{e}	15.54^{d}
HH8	78.24±17.75^{cd}	2.53±0.56^{cd}	75.75±0.93^{a}	21.95^{c}	22.20^{b}	16.73^{d}
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

SH = stingless bee honey; HH = honeybee honey; IC_{50} = half maximal concentration.

Mean ± SD values (average $n = 5$ biological replicates) in a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Potential probiotic properties of honey bacterial isolates

Seven bacterial isolates were collected from the honey samples and further characterized for potential use. Their probiotic potential was evaluated. Here, we tested for the ability of the bacterial isolates to tolerate acidic (pH 2.0 and 3.5) and bile salt conditions. All strains could withstand these conditions, as well as the simulated gastric and pancreatic fluids (Table 5).

Autoaggregation can reflect bacterial adhesive capacity to intestinal epithelial cells and the ability to co-aggregate with other microbes (Del Re et al., 2000). Therefore, this property is essential for retaining the probiotic cells while preventing pathogenic microorganism colonization in the gastrointestinal tract (Alkalbani et al., 2022). Three strains had > 60% autoaggregation after 3 hr, while another strain clumped at the bottom of the stationary test tube within minutes (Table S2). In parallel, the surface hydrophobicity of cells was evaluated through their ability to adhere to solvent (MATS). The results, shown in Table S2, indicated the significantly different adhesion capacities of the honey isolates compared to the *E. coli* control. In particular, H003 and H005 isolates showed > 80% microbial adhesion to xylene, with these levels being higher than those reported in multiple strains of *Lactobacillus* spp. (Benítez-Cabello et al., 2020; Dias et al., 2013).

The hemolytic activity and antibiotic susceptibility of the honey isolates were components of the safety assessment. For the isolates identified as *Bacillus* spp., the hemolytic activity was, to a certain extent, expected, with 1, 1 and 5 isolates with a-, β- and γ- hemolytic activity, respectively (Table S3).

Discussion

The physicochemical properties of the honey samples evaluated in the current study were similar to those reported elsewhere. There was a higher moisture content in the SH samples compared to the HH samples that were consistent with Biluca et al. (2016), who reported that SH typically had a higher moisture content. The higher fructose content in the current SH samples was in line with other studies that described lower reducing sugar levels in SH compared to *Apis mellifera* honey standards (Biluca et al., 2016; Chuttong et al., 2016; de Sousa et al., 2016). However, a generally lower reducing sugar content was observed in the SH samples among the four SH and four HH samples investigated, with one SH sample having a high reducing sugar content (Table 2). Specifically, the SH sample (SH1) collected from Samoeng had the highest glucose and total reducing sugar contents among the SH samples. Notably, this sample was produced by *Lepidotrigona* sp. while the other three SH samples were obtained from *Tetragonula* sp. (Table 1). The connections between bee species and origin and the physicochemical properties of honey have been somewhat examined (Kek et al., 2017). However, the correlation between bee species and the specific sugar content remains unclear. Notably, a significant difference in phenolic components and total antioxidant capacity was reported between Malaysian Kelulut honey and Tualang honey, whose collection sites were geographically and botanically different (Ranneh et al., 2018). In contrast, the current did not find a connection between floral sources and honey properties, though a melissopalynology test could benefit the study in identifying the botanical origin of honey. In the current study, longan honey (SH3 and HH7) was confirmed using the physical properties, including smell, taste and color by an Agricultural Technology Promotion Center's specialist. In contrast, the wildflower honey was not further tested, although *Coffea*, strawberry and white chrysanthemum were documented as predominant flora in the collection sites in Samoeng (SH1 and HH5). While the sucrose content is limited to 5% for finished honey (Mesele, 2021), one sample examined in the current study (SH3) contained exceedingly high sucrose levels. This observation, along with the high moisture content observed in SH samples, suggested an area for improvement in stingless bee honey production in Chiang Mai.

The honey samples showed antioxidant activity (Table 3). A similar observation has been reported in other studies (Al-Hatamleh et al., 2020). In particular, Ahmad et al. (2019a) reported the antioxidant capacity of Malaysian stingless bee honey in terms of vitamin C equivalence that was comparable to some of the SH samples in the current study (SH4 and HH5). Other studies reported antioxidant values using other units, such as gallic acid equivalence (da Silva et al., 2013) and Trolox equivalence (Dżugan et al., 2018). In the current study, the observed antioxidant properties were not correlated with the sugar or amino acid contents. In addition to the phenolic and flavonoid components, the observed antioxidant capacity could stem from other compounds, such as methylglyoxal, as a pyruvic acid derivative (Parmar, 2014). Holistic approaches, such as metabolomic and proteomic analyses, may elucidate the mechanisms underlying the observed bioactivities and provide insights into the characteristics of Chiang Mai honey.

The current study also investigated the antibacterial properties of honey against *Staphylococcus aureus*, *Escherichia coli* and *S. epidermidis* (Table 3). Compared to Yaacob et al. (2020), who reported on *Heterotrigona itama* honey samples, the current SH samples were more varied. Strikingly, four samples (SH1,

SH2, SH3 and HH5) had much larger inhibition zones against *S. aureus* (32.54–40.68 mm) compared to the *H. itama* honey samples (18–25 mm), though the *S. aureus* strains differed in the two studies. Nevertheless, the inhibitory effects against *E. coli* in both studies were assayed using *E. coli ATCC® 11775™, with SH1 in the current study being slightly more potent.*

Though both antibacterial and antioxidant capacities are often found to be associated with phenolic content (Kaya and Yıldırım, 2021), in the current study, these two properties were not necessarily correlated. For example, while SH4 was had a high level of antioxidant based on ABTS radical scavenging assay, it barely showed any inhibitory effects against *S. aureus*, *S. epidermidis* and *E. coli* compared to the other SH samples. The antibacterial capacity of honey could also come from its low pH, H_2O_2 from oxidases (since the samples were unprocessed) or a high sugar content that increases water potential outside the bacterial cells (Nolan et al., 2019).

Consistent with the predominant flora and vegetation in a bees' habitat being reported to influence the physicochemical properties of honey (Se et al., 2018), the current study identified a difference between the biological activities of the SH3 and HH7 samples, with the antioxidant capacity of HH7 being only half that of SH3 and HH7 did not inhibit *E. coli* and *S. aureus* growth (Table 3). Both SH3 and HH7 were collected from San Kamphaeng district with the collection sites both predominated economic longan crops common in Chiang Mai province. The stark difference in the antibacterial activities of these two samples may have stemmed from the different bee species—*Tetragonula* sp. vs. *Apis cerana* (see Tables 1 and 3).

Furthermore, the current study sought to isolate culturable bacteria from the honey samples. *Bacillus* spp. was the predominant bacteria in both the SH and HH samples (Table 4). Some bacterial isolates had promising probiotic potential, based on their acid and bile tolerance. The honey isolates also withstood simulated gastric and pancreatic fluids. In addition, they were sensitive to antibiotics and had autoaggregation and adhesion to solvent capacities (Table S2). These findings aligned with Amin et al. (2020), who isolated *Bacillus* spp. from Malaysian SH, with their isolates (H001-H007) displayed striking auto-aggregation and hydrophobicity (based on MATS) for *Bacillus* spp. and were comparable with some values reported in *Lactobacillus* spp. (Tuo et al., 2013; Del Re et al., 2000; Amin et al., 2020). Specifically, the current study identified a *Bacillus* strain with > 80% autoaggregation and hydrophobicity, while the reported *L. rhamnosus* GG value was roughly 60–70% (Amin et al., 2020). The current study also examined antibiotic susceptibility and the ability of the honey bacterial isolates to break down red blood cells, form biofilms and produce spores (Table S3). Among the honey isolates, H002 and H005 isolates appeared promising for further exploration in food or supplement applications. These two isolates were molecularly identified

Table 4 Molecular identification of honey isolates surviving freeze-thaw cycles

Isolate	Identification using 16S rRNA gene sequencing	% Identity	Identification using 16S-23S ITS gene sequencing	% Identity	Notes
H001	*Bacillus velezensis*	99.88	*B. amyloliquefaciens*	99.12	
	B. amyloliquefaciens	99.88	*B. velezensis*	99.12	
	B. subtilis	99.88			
	uncultured *Lactobacillus* sp. (KU588048.1)	99.88			
H002	*Priestia megaterium* (HM753591.1)	100.00	*P. megaterium*	100.00	
			P. aryabhattai	100.00	
H003	*B. pacificus*	99.89	*Bacillus cereus*	99.52	
	B. thuringiensis	99.89	*Bacillus thuringiensis*	99.29	
	B. paranthracis	99.89			
	B. cereus	99.89			
H004	*Priestia megaterium*	99.89	*P. megaterium*	98.57	Cohabit with *Polyporus umbellatus* (mushroom)
	Bacillus sp. JC301	99.89	*P. aryabhattai*	98.57	
	B. cereus	99.89			
	Bacillus sp. RJA3846	99.89			
H005	*Bacillus* sp. 5SB2	99.65	*P. megaterium*	99.62	Cohabit with *Vitex trifolia* (plant)
	Priestia sp.	99.50			
	Bacillus sp.	99.50			
H006	*Bacillus sp.*	99.88	*Priestia megaterium strain YC4-R4*	100.00	
H007	*B. aerophilus*	100.00	*B. subtilis*	80.18	Mangrove soil bacteria
	B. stratosphericus	100.00			
	B. altitudinis	100.00			
	B. aerius	100.00			
	B. pumilus	100.00			

Table 5 Stress factors tested on honey bacterial isolates

Property tested (% survival)	Honey isolate							*p*-value
	H001	H002	H003	H004	H005	H006	H007	
Acid (pH 2.0)	97.81±0.91^{a}	92.16±0.66^{c}	89.44±1.33^{d}	68.27±2.59^{ef}	70.11±1.67^{e}	67.65±1.54^{f}	94.68±1.77^{b}	< 0.0001
Acid (pH 3.5)	99.80±1.48^{a}	99.51±1.61^{a}	98.34±1.16^{a}	90.36±2.12^{c}	94.40±2.86^{b}	94.32±2.00^{b}	100.07±0.74^{a}	< 0.0001
Bile salt (0.3%)	99.79±1.84^{a}	99.42±1.91^{a}	99.80±0.84^{a}	99.34±1.84^{a}	99.40±0.89^{a}	95.85±1.39^{b}	100.48±1.34^{a}	< 0.001
SGF	98.20±2.49^{a}	94.80±5.26^{a}	65.16±3.21^{b}	2.00±4.47^{d}	26.66±1.49^{c}	7.00±7.21^{d}	97.80±2.28^{a}	< 0.0001
SPF**	95.2±0.77^{a}	88.37±2.63^{b}	77.64±3.58^{c}	34.38±4.32^{e}	87.39±3.89^{b}	49.52±6.59^{d}	94.36±3.26^{a}	< 0.0001
Spore formation (fraction)	0.100±0.005^{bc}	ND	ND	ND	ND	0.200±0.073^{ab}	0.347±0.136^{a}	< 0.01

ND = not detected; SGF = simulated gastric fluid; SPF = simulated pancreatic fluid.

Mean values ± SD (average $n = 5$ biological replicates) in a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

** = percentage survival calculated from N_0, after SGF treatment.

as *Priestia megaterium* (previously *Bacillus megaterium*), as shown in Table 4. This bacterial species has long been used for biotechnological applications, such as small molecule production and heterologous protein expression (Biedendieck et al., 2021). Recently, *P. megaterium* was also reported to increase tolerance to salt and drought stresses in *Arabidopsis* and pak choi (Hwang et al., 2022). Currently, H002 and H005 are being tested for their transformation efficiency and being subjected to whole genome sequencing for further insights.

The current results showed that non-processed (not yet dehumidified, mixed or homogenized) honey locally collected in Chiang Mai had great potential due to antioxidant and antibacterial properties, while also containing environmental bacteria that can be utilized. The ATPC might be able to encourage the consumption of such honey beyond neighboring households and ritual ceremonies. The physicochemical properties of the examined honey were comparable with other reports. Local producers might consider improving some physical properties (such as lowering the moisture and sucrose contents) according to FDA and industrial standards mentioned above. In this regard, some investors are marketing longan honey in domestic and international markets (Chen et al., 2019). Future studies could include investigating the impact of Chiang Mai honey on selected antibiotic-resistant bacteria and systems-level approaches to examining the chemical composition of unadulterated honey.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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