The *in vitro* and *in vivo* antibacterial activities of uniflorous honey from a medicinal plant, *Scrophularia ningpoensis* Hemsl., and characterization of its chemical profile with UPLC-MS/MS

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Abstract

***Ethnopharmacological relevance*:** According to the Compendium of Materia Medica, honey has been used as a traditional medicine in treatment against mucositis, tinea, hemorrhoids and psoriasis. In complementary medicine, due to its significant antimicrobial activity, honey has been widely used as a remedy for skin wounds and gastrohelcosis for thousands of years.

***Aim of the study*:** This study is aimed at exploring the antimicrobial activity and mechanisms of honey sourced from medicinal plants, and revealing the composition-activity relationship, to facilitate their complementary and alternative application in the therapy of bacterial infectious diseases.

***Materials and Methods*:** Eight kinds of medicinal plant-derived uniflorous honey, native to China, were gathered. Their antimicrobial activities were evaluated *in vitro*, and then *in vivo* with the systemically infected mouse model and the acute skin infection model. SYTOX uptake assay, scanning electron microscopy, DNA binding assay, and quantitative real-time PCR, were carried out to elucidate the antibacterial mechanisms. This was followed by an investigation of the componential profile with the UPLC-MS/MS technique.

***Results*:** It was found that *Scrophularia ningpoensis* Hemsl. (figwort) honey (*S. ningpoensis* honey) exhibited broad-spectrum and the strongest antibacterial potency (MICs of 7.81-125.00%, w/v), comparable to manuka honey. In the *in vivo* assays, *S. ningpoensis* honey significantly decreased the bacterial load of the muscles under the acute MRSA-infected skin wounds; the sera level of TNF-α in the *S. aureus* and *P. aeruginosa*-infected mice decreased by 45.38% and 51.75%, respectively, after the treatment of *S. ningpoensis* honey (125 mg/10 g). It was capable of killing bacteria through disrupting the cell membranes and the genomic DNA, as well as down-regulating the expression of genes associated with virulence, biofilm formation and invasion, including *icaA*, *icaD*, *eno*, *sarA*, *agrA*, *sigB*, *fib* and *ebps* in *S. aureus*, and *lasI*, *lasR*, *rhlI*, *rhlR* and *algC* in *P. aeruginosa*. Apart from H_2O_2, some other nonperoxide compounds such as adenosine, chavicol, 4-methylcatechol, trehalose, palmitoleic acid and salidroside, might play a vital role in the antibacterial properties of *S. ningpoensis* honey.

***Conclusions*:** This is the first study to thoroughly investigate the antibacterial activity, mode of action, and componential profile of *S. ningpoensis* honey. It suggested that *S. ningpoensis* honey might be a potential supplement or substitute for manuka honey, for the prevention or treatment of bacterial infections. It will facilitate the precise application of medicinal plant-sourced honey, provide a new thread for the development of antibacterial drugs, and assist in the distinction of different kinds of honey.

Keywords: honey, antibacterial, mechanism, *in vitro*, *in vivo*, untargeted metabolomics

1 Introduction

Honey, a naturally sweet fluid secreted by worker bees following collection of nectar or manna, contains carbohydrates, water, minerals, vitamins, proteins, polyphenols, and some other trace elements. The composition and bioactivity of honey vary depending on its botanical origins (Eteraf-Oskouei and Najafi, 2013). According to the Compendium of Materia Medica, honey can be used in the treatment of constipation, mucositis, tinea, hemorrhoids and psoriasis (Li, 1982). Due to the significant antimicrobial activity of honey, it has been used as a remedy for skin wounds and gastrohelcosis for thousands of years (Eteraf-Oskouei and Najafi, 2013; Mandal and Mandal, 2011; Ratcliffe et al., 2011; Vogt et al., 2020). Modern medicine mainly focuses on its pharmacological properties such as antibacterial, antioxidant, anticancer and wound-healing activities (Vogt et al., 2020).

Honey's antibacterial activity is mainly attributed to the high sugar content, low pH, high osmotic pressure, and the presence of hydrogen peroxide (H_2O_2), phenolic compounds, enzymes (such as lysozyme), and peptides (such as bee defensin-1 peptide, apidaecin, abaecin and hymenoptaecin) (Bucekova et al., 2019; Sojka et al., 2016). For most natural honey, H_2O_2 was produced by glucose oxidase (GOX)-mediated conversion of glucose to gluconic acid under aerobic conditions in the presence of water (Poli et al., 2018). When H_2O_2 is hydrolyzed, it produces oxygen, speeding up the oxidation of polyphenols. Oxidated polyphenols act as pro-oxidant agents facilitating the production of H_2O_2, resulting in the generation of hydroxyl radicals due to the presence of transition metals (Brudzynski et al., 2012). The phenol/H_2O_2-induced oxidative stress is thought to be critical for the DNA degradation and the resultant bacterial death (Brudzynski and Lannigan, 2012; Brudzynski et al., 2012). Bee defensin-1 peptide (Def-1) is one of the most widely studied antimicrobial peptides

(AMPs) identified in honeybee hemolymph and hypopharyngeal glands (Sojka et al., 2016). The mechanisms of action of Def-1 have not been fully elucidated, yet the defensin proteins identified from other species, such as mammals, have been shown to lead a rapid bacterial death through creating a “pore” on the cell membranes (Ganz, 2003).

Manuka honey is a peculiar bee product originated from *Leptospermum scoparium* J.R.Forst. & G.Forst. native to Australia and New Zealand (Allen et al., 1991). It is characterized by an extremely high content of methylglyoxal (MGO) which has been demonstrated to lyse bacterial cells, inhibit flagellation and disrupt cell division (Girma et al., 2019). The Unique Manuka Factor (UMF) grading system reflects the concentration of MGO in commercially available manuka honey. The greater UMF of manuka honey is thought to be more effective against bacteria, and thus is more costly in the consumer market (Carter et al., 2016). Apart from manuka honey, the research on the antimicrobial activity of honey has been mostly concentrated on the samples from a specific origin or honeybee species such as stingless bee honey (Domingos et al., 2021), and honey from China, Greece, Brazil or Pakistan (Wang et al., 2021; Stavropoulou et al., 2022; Azevedo et al., 2021; Mustafa et al., 2022). With respect to the medicinal plant-derived honey, that from buckwheat (*Fagopyrum esculentum* Moehch) (Deng et al., 2018), Shaoka (*Fagonia bruguieri* DC.) (Halawani et al., 2021), fennel (*Foeniculum vulgare* Mill.), agastache (*Agastache rugosa* (Fisch. & C.A.Mey.) Kuntze), pomegranate (*Punica granatum* L.) (Zhang et al., 2021), and some other medicinal plants (Salonen et al., 2017; Chang et al., 2011; Mundo et al., 2004), has been investigated for antimicrobial efficacy. Identification of antibacterial substances from honey has long been a hot topic, while the mode of action is rarely studied in depth (Sateriale et al., 2019; Bucekova et al., 2018; Bucekova et al., 2019; Poli et al., 2018; Wang et al., 2021).

This study is aimed at exploring the antimicrobial activity and mechanisms of honey sourced from medicinal plants, and revealing the composition-activity relationship. Eight kinds of medicinal plant-derived uniflorous honey, native to China, were gathered, and their *in vitro* and *in vivo* antimicrobial activities, as well as the mechanisms of action, were comprehensively investigated. Simultaneously, by employing the UPLC-MS/MS technique, the compositions of *S. ningpoensis* honey and manuka honey were studied to find out the componential difference, facilitating the development of antibacterial drugs and the future distinction of specific honey.

2 Materials and methods

2.1 Harvest of honey samples

Honey samples, produced by worker bees and originating from eight medicinal plants, were collected in six regions of China (Table 1). Genuine honey samples were obtained from reliable beekeepers who placed the bee hives in a region where a kind of medicinal plant predominated the field in the blossom season, accounting for >95% within 1 km^2. During the flowering seasons, it was obvious that massive honeybees were collecting nectar from the specific medicinal plant. Nectar plants were validated taxonomically by Yan Lin with *The Plant List* (http://www.theplantlist.org). The voucher specimens were deposited in the herbarium of Fujian Agriculture and Forestry University, with deposition numbers of SnBz-20201001, FsSjz-20200401, ScJc-20200601, SsSjz-20200601, LjSjz-20200701, AmDx-20200701, DlZz-20210301, and LcZz-20210301.

Table 1: Details of honey samples

Honey	Nectariferous plants	Origin	Florescence
***S. ningpoensis* honey**	*Scrophularia ningpoensis* Hemsl. (figwort)	Bozhou	October 2020
***F. simplex* honey**	*Firmiana simplex* (L.) W. Wight (phoenix tree)	Shijiazhuang	April 2020
***S. chinensis* honey**	*Schisandra chinensis* (Turcz.) Baill. (schizandra)	Jincheng	June 2020
***S. salicifolia* honey**	*Spiraea salicifolia* L. (spiraea)	Shijiazhuang	June 2020

***L. japonicus* honey**	*Leonurus japonicus* Houtt. (motherwort)	Shijiazhuang	July 2020
***A. membranaceus* honey**	*Astragalus membranaceus* (Fisch.) Bunge (milkvetch)	Dingxi	July 2020
***D. longan* honey**	*Dimocarpus longan* Lour. (longan)	Zhangzhou	March 2021
***L. chinensis* honey**	*Litchi chinensis* Sonn. (litchi)	Zhangzhou	March 2021
manuka honey	*Leptospermum scoparium* J.R.Forst. & G.Forst. (manuka)	New Zealand (Comvita)	2021

2.2 Palynological and physicochemical properties of honey samples

The obtained honey was checked with microscopic pollen analysis according to the method recommended by the International Commission for Bee Botany (Louveaux et al., 1978). Briefly, 10 g of fluid honey was dissolved in 20 ml of distilled water and centrifuged at 1000 ×g for 10 min, followed by discarding the supernatant, dissolving precipitation in water and centrifuging again. The remaining sediment was resuspended in 100 μl of water and added onto a microscope slide. After evaporation of water, the pollen preparation was mounted in neutral balsam and observed under a microscope (Nikon, Tokyo, Japan) at 400× magnification. Characteristic pollen grains were counted and those with proportion of more than 45% were considered as the unifloral source of the honey (Thrasyvoulou et al., 2018; Xagoraris et al., 2021; Von Der Ohe et al., 2004; Addi and Bareke, 2021). The moisture content and pH of each honey sample were measured according to the methods proposed by the Association of Official Agricultural Chemists (AOAC International, 1990). The contents of total polyphenol (TPC), H_2O_2 and GOX were determined with the Total Polyphenol Content Test Kit (Congyi Bio, China), the Hydrogen Peroxide Assay Kit (Beyotime, China), and the Glucose Oxidase Activity Assay Kit (Solarbio, China), respectively.

2.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

The antimicrobial activity of honey was evaluated against the strains of model test microorganisms, *Staphylococcus aureus* (*S. aureus*) (NCTC10788), *Enterococcus faecalis* (*E. faecalis*) (ATCC29212), Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC43300), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC27853), *Chromobacterium violaceum* (*C. violaceum*) (ATCC12472), *Escherichia coli* (*E. coli*) (NCTC10418), and *Helicobacter pylori* (*H. pylori*) (ATCC43504) purchased from the China Center for Type Culture Collection, as previously described with minor modifications (Lin et al., 2021). Briefly, 100 μl of each bacterial culture (1.5×10^6 CFU/ml) was incubated with 100 μl of honey solutions (1.95-250%, w/v) prepared in Mueller-Hinton broth (MHB). After 18-h incubation at 37 °C, the optical density values were measured at 595 nm using a microplate reader (Tecan, Männedorf, Switzerland). The lowest concentration without any visible bacterial culture was defined as the MIC. By transferring 10 μl of medium from clear wells onto a Mueller-Hinton agar (MHA) plate for a further 24-h incubation at 37 °C, the concentration without colony growth was considered as the MBC. Ampicillin (Macklin, China) and polymyxin B (Solarbio, China) at concentrations of 1-128 mg/L were used as control.

2.4 Minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) assays

The antibiofilm activity of honey was evaluated by MBIC and MBEC assays as previously described with minor modifications (Lin et al., 2021). Briefly, for the MBIC assay, bacterial culture and honey solutions were incubated as described in the MIC assay for 24 h at 37 °C, 200 rpm. The formed biofilm was fixed with methanol and stained with 0.1% crystal violet, followed by dissolution in 33% glacial acetic acid and measurement of absorbance at 595 nm. For the MBEC assay, prior to the treatment of honey solutions, bacterial culture was incubated in 96-well plates to form biofilm. After

washing off the planktonic bacteria with PBS (10 mM, 200 μl) and a further 24-h incubation, the remaining biofilm at the bottom of the plate was exposed to 1% MTT solution (Macklin, China) for 4 h at 37 °C. Finally, the formazan crystals were dissolved in 100 μl of DMSO and the absorbance was measured at 550 nm.

2.5 Growth curves of bacteria exposed to honey

Bacterial culture and honey samples were incubated as described in the MIC assay, and the growth curves of bacteria were produced using real-time monitoring (Wang et al., 2021). Briefly, *S. ningpoensis* honey, manuka honey, ampicillin and polymyxin B were chosen to generate the growth curves of six bacterial strains. Bacterial culture was treated with 1/2 × MIC of honey samples, and the absorbance was recorded every 30 min at 595 nm, 37 °C, for 15 h. Bacterial culture treated with PBS acted as the control.

2.6 Enzymatic treatment of honey with catalase

The treatment of honey samples with catalase was conducted as previously described (Poli et al., 2018). Briefly, honey was diluted to 250% (w/v) with MHB, and then treated with catalase (1000 U/ml) for 2 h at room temperature. The content of H_2O_2 and MICs/MBCs of each honey were determined as described above.

2.7 Bacterial membrane permeabilization assay

The permeabilization of honey on the bacterial membrane was evaluated by the SYTOX uptake assay as previously described (Lin et al., 2021). Briefly, *S. aureus* or *P. aeruginosa* suspended in HEPES buffer at a density of 1.5×10^8 CFU/ml was incubated with honey solutions (3.95%, 7.81% and 15.63%,

w/v) and 1 μM SYTOX™ Green Nucleic Acid Stain (Invitrogen, USA). It was followed by monitoring the fluorescent intensity over 60 min at 1-min interval (Ex 485 nm / Em 528 nm). Triton X-100 (1%, v/v) was regarded as the positive control.

2.8 Scanning electron microscopy (SEM)

The morphology and membrane integrity of *S. aureus* and *P. aeruginosa* were observed with SEM as previously described (Lin et al., 2021). Briefly, bacteria were treated with honey at 1/4 × and 1/2 × MICs at 37 °C for 18 h, followed by fixation, dehydration, coating with gold-palladium and observation using a JSM-6380LV scanning electron microscope (JEOL, Tokyo, Japan).

2.9 DNA binding assay

The DNA binding ability of honey samples was evaluated as previously described with some modifications (Brudzynski et al., 2012). Briefly, the genomic DNA of the test bacteria was extracted using a TIANamp Bacteria DNA Kit (TIANGEN, China). The resultant DNA (100 ng/μl) was mixed with honey (1.95%-125%) or antibiotics (2-128 mg/L), followed by 5-h incubation at 37 °C, analysis with agarose gel electrophoresis (0.5% agarose gel), and visualization with a trans-illuminator (Peiqing Science and Technology, Peking, China).

2.10 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was conducted as previously described (Selvaraj et al., 2021). Briefly, *S. aureus* and *P. aeruginosa* were treated with honey as described in the MIC assay. Then, bacterial RNA was extracted using an Eastep® Super Total RNA Extraction Kit (Shanghai Promega, China) and reverse-transcribed using the HiScript® II Q RT SuperMix for qRT-PCR (+ gDNA wiper) (Vazyme,

198 China). The qRT-PCR was performed using the 2 × ChamQ SYBR Color qPCR Master Mix (Low
199 ROX Premixed) (Vazyme, China) and specific primers presented in Table 2.

200 **Table 2: Oligonucleotide primers used for qRT-PCR**

Bacteria	Primers	Forward (5'-3')	Reverse (5'-3')
S. aureus	*icaA*	GAGGTAAAGCCAACGCACTC	CCTGTAACCGCACCAAGTTT
S. aureus	*icaD*	ACCCAACGCTAAAATCATCG	GCGAAAATGCCCATAGTTTC
S. aureus	*eno*	GCAGTAGGTGACGAAGGTGGTT	AGCTGCTTCGATTGCTTGGAT
S. aureus	*sarA*	CAAACAACCACAAGTTGTTAAAGC	TGTTTGCTTCAGTGATTCGTTT
S. aureus	*agrA*	CGTGGCAGTAATTCAGTGTATG	AATGGGCAATGAGTCTGTGAG
S. aureus	*sigB*	TGGAGTGTACATGTTCCGAGAC	CACTGATAGAAGGTGAACGCTC
S. aureus	*fib*	ATTGCGTCAACAGCAGATG	GTACCATCATTGTACTCTACGA
S. aureus	*ebps*	GGCAAGGCGAATAACTCGACTG	TTTGAAGCGGCAGAAGCACTTT
S. aureus	*16s rRNA*	GCTGCCCTTTGTATTGTC	AGATGTTGGGTTAAGTCCC
P. aeruginosa	*lasI*	ACCGTAGGCGTGGAGAAGATGA	GCGATCTGGGTCTTGGCATTGA
P. aeruginosa	*lasR*	CTGTGGATGCTCAAGGACTAC	AACTGGTCTTGCCGATGG
P. aeruginosa	*rhlI*	CCATCCGCAAACCCGCTACAT	TTGAGCAGGTAGGCGTCGGT
P. aeruginosa	*rhlR*	GCGACCAGCAGAACATCTCCAG	GGGTTGGACATCAGCATCGGAT
P. aeruginosa	*algC*	GGCTTCGACGATGGCATCTACA	TCCTCGGTGACGGTGATGTTGA
P. aeruginosa	*16s rRNA*	CAAGCGGTGGAGCATGTGGTT	GCAGCACCTGTGTCTGAGTTCC

201 **2.11 Haemolysis assay**

202 The haemolytic activity of honey was examined using horse erythrocytes (Lin et al., 2021), where the
203 erythrocytes treated with 1% (v/v) Triton X-100 and sterile PBS were regarded as the positive control
204 (100% haemolysis) and negative control (0% haemolysis), respectively. A suspension of horse
205 erythrocytes (4%) was incubated with honey (1-128%) at 37 °C for 2 h, followed by measuring the
206 absorbance of the supernatant at 550 nm.

207 **2.12 *In vivo* toxicity assay**

208 The *in vivo* toxicity of *S. ningpoensis* honey was evaluated, together with manuka honey. The

experiment was performed as previously described with some modifications (Zhong et al., 2021). Female BALB/c mice (6-8 weeks, 25-28 g) were purchased from the Animal Research Center of Hubei Province, China. They were housed under a condition of 25 ± 3 °C, 40-70% relative humidity, noise ≤ 60 dB and 12:12 h light-dark cycle, and supplied with a commercial pellet diet and water ad libitum. Prior to the experiment, mice were acclimatized to the environment for 7 days. They were then randomly divided into 7 groups (3 mice/group) and injected intraperitoneally (*i.p.*) with 200 μl of *S. ningpoensis*/manuka honey at doses of 62.5, 125 or 250 mg/10 g in saline, or saline alone (control). The sample size was calculated using the resource equation method. A value E was measured from the sample size, which was the degree of freedom of analysis of variance (ANOVA) and should be in the range of 10 to 20. If E is less than 10, increasing the number of animals will increase the probability of getting more significant results, but if E is greater than 20, increasing the number of animals will not increase the probability of getting significant results. Any sample size keeping E in the range of 10 to 20 should be considered as adequate (Imbroisi Filho et al., 2021; Charan and Kantharia, 2013; Zhou et al., 2022). E = Total experimental animals - Total experimental groups = 3 × 7 - 7 = 14, indicating an adequate sample size. The behavior, weight and survival of mice were recorded for 5 days. The behavior and survival were mainly classified into four types: mice without any abnormal sign, behaving flexibly, reacting quickly and drinking/eating normally; mice showing symptoms of malaise and poor motility; mice with hutching, very ruffled fur and complete immobility even under stimulation; and mice died. Mouse blood was sampled from the orbital vein, followed by being kept at room temperature for about 2 h and centrifuged at 1500 ×g for 20 min to obtain the supernatant serum for biochemistry analysis, including the measurement of alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AKP), with an Aminotransferase Assay Kit (Njjcbio, China) according to the manufacturer's instructions. Mice

treated with physiological saline in the same way as experimental groups were used as the control. Mice were euthanized by asphyxiation with CO_2 followed by cervical dislocation after 5-day administration of honey. The experiment was carried out from July 1 to July 8, 2021. Mice carcasses were bagged and frozen prior to incineration. All the animal studies included in the current research were performed according to the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines (Kilkenny et al., 2010) and the Guidelines for the Care and Use of Medical Laboratory Animals (Ministry of Health, China, 1998). The whole procedure was approved and overseen by the Ethical Review Board of Fujian Agriculture and Forestry University (No. PZCASFAFU2019008).

2.13 *In vivo* antibacterial assay with the systemically infected mouse model

The *in vivo* antibacterial assay with the systemically infected mouse model was performed as previously described (Zhong et al., 2021). Female BALB/c mice (6-8 weeks, 25-28 g, 3 mice/group, 45 mice totally) were housed under the same conditions as above. *S. aureus*- or *P. aeruginosa*-infected mouse model was established by injecting (*i.p.*) the BALB/c mouse with 200 μl of *S. aureus* (9×10^8 CFU) or *P. aeruginosa* (3×10^8 CFU) in saline. After 1 h of bacterial infection, the mice were treated (*i.p.*) with 200 μl of *S. ningpoensis* honey or manuka honey at 62.5, 125 or 250 mg/10 g in saline, or saline alone. Hence, for either *S. aureus*- or *P. aeruginosa*-infected mouse model, 8 groups were included (uninfected mice treated with saline alone, infected mice treated with 62.5, 125, 250 mg/10 g of *S. ningpoensis* honey or manuka honey, and infected mice treated with saline alone), where the E was $3 \times 8 - 8 = 16$, indicating an adequate sample size in accordance with the resource equation method. Mice were euthanized by asphyxiation with CO_2 followed by cervical dislocation after 6-h infection, and then the lungs, spleens, kidneys and livers were harvested. Organs were weighed and separately homogenized in 3 ml of PBS using cell strainers (70 μM, Biosharp, China) to remove

connective tissue or organ capsule. The obtained homogenate was serially diluted and a volume of 5 μl in four replicates was placed onto MHA plates to count the bacterial loads which were expressed as the colony forming unit relative to the weight of organs. Mice sera were collected prior to the euthanasia in the same way as described above for evaluating the level of TNF-α with the ELISA kit (Dakewe, China). After the experiment, mice were euthanized and disposed as described above. The experiment was carried out from July 10 to July 15, 2021. The whole procedure was approved and overseen by the Ethical Review Board of Fujian Agriculture and Forestry University (No. PZCASFAFU2019008).

2.14 *In vivo* antibacterial assay with the acute skin infection model

An acute MRSA infection model was established as previously reported with some modifications (Sun et al., 2020). Female BALB/c mice (6-8 weeks, 25-28 g, randomly divided into 4 groups with 4 mice/group) were housed under the same conditions as above. The number of mice was calculated using the resource equation method, where the E was $4 \times 4 - 4 = 12$, indicating an adequate sample size. An 8 mm full-thickness excisional skin wound was created on the right of the dorsal with a skin biopsy punch when mouse was anesthetized by inhalation with 2% isoflurane in 98% air (surgical duration exposure) (RWD, Dover, USA). MRSA (3×10^7 CFU, concentrated in 5 μl of PBS) was seeded onto the wound. A volume of 100 μl of crude *S. ningpoensis* honey, manuka honey or physiological saline was added onto the surface immediately after the infection and incubated for 10 min. The whole muscle under the wound was removed and homogenized as described above. Then, four replicates of 5 μl of homogenate were placed onto MHA plates following a serial dilution to count the bacterial loads. After the experiment, mice were euthanized and disposed as described above. The experiment was carried out from November 25 to November 30, 2021. The whole procedure was

approved and overseen by the Ethical Review Board of Fujian Agriculture and Forestry University (No. PZCASFAFU2019008).

2.15 Untargeted metabolomic analysis

The chemical substances present in *S. ningpoensis* honey and manuka honey were analyzed by the untargeted metabolomic analysis (Lin et al., 2021). A total of 100 mg (± 1%) of *S. ningpoensis* honey or manuka honey was dissolved in 0.6 ml of 2-chlorophenylalanine (4 ppm in methanol), ground with 100 mg of glass beads for 90 s at 60 Hz, subjected to ultrasound for 10 min at room temperature, centrifuged for 10 min at 12000 rpm, 4 °C, and filtered through a 0.22-μm membrane, for the subsequent quality control and LC-MS/MS analysis. Chromatographic separation was performed using a Vanquish UHPLC (Thermo, Waltham, America) fitted with an ACQUITY UPLC® HSS T3 column (150 × 2.1 mm, 1.8 μm, Waters, Milford, America) maintained at 40 °C. The temperature of the autosampler was 8 °C. Gradient elution of analyte was carried out at a flow rate of 0.25 ml/min with the eluent of 5 mM ammonium formate in water (A) and acetonitrile (B) for the negative mode, or that of 0.1% formic acid in water (C) and 0.1% formic acid in acetonitrile (D) for the positive mode. The linear gradient elution program was set as follows: 0~1 min, 2% B/D; 1~9 min, 2%~50% B/D; 9~12 min, 50%~98% B/D; 12~13.5 min, 98% B/D; 13.5~14 min, 98%~2% B/D; 14~20 min, 2% D for the positive mode and 14~17 min, 2% B for the negative mode. Mass spectrometric analysis was performed with a Q Exactive Plus Orbitrap LC-MS/MS system (Thermo, Waltham, America) in positive and negative modes. In the ESI source, the spray voltage was 3.5 kV and -2.5 kV in positive and negative modes, respectively. Capillary temperature was 325 °C. The Orbitrap analyzer scanned over a mass range of m/z 81-1000 for full scan at a mass resolution of 70000. Fragmentation was realized by the high-energy collisional dissociation (HCD) with the collision energy of 30 eV. Base

peak chromatogram (BPC) was generated by taking the most intense ion from each spectrum at each point in the liquid chromatogram. The raw MS data were converted to mzXML file with Proteowizard software (v3.0.8789), and peaks identification, filtration and alignment were processed using the XCMS package of R (v3.3.2). The data were then subjected to multivariate data analysis, including principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). Components with p-value ≤ 0.05 and variable importance in the projection (VIP) ≥ 1 were considered as statistically significant. Differential components were identified by searching against the Human Metabolome Database (HMDB) (http://www.hmdb.ca), the METLIN (http://metlin.scripps.edu), the MassBank (http://www.massbank.jp/), the LIPID MAPS (http://www.lipidmaps.org), and the mzCloud (https://www.mzcloud.org).

2.16 Statistical Analysis

All data were analyzed using the GraphPad Prism 8.0 software (CA, USA). Significance of differences was calculated by the one-way ANOVA method followed by the Tukey's test. Differences with $p < 0.05$ were considered as statistically significant. All values were presented as mean values ± standard deviation (SD).

3 Results

3.1 Palynological and physicochemical properties of honey samples

The palynological and physicochemical properties of honey samples are shown in Table 3. Micrographs of palynological analysis on honey samples are shown in Figure S1.

Table 3: Palynological and physicochemical properties of honey samples

Honey	Pollen (%)	pH	Moisture (%)	TPC (µg/g) GAE	H_2O_2 (µmol/kg)	GOX (U/g)

***S. ningpoensis* honey**	82.53 ± 1.22	2.47 ± 0.02	19.80 ± 0.20	569.36 ± 16.17	363.24 ± 7.29	1.44 ± 0.23
***F. simplex* honey**	71.40 ± 0.71	2.65 ± 0.06	19.40 ± 0.30	358.51 ± 10.85	405.98 ± 9.33	63.14 ± 4.46
***S. chinensis* honey**	73.48 ± 1.59	2.87 ± 0.04	19.60 ± 0.50	281.28 ± 0.21	391.02 ± 8.76	35.33 ± 6.38
***S. salicifolia* honey**	89.88 ± 0.85	2.89 ± 0.08	19.80 ± 0.20	214.04 ± 2.34	86.12 ± 2.21	3.74 ± 1.70
***L. japonicus* honey**	81.31 ± 1.27	2.20 ± 0.03	23.00 ± 0.70	375.53 ± 19.15	44.26 ± 3.10	0.06 ± 0.02
***A. membranaceus* honey**	73.92 ± 0.86	2.68 ± 0.05	19.60 ± 0.30	418.51 ± 21.92	257.47 ± 5.92	5.94 ± 0.69
***D. longan* honey**	87.46 ± 1.88	3.15 ± 0.03	20.60 ± 0.10	427.02 ± 4.68	245.02 ± 6.48	9.13 ± 3.37
***L. chinensis* honey**	83.35 ± 0.69	2.93 ± 0.07	20.40 ± 0.50	285.32 ± 14.72	319.86 ± 10.41	5.60 ± 0.99
manuka honey	60.89 ±0.83	2.80 ± 0.02	18.80 ± 0.20	845.74 ± 4.89	126.23 ± 5.53	0.06 ± 0.01

TPC, total polyphenol content; GAE, gallic acid equivalents; GOX, glucose oxidase.

3.2 Antibacterial and antibiofilm effects of honey

The MIC and MBC values of honey samples are shown in Table 4 and Figure S2. All of the samples exhibited broad-spectrum antimicrobial activity against test microorganisms, in which *S. ningpoensis* honey possessed the strongest efficacy comparable to manuka honey, while the bacteriostatic and the bactericidal activities of *S. ningpoensis* honey against *H. pylori* (MIC/MBC = 125.00%/250.00%) were weaker than those of manuka honey (MIC/MBC = 62.50%/125.00%). As shown in Table 5 and Figure S3, *S. ningpoensis* honey and manuka honey displayed similar inhibitory activity towards the biofilm formation of test bacteria, with MBICs of 15.63-31.25% and 31.25% against *S. aureus* and *P. aeruginosa*, respectively. In terms of the effects of *S. ningpoensis* honey and manuka honey on biofilm eradication, both of them could destroy the formed *P. aeruginosa* and *S. aureus* biofilms, and manuka honey appeared to be more effective (MBECs of 62.50-125.00% for manuka honey *vs.* 125.00% for *S. ningpoensis* honey, Table 5 and Figure S4).

Table 4: MICs (%) and MBCs (%) of honey samples against model test microorganisms

Honey	Activities	*S. aureus*	*E. faecalis*	MRSA	*P. aeruginosa*	*C. violaceum*	*E. coli*	*H. pylori*
S. ningpoensis	MIC	7.81	7.81	31.25	31.25	7.81	62.50	125.00

honey	MBC	7.81	7.81	31.25	31.25	7.81	62.50	250.00
***F. simplex* honey**	MIC	7.81	31.25	62.50	31.25	15.63	62.50	-
	MBC	7.81	>250.00	>250.00	31.25	31.25	>250.00	-
***S. chinensis* honey**	MIC	15.63	62.50	62.50	62.50	31.25	125.00	-
	MBC	15.63	>250.00	>250.00	125.00	31.25	>250.00	-
***S. salicifolia* honey**	MIC	31.25	125.00	62.50	125.00	31.25	125.00	-
	MBC	62.50	>250.00	>250.00	>250.00	62.50	>250.00	-
***L. japonicus* honey**	MIC	125.00	125.00	>250.00	125.00	62.50	>250.00	-
	MBC	>250.00	125.00	>250.00	>250.00	125.00	>250.00	-
***A. membranaceus* honey**	MIC	62.50	125.00	62.50	125.00	31.25	125.00	-
	MBC	125.00	>250.00	>250.00	125.00	125.00	>250.00	-
***D. longan* honey**	MIC	62.50	125.00	125.00	125.00	62.50	125.00	-
	MBC	>250.00	>250.00	>250.00	>250.00	>250.00	>250.00	-
***L. chinensis* honey**	MIC	62.50	62.50	31.25	62.50	31.25	62.50	-
	MBC	125.00	>250.00	62.50	>250.00	125.00	125.00	-
manuka honey	MIC	15.63	31.25	62.50	31.25	15.63	62.50	62.50
	MBC	31.25	62.50	62.50	62.50	31.25	62.50	125.00
Ampicillin	MIC	1.00 mg/L	2.00 mg/L	4.00 mg/L	2.00 mg/L	8.00 mg/L	4.00 mg/L	-
	MBC	4.00 mg/L	4.00 mg/L	8.00 mg/L	8.00 mg/L	32.00 mg/L	8.00 mg/L	-
Polymyxin B	MIC	-	-	-	1.00 mg/L	128.00 mg/L	8.00 mg/L	-
	MBC	-	-	-	16.00 mg/L	>128.00 mg/L	16.00 mg/L	-

-, represents not tested.

Table 5: MBICs (%) and MBECs (%) of *S. ningpoensis* honey and manuka honey against *S. aureus* and *P. aeruginosa* biofilms

Sample	Activities	*S. aureus*	*P. aeruginosa*
***S. ningpoensis* honey**	MBIC	15.63	31.25
	MBEC	125.00	125.00
manuka honey	MBIC	31.25	31.25
	MBEC	125.00	62.50
Ampicillin	MBIC	1.00 mg/L	128.00 mg/L
	MBEC	128.00 mg/L	128.00 mg/L
Polymyxin B	MBIC	-	1.00 mg/L
	MBEC	-	8.00 mg/L

-, represents not tested.

3.3 Growth curves of bacteria treated with honey

The growth of the six strains of test aerobic bacteria was monitored after treatment of honey over 15 h. Both *S. ningpoensis* honey and manuka honey showed similar antibacterial patterns to antibiotics towards the growth of *S. aureus* and *E. faecalis*, in which the growth rate abated within 7 h but increased afterwards (Figure 1A-B); however, they were much more potent against MRSA, that the OD_{595} of *S. ningpoensis*/manuka honey-treated bacteria was significantly lower than that of ampicillin at 15 h ($p = 0.0008$, *S. ningpoensis* honey *vs.* ampicillin; $p = 0.0014$, manuka honey *vs.* ampicillin; n = 4, Figure 1C). For Gram-negative bacteria, the suppressive impact of test honey samples seemed not as effective as that of antibiotics (Figure 1D-F), especially against *E. coli* ($p = 0.770$, *S. ningpoensis* honey *vs.* control; $p = 0.999$, manuka honey *vs.* control; 15 h, n = 4). It is noteworthy that although *S. ningpoensis* honey could restrain the growth of most test bacteria, it was only effective within the first 7 h and the function was not sustainable at later stages (Figure 1).

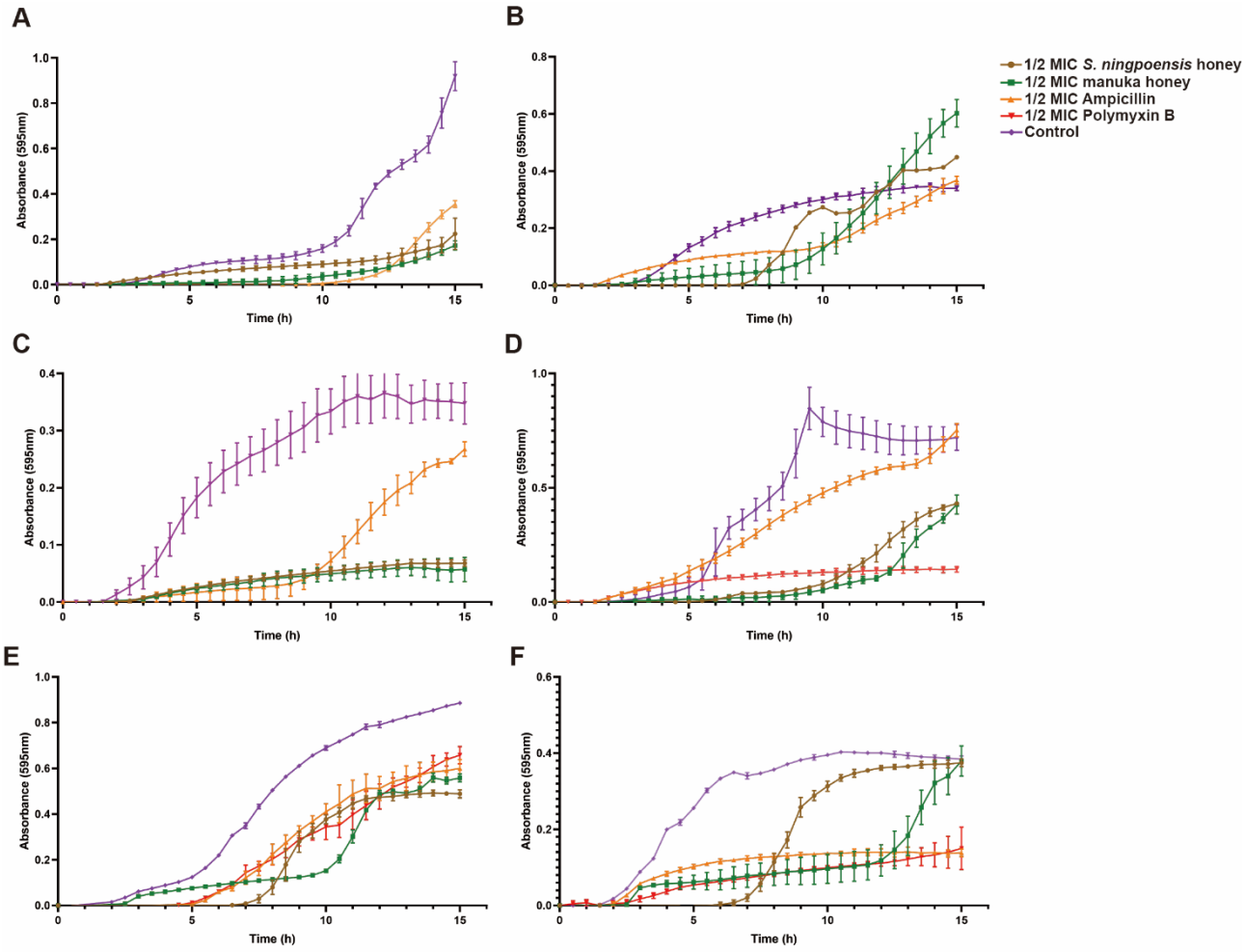


Figure 1: Growth curves of test bacteria treated with honey. Effects of 1/2 × MICs of *S. ningpoensis* honey, manuka honey, ampicillin and polymyxin B on the growth of *S. aureus* (A), *E. faecalis* (B), MRSA (C), *P. aeruginosa* (D), *C. violaceum* (E) and *E. coli* (F). Control, bacteria treated with PBS.

3.4 Effect of catalase on the antimicrobial activity of honey

Hydrolyzed by catalase, the H_2O_2 present in honey samples, except for *L. japonicus* honey, reduced remarkably ($p < 0.001$, n = 3, Table 3 and 6). After exposure to catalase, aside from *S. ningpoensis* honey, *F. simplex* honey and manuka honey which retained a relatively potent antimicrobial effect (MICs ≤ 62.50%), the efficacy of other honey samples towards *S. aureus* and *P. aeruginosa* declined to a similar level (MIC ≥ 125.00%, MBC > 250.00%). The bacteriostatic and the bactericidal effects of manuka honey on *S. aureus* and *P. aeruginosa* were 2-fold weaker than the catalase-untreated replicate (Table 6).

Table 6: H_2O_2 content and MICs/MBCs (%) of honey treated with catalase

Honey		*S. ningpoensis* honey	*F. simplex* honey	*S. chinensis* honey	*S. salicifolia* honey	*L. japonicus* honey	*A. membranaceus* honey	*D. longan* honey	*L. chinensis* honey	manuka honey
H_2O_2 (μmol/kg)		62.14 ± 5.16	51.42 ± 7.14	59.75 ± 4.03	62.52 ± 6.39	43.63 ± 2.96	61.07 ± 4.48	56.58 ± 9.32	43.13 ± 3.72	43.91 ± 6.88
S. aureus	MIC	62.50	62.50	125.00	125.00	125.00	>250.00	125.00	125.00	31.25
	MBC	250.00	250.00	>250.00	>250.00	>250.00	>250.00	>250.00	>250.00	62.50
P. aeruginosa	MIC	62.50	62.50	125.00	125.00	125.00	125.00	125.00	125.00	62.50
	MBC	>250.00	>250.00	>250.00	>250.00	>250.00	>250.00	>250.00	>250.00	250.00

3.5 Membrane permeabilization of honey on bacteria

As indicated in the SYTOX uptake assay, 3.95-15.63% of *S. ningpoensis* honey induced significant membrane permeability on *S. aureus* from 3 min post-treatment ($p < 0.01$ *vs.* Blank, n = 4, Figure 2A); for *P. aeruginosa*, such a phenomenon occurred at 21 and 39 min post-treatment of 7.81% and 15.63% of *S. ningpoensis* honey ($p = 0.015$, 0.041 *vs.* Blank, n = 4), respectively, and the effect increased over the period of monitoring (Figure 2B). When *S. aureus* was treated with manuka honey, remarkable membrane permeability was induced by 3.95 and 7.81% of manuka honey at 21 and 30 min ($p < 0.05$ *vs.* Blank, n = 4, Figure 2C), respectively. Additionally, manuka honey could only lead to the dramatical membrane permeability of *P. aeruginosa* at 15.63% from 55 min post-treatment ($p = 0.049$ *vs.* Blank, n = 4, Figure 2D). Hence, compared with manuka honey, the membrane permeabilization capability of *S. ningpoensis* honey was stronger towards both of the test Gram-positive and -negative bacteria, implying a marked bacterial killing potency.

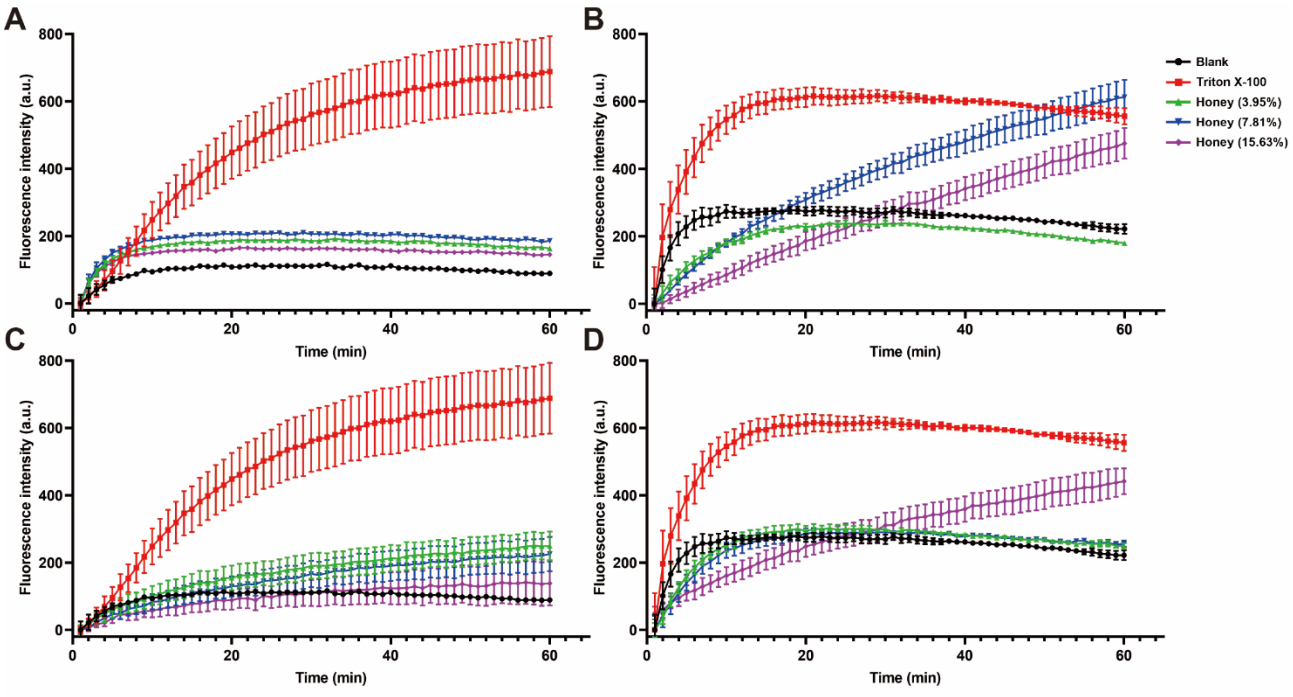


Figure 2: Membrane permeability of test bacteria treated with honey. *S. aureus* and *P. aeruginosa* treated with *S. ningpoensis* honey (A and B) and manuka honey (C and D), respectively.

3.6 SEM

The effects of *S. ningpoensis* honey on the membrane integrity and morphology of *S. aureus* and *P. aeruginosa* are shown in Figure 3. In contrast to the normal bacteria (Figure 3A and D) presenting a smooth and regular surface, as well as a plump morphology, those treated with 1/4 × MIC of *S. ningpoensis* honey generally retained an integrate morphology but with crenulate, scabrous and irregular membranes (Figure 3B and E). When treated with 1/2 × MIC of *S. ningpoensis* honey, *S. aureus* and *P. aeruginosa* displayed obvious leakage of cell inclusion, in which the damage in the membrane of *P. aeruginosa* appeared to be more severe, indicating significant cell lysis (Figure 3C and F).

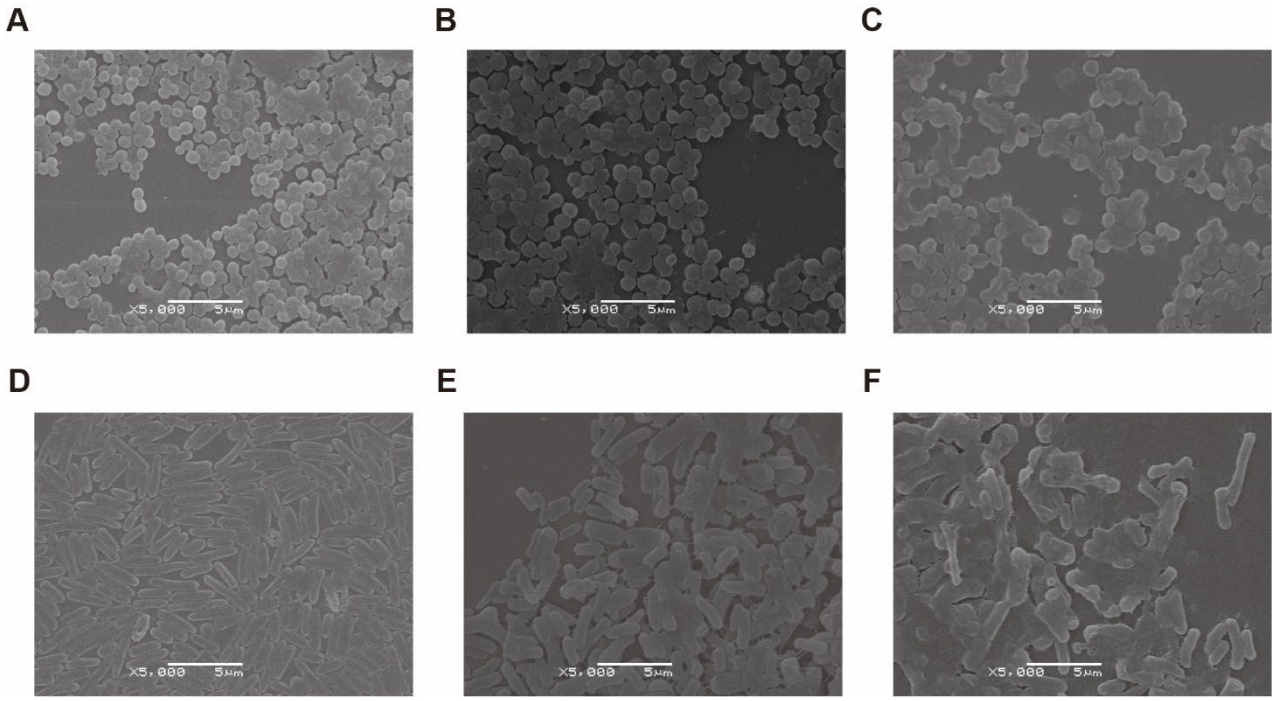


Figure 3: Micrographs of *S. aureus* and *P. aeruginosa* observed with SEM. Untreated *S. aureus* (A) and *P. aeruginosa* (D) as control. (B-C) *S. aureus* treated with *S. ningpoensis* honey at 1/4 × and 1/2 × MICs. (E-F) *P. aeruginosa* treated with *S. ningpoensis* honey at 1/4 × and 1/2 × MICs.

3.7 DNA binding ability

S. ningpoensis honey showed a strong ability to bind to the DNA of all test bacteria at concentrations

of ≥ 31.25% (Figure 4A). Notably, *S. ningpoensis* honey could bind to the DNA of *E. coli* at a concentration (15.63%) lower than the corresponding MIC (62.50%). On the contrary, manuka honey only bound to the DNA of *E. coli* at higher concentrations (≥ 62.50%) and exhibited very weak binding ability to the DNA of *P. aeruginosa*, *C. violaceum*, *E. faecalis* and MRSA (Figure 4B). Meanwhile, none of the test antibiotics possessed a distinct DNA binding ability to sensitive bacteria (Figure 4C-D).

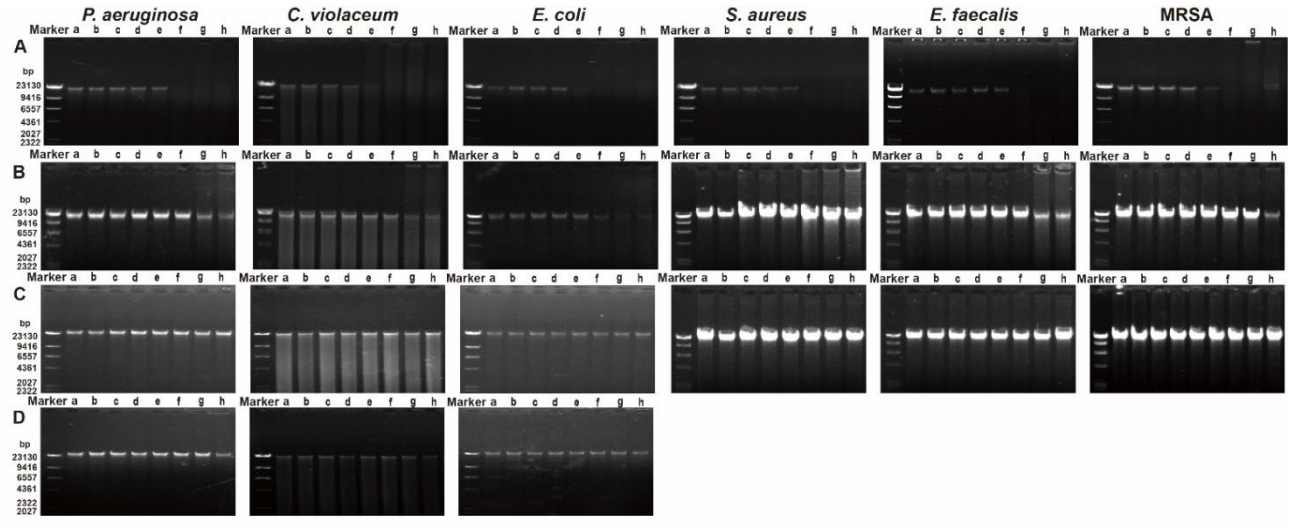


Figure 4: DNA binding ability of honey to sensitive microbes as analyzed with electrophoresis. Binding ability of *S. ningpoensis* honey (A), manuka honey (B), ampicillin (C) and polymyxin B (D) to the genomic DNA of *P. aeruginosa*, *C. violaceum*, *E. coli*, *S. aureus*, *E. faecalis* and MRSA. Lane a-h represents 0, 1.95-125% of *S. ningpoensis* honey (A)/manuka honey (B), and 0, 2-128 mg/L of ampicillin (C)/polymyxin B (D).

3.8 Modulatory effects of honey on bacterial gene expression

After 18-h incubation with honey, the expression of *icaA*, *icaD*, *eno*, *sarA*, *agrA*, *sigB*, *fib* and *ebps* in *S. aureus*, and that of *lasI*, *lasR*, *rhlI*, *rhlR* and *algC* in *P. aeruginosa*, were evaluated. In comparison with the control group, it was obvious that the expression of *icaA*, *icaD*, *eno*, *sarA*, *agrA*, *sigB*, *fib* and *ebps* in *S. aureus* was significantly downregulated after exposure to 1/2 × MICs of *S. ningpoensis* honey and ampicillin ($p < 0.01$, n = 4), and that of *icaD*, *eno*, *agrA*, *sigB*, *fib* and

ebps was also downregulated by 1/2 × MIC of manuka honey ($p < 0.01$, n = 4), while the expression of *icaA* and *sarA* was upregulated after treatment of 1/2 × MIC of manuka honey ($p < 0.001$, n = 4). Apart from *agrA*, the expression of other genes after the treatment of 1/2 × MIC of *S. ningpoensis* honey was significantly lower than that of manuka honey ($p < 0.001$, n = 4, Figure 5A). In addition, *S. ningpoensis* honey significantly downregulated the expression of *lasI*, *lasR*, *rhlI*, *rhlR* and *algC* in *P. aeruginosa* ($p < 0.001$ *vs.* control, n = 4). What is noteworthy is that when *P. aeruginosa* was treated with 1/2 × MIC of manuka honey and ampicillin, only *rhlI* expression was downregulated ($p < 0.001$ *vs.* control, n = 4, Figure 5B).

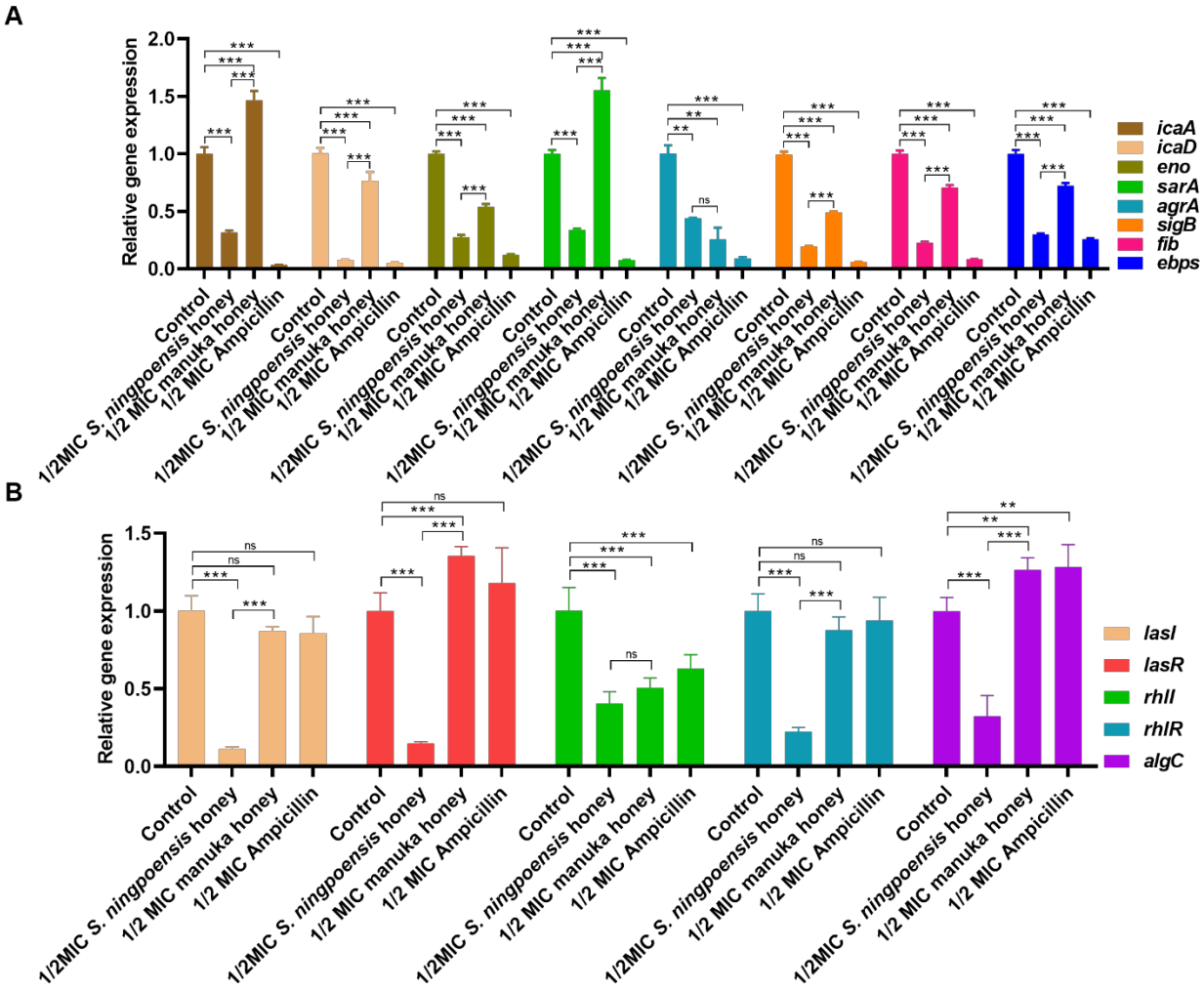
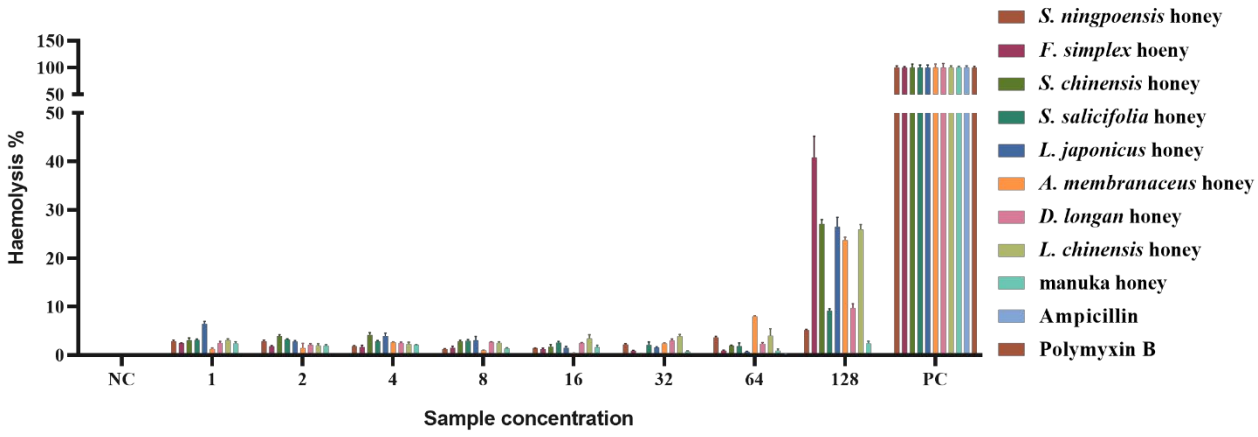


Figure 5: Gene expression in honey-treated *S. aureus* (A) and *P. aeruginosa* (B). Control, bacteria treated with MHB. The *16s rRNA* gene was used as a reference gene. *, $p < 0.05$; **, $p < 0.01$; ***, p

422 <0.001, n = 4.

423 ### 3.9 Haemolytic activity

424 As some kinds of honey presented remarkable membrane permeant activity towards bacteria, their
425 effects on mammalian erythrocytes were investigated. Apart from *A. membranaceus* honey, which
426 caused 7.97 ± 0.31% haemolysis at concentration of 64.00%, the majority of honey samples displayed
427 less than 5% haemolysis at that concentration (Figure 6). In combination with the antimicrobial
428 activity (Table 4), *S. ningpoensis* honey and manuka honey seemed to possess superior antibacterial
429 potency but without distinct haemolysis, thus they were selected for the subsequent *in vivo* evaluation.



430

431 **Figure 6: Haemolytic activity of honey on horse erythrocytes.** Honey (1-128%) and antibiotics (1-
432 128 mg/L) were tested. PC and NC represent positive and negative control that red blood cells were
433 incubated with 1% TritonX-100 and PBS, respectively.

434 ### 3.10 *In vivo* toxicity of honey

435 After intraperitoneal injection of honey, mice behavior and survival were observed for 5 days. As
436 shown in Figure 7A, abnormal behavior appeared within 30 min post-injection, but it generally
437 vanished after 48 h. The higher (250 mg/10 g) and medium (125 mg/10 g) doses of manuka honey
438 led to 100% and 33% mortality in mice, respectively. All the test mice survived at the medium (125

mg/10 g) and lower (62.5 mg/10 g) doses of *S. ningpoensis* honey. The weight of mice treated with either *S. ningpoensis* honey or manuka honey decreased in the first 24 h, while they began to put on weight after 48 h (Figure 7B). After 5-day treatment of honey, the levels of ALT, AST, and AKP in the sera of all survival mice were measured to evaluate the toxicity of honey towards liver. As a result, when medium dose (125 mg/10 g) of honey was administered, the levels of ALT, AST and AKP in *S. ningpoensis* honey-treated mice were 42.54%, 79.40% and 76.63% relative to the control group ($p <$ 0.0001, $p = 0.2234$ and 0.0004, n = 3); the levels in manuka honey-treated mice were 69.06%, 47.15% and 77.17% relative to the control group ($p = 0.0461$, 0.0004 and 0.0015, n = 2, Figure 7C).

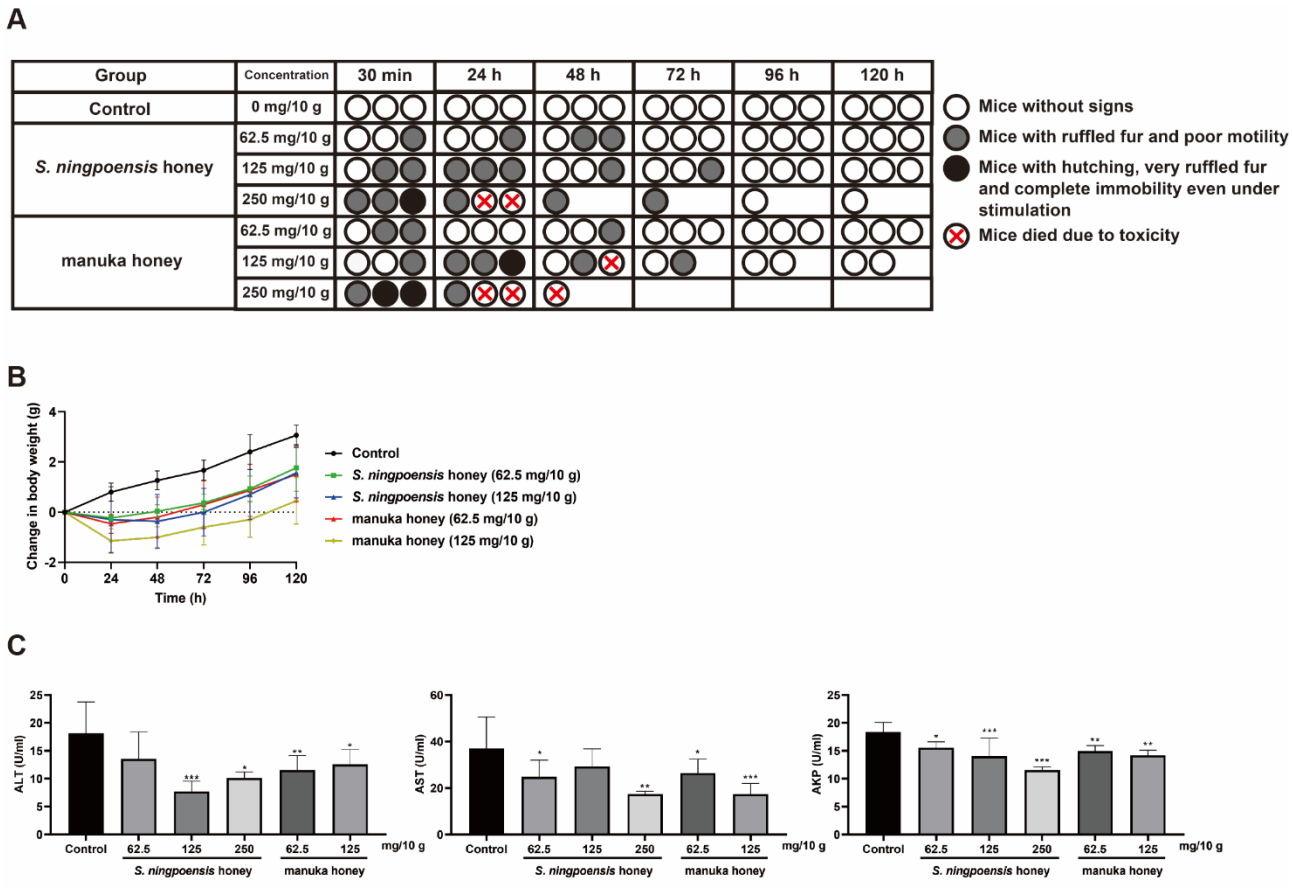


Figure 7: Evaluation of the *in vivo* toxicity of honey. (A) Observation of the survival and behavior of BALB/c mice treated with *S. ningpoensis* honey and manuka honey over 120 h. Each circle represents one mouse and the status of the mouse is indicated by different circle shading as outlined in the legend. (B) Changes in the body weight of the mice administered (*i.p*) with different doses of honey. Each data point represents the mean ± standard deviation. (C) Levels of ALT, AST and AKP in the sera of mice. Control, mice administered with physiological saline. *, p <0.05; **, p <0.01;

***, p <0.001, compared with control.

3.11 Systemic antibacterial activity of honey in bacteria-infected mice

According to Figure 8A, after injection of *S. aureus* or *P. aeruginosa*, the distribution of bacteria to lungs, spleens, kidneys and livers could not be efficiently restrained by the treatment of *S. ningpoensis* honey or manuka honey, expect that manuka honey demonstrated modest inhibition towards the invasion of *S. aureus* to spleens at concentrations of 62.50 mg/10 g (p = 0.023, n = 3) and 250.00 mg/10 g (p = 0. 021, n = 3). Despite this, both *S. ningpoensis* honey and manuka honey dramatically reduced the level of TNF-α in the sera of *S. aureus* or *P. aeruginosa*-infected mice models. The level of TNF-α in *S. aureus* and *P. aeruginosa*-infected mice decreased by 45.38% and 51.75% with significant difference after treatment of 125 mg/10 g of *S. ningpoensis* honey (p < 0.05, n = 3), indicating a potential anti-inflammatory activity (Figure 8B).

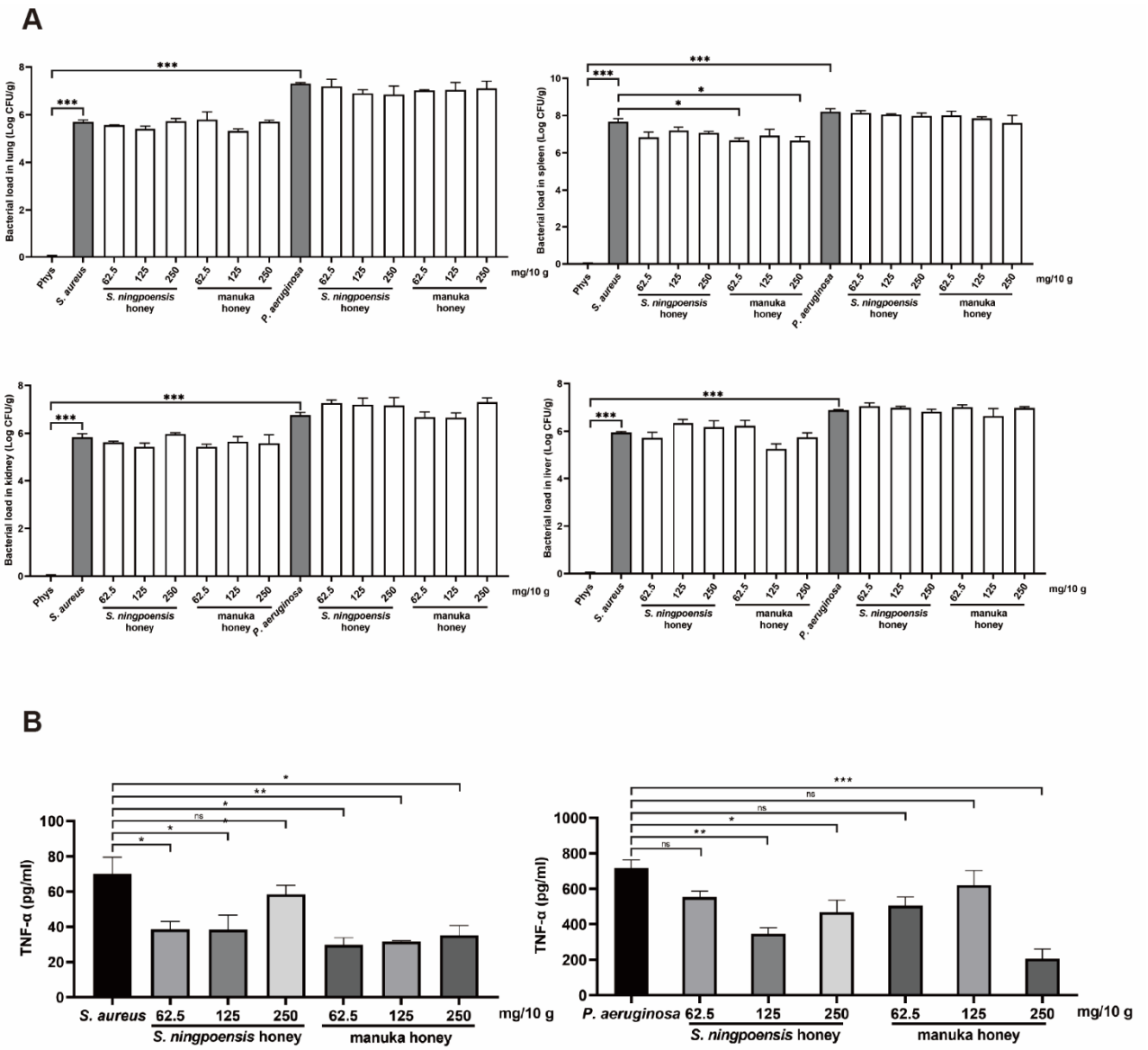


Figure 8: Evaluation of the systemic antibacterial activity of honey in bacteria-infected mice. (A) Bacterial load in lungs, spleens, kidneys and livers of mice. (B) Level of TNF-α in the sera of *S. aureus* or *P. aeruginosa*-infected mice. Phys, mice treated with physiological saline alone. *S. aureus*/*P. aeruginosa*, mice treated with saline at 1 h post-infection of *S. aureus*/*P. aeruginosa*. *, p <0.05; **, p <0.01; ***, p <0.001, n = 3.

3.12 Antibacterial activity of honey towards acute skin infection model

As shown in Figure 9, when the full-thickness excisional skin wounds of mice were infected with MRSA, the immediate treatment of *S. ningpoensis* honey and manuka honey reduced the bacterial load of the local tissue by 79.57% and 63.44% compared with the saline-treated mice, respectively, with significant difference (p < 0.01, n = 4). The efficacy of *S. ningpoensis* honey appeared to be

more significant than that of manuka honey in such an acute skin infection model (p = 0.048, n = 4).

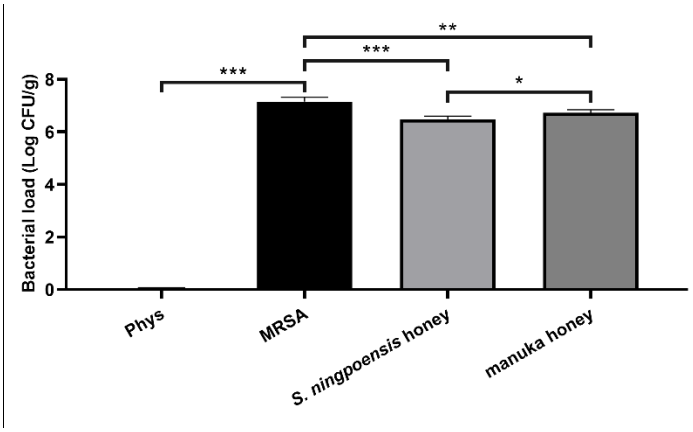


Figure 9: Bacterial load of the muscle under the acute MRSA-infected skin wounds following treatment of honey. Phys, mice treated with physiological saline alone. MRSA, mice treated with saline after bacterial infection. *, p <0.05; **, p <0.01; ***, p <0.001, n = 4.

3.13 Untargeted metabolomic analysis of the components present in *S. ningpoensis* honey and manuka honey

Untargeted metabolomic technique was employed to reveal the differential compounds present in *S. ningpoensis* honey and manuka honey. BPCs of UPLC/MS analysis representing the intensity of the most intense peak at each point are shown in Figure 10. As indicated in the principle component analysis (PCA) (Figure 11A-B) and the orthogonal partial least squares discriminant analysis (OPLS-DA) (Figure 11C-D), *S. ningpoensis* honey could be clearly distinguished from manuka honey. A total of 249 differential metabolites were identified, with 100 up-regulated and 149 down-regulated in *S. ningpoensis* honey *vs.* manuka honey (Table S1-2). Hierarchical clustering also showed a different chemical profile between *S. ningpoensis* honey and manuka honey (Figure S5). According to KEGG analysis, the differential compounds present in *S. ningpoensis* honey were significantly enriched in the pathway of bacterial chemotaxis (map02030), pantothenate and CoA biosynthesis (map00770), oxidative phosphorylation (map00190), biosynthesis of siderophore group nonribosomal peptides

(map01053), biofilm formation-Vibrio cholera (map05111), ferroptosis (map04216), and inflammatory mediator regulation of TRP channels (map04750) (Table S3).

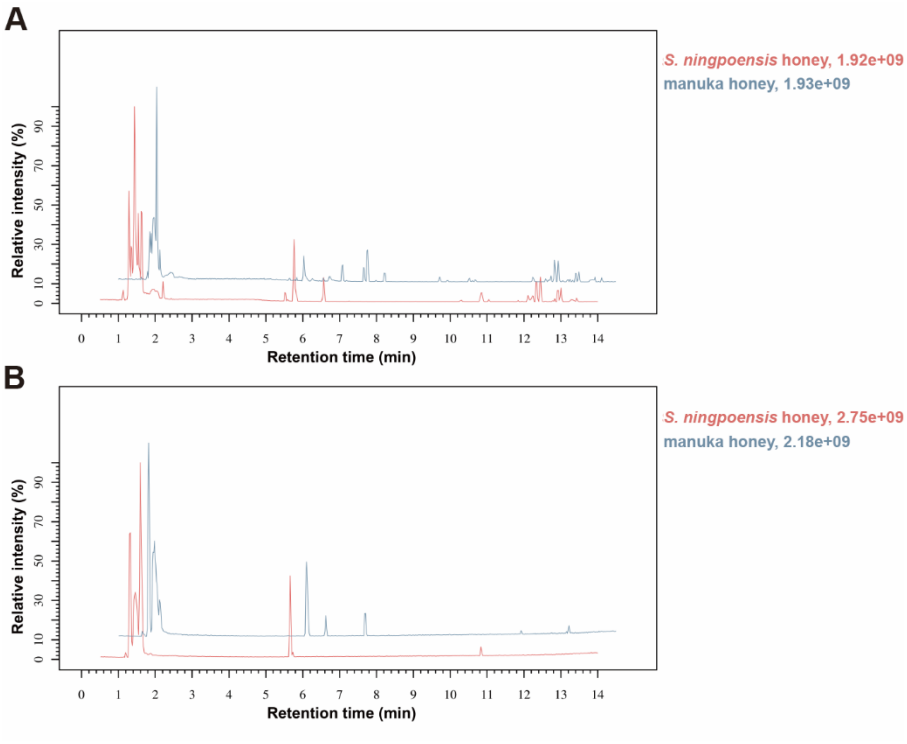


Figure 10: Base peak chromatograms of *S. ningpoensis* honey and manuka honey in positive (A) and negative (B) ion modes.

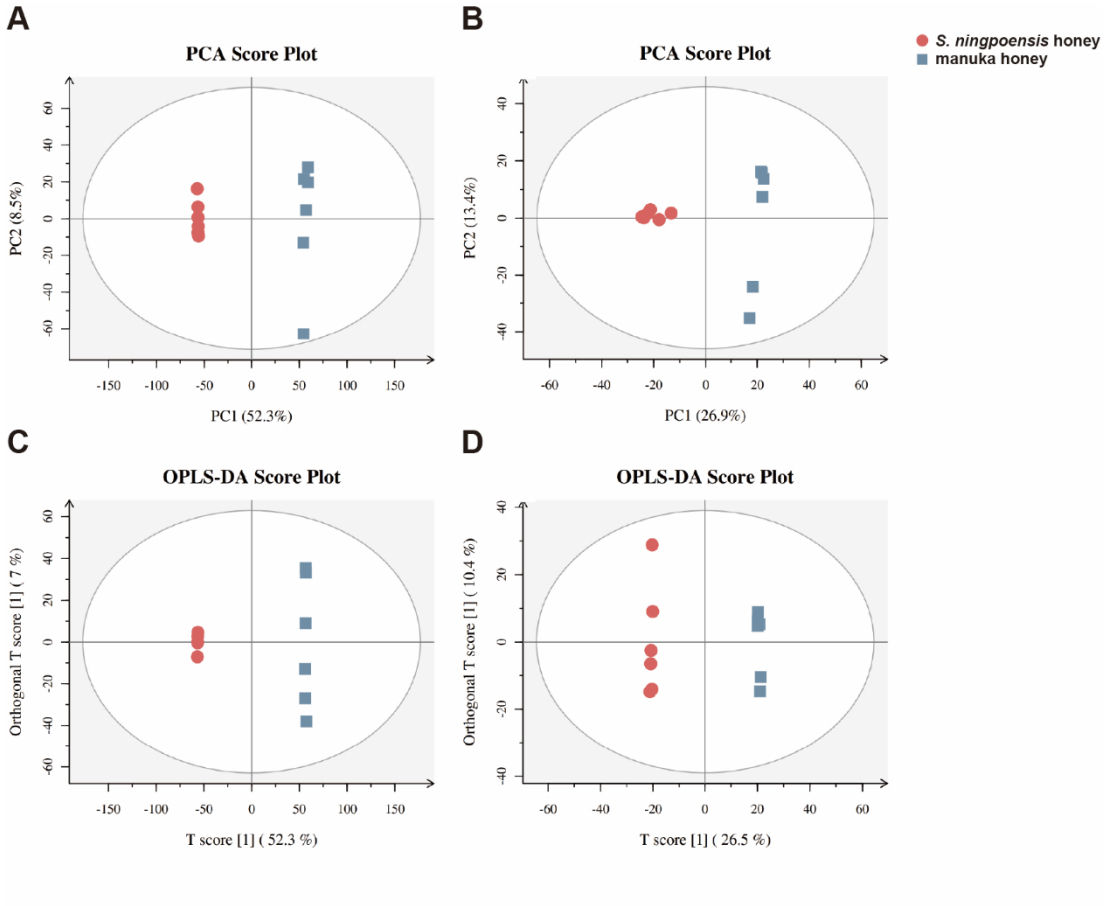


Figure 11: Untargeted metabolomic analysis of compounds in *S. ningpoensis* honey and manuka honey. PCA plots (A-B) and OPLS-DA plots (C-D) showing clear distinction between *S. ningpoensis* honey and manuka honey, and the efficiency of the model, in positive (left panel) and negative (right panel) ion modes.

4 Discussion

Honey's antibacterial mechanism has been proven to be related to a variety of factors. It is attributed to the synergistic impact of the intrinsic properties of honey itself and a wide range of exogenous bioactive substances in honey. Low moisture and high sugar content of honey form a hypertonic circumstance for the survival of bacteria, leading to the loss of water and then suppression of growth and proliferation (Albaridi, 2019). The low pH of honey, detrimental to bacteria, is formed primarily from the hydrolysis of glycose, and the resultant gluconic acid (Karabagias et al., 2014). The synergy between polyphenols and H_2O_2 was also shown to be responsible for honey's antibacterial activity (Brudzynski et al., 2012).

In this study, seven strains of model microorganisms were employed to evaluate the antimicrobial effects of the medicinal plant-derived honey native to China. These pathogens can invade many parts of the human body, such as the intestine, urinary tract and respiratory tract, causing severe systemic infections and even mortality (Gravina et al., 2018; Bretón et al., 2002; Sachu et al., 2020; Sindeldecker and Stoodley, 2021; Tang et al., 2020). Almost all of them have developed multidrug resistance, making clinical therapeutics increasingly difficult (Yang et al., 2014; Krawczyk et al., 2021; Poirel et al., 2018). As shown in Table 3-4, the antibacterial potency of honey was generally positively correlated with the concentration of H_2O_2, except for manuka honey. At the same time, all the honey samples suffered from a decline in the antimicrobial potency towards the test microorganisms after exposure to catalase (Table 4 and 6), suggesting that H_2O_2 played a vital role in the honey's antibacterial activity. Among these samples, the retained relatively strong bacteriostatic effect of *S. ningpoensis* honey, after the treatment of catalase (Table 6), implied that its antibacterial activity might rely partly on the nonperoxide activity (NPA) (Bouzo et al., 2020). Thus, analyzing the componential profile of *S. ningpoensis* honey may facilitate the discovery of nonperoxide antimicrobial substances from honey and the corresponding target sites in bacteria. In addition, the current study showed that honey with more polyphenols tended to exhibit stronger antibacterial activity, indicating that polyphenols contributed to the antibacterial function to some degree. In contrast, GOX was irrelevant to honey's antibacterial activity, implying that GOX was not directly involved in the antibacterial process (Table 3-4).

Bacteria will be subjected to irreversible damage if their membranes are destroyed. The stronger and more sustainable membrane permeabilization of *S. ningpoensis* honey towards Gram-negative bacteria than that towards Gram-positive bacteria over the period of monitoring (Figure 2A-B) suggested that *S. ningpoensis* honey could penetrate the outer membrane of Gram-negative bacteria,

while it was relatively difficult to disrupt the thick peptidoglycans of Gram-positive bacteria, as also demonstrated in the SEM micrographs (Figure 3). Together with Figure 4 in which *S. ningpoensis* honey exhibited similar binding ability to the DNA of Gram-positive and -negative bacteria, it was deemed that *S. ningpoensis* honey could inhibit the growth of bacteria through disrupting the cell membranes as well as the genomic DNA.

The virulence and biofilm construction of *P. aeruginosa* are regulated by Quorum Sensing (QS) which relies on the density of bacteria (Kong et al., 2021). RhlIR and LasIR are two important systems related to the QS of *P. aeruginosa*. RhlI and LasI aim at producing N-(3-oxododecanoyl)-L-homoserine lactone (N-(3-oxo-C12-HSL)) and N-butanoyl homoserine lactone (C4-HSL), while RhlR and LasR are responsible for reading N-(3-oxo-C12-HSL) and C4-HSL (Sampathkumar et al., 2019). The reading process will result in the production of transcription regulatory factors, boosting the expression of virulence genes in *P. aeruginosa* (Waters and Bassler, 2005). In the present study, we found that the expression of QS-related genes (*RhlI*, *LasI*, *RhlR* and *LasR*) was suppressed obviously in *P. aeruginosa* after being treated with *S. ningpoensis* honey ($p < 0.001$ *vs.* control, n = 4, Figure 5B), indicating that *S. ningpoensis* honey could decrease the production of virulence in *P. aeruginosa* during biofilm growth and adhesion. Moreover, *P. aeruginosa* biofilm maturation requires the production of the extracellular polymeric substances (EPS) such as polysaccharide synthesis locus (Psl), exopolysaccharide (Pel), and alginate (Alg) (Seder et al., 2021). The biosynthesis of Alg is basically mediated by *algC*-encoded Phosphomannomutase (Miari et al., 2020). Combining the decreased expression of *algC* in *S. ningpoensis* honey-treated *P. aeruginosa* ($p < 0.0001$ *vs.* control, n = 4) and its antibiofilm activity (Figure 5B and Table 5), it is obvious that *S. ningpoensis* honey could also reduce the biofilm formation of *P. aeruginosa* by reducing the synthesis of Alg, weakening its pathogenicity (Wang et al., 2014).

559 Polysaccharide intercellular adhesin (PIA), synthesized by the intercellular adhesion (*ica*) operon-
560 encoded glycosyltransferase enzymes (Wu et al., 2019), is essential for the formation of biofilm and
561 the adhesion of *S. aureus* (Liu et al., 2017). Co-expression of *icaA* and *icaD* would enhance the
562 activity of N-acetylglucosaminyl transferase and slime production, facilitating the adhesion of *S.*
563 *aureus* during biofilm formation (Arciola et al., 2006). *Ica* genes are regulated by several genes such
564 as staphylococcal accessory regulator A (*sarA*) and virulence genes accessory gene regulator (*agrA*)
565 (El-Far et al., 2021). The coaction of *sarA* and *agrA* could regulate the biofilm formation, increasing
566 the secretion of virulence factor observably (Farha et al., 2020). Simultaneously, *sigB* regulated the
567 expression of *sarA* and *agrA* during stress response (Kim et al., 2020). Hence, in this study, the
568 reduced survival and biofilm generation of *S. aureus* after the treatment of *S. ningpoensis* honey
569 (Table 4-5) might also be attributed to the significant down-expression of *icaA*, *icaD*, *sarA*, *agrA* and
570 *sigB* induced by *S. ningpoensis* honey ($p < 0.01$ *vs.* control, n = 4, Figure 5A). On the other hand,
571 manuka honey only down-regulated the expression of *icaD*, *agrA* and *sigB* ($p < 0.01$ *vs.* control, n =
572 4), and up-regulated that of *icaA* and *sarA* ($p < 0.001$ *vs.* control, n = 4, Figure 5A), which explained
573 the higher toxicity of *S. ningpoensis* honey towards *S. aureus* than that of manuka honey (Table 4-5).

574 Additionally, some other genes play an important role in the invasion of bacteria. For instance, α-
575 enolase, encoded by *eno* gene, could actuate *S. aureus* attaching to lamin, and facilitate its diffusion
576 through blood and the invasion into host tissues (Carneiro et al., 2004). The elastin binding protein
577 of *S. aureus* (EbpS) encoded by *ebps* gene contributed to the colonization of *S. aureus* in mammalian
578 tissues (Downer et al., 2002). And the fibrinogen binding protein (Fib) encoded by *fib* gene could
579 bind to the fibrinogen protein to prevent platelet adherence, aggregation, and clotting at the sites of
580 injury (Tristan et al., 2003). As *S. ningpoensis* honey remarkably reduced the expression of *eno*, *ebps*
581 and *fib* genes ($p < 0.001$ *vs.* control, n = 4) and such an effect was significantly stronger than manuka

honey ($p < 0.001$, n = 4, Figure 5A), it further explained the superior antibacterial/antibiofilm effects of *S. ningpoensis* honey on *S. aureus* than those of manuka honey (Table 4-5). Moreover, it also explained the protective effect of *S. ningpoensis* honey on the MRSA-infected acute skin wound model (Figure 9).

Before investigating the *in vivo* antibacterial effects of *S. ningpoensis* honey, its toxicity was evaluated in the first place. It did not induce obvious haemolysis in mammalian erythrocytes *in vitro* (Figure 6); and *in vivo*, although *S. ningpoensis* honey affected the behavior and the weight of mice to some extent, the abnormalcy eliminated after 48 h, and it did not cause hepatotoxicity (Figure 7). It suggested that administration of *S. ningpoensis* honey might be relatively safe, and its effect of reducing the levels of aminotransferase, consistent with the reported liver protective function of honey (Abd Rashid et al., 2021), implied that *S. ningpoensis* honey might also have the potential to protect liver or reduce hepatotoxicity. In fact, the *in vivo* toxicity of *S. ningpoensis* honey at the early stage might be attributed to the presence of H_2O_2 as it was reported that cells exposed to a relatively higher concentration of H_2O_2 were subjected to growth arrest and even cell death (Gülden et al., 2010). Nonetheless, cells could develop self-protection against such oxidative stress and adaptability after several hours of H_2O_2 stimulation (Davies, 1999), which might explain the recovery of mice's behavior after being injected with honey for several hours or days (Figure 7A). In this study, it was also found that manuka honey displayed stronger *in vivo* toxicity than *S. ningpoensis* honey (Figure 7A), which might be owing to the MGO in manuka honey as it was reported to induce membrane fragility, haemolysis, and amino group depletion in erythrocytes, as well as DNA damage, loss of cell viability, and elevated glycated products in leukocytes (Prestes et al., 2017).

In terms of the *in vivo* antibacterial activity of *S. ningpoensis* honey, although it could not reduce the

bacterial load in organs via intraperitoneal injection, it significantly reduced the sera level of TNF-α in both *S. aureus* and *P. aeruginosa*-infected mice (Figure 8). In addition to playing a vital role in host defense against bacteria and being an inflammatory marker for bacterial infection, it is noteworthy that TNF-α could exacerbate some bacterial infections, and excess TNF-α is associated with pathology such as atherosclerosis and cancer (Mizgerd, 2003; Gonçalves et al., 2001). Hence, the ability of *S. ningpoensis* honey to reduce the level of TNF-α suggested that it might potentially prevent the exacerbation of bacterial infection and inflammation-associated pathology through controlling the level of TNF-α, supplementing its antibacterial activity. Moreover, in the acute MRSA-infected skin wound model, *S. ningpoensis* honey significantly decreased the bacterial load of the muscles ($p < 0.0001$ *vs.* MRSA group, n = 4, Figure 9), suggesting that it might be a promising antibacterial medication for acute wounds. In combination with the growth curves of the *S. ningpoensis* honey-treated bacteria (Figure 1), it implied that *S. ningpoensis* honey might be effective against the early acute infection, and in clinical practice, the viscidity of *S. ningpoensis* honey might also protect wounds from bacteria invasion and create a moist environment, hastening wound healing (Su et al., 2021). Remarkably, the bacteriostatic and the bactericidal effects of *S. ningpoensis* honey on some common clinical pathogens such as MRSA and *P. aeruginosa* were stronger than manuka honey (Table 4), suggesting that *S. ningpoensis* honey might be a prospective substitute for manuka honey for bacterial infection. As the infection of MRSA basically occurs in the skin and soft tissue (Yue et al., 2016), *P. aeruginosa* can infect the airway, urinary tract, gastrointestinal tract and blood (Wu et al., 2021), and the infection of *H. pylori* usually happens in the gastrointestinal tract or tissues around the eyes (de Brito et al., 2019), it is speculated that *S. ningpoensis* honey may have the potential to be used for the prevention or treatment of cutaneous and gastrointestinal infections according to its effects on the MRSA-infected skin wound model (Figure 9) and the MICs/MBCs against MRSA, *P.*

627 *aeruginosa* and *H. pylori* (Table 4).

628 According to the metabolomic analysis, it was found that among the significantly upregulated
629 compounds in *S. ningpoensis* honey (p-value ≤ 0.05 and VIP ≥ 1, Table S2), adenosine (C00212),
630 chavicol (C16930), pyrimidodiazepine (C02587), trehalose (C01083), palmitoleic acid (C08362),
631 linoleic acid (C04717), salidroside (C06046), D-xylitol (C00379) and perillyl alcohol (C02452)
632 might contribute to its antibacterial function as they were reported to possess antimicrobial activity
633 to different extents (Crippen et al., 2021; El-Kalyoubi et al., 2017; Figueiredo et al., 2020; Hossain et
634 al., 2010; Jain et al., 2016; Kim et al., 2020; Li et al., 2018; Subramanian et al., 2019; Vitali et al.,
635 2012). Furthermore, the KEGG analysis (Table S3 and Figure S6) revealed that some significantly
636 variant compounds in *S. ningpoensis* honey were involved in the pathways associated with the
637 antibacterial process. For example, the down-regulation of some compounds (pyruvic acid, C00022;
638 L-aspartic acid, C00049; pantothenate, C00864; salicylic acid, C00805; isochorismate, C00885)
639 participating in the pantothenate and CoA biosynthesis, or biosynthesis of siderophore group
640 nonribosomal peptides, could influence the survival and propagation of pathogenic bacteria (Crosa
641 and Walsh, 2002; Spry et al., 2008). Ferroptosis and oxidative phosphorylation, involving up-
642 regulation of L-glutamic acid (C00025) and fumaric acid (C00122), were related to oxidative stress
643 and oxidative damage to bacteria (Baecker et al., 2021). Inflammatory mediator regulation of TRP
644 channels, involving up-regulation of histamine (C00388) and 12-keto-tetrahydro-leukotriene B4
645 (C02165), could explain the potential anti-inflammatory activity of *S. ningpoensis* honey (Kono et
646 al., 2021). Biofilm formation-Vibrio cholerae, involving down-regulation of glucose 6-phosphate
647 (C00092), was related to the antibiofilm activity of *S. ningpoensis* honey. Commotion of bacterial
648 chemotaxis, caused by the down-regulation of L-aspartic acid (C00049), D-ribose (C00121) and D-
649 maltose (C00208), was related to the negative influence of *S. ningpoensis* honey on the bacterial

activity (Hou et al., 2021). Thus, the presence of these chemicals in *S. ningpoensis* honey, as well as their participation in the relevant signaling pathways, explained its pronounced antibacterial properties.

5 Conclusion

In this study, we investigated the antibacterial properties of eight kinds of medicinal plant-derived uniflorous honey native to China, and found that *S. ningpoensis* honey possessed the strongest antibacterial effect, comparable to manuka honey. It could induce bacterial death by disrupting the cell membranes and the structures of genomic DNA, as well as regulating the expression of virulence, biofilm formation, adhesion and invasion-related genes in pathogenic microorganisms. In addition, *S. ningpoensis* honey could exert antibacterial function in the acute MRSA-infected skin wounds. Such a significant antibacterial efficacy of *S. ningpoensis* honey indicates its potential for clinical application. Physicochemical and metabolomic analysis further revealed that, apart from H_2O_2, some other nonperoxide compounds present in *S. ningpoensis* honey such as adenosine, chavicol, 4-methylcatechol, trehalose, palmitoleic acid and salidroside, might play a vital role in the antibacterial activity. Therefore, this is the first study that finds the marked antibacterial properties of *S. ningpoensis* honey, and thoroughly elucidates its mode of action and componential profile. It provides an insight into the mechanisms of action of honey in inhibiting the bacterial growth, and facilitates the exploitation and application of medicinal plant-derived honey in place of manuka honey. Meanwhile, it offers clues towards the discovery of antibacterial substances from medicinal plant-derived honey and the corresponding novel target sites in bacteria. Nevertheless, there are several limitations to this study. For example, the doses of honey used for the evaluation of the *in vivo* toxicity appeared to be too high, leading to a relatively high mortality rate of experimental animals; only a

kind of inflammatory marker, TNF-α, was analyzed in the sera of *S. aureus*- or *P. aeruginosa*-infected mice to evaluate the anti-inflammatory effect of *S. ningpoensis* honey. In the future, apart from optimizing the doses of honey applied in the *in vivo* assays and analyzing some additional inflammatory markers, it will be worthwhile investigating the combinational effects of *S. ningpoensis* honey with antibiotics on the multidrug resistant pathogens, as well as isolating and characterizing antimicrobial substances from *S. ningpoensis* honey, in order to facilitate the application of *S. ningpoensis* honey and to provide new strategies for the therapy of infectious diseases.

Ethics approval and consent to participate

Animal studies were performed according to the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines and the Guidelines for the Care and Use of Medical Laboratory Animals (Ministry of Health, China, 1998). The whole procedure was approved and overseen by the Ethical Review Board of Fujian Agriculture and Forestry University (No. PZCASFAFU2019008).

CRediT authorship contribution statement

Tianxing Lin: Conceptualization; Formal analysis; Investigation; Methodology; Visualization; Writing - original draft. **Lei Huang**: Conceptualization; Formal analysis; Investigation; Methodology; Visualization; Validation. **Ningna Cheng**: Investigation; Validation. **Yuzhen Wang**: Methodology; Validation. **Zhen Ning**: Methodology; Validation. **Shaokang Huang**: Funding acquisition; Formal analysis; Resources. **Yuanhua Wu**: Visualization; Writing - review & editing; **Tianbao Chen**: Supervision; Writing - review & editing. **Songkun Su**: Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Writing - review & editing. **Yan Lin**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project

administration; Resources; Supervision; Visualization; Writing - review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interests.

Acknowledgements

This work was supported by the Natural Science Foundation of Fujian Province [No. 2019J01408]; the Outstanding Young Scientist Program of Fujian Agriculture and Forestry University [No. xjq201916]; the National Natural Science Foundation of China [No. 31772684]; the Fuzhou Science and Technology Project [2019-N-3, KH190316A, KH190025A]; and the earmarked fund for CARS [No. CARS-44-KXJ4].

Appendix A. Supplementary data

Figure S1: Micrographs of palynological analysis on honey samples. Pollen grains of *S. ningpoensis* honey (A), *F. simplex* honey (B), *S. chinensis* honey (C), *S. salicifolia* honey (D), *L. japonicus* honey (E), *A. membranaceus* honey (F), *D. longan* honey (G), *L. chinensis* honey (H), and manuka honey (I) observed at 400× magnification.

Figure S2: Inhibitory effects of honey on the growth of model test microorganisms. Effects of honey samples on the growth of *S. aureus* (A), *E. faecalis* (B), MRSA (C), *P. aeruginosa* (D), *C. violaceum* (E), *E. coli* (F), *H. pylori* (G). *, p <0.05; **, p <0.01; ***, p <0.001, compared with the bacterial group, n = 8.

Figure S3: Effects of honey on the formation of *S. aureus* and *P. aeruginosa* biofilms. Biofilm inhibition curves of *S. ningpoensis* honey (A), manuka honey (B) and ampicillin (C) against *S. aureus*.

Biofilm inhibition curves of *S. ningpoensis* honey (D), manuka honey (E), ampicillin (F) and polymyxin B (G) against *P. aeruginosa*. *, p <0.05; **, p <0.01; ***, p <0.001, compared with the control, n = 8.

Figure S4: Eradication effects of honey on the formed biofilms of *S. aureus* and *P. aeruginosa*. Biofilm eradication curves of *S. ningpoensis* honey (A), manuka honey (B) and ampicillin (C) against *S. aureus*. Biofilm eradication curves of *S. ningpoensis* honey (D), manuka honey (E), ampicillin (F) and polymyxin B (G) against *P. aeruginosa*. *, p <0.05; **, p <0.01; ***, p <0.001, compared with the control, n = 8.

Figure S5: Hierarchical clustering of the differential metabolites between *S. ningpoensis* honey and manuka honey.

Figure S6: KEGG enrichment of the differential compounds in *S. ningpoensis* honey *vs.* manuka honey.

Table S1: Compounds identified from *S. ningpoensis* honey and manuka honey.

Table S2: Differential compounds in *S. ningpoensis* honey and manuka honey.

Table S3: KEGG enrichment of the differential compounds in *S. ningpoensis* honey *vs.* manuka honey.

Abbreviations

H_2O_2 hydrogen peroxide

GOX glucose oxidase

Def-1 bee defensin-1 peptide

AMPs antimicrobial peptides

MGO methylglyoxal

UMF Unique Manuka Factor

UPLC-MS/MS high performance liquid chromatography-tandem mass spectrometry

TPC total polyphenol content

GAE, gallic acid equivalents

MIC minimum inhibitory concentration

MBC minimum bactericidal concentration

MRSA methicillin-resistant *Staphylococcus aureus*

CFU colony forming units

MBIC minimum biofilm inhibitory concentration

MBEC minimum biofilm eradication concentration

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

DMSO dimethyl sulfoxide

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

SEM scanning electron microscope

PBS phosphate buffered saline

ALT alanine transaminase

AST aspartate aminotransferase

AKP alkaline phosphatase

TNF-α tumor necrosis factor-α

ELISA enzyme linked immunosorbent assay

PCA principal component analysis

OPLS-DA orthogonal partial least-squares discriminant analysis

VIP variable importance in the projection

GAE Gallic Acid Equivalents

KEGG Kyoto Encyclopedia of Genes and Genomes

NPA nonperoxide activity

QS Quorum Sensing

EPS extracellular polymeric substances

PIA polysaccharide intercellular adhesin

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