HONEY MICROBIOME AND METABOLOME: A VAST RESERVOIR OF NATURAL ANTIMICROBIALS

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Zirui Ray Xiong

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HONEY MICROBIOME AND METABOLOME: A VAST RESERVOIR OF NATURAL ANTIMICROBIALS

Zirui Ray Xiong, Ph. D.

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Raw honeys contain diverse microbial communities, which have the potential to produce antimicrobial secondary metabolites. These naturally occurring antimicrobials are highly valuable for industry application. Investigating honey microbiome and metabolome can provide important information on factors influencing the microbial communities and antimicrobials produced. In our study, amplicon metagenomics was used to analyze the composition of microorganisms in raw honey and investigate environmental and physicochemical variables that are associated with different microbial communities. The analyzed honey samples had relatively similar bacterial communities but more distinct and diverse fungal communities. Honey type was determined as a significant factor influencing alpha and beta diversity metrics of bacterial and fungal communities. Important bacterial and fungal amplicon sequence variants (ASVs) that influenced the overall community were identified. To obtain novel antimicrobials from natural sources, bacteria from raw honey were isolated and their antifungal-producing potential was evaluated. Naturally occurring antifungal secondary metabolites from these bacteria were further purified and identified. Using mass spectrometry and whole-genome sequence data, the main antifungal compound produced by two *Bacillus velezensis* isolates was determined as iturin A, a lipopeptide

exhibiting broad spectrum antifungal activity. Results from this study provide important insights into the microbial communities associated with different types of raw honey and their antifungal metabolites. This research could improve our understanding of microbial dynamics in beehives, improve honey production, and prevent honeybee disease. Currently, there is a high demand for natural, broad-spectrum, and eco-friendly bio-fungicides in the food industry. Naturally occurring antifungal products from food-isolated bacteria are ideal candidates for agricultural and food applications.

BIOGRAPHICAL SKETCH

Zirui Ray Xiong was born in Wuhan, China. He received his Bachelor of Science degree in Biotechnology from University of Science and Technology of China. Following the guidance of a mentor, he developed a strong interest in Food Science and Microbiology. He decided to pursue a master's degree in Food Science at Cornell University in 2016. After receiving the master of professional sciences degree, he worked as a laboratory technician at the Cornell AgriTech HPP Validation Center. Under the influence of great mentors, he started his PhD journey in 2018 spring. Over the past six years at Cornell University, he has worked on several research projects related to food safety, food quality, fermentation, protein chemistry, and bioinformatics. After graduating in summer 2022, he plans to work in the industry as a research scientist.

To my parents, Wang Ping and Xiong Zhizhong, for their love, sacrifice, and dedication.

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Part I. Honey

Physical properties, chemical composition, and biological activities

Honey is produced by honey bees, *Apis melifera*, which collect and transform nectar, plant excretions, and bee secretions into a natural sweet substance (Olaitan et al., 2007). Excess water evaporates during the process, which takes several days. Bees produce invertase, an enzyme that digests sucrose, the predominant sugar in nectar, into glucose and fructose. Honey is a nutritious food that has been consumed by humans for thousands of years. It is viscous, aromatic, sweet, and commonly used as a sweetener for food. Honey has a variety of nutritional and health benefits, making it popular among consumers.

Sugar accounts for about 85% of the total solids in honey. Fructose and glucose are the two major simple sugars, accounting for 85-95% of total sugar (Olaitan et al., 2007). Other monosaccharides, disaccharides, and oligosaccharides are present, including galactose, maltose (7%), isomaltose, and sucrose (1%), accounting for 5-15% of honey (Lazaridou et al., 2004; Ouchemoukh et al., 2010; Ruiz-Matute et al., 2010; Val et al., 1998). Honey crystalizes when monohydrate glucose crystallizes. The crystallization process is influenced by several factors including water and glucose content, micro-chemical composition, and storage conditions.

As a supersaturated solution of sugar, honey has a high viscosity and hygroscopicity. Honey with water content less than 18.8% will absorb moisture from

the environment with a relative humidity of 60% or higher (Olaitan et al., 2007). As a product containing mostly sugar, honey has a low enough water activity to suppress the growth of most bacteria and fungi (Costa et al., 2013; Machado De-Melo et al., 2018). The mean water activity of honey ranges between 0.49 and 0.65 (Cavia et al., 2004; Costa et al., 2013). The growth of most bacteria is suppressed in foods when the water activity is below 0.91, while most yeast and molds are suppressed when the water activity is below 0.7 (Machado De-Melo et al., 2018). The threshold for microorganism growth in foods is 0.6 (Fontana Jr., 2007).

For the nitrogen compounds in honey, total protein ranges from 0.2% to 1.6%, most of which come from bee glands and plant pollen (Chua et al., 2013; Won et al., 2009). Some amino acids in free or bound forms are also detected in honey (Paramás et al., 2006). Invertase (alpha-glucosidase), glucose oxidase, and diastase are the three most common and important enzymes in honey. Invertase and glucose oxidase are produced by the hypopharyngeal glands of honeybees, which help the transition of nectar to honey (Machado De-Melo et al., 2018). Diastase (amylase) originates from either bees or plants, and is used as a measure of honey freshness and an indicator of adulteration (Machado De-Melo et al., 2018). Due to the presence of reducing sugar and amino acids, the Maillard reaction may occur depending on storage and processing conditions, which may yield undesirable compounds like 5-hydroxymethylfurfural (5-HMF) (Iglesias et al., 2006).

Glucose is oxidized by glucose oxidase to produce gluconic acid and hydrogen peroxide. As a slow-release antiseptic, hydrogen peroxide can inhibit the growth of microorganisms in honey, especially prior to sufficient water evaporation to reach low

water activity (al Somal et al., 1994). Honey has a pH between 3.2 and 4.5. Due to the buffering capacity of honey, its pH is not correlated to its acidity. Gluconic acid contributes to the acidity and characteristic taste of honey (Olaitan et al., 2007), and comprises 70-90% of organic acids. More than 30 non-aromatic organic acids are found in different types of honey, which originate from plants or enzymatic reactions and contribute to the color and flavor of honey (Mato et al., 2003; Mato et al., 2006). Acidity in honey can act as a hurdle to the growth of spoilage and pathogenic microorganisms, and free acidity is used for honey quality control (Leistner & Gorris, 1995; Ojeda de Rodríguez et al., 2004; Terrab et al., 2002).

Honey contains flavonoids and phenolic acids, mainly originating from plant nectar, honeydew, and pollen (Ferreres et al., 1992). Phenolic compounds constitute approximately 56-500 mg per kg of honey (Al-Mamary et al., 2002). The most abundant phenolic compounds in honey are myricetin, quercetin, luteolin, protocatechuic acid, and p-hydroxybenzoic acid (Olas, 2020). Phenolic compounds contribute to the functional properties of honey as a healthy sweetener. These bioactive antioxidants can scavenge free radicals and reactive oxygen species (ROS), preventing their damage to cell membrane, enzymes, lipids, and DNA (Samarghandian et al., 2017). Flavonoids, phenolic acids and derivatives, and other organic acids are stable during honey storage and dilution, contributing to the antimicrobial, anti-inflammatory, and immunomodulatory properties of honey (Ben Sghaier et al., 2011).

Additionally, honey contains different vitamins and minerals originating from plants, which are important for human diet (Madejczyk & Baralkiewicz, 2008). Some examples are potassium, calcium, copper, iron, manganese, phosphorus, vitamin C,

thiamine, riboflavin, nicotinic acid, pantothenic acid and so on (Machado De-Melo et al., 2018; Olaitan et al., 2007). In terms of absolute value, the content of these minerals and vitamins is low. But compared to sugar, honey is a healthier sweetener (Bogdanov et al., 2008).

Liquid honey color ranges from clear and colorless to dark amber or black (Olaitan et al., 2007). Some factors influencing honey color include botanical origin, honey age, storage conditions, suspended particles (pollen), and enzymatic reactions (Maillard reaction, lipid oxidation). In terms of chemical composition, sugar, carotenoids, xanthophylls, anthocyanins, phenolic compounds, minerals can have an influence on honey color (de Almeida-Muradian et al., 2014).

Overall, several environmental factors determine the chemical composition and physicochemical properties of honey. These factors include botanical source, geographic origin, soil composition, climate, harvest season, and nectar flux intensity (Ojeda de Rodríguez et al., 2004). Maturation degree (ripeness) of honey and human factors, like extraction, processing, and storage will also influence honey chemical and biological composition (Machado De-Melo et al., 2018). Honeys from different floral sources, season, and location have varied levels of antimicrobial activity (Grabowski & Klein, 2017).

Honey history as traditional medicine

Honey has been used for its medicinal properties by different cultures for millennia (Allsop & Miller, 1996). The first record of humans collecting honey from wild bees dates to 6000 BC, while the first written record of using honey as a drug and

ointment dates back to 2000 BC (Crane, 1975). It was used as a remedy for infection long before bacteria were identified as the causative agents. Early records by Aristotle (*c.* 350 BC) mentioned honey as salve for wounds and sore eyes. Dioscorides from *c.* 50 AD described honey as "good for sunburn and spots on the face" and "good for all rotten and hollow ulcers" (Molan, 1999). More recently, honey was described to effectively clear infection and promote healing (Subrahmanyam, 2005). For wounds without infection, honey can reduce inflammation by quenching free radicals and reducing inflammatory mediators (Al-Waili & Boni, 2003; Bilsel et al., 2002; Postmes, 2001). For infected wounds, honey is a broad-spectrum antimicrobial agent that is effective against both bacteria and fungi (Molan et al., 1988; Radwan et al., 1984). Researchers found that honey can clear the infection of *Pseudomonas* in patients suffering from wounds (Cavanagh et al., 1970). Honey was found to be inhibitory towards a variety of bacterial and fungal pathogens, including *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Streptococcus*, *Salmonella*, *E. coli*, *Vibrio*, *Yersinia*, *Plesiomonas*, *Shigella*, *Clostridium*, *Aspergillus*, *Penicillium*, *Candida*, and others (Efem, 1993; Molan, 1992; Mundo et al., 2004; Obaseiki-Ebor & Afonya, 1984; Obi et al., 1994). In addition to its bacteriostatic and bactericidal effects, honey has also been demonstrated as antiviral and antiparasitic (Maddocks & Jenkins, 2013). Honey is antimicrobial due to its osmotic effect, high acidity, presence of hydrogen peroxide and phytochemicals (Olaitan et al., 2007). Hydrogen peroxide produced by glucose oxidase was determined as the main antimicrobial component of honey (Adcock, 1962; Molan, 1992; White et al., 1963). Non-peroxide antimicrobial activity of honey comes from antioxidants like phenolic compounds, antimicrobial

peptides, and proteinaceous substances (Lee et al., 2008a, 2008b; Molan, 1992; Mundo et al., 2004; Truchado et al., 2009). The factors that may influence the antimicrobial activity of honey include botanical source, bee metabolism, seasonality, geographic location, climate, and other environmental factors (Basualdo et al., 2007).

Honey microbiota

Most microorganisms in honey are dormant and cannot grow or reproduce (Olaitan et al., 2007). These microorganisms originate from pollen, flowers, air, dust, dirt, and the honeybee digestive tract. Spore-forming bacteria, yeasts and molds can be introduced through various sources and survive in honey. Microbial contamination can introduce yeasts and bacteria into honey during human processing. The two most prevalent bacterial families are considered the core microbiota of honey: *Bacillaceae* and *Lactobacillaceae*. Other prevalent bacterial families include *Enterobacteraceae*, *Acetobacteraceae*, *Microbacteriaceae*, and *Bifidobacteriaceae* (Brudzynski, 2021). Lactic acid bacteria are frequently found in plant nectar and honey, including *Lactobacillus* and *Fructobacillus*. Strains of these genera possess antibacterial, antifungal, and anti-biofilm potentials (Berríos et al., 2018; Olofsson & Vásquez, 2008; Ramos et al., 2020). Honey contains probiotic bacteria like *Bifidobacterium* and *Lactobacillus*, originating from honeybee digestive tract. These probiotic bacteria are beneficial to human gut (Olofsson & Vásquez, 2008). Honey is also considered prebiotic due to the presence of oligosaccharides, which increase the population of lactobacilli and bifidobacteria in human gut microbiome (Sanz et al., 1995; Ustunol & Gandhi, 2001; Yun, 1996).

The potential presence of *Clostridium botulinum* spores is a risk associated with raw honey (Brown, 2000). Infants should not consume raw honey because these spores can germinate and grow inside their stomach and produce toxins, resulting in infant botulism (Machado De-Melo et al., 2018). Yeasts and molds that are osmotolerant, xerotolerant, and acidotolerant are also found in honey, including *Bettsia*, *Ascosphaera*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Zygosaccharomyces* (Kačániová et al., 2012; Rodríguez-Andrade et al., 2019). Many of these fungi are considered contaminants and may spoil the honey when the honey moisture content is above 18% (Chaven, 2014). Studying the honey microbiome can reveal bacterial and fungal communities originally from bee digestive tract, flower, and pathogens introduced during harvesting, processing, and storage (Bovo et al., 2020; Kňazovická et al., 2020; Wen et al., 2017). These studies further contribute to our understanding of the nutritional value and health benefits of this highly valuable food products.

Part II. Food microbiome and foodomics studies

Definition, previous investigation, and significance

Microbiome is defined as the total population of all microorganisms and their genomes inhabiting a particular environment, while microbiota is defined as all microorganisms of an ecosystem or a specific niche (Berg et al., 2020). Under this definition, microbiome is the sum of microbiota in an ecological niche and their activities, including structural elements, microbial metabolites, and surrounding environmental conditions.

Microorganisms in foods play important roles, including fermentation, contamination, and spoilage. The rapid development of easy-to-use next-generation sequencing (NGS) technologies along with the lowering of costs to access those technologies has allowed more researchers than ever before to explore the microbiome of foods. Using high-throughput and high-resolution genomics, transcriptomics, proteomics, and metabolomics technologies to study food microbiomes is an emerging research field called “foodomics” (García-Cañas et al., 2012; Herrero et al., 2012). Food microbiomes are important to human health and food production. In-depth sequencing of food microbiomes can provide knowledge of community composition, functional potential, microbial activities and interactions in the environment, which can contribute to fermentation control, food safety and quality improvement, food adulteration prevention, identification of bioactive compounds in complex food systems, and elucidation of agricultural and economic values of these food products (Kafantaris et al., 2021). Foodomics research connects food components, diet, individual health, and diseases (Capozzi & Bordoni, 2013). Some specific applications

include early, rapid, reliable detection of pathogens, antimicrobial resistance genes, toxins, allergens, and other adulterants in foods (Andjelković et al., 2017). Foodomics research increases our understanding of biochemical, molecular, and cellular mechanisms of food microbiomes and their implications on the human microbiome and human health.

Fermented foods were the first type of food products subjected to genomic analysis for microbiome investigation, including cheese, sausages, and kimchi (Ahn et al., 2014; Jung et al., 2013; Lessard et al., 2014; Połka et al., 2015). These studies revealed the dynamics of different microorganisms in food during the fermentation process over time, provided important information on starter cultures and spoilage organisms, and offered guidance on fermentation control. Additionally, the expression of metabolite genes was analyzed to elucidate the competition and survival strategies of these microorganisms as well as their contribution to flavor, nutrition, and human health (Jung et al., 2013; Lessard et al., 2014). Food microbial community structure and dynamics are associated with physicochemical properties of food products (De Filippis et al., 2017). One specific example is the survey of 60 Irish cheeses to evaluate their bacterial diversity (Quigley et al., 2012). Microbial composition was influenced by cheese type, milk origin, ingredients, salt content, and processing conditions. The distribution of microorganisms is spatially heterogeneous, with certain bacteria dominating rind, crust, and core of cheese. Studies on the cheese microbiome provided important information on the roles the microbiota play in cheese ripening, flavor, preservation, spoilage, and ecological dynamics (Ercolini, 2013).

Identifying food microbiota with 16S rRNA gene

The “gold standard” to elucidate the food microbiome is to isolate and identify individual strains via culture-based methods, which are of low efficiency and biased. Some researchers estimate that only 0.1% of the microbial community can be identified with culture-based methods (Cao et al., 2017). The shift from using traditional culture-based methods to NGS technologies to characterize microbial communities in ecological systems is evolutionary, providing insights into diverse and dynamic systems that were previously uncharacterized or only partially characterized.

At present, the most common high-throughput sequencing technology used in food-related research is amplicon sequencing. Marker genes, like the 16S rRNA gene (for bacteria identification), are amplified with primers using metagenomic DNA as templates and sequenced on massively parallel high-throughput platforms.16S rRNA gene sequencing can be used to characterize food microbiota composition and analyze the relative abundance and taxonomy of microorganisms. The 16S rRNA gene is comprised of 9 hypervariable regions flanked by conserved sequences (Neefs et al., 1993). This region is ideal for designing primers for DNA amplification of hypervariable regions and bacterial taxonomic classification. Traditional Sanger sequencing has been used extensively to amplify the 16S rRNA gene region and to investigate the food microbiota when combined with culture methods. However, this method is of low throughput and misses microbial population of low abundance. Using NGS platforms to amplify 16S rRNA gene region can significantly increase the sequencing capacity and thoroughly identify microbial population with a reasonable cost. The requirement for input DNA template is relatively low, making it possible to

use on foods with low bacterial abundance (Cao et al., 2017). However, this method has its limitations. The taxonomic and phylogenetic resolution from 16S rRNA gene sequencing is relatively low, and it cannot be used to classify taxonomy beyond species level. Moreover, the primer selection is complicated. There are 9 hypervariable regions for the 16S rRNA gene, and these regions do not perform equally well for amplicon sequencing. Some studies showed that V4/V5 region performs better than standard V3/V4 region in terms of sequencing efficiency and reducing amplification biases (Claesson et al., 2010). Regardless of the primer pair selection, longer amplicon fragments, longer read length and higher coverage will likely yield better classification results, but the amplification biases persist (Cao et al., 2017).

16S rRNA amplicon sequencing has been used by researchers to evaluate food microbiome composition and provide evidence on the impact of environment on food microbiome. Researchers evaluated the core microbiome of raw milk with 16S rRNA gene sequencing (Rodrigues et al., 2017). Spoilage organisms and pathogens were found in raw milk, including *Acinetobacter*, *Thermoanaerobacterium*, *Enterobacteriaceae*, and *Streptococcus*. A cheese microbiome study using 16S rRNA gene amplicon sequencing found that environmental microbiota from the cheese production site dominated the cheese samples, confirming that processing environment influenced food microbial community and may shape site-specific product characteristics (Bokulich et al., 2018). The microbiome analysis of powdered infant formula with high throughput 16S rRNA gene sequencing found that the most prevalent genera were *Pseudomonas*, *Acinetobacter*, and *Streptococcus* (Anvarian et

al., 2016). Microbiota with the highest diversity were found in areas with low care, and most microorganisms were associated with soil. The microbiota of ready-to-eat fruits and vegetables were found to be influenced by season, irrigation water, and soil using high throughput 16S rRNA gene sequencing (Telias et al., 2011; Williams et al., 2013). These studies provided important insights into the origin of spoilage and pathogenic organisms in fresh produce, contributing to the efforts on spoilage prevention and outbreak investigation (van Dyk et al., 2016).

Whole-genome metagenomic sequencing

To avoid primer and amplification biases, metagenomic sequencing (also called shotgun metagenomic sequencing) can be used to evaluate the microbiome composition by sequencing the entire DNA content in the sample without PCR amplification (Ercolini, 2013). Metagenomic sequencing obtains genetic information of all members within the sample community and provides in-depth taxonomic classification beyond the species level. The metagenomic data can be used to elucidate evolutionary history, community structure, metabolism, and function capabilities. For example, metagenomic analysis of food fermentation process can allow for monitoring of microbiota on strain-level and identifying key enzymes and metabolic activities that facilitate the fermentation process, like sugar and amino acid metabolism and production of flavor compounds (Scholz et al., 2016; Siezen et al., 2008). Furthermore, industrial strains with desirable traits, like high stress tolerance and high metabolism efficiency, can be selected based on metagenomic data and used to produce high quality products (Hao et al., 2011). However, metagenomic sequencing

is substantially more expensive than amplicon sequencing, and produces large amount of data that include virus, bacteria, archaea, fungi, protozoa, algae, and other DNAs present in the sample, making it computationally intensive to analyze and interpret (De Filippis et al., 2017).

Other foodomic approaches

In terms of transcriptomics, high-throughput microarray and RNA-seq have been used in foods to evaluate the presence, growth, and metabolism of foodborne pathogens (Lamas et al., 2019). Other applications include food authentication, detection of adulteration and genetically modified ingredients, and analysis of herbal food metabolites (Kafantaris et al., 2021; Ko et al., 2018; Lancova et al., 2011; Roy et al., 2018). Metatranscriptomic analysis can help us characterize the complex interactions between different microbial communities within a sample. For example, amino acid metabolism during cheese ripening is an important indicator of the roles that fungi and bacteria play in the flavor development and cheese maturation process (Dugat-Bony et al., 2015; Lessard et al., 2014; Monnet et al., 2016). Transcriptomic data provide insights into the metabolic activities of complex microbial communities and shed light into their interaction and dynamics. This information can help us control and manage the fermentation process and improve product quality.

High-throughput proteomic and metabolomic tools can also be used on food products (Andjelković et al., 2017). Some applications include detecting adulteration of pathogens, toxins, and allergens; ensuring proper ingredient composition; studying potential biomarkers for authentication/traceability and bioactive compounds (Bordoni

& Capozzi, 2015; D'Alessandro & Zolla, 2012; Rešetar et al., 2015; Rezzi et al., 2007). Previous studies analyzed the proteome of honey using 2D electrophoresis with mass spectrometry (Borutinskaite et al., 2018; Rossano et al., 2012; Zhang et al., 2019). Glucose oxidase, alpha-glucosidase, and other antimicrobial peptides were identified in honey. Moreover, the proteomic profile of honey could be used as an identifier to differentiate honeys (Azevedo et al., 2017). For the metabolomic profile of honey, researchers used NMR spectroscopy to classify different types of honey (Schievano et al., 2010; Schievano et al., 2012). Metabolite fingerprinting with specific chemical markers can be used to distinguish honey from different geographic origins, floral source, and composition (Boffo et al., 2012; Razali et al., 2018). However, effectively analyzing the proteomic and metabolomic data and combining these analyses with transcriptomic and genomic data is computationally extremely difficult.

With the advancement of NGS and the continuous expansion of the genome database of microorganisms in food, we can develop standardized global surveillance of foodborne pathogens, virulence factors, and antimicrobial resistance genes, making it possible to quickly identify and track contaminated food products and reduce adverse impacts on human health (Schlundt et al., 2020). These tools provide us access to minimize food safety and security problems and improve global public health.

Part III. Controlling microbial contamination in food systems

Overview

Microbial contamination of food products can cause serious health problems, food security issues, and environmental impacts. Food loss and waste generated by foodborne pathogens and spoilage organisms pose risks to a safe and efficient food system. Food waste at the retail and consumer levels was estimated to be 133 billion pounds in 2010, with an estimated value of $162 billion (Thakali & MacRae, 2021). Spoilage and pathogenic microorganisms are ubiquitous in the environment, and can be introduced to food products during production, processing, transportation, retail, and consumer stages (Thakali & MacRae, 2021).

Spoilage microorganisms produce enzymes and by-products that cause the deterioration of odor, appearance, and taste of foods, making products undesirable and unacceptable for consumption, leading to economic loss and food waste (Nychas & Panagou, 2011). Some common spoilage organisms include nonspore-forming lactic acid bacteria, spore-forming *Bacillus* and *Clostridium* species, yeasts and molds (Lorenzo et al., 2018).

Ingestion of food contaminated with pathogens causes foodborne illnesses. Some of the leading bacterial pathogens that cause illnesses, outbreaks, hospitalizations, and deaths in the US include pathogenic *E. coli* (ETEC), *Shigella* spp., *Campylobacter* spp., and *Salmonella* spp.. Foodborne diseases have significant economic and social costs. There are more than 600 million cases of foodborne illnesses and 420,000 deaths caused by 31 foodborne pathogens annually as estimated by WHO (Havelaar et al., 2015). Global burden of foodborne disease is estimated by

the disability adjusted life year (DALY) metric established by WHO, and it was 33 million DALYs in 2010 (Havelaar et al., 2015).

Food safety and spoilage issues caused by pathogens and spoilage organisms are interrelated from ecological and microbiologic perspectives (Petruzzi et al., 2017). Chemical preservatives, such as sodium benzoates, sodium propionates, potassium sorbates, sodium nitrites and nitrates, sulfur dioxide, and other organic acids are commonly used in food products to control microorganism growth and extend shelf life. However, these synthetic chemical preservatives have low consumer acceptance, and may have adverse health effects, especially after long-term exposure (Sharma, 2015; Trasande et al., 2018; Zhong et al., 2018). As an alternative, natural antimicrobial agents produced by plants, animals, mushrooms, bacteria, and other natural sources are highly desirable (Villalobos-Delgado et al., 2019). These bioactive compounds can be incorporated into food formulation, films, coatings, and packaging to increase product shelf life.

Honey is an ecological reservoir of antimicrobial compounds produced by microorganisms originating from plants and honeybees (Brudzynski, 2021). These antimicrobial compounds are considered secondary metabolites, defined as auxiliary metabolites not required for growth and survival of microorganisms, including antibiotics, pigments, hormones, and others (Singh et al., 2019). Some researchers consider honey as a stable colloidal system that preserves the structure and function of bioactive compounds, while releasing them upon honey dilution (Brudzynski & Sjaarda, 2021). Antimicrobial compounds produced by microorganisms in honey are mostly nonselective, partially explaining honey's broad-spectrum inhibition of

bacteria and fungi. Some common antimicrobial compounds produced by bacterial strains include bacteriocins, surfactants, siderophores, and secreted enzymes. Studying these secondary metabolites can lead to discovery of new antimicrobial compounds in honey, with potential application in agricultural and medical fields. Here is a discussion of natural antimicrobials produced by bacteria with potential food applications.

Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial proteins or peptides produced by bacteria like *Lactobacillus*, *Lactococcus*, and *Pediococcus*. Some of these bacteriocins have a highly specific target, while others are broad-spectrum and effective against a variety of bacteria.

Class I bacteriocins are small antimicrobial proteins with 19 to 38 amino acids. All class I bacteriocins undergo post-translational modifications to include uncommon amino acids and structures, such as lantibiotics with inter-residual thioester bonds (Alvarez-Sieiro et al., 2016). Some examples of class I bacteriocins are cyclized peptide enterocin AS-48, negatively charged circular sactipeptide subtilosin A, linear azole/azoline-containing peptides (LAPs) streptolysin S, glycocins, and lasso peptides. One of the most studied bacteriocins is nisin, a class IA lantibiotic produced by *Lactococcus lactis* subsp. *lactis* (Dodd et al., 1990). Nisin is a broad-spectrum bacteriocin that is active against a variety of Gram-positive bacteria including *Staphylococcus*, *Listeria, Lactobacillus*, *Bacillus* etc. It contains thioether amino acids lanthionine and methyllanthionine. Nisin is cationic because of the N-terminal

lanthionine ring. It can bind to the anionic phosphate group on the lipid II of Gram-positive bacterial cell wall, forming peptide-lipid II complex and initiating pore formation (Breukink et al., 1999). Subsequently, the C-terminal peptide is inserted into the cytoplasmic membrane to form transmembrane pore, causing ion efflux, collapse of proton motive force, cell permeabilization, and rapid cell death (Moll et al., 1999; van Heusden et al., 2002). Additionally, nisin can inhibit cell wall biosynthesis by binding to lipid II and disrupting the formation of peptidoglycan chain (Moll et al., 1999; Wiedemann et al., 2001). Nisin is used in food products like milk, cream, yogurt, cheese, canned vegetables, bakery products, cured meat, and others (Delves-Broughton et al., 1996; Silva et al., 2018). However, due to the presence of an outer membrane for Gram-negative bacteria, nisin and other lantibiotics are not able to penetrate and access lipid II on the cytoplasmic membrane and are generally not effective against Gram-negative bacteria, unless at extremely high concentration or combined with chelating agents to compromise the outer membrane (Stevens et al., 1991). Nisin is also limited to products with pH lower than 7 since it loses its activity at high pH (de Arauz et al., 2009).

Class II bacteriocins are small, linear proteins that contain unmodified peptides. These bacteriocins are heat and pH stable (Abriouel et al., 2011). Pediocin is a class IIA bacteriocin produced by *Pediococcus* spp.. Pediocin is broad-spectrum antimicrobial and effective against both Gram-positive and Gram-negative bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *Pseudomonas* and *E. coli* (Silva et al., 2018). Pediocin functions by binding to the receptor of sugar transporter mannose phosphotransferase system and inserting into

target cell cytoplasmic membrane, which leads to pore formation and cell lysis (Diep Dzung et al., 2007). Pediocin is generally recognized as safe (GRAS) and widely used in milk and dairy products, like cream, cottage cheese, and cheese sauce to extend their shelf life (Pucci et al., 1988). Some other examples of class II bacteriocins include two-peptide bacteriocin lactococcin G, leaderless plasmid-encoded two-peptide enterocin L50, and single linear peptide lactococcin A.

Class III bacteriocins are large heat-labile antimicrobial proteins with a molecular weight larger than 10 kDa. These bacteriocins usually have phospholipase activity. Examples include enterolysin A (34.5 kDa), zoocin A (29.2 kDa), and megacin A-216 (66 kDa). Both enterolysin A and zoocin A target the bacterial cell wall by cleaving the peptidoglycan and disrupting the cell wall structure (Khan et al., 2013; Simmonds et al., 1996). Megacin A-216 has a narrow antibacterial spectrum. It functions like phospholipase, converting phospholipids to lysophospholipids and impairing cell membrane integrity (Kiss et al., 2008). Since these bacteriocins are large and heat sensitive, they have yet to be used for food applications.

Nonribosomal peptides (NRPs) and polyketides

Nonribosomal peptides (NRPs) are peptide secondary metabolites synthesized by multidomain mega-enzymes called nonribosomal peptide synthetases (NRPSs). Their synthesis is independent of ribosomes and messenger RNAs (Evans et al., 2011). Bacteria and fungi synthesize these NRPs naturally. The peptide chain of NRPs are usually 3-15 amino acids in linear, cyclic, or branched forms (Mootz et al., 2002). Lipopeptides and siderophores are examples of thiotemplate NRPs. Lipopeptides have

a hydrophilic peptide moiety and a hydrophobic alkyl chain, forming a linear or cyclic structure. These lipopeptides are amphiphilic and can disrupt target cell membranes. Some examples include surfactin, fengycin, and iturin. Surfactins are mainly antibacterial and antiviral, while fengycin and iturin are antifungal. Polymyxin is one of the most used and well-studied cyclic lipopeptide produced by *Paenibacillus polymyxa* and *P. alvei*. Polymyxin can bind to the lipid A of lipopolysaccharide on the outer membrane of Gram-negative bacteria and destabilize the membrane, resulting in membrane permeabilization and cell lysis (Abriouel et al., 2011). In addition to their antimicrobial activities, lipopeptides have also been demonstrated to disrupt biofilm formation, cell motility, virulence expression, and other functions associated with plant defense and root colonization (Raaijmakers et al., 2010). On the other hand, siderophores function by sequestering and depleting iron from the environment and inhibit the proper function of other microorganisms in the niche. Bacillibactin is a common siderophore produced by *Bacillus* spp. and can efficiently chelate ferric iron and reduce its bioavailability, suppressing surrounding microorganisms (Caulier et al., 2019).

Polyketides are a group of natural bioactive secondary metabolites with diverse structure and function. Polyketides are synthesized by multi-domain enzymes polyketide synthases (PKSs). PKSs are categorized into three groups: large and highly modular type I PKS, monofunctional type II PKS, and type III PKS with no acyl carrier protein domains (Ridley et al., 2008). Polyketides are produced by bacteria, fungi, and plants. They act as antibacterial and antifungal agents. The three types of antimicrobial polyketides produced by *Bacillus* spp. are bacillaene, difficidin, and

macrolactin (Caulier et al., 2019). Bacillaene is a polyene polyketide and exhibits inhibition against a variety of bacteria and fungi, including *E. coli*, *B. thuringiensis*, *S. aureus* and *Fusarium*. Difficidin is also a polyene polyketide and is active against bacterial pathogens including *E. coli* and *C. perfringens*. Macrolactin is both antibacterial and antifungal, and is active against *E. coli*, *B. subtilis*, *S. aureus*, *Fusarium* and others.

The biosynthesis of NRPs and polyketides is highly similar, and hybrid NRPS-PKS gene clusters are widespread in bacterial and fungal genome (Wang et al., 2014). Peptide-polyketide hybrids have great structural diversity. Examples include antibiotic bacillaene, mycotoxin fusarin C, cyclic antifungal lipopeptide mycosubtilin, and paenilamicin (Aleti et al., 2015; Fisch, 2013; Van Lanen & Shen, 2006). These natural metabolites have a broad range of biological activities and enormous pharmaceutical potentials.

Enzymes and other compounds

Some other proteins synthesized by bacteria through ribosomes that exhibit antibacterial activities are cell wall degrading enzymes, like cellulase, glucanases, proteases and chitinases, and quorum quenching enzymes that disrupt bacterial quorum sensing, like lactonase, decarboxylase, acylase, and deaminase (Caulier et al., 2019). There are other small antimicrobial secondary metabolites produced by bacteria. Volatile organic compounds (VOCs) that containing sulfur or nitrogen, fatty acids and their derivatives, like benzenoid, terpene, and isoprenoid, are all important for the antimicrobial activities of these bacteria.

Food contamination is a constant threat to the public health and social-economic development around the world, and specific measures must be taken to improve food safety and quality throughout supply chain (Havelaar et al., 2015). Natural antimicrobials produced by food-grade microorganisms, like bacteriocins produced by lactic acid bacteria, are potentially safe for human consumption. These natural antimicrobials can be used as food additives to meet the consumer's expectation of healthy, natural, and safe food products. Currently, nisin and pediocin PA-1 have been used extensively in the food industry to increase product shelf life. Many other enzymes, bacteriocins, nonribosomal peptides, and polyketides have the potential to be used as food preservatives. With the increase of bacterial resistance to conventional antibiotics and the growing consumer demands of natural foods with minimal processing and no added chemical preservatives, antibiotic-producing bacterial strains isolated from food sources and purified antimicrobials produced by these strains have great potentials for food industry applications. Continuous search for novel, safe, broad-spectrum antimicrobials from natural sources with stabilities across a wide range of pH, temperature, and other environmental conditions is necessary.

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CHAPTER 2

MICROBIOME ANALYSIS OF RAW HONEY REVEALS IMPORTANT FACTORS INFLUENCING THE BACTERIAL AND FUNGAL COMMUNITIES

Abstract

Raw honeys contain diverse microbial communities. Previous studies have focused on isolating bacteria and fungi that are culturable, while missing a large proportion of the microbial community due to culture-based constraints. This study utilized next-generation sequencing (NGS) to analyze the composition of microorganisms in raw honey; these data can reveal environmental and physicochemical variables that are associated with different microbial communities. To examine the microbial composition (bacteria and fungi) of raw honey and analyze its association with physicochemical properties, four types of honey (monofloral, wildflower, manuka, and feral; $n_{total} = 36$) were analyzed via amplicon metagenomics. The analyzed honey samples had relatively similar bacterial communities but more distinct and diverse fungal communities. Honey type was determined as a significant factor influencing alpha and beta diversity metrics of bacterial and fungal communities. For the bacterial communities, titratable acidity (TA) was associated with community richness and diversity. For the fungal communities, Brix, TA, and color were associated with community richness, while water activity and color were associated with community diversity. Additionally, important bacterial and fungal amplicon sequence variants (ASVs) that influenced the overall community were identified. Results from this study provide important insights into the microbial communities associated with different types of raw honey, which could improve our understanding of microbial dynamics in

beehives, improve honey production, and prevent honeybee disease.

Introduction

Honey has a diverse microbiome, most of which originates from pollen, flowers, soil, air, dust, and the honeybee digestive tract (Snowdon & Cliver, 1996). Additionally, some secondary microbial contaminants may be introduced into honey during human processing (Snowdon & Cliver, 1996). Honey has a water activity between 0.50 - 0.65. It is generally acidic, with pH ranging from 3 - 5 due to the presence of organic acids like gluconic acid (Balzan et al., 2020; Olaitan et al., 2007). The physicochemical properties of honey have an influence on the microbial communities. The low water activity, low pH, and antimicrobial components (including hydrogen peroxide, antioxidants, and antimicrobial peptides) of honey inhibit the growth of vegetative bacterial cells (Olaitan et al., 2007). Few organisms can survive the osmotic stress of honey; those that do are mainly spore-forming bacteria and yeasts. Previous studies found osmotolerant bacteria that were transmitted to honey from flower nectar through bee pollination (Álvarez-Pérez et al., 2012; Fridman et al., 2012).

Honey-associated microorganisms can be grouped into three types based on origin and ecological niche: bee gut microorganisms, bee pathogens, and plant-associated microorganisms (Bovo et al., 2018). *Lactobacillus* and *Bifidobacterium* lactic acid bacteria (LAB) are major components of the bee gut microbiome and are relatively conserved in honeybee digestive tracts globally (Anderson et al., 2013; Raymann & Moran, 2018). These genera have been found in bee-collected nectar and honey (Olofsson & Vásquez, 2008). Up to 10^8 CFU per gram of viable LAB have been found in different honey samples (Vásquez et al., 2012). A few other bacterial

genera are frequently, though not ubiquitously, found in honeybee digestive tracts; these include *Apibacter*, *Acetobacter*, and *Asaia*. Some rarer bacteria that can cause disease in and death of honeybees may also be found in honeybee digestive tracts; these include *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Serratia* (Raymann & Moran, 2018). Fungal genera found in honeybee digestive tracts include *Saccharomyces*, *Zygosaccharomyces*, and *Candida* (Yun et al., 2018).

Overall honeybee health can be threatened by bacterial and fungal pathogens, which may contribute to colony collapses (Schwarz et al., 2015). Common bacterial pathogens include *Melissococcus*, *Paenibacillus*, and *Spiroplasma*. Fungal pathogens for honeybees include *Ascosphaera*, *Aspergillus*, and *Nosema*. As a common mold found in the environment, some *Aspergillus* spp. are opportunistic pathogens that can infect honeybee larvae and cause stonebrood disease. The common chalkbrood disease is caused by *Ascosphaera apis*, while nosema disease is caused by spore-forming fungi *Nosema apis* and *Nosema ceranae* (Jensen et al., 2013; Schwarz et al., 2015). Bacteria and fungi that are commonly found in plants and soil can be transmitted to beehives through pollination. These plant-associated microorganisms are present in honey and other bee products like bee bread (a fermented mixture of pollen and nectar used as food for bees), and some of these microorganisms are beneficial to the bee colonies (Kurek-Górecka et al., 2020). One example is *Actinobacteria*. Even though some *Actinobacteria* spp. are plant pathogens, many of them are protective microbes for honeybees and other insects because they produce secondary metabolites which prevent fungal growth and spoilage (Anderson et al., 2013; Barke et al., 2010; Mohr & Tebbe, 2006). A variety of *Enterobacteriaceae* and *Firmicutes* were found in flowers

including *Lactobacillus, Bacillus*, and *Weissella* spp., many of which are present in honeybee digestive tracts and honey products. *Lactobacillus kuneei* has been found in flowers, honeybee gut, and bee bread (Anderson et al., 2013). The ubiquitous presence of LAB across different bee species is the result of horizontal transmission between beehives and environment. The high similarity between *Firmicutes* found in flower nectar and those isolated from honeybee hives is further indication that horizontal transmission of these bacteria happens through honeybee pollination (Vásquez et al., 2012). The plant-associated bacteria *Paenibacillus* spp. are commonly found in soil. Some species of *Paenibacillus* are bee pathogens: *P. larvae* is the causative agent for American foulbrood disease; *P. alvei* is commonly found as a secondary invader of European foulbrood disease caused by *Melissococcus plutonius* (Genersch, 2010). As for the common plant-associated fungi found in honey, *Cladosporium* is a filamentous fungus that is common in the environment, and some species are potential plant pathogens (Bensch et al., 2012). It was proposed that *Cladosporium* could cohabit with bees and transmit from plant or bees to persist in bee products (E. O. Martinson et al., 2012). Other filamentous fungi that are commonly found in plant pollen include *Botrytis*, *Penicillium*, and *Mucor*, which are transmitted to honeybees and frequently found in bee bread (Disayathanoowat et al., 2020). Some common genera of yeast that were isolated from pollen and bee bread include *Candida, Cryptococcus*, *Kloeckera*, *Metschnikowia*, and *Rhodotorula* (Gilliam et al., 1974). Flower-derived microorganisms are subjected to environmental changes, which in turn contribute to the variation, growth, and secondary metabolite production of other environment-derived microorganisms in honeybees (Vásquez et al., 2012).

As previous studies suggest, microbial and honeybee DNA present in honey reflect the hive microbiome and honeybee hologenome; these data may reveal the bee pathosphere and indicate overall bee colony health (Bovo et al., 2020). Analyzing the honey microbiome can potentially help in the understanding of microbial hive dynamics, which may improve honey production and prevent honeybee diseases. However, most previous honey microbiome studies use traditional culture-based methods to isolate and identify microorganisms in honey, which is subject to culture biases (Anderson et al., 2013). Using culture-independent methods to investigate the microbiome of honey avoids biases induced by researcher-selected growth conditions. Recent studies have used next-generation sequencing (NGS) methods to study the microbiome of honeybee gastrointestinal tracts, pollen, and bee bread, while metagenomic analyses of honey are limited (Disayathanoowat et al., 2020; Engel et al., 2012; Jones et al., 2018; Moran et al., 2012; Powell et al., 2014; Yun et al., 2018).

In our study, we used 16S and ITS metabarcoding method to evaluate and compare the microbiomes of raw honey derived from different sources. We selected monofloral honey and multifloral honey from central NY region. To compare the differences between different honey types, we also chose to include two special types, manuka honey and feral honey, that have not been studied previously and could potentially have distinct and interesting microbial communities. Manuka honey is a highly valuable New Zealand monofloral honey with antimicrobial and antioxidant capabilities (Niaz et al., 2017). The high content of antioxidants could be produced by certain microorganisms in honey, which would deter the growth of other microorganisms in the environmental niche (Brudzynski, 2021). Feral honey is

produced by domesticated western honey bees *Apis mellifera* that swarmed and established wild colonies (Hinshaw et al., 2021). Feral colonies are able to survive in the wild without human management and develop mechanisms to defend against varroa mites and other pathogens (Youngsteadt et al., 2015). The microbiome of feral honeybees is potentially associated with the strong immune systems and mite survival strategies of these bee colonies, which could potentially be reflected in the honey.

Previous studies have reported the association between honey microbiomes and parameters like moisture, electrical conductivity, and botanical origin (Balzan et al., 2020; Kňazovická et al., 2020; Wen et al., 2017). To further evaluate different physicochemical parameters of raw honey and their association with the microbiome of different types of honey, we measured honey pH, water activity, Brix, titratable acidity, color, and evaluated their association with microbial community diversity. We found that the bacterial communities among honey samples were relatively conservative, while fungal communities were more diverse. Some physicochemical properties of honey, including titratable acidity, water activity, and color, were associated with microbiome composition. To the best of our knowledge, this is the first article assessing the microbiome of manuka honey and feral honey via amplicon metagenomics.

Materials and methods

Honey sample collection

In this study, we performed physicochemical and microbiome analysis on four types of honey: monofloral, wildflower, manuka, and feral. Monofloral honey was purchased from two local honey shops (Ithaca, NY). Different floral sources were selected, including basswood, bamboo, buckwheat, orange blossom, goldenrod, and black locust. Wildflower honey was purchased from three honey shops in the central NY region. For the New Zealand manuka honey, three different brands were purchased online. Three feral honey samples were provided by a local beekeeper (Utica, NY), where the honeys were collected from swarmed honeybees. Honey samples were stored at room temperature until processing, due to honey's shelf-stable nature. A total number of 36 honey samples were analyzed in this study.

Physicochemical analysis of raw honey

All honey samples were subject to physicochemical analysis. pH, titratable acidity, and Brix were measured using pH meter (pHi 470, Beckman Coulter, Brea, CA), automatic titrator (Ti-Note EasyPlus Titrators AP002, Mettler Toledo, Columbus, OH), and pocket digital refractometer (Sper Scientific, Scottsdale, AZ). Water activity was measured with water activity meter (AQUALAB 4TE, METER Group, Pullman, WA) and color was measured with Chroma Meter (Konica Minolta CR-400, Tokyo, Japan) using CIELAB scale. All measurements were performed in triplicate.

DNA extraction, library preparation, and Illumina amplicon sequencing

Honey was dissolved in phosphate-buffered saline (PBS) and treated with 1500

U/mL catalase to remove hydrogen peroxide that could be produced during dilution (Brudzynski et al., 2011; Chen et al., 2012). The 50% (w/w) honey solution was incubated at room temperature for 2 hours and centrifuged at 10,000 rpm, 4 °C for 15 min. The pellet was resuspended in 10 mL PBS and centrifuged at 10,000 rpm, 4 °C for another 15 min. DNA was extracted from this pellet with the DNeasy PowerSoil Pro Kit according to the manufacturer's recommendation. Illumina MiSeq library preparation for 16S rRNA and ITS gene amplicon was performed. The 16S V3-V4 region was amplified with primers IL_Bakt341F (CCTACGGGNGGCWGCAG) and IL_Bakt805R (GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011; Klindworth et al., 2013). A 0-4 bp heterogeneity spacer between Illumina index sequence and the 16S locus-specific primer was included to improve sequencing quality on the flow cell (Fadrosh et al., 2014). The ITS 5.8S-ITS2 region was amplified with primer IL_5.8SFungF (AACTTTYRRCAAYGGATCWCT) and IL_ITS4FungR (AGCCTCCGCTTATTGATATGCTTAART) (Taylor et al., 2016). Similarly, a 0-4 bp heterogeneity space was added between Illumina index sequence and the ITS primer. A two-step library preparation was adapted from a previous study by Holm et al. (2019). Successful target amplification from the first PCR was verified by gel electrophoresis and samples were then submitted to the Cornell Biotechnology Resource Center, Cornell Institute of Biotechnology (Ithaca, NY), for indexing and sequencing. Samples were quantified by Qubit 4 Fluorometer and then normalized prior to performing unique dual indexing. After dual indexing, samples were pooled and the library was cleaned using AMPure XP beads. Quality control with fragment analysis confirmed the correct distribution of fragment lengths. An Illumina MiSeq 2 x

250 bp (V2 chemistry) reagent kit was used to sequence the library. Two PCR negative controls and 4 extraction negative controls were included in this study. A total number of 84 amplicon samples were sequenced.

Data Analysis

QIIME 2 2021.11.0 was used to process and analyze the demultiplexed 16S and ITS amplicon sequencing data (Bolyen et al., 2019). Primers were trimmed from raw reads of 16S and ITS sequences using q2-cutadapt plugin. To achieve more accurate fungal taxonomic classification, demultiplexed ITS sequences were trimmed and conserved regions were removed using the q2-ITSxpress plugin (Rivers et al., 2018). DADA2 was used to filter, denoise, and merge trimmed reads to identify all observed amplicon sequence variants (ASVs) (Callahan et al., 2016). The chimeric sequences identified by DADA2 were removed. Taxonomy assignment was performed using a precomputed naïve Bayesian classifier (SILVA version 138 reference alignment for 16S rDNA sequences and UNITED version 8.3 database for ITS sequences) using q2-feature-classifier (Bokulich et al., 2018).

Downstream analyses and visualization, including diversity analysis, statistical testing, and microbial community composition were performed in R (version 4.1.1). For 16S and ITS sequences, ASVs identified as mitochondria or chloroplast by the classifier were treated as contaminants and removed. Unknown ASVs at the phylum level were removed; these were typically unassigned mitochondria or chloroplast sequences (data not shown). Further decontamination of the sequences was based on extraction control and PCR control using “decontam” package in R (Davis et al., 2018). Sequences were rarefied and normalized with “phyloseq” package in R by

resampling the abundance values to achieve parity between samples (McMurdie & Holmes, 2013). The most abundant bacterial and fungal ASVs in honey samples were visualized with "ggpubr" package (version 0.4.0). Alpha diversity metrics, including Shannon diversity, Simpson and inverse Simpson diversity, Pielou's evenness, ACE and Chao richness metrics, were calculated with "vegan" R package (version 2.5-7). For normally distributed alpha diversity metrics, ANOVA with Tukey's honest significance test was used to perform pairwise comparisons between groups of categorical variables. General linear model with normal distribution was used to fit alpha diversity metrics to continuous variables. For non-normally distributed alpha diversity metrics, Wilcoxon rank sum test with false discovery rate (FDR) corrections for multiple comparisons. Kruskal-Wallis tests were performed on categorical variables. General linear model with quasipoisson distribution was used to fit continuous variables. For beta diversity, Bray-Curtis dissimilarity, Jaccard distance, and phylogeny-based UniFrac (weighted and unweighted) metrics were calculated (Beals, 1984; Jaccard, 1912; Lozupone & Knight, 2005; Lozupone et al., 2007). To visualize the differences in microbiome composition, beta diversity metrics were plotted with non-metric multidimensional scaling (NMDS). The multivariate homogeneity of group dispersion was tested by beta dispersion and the community composition was compared with permutation analysis of variance (PERMANOVA) using the "adonis" function in the "vegan" package with 1000 permutations. Additional visualizations, including heat maps and Venn diagrams, were created in R.

Results

A total of 36 honey samples were subjected to physicochemical analysis and genome extraction for amplicon sequencing and microbiome analysis. The physicochemical data, including Brix, pH, titratable acidity, color, and water activity, were summarized in Table 2.1. Two feral honey samples, FF1 and FF2, were missing physicochemical data due to limited sample quantity. These two samples were subjected to 16S and ITS amplicon sequencing but were removed when performing diversity analyses.

A total of 2,040,648 raw 16S amplicons and 4,084,874 raw ITS amplicons were sequence for 42 samples, including 4 extraction controls and 2 PCR controls. After trimming adapter sequences and primers, filtering low quality reads, denoising, and removing chimeric sequences, a total of 1,317,356 16S and 2,308,930 ITS reads remained for downstream analyses. After removing sequences unidentified at phylum level and decontamination using PCR and extraction controls, a total number of 1,285,423 16S reads remained. For ITS sequence, there were 2,305,376 reads retained after removing sequences unidentified at class level. After careful consideration, we removed two extraction contaminants identified as *Yarrowia lipolytica* and only used 830,110 reads of ITS sequence for downstream analysis. Before rarefaction and normalization, samples with low reads were removed: 16S sequences of 2 monofloral honey (HRG2 and HRO2) and 1 wildflower honey (W21S2), ITS sequences of 1 monofloral honey (HRO2) and 2 feral honeys (FF1 and FF2). Rarefaction curves for 16S and ITS sequences were visualized in Supplemental Figure 2.1. All samples reached plateau after resampling, indicating that the sequencing depth was sufficient

to capture microbial community diversity of the samples. To visualize the most abundant genera for bacterial composition, bacterial ASVs were agglomerated to the genus level, and genera with abundance higher than 0.05% were selected for honey bacterial composition plot (Figure 2.1). Similarly, fungal ASVs were agglomerated to the species level, and species with relative abundance higher than 0.3% were selected for honey fungal composition plot to illustrate the most abundant fungal species (Figure 2.2). The bacterial community showed less variability compared to fungal community, and it was dominated by *Lactococcus lactis*. Some other common genera of bacteria include *Citrobacter*, *Pseudomonas*, *Serratia*, and *Cedecea*. Specific fungal species were dominant in certain samples. *Yarrowia lipolytica* and *Bettsia alvei* were dominant in some of the monofloral, manuka, and wildflower honey, while feral honey was dominated by *Zygosaccharomyces mellis.* Some other fungal species were found in particular types of wildflower honey, such as *Skoua* sp., *Zygosaccharomyces rouxii*, *Ascosphaera celerrima*, and *Saccharomyces* sp. The core honey microbiome can be represented by shared taxa among different types of honey. As shown in the Venn diagrams (Figure 2.3), 66 bacterial ASVs were shared amongst all four types of honey, while there was 0 fungal ASV shared by all four types of honey. Based on our result, we can presume that there is a core bacterial microbiome for honey. Additionally, 167 fungal ASVs were present only in wildflower honey and 80 fungal ASVs were found only in monofloral honey, which further demonstrated that honey has a diverse and distinct fungal community.

Table 2.1. Physicochemical properties of honey. Each value is the mean of three measurements.

Table 2.1.

Sample ID	Honey Type	Brix	pH	TA	Color_L*	Color_a*	Color_b*	Aw
S1	Manuka	77.33	3.91	0.03427	29.67	2.26	10.62	0.6087
NZ1	Manuka	77.93	4.08	0.02843	30.45	0.94	11.64	0.5985
A1	Manuka	76.03	3.74	0.03327	30.72	2.23	12.42	0.6002
HRB1	Monofloral	81.77	4.06	0.03222	32.77	0.16	9.25	0.5433
HRO1	Monofloral	79.2	3.69	0.03465	31.56	0.37	11.85	0.5285
HRG1	Monofloral	78.3	4.07	0.03442	30.79	1.06	12.34	0.5425
WBB1	Monofloral	79.73	4.05	0.01967	29.1	3.59	12.75	0.5465
WBW1	Monofloral	80.37	4.2	0.03327	32.8	-0.01	8.51	0.5487
WBU1	Monofloral	79.63	3.79	0.03756	22.65	3.91	3.8	0.5602
WL1	Monofloral	78.73	4.11	0.02223	32.8	0.09	8.84	0.5226
W20F1	Wildflower	81.8	4.23	0.01302	29.04	2.78	12.22	0.561
W21S1	Wildflower	82.27	4.3	0.01152	30.84	0.45	10.87	0.5378
W21F1	Wildflower	80.03	3.93	0.01317	32.86	-1	7.38	0.5761
JS1	Wildflower	77.77	3.75	0.01245	41.05	-1.04	7.84	0.5651
KH1	Wildflower	82.8	4.05	0.01352	32.14	0.84	11.35	0.5287
FR1	Feral	77.4	4.29	0.0119	29.6	0.16	3.21	0.6337

FF1	Feral	NA*	NA	NA	NA	NA	NA	NA
FH1	Feral	77.03	3.84	0.0208	35.64	-0.62	8.1	0.6168
S2	Manuka	77.33	3.91	0.03427	29.67	2.26	10.62	0.6087
NZ2	Manuka	77.93	4.08	0.02843	30.45	0.94	11.64	0.5985
A2	Manuka	76.03	3.74	0.03327	30.72	2.23	12.42	0.6002
HRB2	Monofloral	81.77	4.06	0.03222	32.77	0.16	9.25	0.5433
HRO2	Monofloral	79.2	3.69	0.03465	31.56	0.37	11.85	0.5285
HRG2	Monofloral	78.3	4.07	0.03442	30.79	1.06	12.34	0.5425
WBB2	Monofloral	79.73	4.05	0.01967	29.1	3.59	12.75	0.5465
WBW2	Monofloral	80.37	4.2	0.03327	32.8	-0.01	8.51	0.5487
WBU2	Monofloral	79.63	3.79	0.03756	22.65	3.91	3.8	0.5602
WL2	Monofloral	78.73	4.11	0.02223	32.8	0.09	8.84	0.5226
W20F2	Wildflower	81.8	4.23	0.01302	29.04	2.78	12.22	0.561
W21S2	Wildflower	82.27	4.3	0.01152	30.84	0.45	10.87	0.5378
W21F2	Wildflower	80.03	3.93	0.01317	32.86	-1	7.38	0.5761
JS2	Wildflower	77.77	3.75	0.01245	41.05	-1.04	7.84	0.5651
KH2	Wildflower	82.8	4.05	0.01352	32.14	0.84	11.35	0.5287
FR2	Feral	77.4	4.29	0.0119	29.6	0.16	3.21	0.6337
FF2	Feral	NA	NA	NA	NA	NA	NA	NA
FH2	Feral	77.03	3.84	0.0208	35.64	-0.62	8.1	0.6168

*Physicochemical data were not available for FF1 and FF2 due to limited amount of honey samples collected.

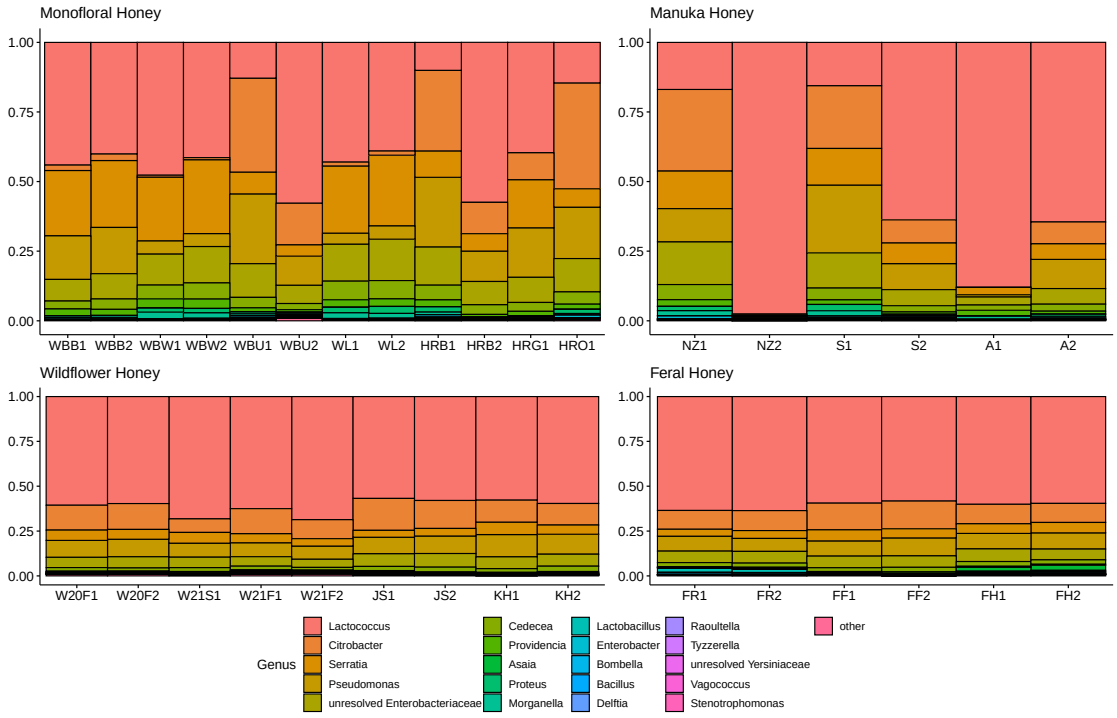

Figure 2.1. Honey bacterial composition plot. 16S ASVs were agglomerated to the genus level for each honey sample. Genera with relative abundance higher than 0.05% across samples were selected and plotted. Genera with less than 0.05% were agglomerated as “others”.

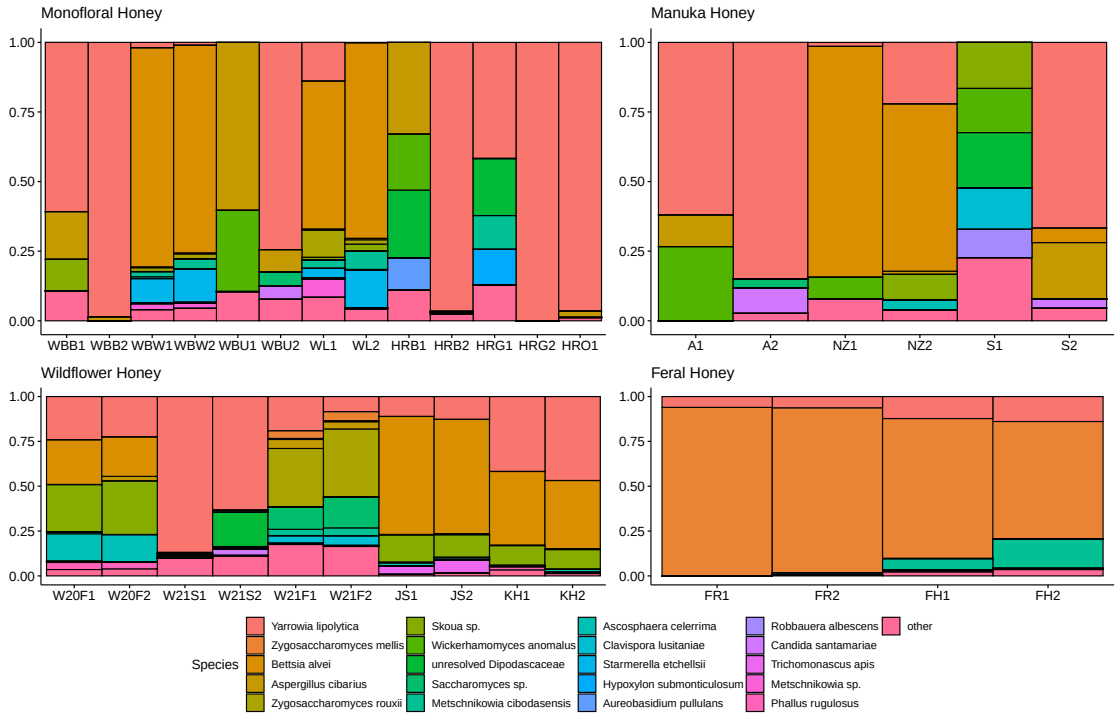

Figure 2.2. Honey fungal composition plot. ITS ASVs were agglomerated to the species level for each honey sample. Species with relative abundance higher than 0.3% across samples were selected and plotted. Species with less than 0.3% were agglomerated as “others”.

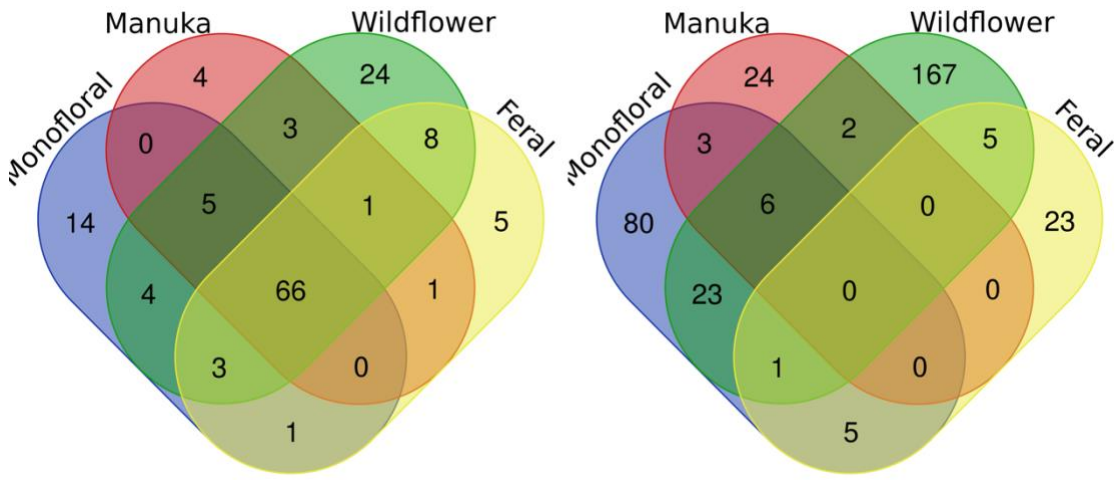

Figure 2.3. Venn diagrams for bacterial and fungal ASVs of four honey types. Bacterial and fungal ASVs were grouped based on four types of honey: monofloral, manuka, wildflower, and feral. ASVs shared between different types of honey were labeled in the overlapping area in the diagram. Left: bacterial. Right: fungal.

To evaluate the species diversity within each honey type, alpha diversity of bacterial composition was assessed with Shannon diversity, inverse Simpson diversity, Chao richness and ACE richness indices, while alpha diversity of the fungal composition was measured with Shannon diversity, inverse Simpson diversity, Chao richness and Pielou's evenness indices. Bar plots of alpha diversity indices grouped by honey types were visualized in Figure 2.4 and 2.5. ANOVA analysis reported p-value below 0.05 for all 4 metrics of bacterial alpha diversity and 1 metric of fungal alpha diversity (Chao richness), indicating that there were differences in the mean of these

indices between honey types. Pairwise comparisons using Tukey's HSD test showed that there were significant differences between the bacterial community richness of monofloral and wildflower honey as measured by Chao and ACE indices ($p < 0.05$). In terms of the bacterial community diversity, there were significant differences between wildflower and monofloral honey as estimated by Shannon diversity metric ($p = 0.0144$) and between wildflower and manuka honey as measured with inverse Simpson diversity ($p = 0.0416$). Considering that Shannon diversity and inverse Simpson diversity metrics were not normally distributed, we thus performed Kruskal-Wallis rank sum test on these two metrics and found that they also differed by honey types ($p < 0.01$). Pairwise comparison was performed using Wilcoxon rank sum exact test and p-value was adjusted with false discovery rate (FDR) correction. Monofloral honey was found to be significantly different from both feral and wildflower honey as estimated by Shannon and inverse Simpson diversity metrics ($p < 0.01$). Fungal diversity metrics (Shannon and inverse Simpson) were relatively similar between different honey types. For the fungal community richness, only wildflower honey showed significant difference from the other three types of honey as estimated by Chao richness ($p < 0.05$). Similarly, Kruskal-Wallis rank sum test was used for non-normally distributed Chao richness metric and significant differences were found between groups ($p < 0.05$). Wildflower honey had a significantly different richness compared to the other three types of honey ($p < 0.05$) using Wilcoxon rank sum exact test with FDR adjustment.

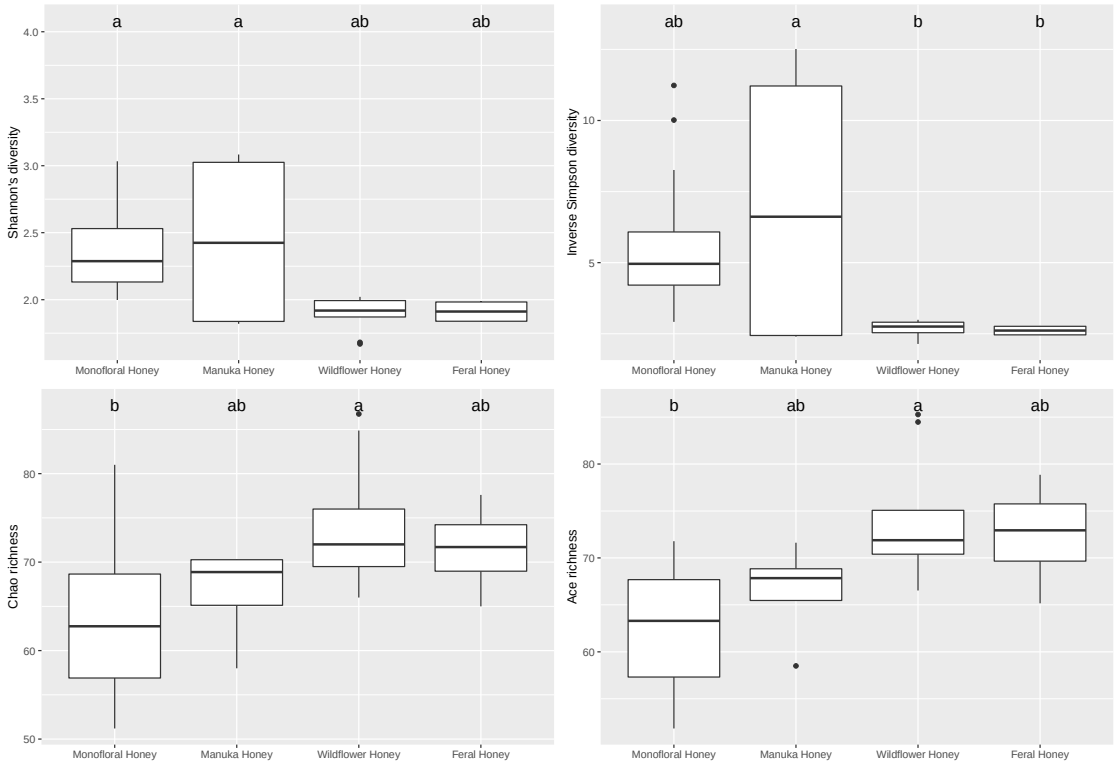

Figure 2.4. Alpha diversity metrics of honey bacterial community. Diversity was measured with Shannon and inverse Simpson indices. Richness was measured with Chao and ACE indices. Comparison between honey sample types was performed with ANOVA and Tukey's honest significance test. Letters above the bar plots represented shared significance groups (p-value cutoff is 0.05).

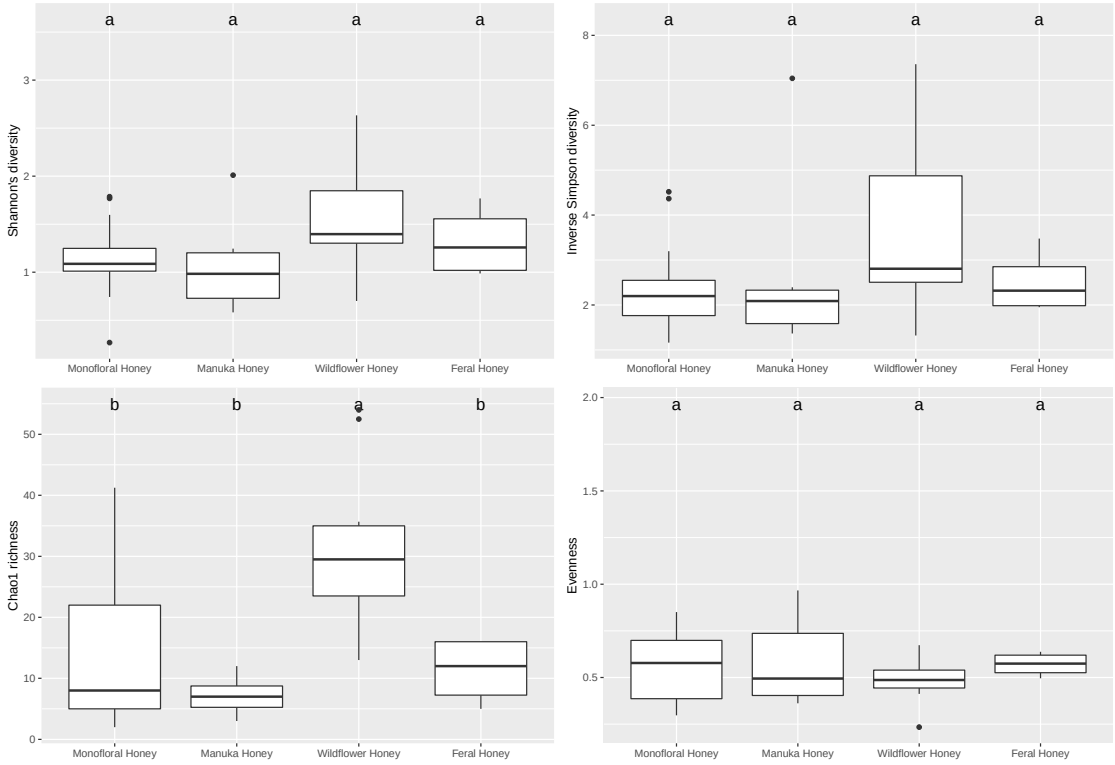

Figure 2.5. Alpha diversity metrics of honey fungal community. Diversity was measured with Shannon and inverse Simpson indices. Richness was measured with Chao index. Evenness was measured with Pielou's evenness index. Comparison between honey sample types was performed with ANOVA and Tukey's honest significance test. Letters above the bar plots represented shared significance groups (p-value cutoff is 0.05).

Physicochemical properties were tested for their correlations to alpha diversity metrics. For the bacterial community, titratable acidity (TA) was found to be associated with ACE richness by fitting the data to a general linear model (p-value = 0.0445). For the non-normal diversity metrics, TA was also found to be significantly

correlated with Shannon (t value = 4.025, Pr(>|t|) = 0.000414) and inverse Simpson (t value = 3.860, Pr(>|t|) = 0.000641) metrics using quasipoisson distribution (p-value < 0.001). For the fungal community composition, Brix, TA, and color (L* and a*) were found to be significantly associated with Chao richness estimator using quasipoisson distribution ($p < 0.05$). However, Pielou's evenness index and diversity metrics were not correlated with any physicochemical properties.

To evaluate the degree of differentiation among microbial communities of different honey types, Bray-Curtis and Jaccard beta diversity indices were calculated for both bacterial and fungal community of each sample. Weighted and unweighted UniFrac distance metrics were calculated only for the bacterial community but not for the fungal community, because ITS sequences cannot be used to inform evolutionary distances among distantly related species (Lücking et al., 2020; Schoch et al., 2012). The differences of Bray-Curtis index between samples were visualized with heatmap for both bacterial and fungal community (Figure 2.6). Honey samples were separated into three clusters based on bacterial composition, while fungal composition was divided into 12 clusters. Overall, honey samples used in this study had similar bacterial composition, but the fungal composition was more diverse. Varying degrees of overlap can be observed for clusters of each honey type in NMDS plots, especially for the fungal community of monofloral, wildflower, and manuka honey (Figure 2.7 and 2.8). PERMANOVA analysis showed significant differences in microbial community composition for different honey types using Bray-Curtis dissimilarity (pseudo $F = 4.2385$, $R^2 = 0.33714$, $p = 0.001998$ for bacterial community, pseudo $F = 2.7998$, $R^2 = 0.22459$, $p = 0.000999$ for fungal community). Pairwise comparison

between honey types was performed to further evaluate the differences. Results showed significant differences between the bacterial community of monofloral and wildflower honey using Bray-Curtis index (pseudo F = 9.0657543, R^2 = 0.32301837, p = 0.001, adjusted p-value = 0.006). The differences of bacterial community between monofloral and feral honey was also significant (pseudo F = 4.3954513, R^2 = 0.23894229, p = 0.008, adjusted p-value = 0.048). Other distance metrics for bacterial community, including Jaccard, weighted UniFrac and unweighted UniFrac showed similar results. As for the fungal community, pairwise comparison between different honey types showed significant differences between monofloral honey and feral honey using Bray-Curtis index (pseudo F = 4.8119958, R^2 = 0.24288294, p = 0.001, adjusted p-value = 0.006). The fungal composition differences between wildflower and feral honey were also significant (pseudo F = 7.1475886, R^2 = 0.37328923, p = 0.002, adjusted p-value = 0.012). Although the adjusted p-value was higher than 0.05 for the pairwise PERMANOVA analysis between manuka honey and feral honey using Bray-Curtis distance metric (pseudo F = 7.0767424, R^2 = 0.46938140, p = 0.009, adjusted p-value = 0.054), the difference of Jaccard distance metric for these two honey types was significant, with an adjusted p-value lower than 0.05 (p = 0.005, adjusted p-value = 0.030).

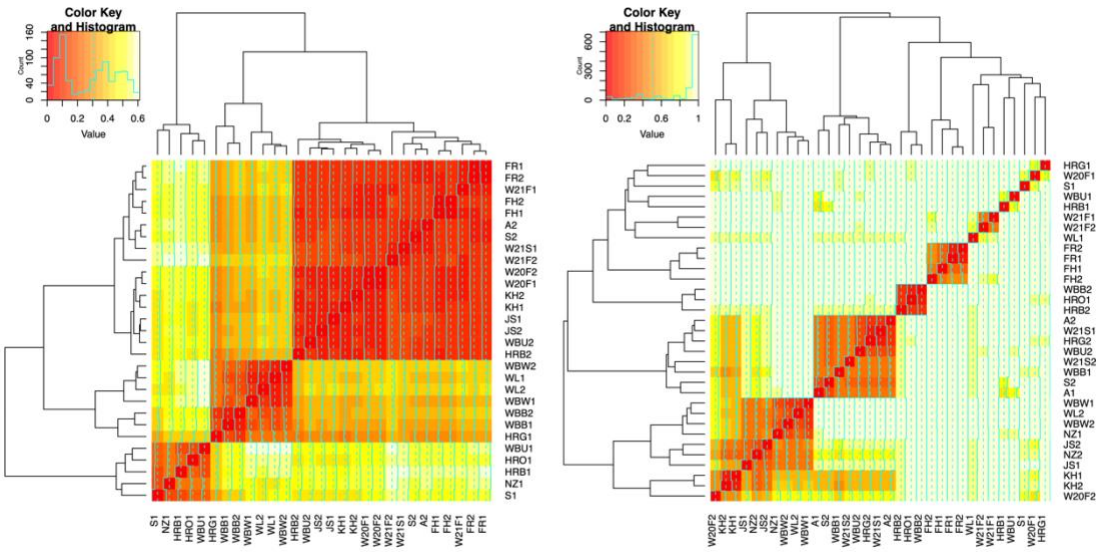

Figure 2.6. Heatmaps of Bray-Curtis distances between honey bacterial and fungal community. Left: bacterial community. Right: fungal community. Each line and column represented a honey sample. The degree of similarity based on Bray-Curtis distances was represented by the color and dendrogram. Color red represented high similarity while light yellow represented low similarity. Samples grouped together in the dendrogram were highly similar. Color key and histogram in the top left corner of the heatmap represented the distribution of Bray-Curtis distances.

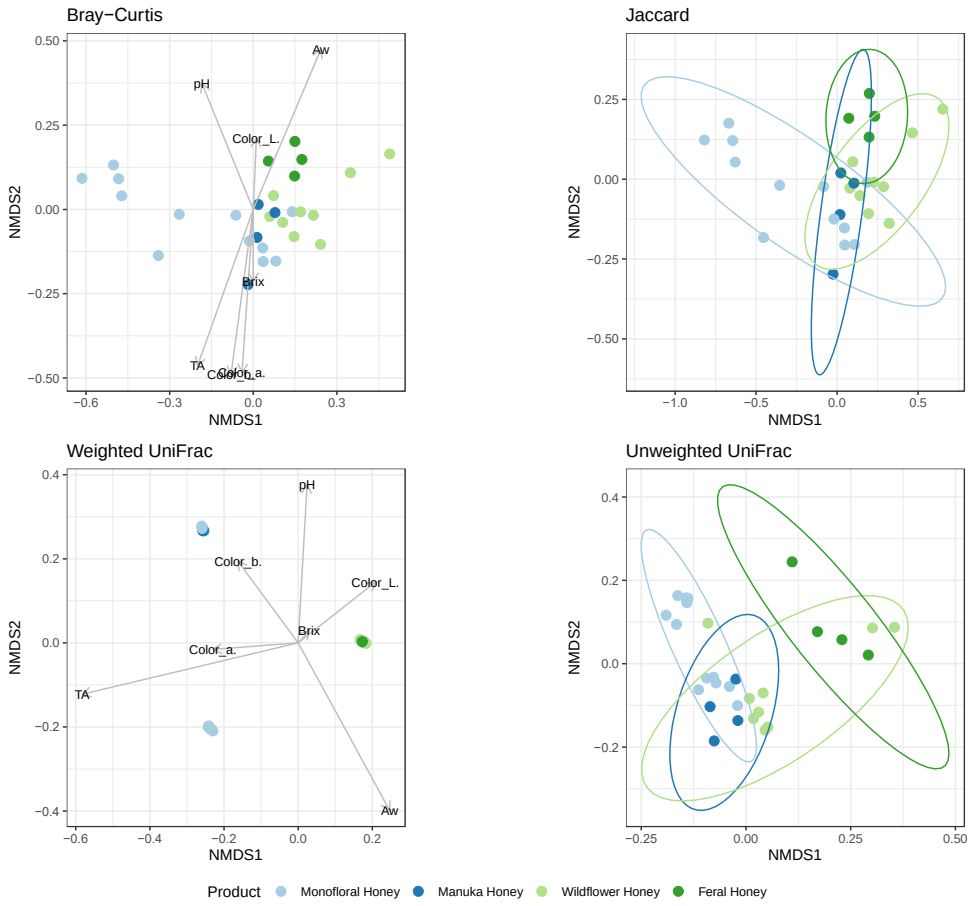

Figure 2.7. Non-metric multidimensional scaling (NMDS) ordination for bacterial community structure based on the relative abundance of 16S ASVs. Community dissimilarity was evaluated with four metrics: Bray-Curtis, Jaccard, weighted UniFrac, and unweighted UniFrac. Arrowed lines (vectors) showing correlation between physicochemical properties and community dissimilarity were plotted for Bray-Curtis and weighted UniFrac metrics. The vectors represented mean direction and strength of correlation. Ellipses indicating confidence intervals of 95% for all honey types were plotted for Jaccard and unweighted UniFrac metrics.

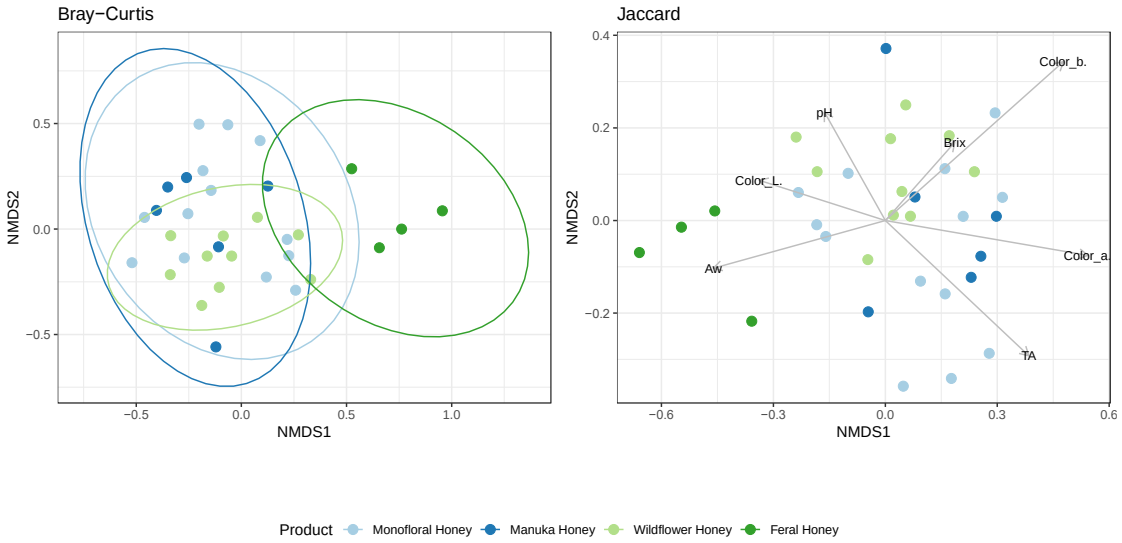

Figure 2.8. Non-metric multidimensional scaling (NMDS) ordination for fungal community structure based on the relative abundance of ITS ASVs. Community dissimilarity was evaluated with Bray-Curtis and Jaccard metrics. Ellipses indicating confidence intervals of 95% for all honey types were plotted for Bray-Curtis dissimilarity. Arrowed lines (vectors) showing correlation between physicochemical properties and Jaccard dissimilarity were plotted. The vectors represented mean direction and strength of correlation.

Based on the visualization of the beta diversity metrics and the beta dispersion test, these differences in beta diversity can be attributed to the non-homogeneous distribution of each honey group. Permutation test for homogeneity of multivariate dispersions showed that the group distances of bacterial Bray-Curtis index were significant ($F = 6.4887$, $Pr(>F) = 0.002997$). Pairwise comparison further

demonstrated that the dispersion of wildflower and feral honey was significantly different from manuka and monofloral honey ($p < 0.05$). Similarly, the group distances of fungal Bray-Curtis index were significant ($F = 6.6999$, $Pr(>F) = 0.002997$). Further pairwise comparison showed that the beta dispersion of feral honey was significantly different from the other 3 types of honey ($p < 0.05$).

To elucidate the relationship between physicochemical properties and the microbial community, all physicochemical parameters were treated as continuous variables and fitted to the bacterial Bray-Curtis metric, bacterial weighted Unifrac, and fungal Jaccard metric. Vectors of Brix, pH, TA, water activity and CIELAB color were visualized in NMDS plots for bacterial Bray-Curtis, bacterial weighted Unifrac, and fungal Jaccard indices (Figure 2.7 and 2.8). PERMANOVA analysis was performed on physicochemical data to evaluate the correlation between these variables and the microbial composition. Titratable acidity was determined as a factor that was significant for bacterial Bray-Curtis dissimilarity (pseudo $F = 7.1182$, $R^2 = 0.20863$, $p = 0.001998$) and weighted UniFrac (pseudo $F = 13.242$, $R^2 = 0.32906$, $p = 0.000999$). The fungal community measured by Jaccard distance was determined to be significantly associated with water activity (pseudo $F = 2.6309$, $R^2 = 0.07823$, $p = 0.01199$) and color (L*: $p = 0.03497$, a*: $p = 0.007992$, b*: $p = 0.005994$).

The differences in beta diversity of different types of honey can be attributed to the taxonomic composition of the microbiota, and the taxa with the highest coefficient values were visualized in Figure 9. For the bacterial community measured with Bray-Curtis metric, the top 5 ASVs with the largest effects on PERMANOVA coefficient were in the genera of *Lactococcus*, *Serratia*, *Citrobacter*, *Serratia*, and *Pseudomonas*.

For the fungal community, the top 5 ASVs with the largest effects on PERMANOVA coefficient were under the species of *Zygosaccharomyces mellis*, *Yarrowia lipolytica*, *Bettsia alvei*, *Zygosaccharomyces mellis*, and *Skoua* sp.

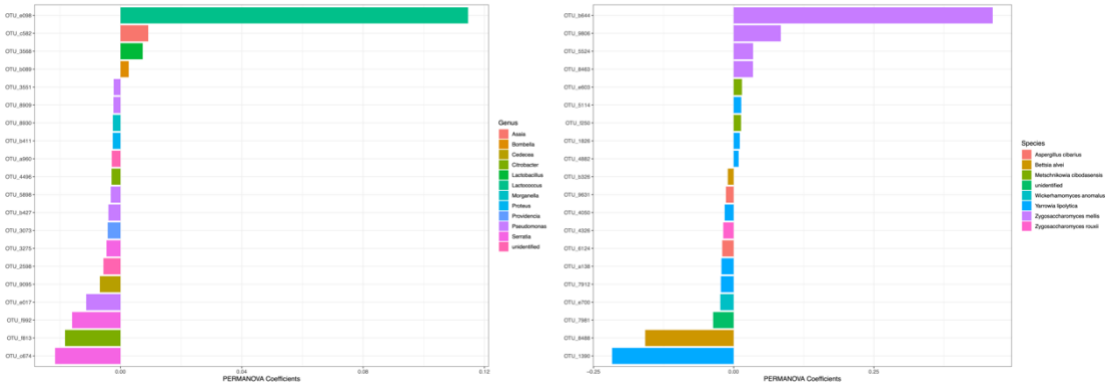

Figure 2.9. Top coefficient amplicon sequence variants (ASVs) for beta diversity. Top bacterial and fungal ASVs that were associated with community differences between samples as estimated by Bray-Curtis dissimilarity were plotted. Color of each bar represented the genera for 16S ASVs and species for ITS ASVs. The top 20 ASVs with the highest PERMANOVA coefficient values were plotted.

Discussion

Honey microbiota is a complex matrix that contains ecological information regarding the host microenvironment, the hive pathosphere, and the honeybee hologenome (Schwarz et al., 2015). Some bioindicators, including the agricultural and urban landscape, microbial environment that honeybees are exposed to, and the chemical pollutants in the foraging routes, can be reflected in the honey microbiota (Bargańska et al., 2016; Lambert et al., 2012; Rissato et al., 2007). NGS methods, including metabarcoding, can elucidate the complicated mutualism and symbiotic ecological relationships between honeybees and the environment (Bovo et al., 2018; Bovo et al., 2020). The information we obtained from next-generation sequencing can provide taxonomic classification of honey microbiota and potentially be used as an indicator for the overall beehive health and honey origin (Bovo et al., 2020).

Most of the bacterial species identified in honey were osmotolerant, xerotolerant, and acidotolerant, considering that honey has a relatively high sugar content, low water activity, and low pH (Brudzynski, 2021). One of the most abundant bacterial species we found in our honey samples is *Lactococcus lactis*, which is consistently present in all honey samples we sequenced (Fig. 1). *Lactococcus* is a member of the lactic acid bacteria (LAB), which are able to ferment carbohydrates in honey (fructose and glucose) and produce lactic acid. As a ubiquitous group of bacteria that are commonly found in plant materials, LAB have been isolated from honeybee hives and bee products in previous research (Kňazovická et al., 2020; Sinacori et al., 2014). Some secondary metabolites produced by LAB strains can inhibit spoilage organisms and pathogens and contribute to the overall beehive health.

One example is *Lactobacillus kunkeei*, which is beneficial to the bee colony by protecting the hive from potential pathogens like *Paenibacillus larvae* and *Nosema ceranae* (Arredondo et al., 2018). As a ubiquitous species that is commonly found in flower, fruits, and soil, *L. kunkeei* is commonly associated with honeybee hive environment and bee products. *L. kunkeei* was found in honey bee bread using both culture-dependent and culture-independent method (Anderson et al., 2013). For honey samples in our study, *L. kunkeei* is one of the fructophiles that can be found in some but not all honey samples (Fig. 1). Comparatively, another study on the microbiome of stingless bee honey revealed that the most abundant species is *Lactobacillus malefermentans*, and the top 7 OTUs in this study were all members of the genus *Lactobacillus* (Rosli et al., 2020). One of the possible reasons that *Lactobacillus* is missing in some of our honey samples is that *Lactobacillus* disappears below moisture content of 18% during honey ripening process (Ruiz-Argueso & Rodriguez-Navarro, 1975; Wen et al., 2017). Other studies also suggested that the presence of *L. kunkeei* is sporadic and its detection is dependent on the factors like floral source and season (Vásquez et al., 2012). Distinct differences can be seen when comparing our bacterial profile with the bacterial profile of vitex honey during ripening, which was dominated by *Bacillus* spp. (Wen et al., 2017). However, some bacteria with high abundance in vitex honey can be found in our honey samples, including *Lactococcus* and *Pseudomonas*. Some unresolved Enterobacteriaceae were present in our honey samples, which are likely from the pollination environment since they are frequently isolated from crops of forager bees (Corby-Harris et al., 2014). Even though gut microbiota could be a source of microbial community members in honey, many gut

bacteria are considered gut-specific and do not survive well in other environments. Only *L. kunkeei* and *Acetobacteraceae* (*Asaia* spp.) were found in extreme conditions like honey and royal jelly (Anderson et al., 2013; V. G. Martinson et al., 2012; Vojvodic et al., 2013). *Serratia* is one of the most abundant genera found in our honey samples, which is consistent with a previous microbial metabarcoding study on three polyfloral honeys from Italy, where *Serratia symbiotica* was the fourth most abundant bacteria accounting for 4.8% of the bacteria reads (Bovo et al., 2020). The origin of *Serratia* is somewhat puzzling, since it is commonly associated with aphids as a secondary endosymbiont. It is possible that *Serratia* originated from honeydew produced by aphids, which was then fed to honeybees to produce honey (Bovo et al., 2020).

For the fungal communities, diverse profiles can be observed across different types of honey. The most abundant fungal genera in our honey samples were *Bettsia*, *Yarrowia*, *Skoua*, *Zygosaccharomyces*, and *Metschnikowia*. Similar to our study, the fungal profile of vitex honey is also heterogeneous, with *Waitea*, *Phoma*, *Metschnikowia*, *Cryptococcus* being the most predominant genera. *Metschnikowia* was found to be relatively stable in mature vitex honey and dominant in vitex flower. We propose that *Metschnikowia* and other yeasts in our honey samples originated from nectar, which can be transmitted from flower and fruits to honeybee products (Hong et al., 2001; Lievens et al., 2015). The absence of *Waitea* and *Cryptococcus* in our honey samples could be due to flower origin, since these two genera were found to be dominant in vitex flower (Wen et al., 2017). Culture-based methods identified yeasts like *Zygosaccharomyces* and *Debaryomyces* as the most prevalent genera in honey

(Sinacori et al., 2014). In a culture-independent study with ITS2 metabarcoding, *Zygosaccharomyces* was the only species shared among almost all honey samples (Balzan et al., 2020). Filamentous fungi like *Aspergillus* are considered environmental contaminants for honey (Kacániová et al., 2009). A shotgun metagenomic study found that the second most represented fungus in polyfloral Italian honey was *Aspergillus flavus* (Bovo et al., 2020). *Aspergillus flavus* is a potential honeybee pathogen that could cause stonebrood disease, and was found to be abundant in some of our monofloral, wildflower, and manuka honey samples (Fig. 2). Similarly, *Ascosphaera apis* is the causative agent for chalkbrood disease (Vojvodic et al., 2011). *Ascosphaera* sp. was found to be prevalent in some of the wildflower honey samples in our study (Fig. 2). However, the presence of pathogenic fungi does not necessarily mean that the beehives are infected. Indeed, as shown in the study by Bovo et al, none of the sampled colonies that contained pathogenic fungi DNA in metagenomic analysis displayed any of these symptoms over two years (Bovo et al., 2020). The onset of these diseases requires specific environment factors, and most of the pathogenic fungi only survive in honey as dormant spores.

In our study, we chose to not perform culture-based isolation methods due to culture biases. Performing bacterial and fungal culture isolation could not give us a whole picture of the microbiota, nor could it provide proof of the absence of certain species. As previous studies shown, species from genera *Bacillus* and *Paenibacillus* were considered dominant when evaluating the honey bacterial composition with culture-based method because aerobic plate counts were usually dominated by fast-growing bacteria like *Bacillus* spp., *Staphylococcus* spp. and *Paenibacillus* spp., while

the dominant bacteria identified using amplicon sequencing were under-represented in culture-based methods due to various factors, like injured cells, persister cells, improper culture environment, or failing to compete with other organisms in culture (Balzan et al., 2020; Iurlina & Fritz, 2005; Sinacori et al., 2014). Moreover, plate count methods overestimated the bacteria abundance in honeybee stomach by over one order of magnitude, and core crop bacteria identified using culture-based method were inconsistent and occurred at low frequency when using qRT-PCR or NGS methods (Corby-Harris et al., 2014). In our opinion, using culture-independent methods to investigate the microbiome of honey avoids the growth condition and culture biases, and culture-based methods should not be performed as a complement to culture-independent amplicon sequencing or metagenomic studies. Alternatively, designing strain-specific primers and performing qRT-PCR is the proper way to confirm the presence/absence of certain species identified by amplicon sequencing.

The physicochemical properties of different honey types in this study were highly comparable, especially for pH and Brix (Table 1). Color is one of the parameters that can be used to distinguish different honeys. Ecological diversity indices can be assessed based on the ASVs in different honey products, and certain hypotheses can be drawn based on statistical analysis. In our study, titratable acidity was found to be correlated with bacterial alpha diversity metrics, including ACE richness, Shannon diversity, and inverse Simpson diversity. A few physicochemical factors were also found to be correlated with fungal Chao richness metric, including TA, Brix, and color. Furthermore, based on beta diversity metric correlation analysis, we determined that TA was a significant factor associated with the differences in

bacterial communities, while water activity and color were associated with the differences in fungal communities. Previous studies showed that honey pH and acidity were independent of geographic origins but associated with nectar composition and botanical source (da Silva et al., 2016; Scholz et al., 2020). Honey age, moisture, and purchase source were considered as relevant factors for the microbial community in raw honey, while botanical origin only affected the fungal composition (Balzan et al., 2020). pH, water activity, and country of origin were considered as minor factors. In our study, moisture was not a significant factor shaping the bacterial or fungal community. Conversely, several previous studies showed that honey microbial profile was associated with its moisture. In the study by Wen et al. (2017), the fungal community of vitex honey was correlated with moisture. Honey with high moisture content is more likely to ferment and spoil. However, the moisture content variation in our honey samples was relatively small, which may be the reason that the moisture content was not a significant factor influencing the microbial community of our honey samples. Another group of researchers evaluated physicochemical parameters including pH, water content, free acidity and electrical conductivity and determined that only electrical conductivity was associated with bacterial community of honey based on RDA analysis and permutation test (Kňazovická et al., 2020). In the study by Rosli et al, the authors considered that the microbiome of stingless bee honey was associated with physicochemical factors including pH, acidity, and moisture content (Rosli et al., 2020). The marginal effects of limited sample size may contribute to the discrepancy among different studies. Some other authors also mentioned the geographic region may be an important factor influencing the microbial community in

honeybee products (Disayathanoowat et al., 2020). We only included two geographic regions in our study, which is why we cannot draw any conclusions on its effect on the microbial community. Future metagenomic studies should take geographic location into consideration when evaluating factors that may impact the microbial community of honey. To fully understand the effects of geographic location and other relevant variables on the microbiome diversity, samples collected in different regions of US or world need to be included, with specific details on the geographic distribution, local flowering plants diversity, and the honeybee genetic background.

Next-generation sequencing tools provide a higher level of resolution of the community composition compared to traditional culture methods. Species with low abundance can be detected with in depth sequencing, which enables us to evaluate the microbiome composition more precisely (Claesson et al., 2010; Gupta et al., 2019). Some studies have been performed to evaluate the floral source of honey using DNA metabarcoding for authentication, and the digestive tract microbiome of honeybees with metagenomic tools (de Vere et al., 2017; Graystock et al., 2017; Jones et al., 2018; Utzeri et al., 2018; Yun et al., 2018). Many of these studies used 16S rRNA amplicon sequencing, which is what we chose to use in our study to evaluate the composition of bacterial community in honey. Metabarcoding methods have high coverage, high sequencing depth, and is non-selective (Cao et al., 2017). However, the disadvantage is that most of the sequences are assigned to the taxon with high abundance, which may neglect some of the less common species in a community with high complexity (Clooney et al., 2016). The bacterial classification is also identified at genus level or above (Claesson et al., 2010). To avoid using a pre-defined percentage

threshold to determine variants, we chose to use amplicon sequence variants (ASVs), which considers amplicon abundance and error rates to discard spurious sequences and retain biologically meaningful sequences (Callahan et al., 2017). This method has a finer resolution and identifies microorganisms at phenotypic levels (Rognes et al., 2016). Using ASVs to represent original sequences is considered a step forward compared to previous studies using operational taxonomic units (OTUs) to construct consensus sequences with 97% similarity, which inevitably loses some taxonomic information (Strube, 2021). In our study, we chose the 16S V3-V4 region and 5.8S-ITS2 regions considering the limited read length of Illumina MiSeq. The potential sequencing biases from Illumina MiSeq is also the reason we condensed ASVs to the genus level for 16S amplicons and species level for ITS amplicons instead of using ASVs as individual units (Strube, 2021). Future studies should use the full 16S V1-V9 region and full-length ITS1-5.8S-ITS2 region to get better resolution of the honey bacterial and fungal population. Using sequencing platforms with higher read length and choosing proper primers for multiple barcode sequences will yield results with higher resolution.

This study contributes to the knowledge of environmental effects on microbial biodiversity and ecosystem associated with different types of honey. Investigation on the microbiome of honey and other bee products could shed light into Colony Collapse Disorder (CCD), a common disease in honeybee colonies that causes significant ecological and economic damage (Cox-Foster et al., 2007). By comparing the microbiome of honey produced by different bee colonies, we can investigate the correlation between the microbiome and honeybee diseases. Even though the presence

of pathogenic microbial DNA may not directly correlate to honeybee diseases, using metagenomic tools to determine the relative abundance of these pathogens can provide information on possible hive diseases and overall beehive health. Future studies should focus on the shift of certain bacterial and fungal species in honey and other bee products to decipher the implication of honey microbiome on honeybee diseases and overall bee colony health.

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CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF ANTIFUNGAL LIPOPEPTIDE PRODUCED BY BACILLUS VELEZENSIS ISOLATED FROM RAW HONEY

Abstract

Raw honey contains a diverse microbiota originating from honeybees, plants, and soil. Some gram-positive bacteria isolated from raw honey are known for their ability to produce secondary metabolites that have the potential to be exploited as antimicrobial agents. Currently, there is a high demand for natural, broad-spectrum, and eco-friendly bio-fungicides in the food industry. Naturally occurring antifungal products from food-isolated bacteria are ideal candidates for agricultural applications. To obtain novel antifungals from natural sources, we isolated bacteria from raw clover and orange blossom honey to evaluate their antifungal-producing potential. Two *Bacillus velezensis* isolates showed strong antifungal activity against food-isolated fungal strains. Antifungal compound production was optimized by adjusting the growth conditions of these bacterial isolates. Extracellular proteinaceous compounds were purified via ammonium sulfate precipitation, solid phase extraction, and RP-HPLC. Antifungal activity of purified products was confirmed by deferred overlay inhibition assay. Mass spectrometry (MS) was performed to determine the molecular weight of the isolated compounds. Whole genome sequencing (WGS) was conducted to predict secondary metabolite gene clusters encoded by the two antifungal-producing strains. Using MS and WGS data, we determined that the main antifungal compound produced

by these two *Bacillus velezensis* isolates was iturin A, a lipopeptide exhibiting broad spectrum antifungal activity.

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Introduction

Antifungal resistance in medically and agriculturally relevant fungi is increasing globally, straining the limited selection of safe and effective antifungal agents. The development of novel antifungal agents is much slower than the spread of antifungal resistant strains, which presents a serious human health and food security problem (Fisher, Hawkins, Sanglard, & Gurr, 2018). In the medical field, fungal infections are extremely difficult to treat. Fungicides that are broad spectrum, effective, and safe to use, are limited. Furthermore, the prevalence of multi-drug resistant fungal pathogens has been increasing in hospitals and nursing homes (Slifka, Kabbani, & Stone, 2020). For the widely deployed azole family, resistance has been observed in common fungal pathogens (Fisher, Hawkins, Sanglard, & Gurr, 2018). For example, multi-azole-resistant strains of the opportunistic pathogen *Aspergillus fumigatus* have been isolated from patients with invasive aspergillosis (van Paassen, Russcher, In 't Veld-van Wingerden, Verweij, & Kuijper, 2016). Fluconazole-resistant *Candida glabrata* with increased resistance to the other first-line antifungal drug echinocandin was also observed, which further limited the available options to treat this infection. (Alexander, Johnson, Pfeiffer, Jimenez-Ortigosa, Catania, Booker, Castanheira, Messer, Perlin, & Pfaller, 2013). Additionally, multidrug-resistant *Candida auris*, first isolated in 2009, has invasively infected patients worldwide through hospital-acquired transmission (Chowdhary, Sharma, & Meis, 2017). In the agricultural field, fungal plant pathogens have also acquired resistance against antifungal agents. Even though more fungicides are available for field application, the rapid rate of antifungal resistance development is alarming. A classic example of an

organism with high risk of antifungal resistance development is *Botrytis cinerea*, which is able to adapt to new fungicide classes. Multidrug-resistant *B. cinerea* strains have been isolated in strawberry fields around the world (Hahn, 2014). The predominant class of chemicals used for antifungal treatment of crops is azoles. Scientists have urged to restrict the use of azoles in agriculture, as resistant fungal strains are being continuously isolated from environmental and clinical settings at an increasing rate (Denning & Bromley, 2015). However, due to the lack of alternatives, it is still being widely used in economically important crops to avoid crop losses. In contemporary food systems, spoilage caused by fungi is no less serious. Food loss due to fungal spoilage was estimated to account for 5-10% of the world food supply, and post-harvest microbial spoilage was estimated to contribute to 25% of global food waste (Cook & Johnson, 2009; Gram, Ravn, Rasch, Bruhn, Christensen, & Givskov, 2002). In a survey of 51 juice manufacturers, 92% reported experiencing yeast or mold spoilage in their finished product and 89% reported previous occurrences of yeast or mold spoilage of their ingredients (Abigail B. Snyder & Randy W. Worobo, 2018). Spoilage fungi are difficult to control due to their ability to survive extreme conditions, like low water activity, limited nutrients, high acidity, and extreme heat treatment. Moreover, the trade-off of common fungal-controlling approaches in the food industry is the negative environmental impact, such as food waste, unsustainable packaging, and environmental damage by synthesized chemicals (A. B. Snyder & R. W. Worobo, 2018). Natural bio-fungicide could be a beneficial addition to traditional fungal-controlling approaches and mitigate the environmental impact.

The urgent need for natural, novel, safe, and potent antifungal compounds lead

us to seek solutions from natural products, like honey. Raw honey is inhibitory to fungi, partially due to its high sugar content and low water activity (Molan, 2015). However, a survey comparing the antifungal effects of raw monofloral honey with synthetic honey demonstrated that heather and lavender honey exhibited higher antifungal activity than sugar-based synthetic honey (Estevinho, Afonso, & Feas, 2011; Feas & Estevinho, 2011). Other than osmotic inhibition, some chemical components in raw honey are also antifungal: hydrogen peroxide, flavonoids, phenolic acids, lysozymes, and other antioxidant compounds (Wahdan, 1998). Additionally, antifungal bacteria are present in raw honey. In previous studies, *Bacillus* spp. strains isolated from raw honey were able to produce a variety of secondary metabolites to inhibit the growth of other microorganisms and gain survival advantages. *B. subtilis* H215 was isolated from raw honey and it was inhibitory to *Byssochlamys fulva* H25 (H. Lee, Churey, & Worobo, 2008b). Another isolate found in US domestic honey, *B. thuringiensis* SF361, showed broad spectrum antifungal activity against *Aspergillus*, *Penicillium*, *Byssochlamys*, and *Candida albicans* (H. Lee, Churey, & Worobo, 2008a; Manns, Churey, & Worobo, 2012). Additionally, lactic acid bacteria isolated from honey samples including *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus* showed inhibition against pathogenic *Candida* species (Bulgasem, Lani, Hassan, Wan Yusoff, & Fnaish, 2016). Both lactic acid bacteria and *Bacillus* spp. produce a variety of antifungal secondary metabolites including organic acids, volatile compounds, ribosomally synthesized peptides, and nonribosomal peptides (Caulier, Nannan, Gillis, Licciardi, Bragard, & Mahillon, 2019; Reis, Paula, Casarotti, & Penna, 2012; Schnürer & Magnusson,

2005). The potential application of these microbial natural products in the food industry, agricultural and medical field is promising. One example is nisin, a bacteriocin isolated from *Lactococcus lactis* subsp. *lactis* strain and exhibits broad-spectrum antibacterial activity (Reis, Paula, Casarotti, & Penna, 2012). Nisin is used in dairy and meat products as a biopreservative compound to inhibit foodborne pathogen *Listeria monocytogenes* (Martinez & Rodriguez, 2005). Additionally, several strains of *B. subtilis*, *B. thuringiensis*, and *B. amyloliquefaciens* were approved as commercial biopesticides by the Environmental Protection Agency (EPA) (U.S. Environmental Protection Agency, 2021). Lipopeptides secreted by these *Bacillus* species were used commercially as antifungal agents to control plant diseases caused by phytopathogens (Fira, Dimkic, Beric, Lozo, & Stankovic, 2018).

In an effort to isolate novel antifungal compounds as candidates for medical and/or agricultural applications, we designed this study to isolate, purify, and characterize antifungal proteinaceous compounds from raw honey. Several *Bacillus* strains were isolated from raw clover and orange blossom honey. Extracellular antifungal compounds were purified via ammonium sulfate precipitation, solid phase extraction (SPE), and reversed-phase high performance liquid chromatography (RP-HPLC). Whole genome sequencing was performed on two antifungal producing strains identified as *B. velezensis*. Using a combination of genome secondary metabolite gene cluster analysis and mass spectrometry (MS), we determined that the antifungal compound belonged to the iturin family.

Materials and methods

Antifungal isolates selection

Raw clover honey and orange blossom honey were purchased from a local honey shop (Dundee, NY). Honey samples were diluted with 0.1% peptone water, and 100 μL of 10^{-1} and 10^{-2} dilutions were spread plated on tryptic soy agar (TSA) (BD Difco, Franklin Lakes, NJ). Plates were incubated at 30 °C for 24 hours. Visually distinct colonies were selected to test their antifungal activity. Eight fungal strains isolated from commercially processed food products were used as antifungal activity indicators (Snyder, Churey, & Worobo, 2019). Food-isolated fungal strains were incubated at ambient temperature on potato dextrose agar (PDA, BD Difco, Franklin Lakes, NJ) for at least 4 weeks prior to harvest. Fungal spores were harvested by flooding the surface of fully grown plates with 10 mL 0.1% Tween 80 (Sigma, St. Lois, MO). Spore suspension was filtered with several layers of sterile cheese cloth to remove debris and stored at -80 °C.

Antifungal assays

Antifungal activities of bacterial isolates were determined by deferred overlay inhibition assay: fungal spore suspensions were mixed with 10 mL 0.75% soft TSA and overlaid on PDA plates. Bacterial isolates were spotted with sterile toothpicks on the surface of solidified soft agar with fungal indicators. Plates were incubated at ambient temperature for 48 to 72 hours and inhibition zones were recorded. Bacterial colonies that showed antifungal properties were selected for further analysis. Bacterial isolates were stored in 20% glycerol at -80 °C.

Bacterial classification through 16S rRNA gene sequencing

Bacterial isolates exhibiting strong inhibition toward fungal indicators were initially identified by 16S rRNA gene sequencing. DNA was obtained using the

Genomic DNA extraction kit (Qiagen, Germantown, MD) and 16S rRNA genes were amplified through polymerase chain reaction (PCR). A set of primers (IDT, Coralville, IA) were used to amplify the conserved region in bacteria. 16S forward primer sequence: 5'-AGAGTTTGATCCTGGCTCAG-3'. 16S reverse primer sequence: 5'-AAGGAGGTGATCCAGCC-3'. PCR procedures were as follows: 3 μL DNA template, 1 μL forward primer and 1 μL reverse primer, 0.3 μL GoTaq Flexi DNA polymerase (Promega, Madison, WI), 10 μL 5X Colorless GoTaq Flexi buffer (Promega, Madison, WI), 4 μL 25 mM $MgCl_2$ (Promega, Madison, WI), 2 μL 10 mM dNTP (New England Biolabs, Ipswich, MA), 29 μL dH_2O. Total volume was 50 μL per PCR tube. Thermal cycling conditions were as follows: 1 cycle of 94 °C for 5 minutes, 35 cycles of 94 °C for 30 seconds, 50 °C for 1 minute, 72 °C for 2 minutes, 1 cycle of 72 °C for 10 minutes. PCR products were purified by QIAquick PCR purification kit (Qiagen, Germantown, MD). Purified DNA products were sent to Cornell University Biotechnology Resource Center (Ithaca, NY) for Sanger sequencing. The sequencing data were analyzed using NCBI Nucleotide Blast homology search to determine the species of those antifungal bacterial isolates (Altschul, Gish, Miller, Myers, & Lipman, 1990).

Optimized production of antifungal compounds

Different growth conditions were tested to optimize antifungal production by the honey isolates. Four media were selected for growth optimization: tryptic soy broth (TSB) (BD Difco, Franklin Lakes, NJ), brain-heart infusion (BHI) (BD Difco, Franklin Lakes, NJ) broth, 1.5% casamino acids (CAA) (BD Difco, Franklin Lakes, NJ) with 0.5% yeast extract (BD Difco, Franklin Lakes, NJ) broth, and potato dextrose

broth (PDB) (BD Difco, Franklin Lakes, NJ). Selected growth times were 24 hours or 48 hours, and selected incubation temperature and shaking speed combinations were 37 °C at 250 rpm or 30 °C at 150 rpm. Following the growth of each strain under each condition, the cell-free supernatant was tested for antifungal activity. Cultivated media was first centrifuged at 4 °C, 13000 x *g* for 10 minutes. Supernatant was then filtered through a 0.22 μm polyethersulfone (PES) bottle top filter (250 mL, Celltreat, Pepperell, MA). The cell-free filtrate was tested for antifungal activity using a well diffusion overlay inhibition assay. Wells were made on 25 mL PDA plates using the wide end of sterile 1000 μL pipette tips (diameter: 8.8 mm). A total volume of 600 μL filtrate was added to each well and dried in a biosafety cabinet. Fungal spores were suspended and mixed with 10 mL 0.75% soft TSA and poured onto PDA plates. Plates were incubated at ambient temperature for 48-72 hours, until the complete growth of fungi or the inhibition zone could be visualized. Clear inhibition zones were observed and recorded.

Purification of antifungal proteinaceous compounds

Two bacterial isolates WRB-ZX-001 and WRB-ZX-002 that showed the ability to excrete antifungal compounds into the broth media were selected for purification. Supernatant of the cell culture grown at optimized condition was treated with ammonium sulfate to precipitate proteins. Solid ammonium sulfate was added to the supernatant at 4 °C to reach saturation of 20%, 40%, 60%, 80% and 100%. Ammonium sulfate precipitates of each percentage saturation were collected separately by centrifugation at 13000 x *g*, 4 °C for 20 min and re-dissolved in sterile Milli-Q H_2O. Precipitates were tested against fungal indicator strain *A. fumigatus* and

fractions showed antifungal activity were further purified by reversed-phase solid phase extraction (SPE) using a C18 sorbent cartridge (Sep-Pak Classic, Waters, Milford, MA) with acetonitrile as solvent. Acetonitrile with gradient concentrations from 0% to 100% with an increment of 10% was added to eluate the antifungal compounds. All fractions were tested against fungal indicator strain *A. fumigatus* through the well diffusion overlay inhibition assay as described before. Antifungal fractions from SPE were purified via high-performance liquid chromatography (HPLC, Agilent 1200 Series Gradient System, Santa Clara, CA). The following HPLC elution condition was used: 0–10 min mobile phase A (0.05% TFA in dH_2O); 10–40 min a gradient of 0–100% mobile phase B (0.05% TFA in acetonitrile); and 40–50 min mobile phase B, with a flow rate of 1 mL/min. Fraction collection from HPLC was performed every 1.5 min. The active fractions were re-injected onto HPLC with the same elution condition to confirm its purity. The antifungal activity of HPLC collected fractions was determined by well diffusion overlay inhibition assay as mentioned previously. Antifungal activity units (AU/mL) of active ammonium sulfate precipitate, SPE fractions and HPLC collected fractions, defined as the reciprocal of the highest dilution yielding a clear inhibition zone, were calculated.

Growth curve and antifungal production

The growth curves of two selected bacterial isolates, WRB-ZX-001 and WRB-ZX-002, and their antifungal production over time were determined. These two isolates were pre-grown in 5 mL BHI broth at 30 °C, 150 rpm for 12 hours. Pre-growth cell culture (500 µL) was inoculated into 50 mL BHI broth. Samples were taken every two hours from 0 h to 96 h for cell density and antifungal activity

measurement. The absorbance of the samples was measured at 600 nm using a spectrophotometer (Spectronic 20D+, Thermo Scientific, Waltham, MA); absorbance values were used to plot growth curves for the two isolates. Antifungal activity was tested by well diffusion overlay inhibition assay of sterile-filtered supernatant against fungal indicator strain *A. fumigatus*. Cell-free supernatants were diluted two-fold and antifungal activity units were calculated as the reciprocal of the highest dilution showing a clear inhibition zone. Biological duplicates were performed. Data was analyzed and visualized in R version 4.0.2. R package growthcurver 0.3.0 was used to fit the microbial growth data to a standard form of logistic equation (Sprouffske & Wagner, 2016).

Heat stability and protease stability test

To measure the heat stability and protease stability of the antifungal compounds produced by WRB-ZX-001 and WRB-ZX-002, active antifungal fractions of ammonium sulfate precipitate were selected for testing. For heat stability, samples were treated by steam sterilization at 121 °C for 15 min in an autoclave. Antifungal activity was measured by deferred overlay inhibition assay of 10 µL 2-fold diluted heat-treated samples. The protease stability was tested by incubating the samples individually with 100 µg of pronase E (10 mg/mL, Sigma, St. Lois, MO), α-chymotrypsin (25 mg/mL, Sigma, St. Lois, MO), pepsin (20 mg/mL, Sigma, St. Lois, MO), and trypsin (2.5%, Sigma, St. Lois, MO) at 37 °C for 30 min. Antifungal activity was measured by deferred overlay inhibition assay of 10 µL 2-fold diluted protease-treated samples. Antifungal activity units of heat-treated and protease-treated samples were calculated.

Protein molecular weight determination via mass spectrometry

Active fractions from SPE were analyzed with direct-infusion mass spectrometry (DIMS) to determine the molecular weight of the antifungal compounds. DIMS was performed on a Triversa Nanomate nanospray direct infusion robot (Advion, Ithaca, NY) attached to a Orbitrap Fusion Lumos Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA). Samples were diluted in 50 mM ammonium formate followed by centrifugation prior to direct infusion. Spectra were acquired in positive ion mode with a resolution setting of 500,000 (at *m/z* 200). Active fractions collected from HPLC were analyzed by liquid chromatography-mass spectrometry (LC-MS) to measure accurate mass of intact protein. Each sample was diluted with 0.1% formic acid and analyzed by LC-MS with a Dionex RSLCnano HPLC coupled to an OrbiTrap Fusion Lumos (Thermo Fisher Scientific, Waltham, MA) mass spectrometer using a 60 min gradient (2-90% acetonitrile). Sample was resolved using a 75 µm x 150 cm PepMap C4 column (Thermo Scientific, Waltham, MA). MS spectra of protein ions of different charge-states were acquired in positive ion mode with a resolution setting of 120,000 (at *m/z* 200) and accurate mass was deconvoluted using Xcalibur (Thermo Scientific, Waltham, MA). DIMS and LC-MS analyses were performed at Donald Danforth Plant Science Center, Proteomics & Mass Spectrometry Facility (St. Louis, MO).

Whole genome sequencing and genome analysis

Cell pellets from overnight BHI culture of the isolates were treated with lysozyme (20 mg/mL, Millipore Sigma, St. Lois, MO) and RNase A (Qiagen, Germantown, MD). Genomic DNA was extracted using QiaAMP DNA Minikit

(Qiagen, Germantown, MD). Library preparation, quality control, and sequencing were conducted by Cornell University Biotechnology Resource Center (Ithaca, NY) using Nextera XT DNA library preparation and indexing kits (Illumina, San Diego, CA). Illumina MiSeq (Illumina, San Diego, CA) was used to obtain 2 × 250 bp paired-end reads. Reads were trimmed using Trimmomatic (version 0.39) and *de novo* assembled with SPAdes (version 3.13.1) using the default k-mer settings for bacterial genome assembly (Bankevich, Nurk, Antipov, Gurevich, Dvorkin, Kulikov, Lesin, Nikolenko, Pham, Prjibelski, Pyshkin, Sirotkin, Vyahhi, Tesler, Alekseyev, & Pevzner, 2012; Bolger, Lohse, & Usadel, 2014). Scaffolds less than 500 bp were trimmed and assembly quality was assessed using QUAST (version 4.0) (Gurevich, Saveliev, Vyahhi, & Tesler, 2013). Average genome coverage was determined using BBmap (version 38.45) and SAMtools (version 1.11) (Li, Handsaker, Wysoker, Fennell, Ruan, Homer, Marth, Abecasis, Durbin, & Genome Project Data Processing, 2009). Genome assemblies of *B. amyloliquefaciens* group type strains were downloaded from the National Center for Biotechnology Information (NCBI) assembly database and average nucleotide identity (ANI) analysis of the isolates was conducted via the OrthoANI method using OAT (version 1.40) with BLAST+ (version 2.9.0) (I. Lee, Ouk Kim, Park, & Chun, 2016). The draft genomes of *B. velezensis* WRB-ZX-001 and WRB-ZX-002 sequenced in this study, the complete genome of *B. subtilis* 168 as an outgroup, and other 42 genomes of *B. amyloliquefaciens* group extracted from NCBI were used to construct a SNP-based phylogeny. The program kSNP v3.0 was used with a kmer size of 19 as determined by Kchooser (Gardner, Slezak, & Hall, 2015). The core SNPs were used to build the maximum likelihood

phylogeny in RAxML v8.2.12 under general time-reversible model with gamma distributed sites (GTRGAMMA) and 1000 bootstrap repetitions (Stamatakis, 2014). The phylogenetic tree was edited in FigTree v1.4.4 and deposited on Figshare (https://doi.org/10.6084/m9.figshare.16688839). The absolute core SNP distance matrix was calculated using Geneious v2020.2.4. Rapid annotation of the genomes was performed using prokka v1.12 (Seemann, 2014). Functional annotation of the predicted proteins was performed with BLAST2GO v1.4.4 (Conesa, Gotz, Garcia-Gomez, Terol, Talon, & Robles, 2005). Additionally, genome annotation was performed by the NCBI using the Prokaryotic Genome Annotation Pipeline (PGAP) database (Tatusova, DiCuccio, Badretdin, Chetvernin, Nawrocki, Zaslavsky, Lomsadze, Pruitt, Borodovsky, & Ostell, 2016). Putative bacteriocin genes were identified using BAGEL4 (van Heel, de Jong, Song, Viel, Kok, & Kuipers, 2018). Secondary metabolite genome mining pipeline (antiSMASH) was used to identify potential secondary metabolite synthesis gene clusters (Blin, Shaw, Steinke, Villebro, Ziemert, Lee, Medema, & Weber, 2019). Genome alignment between our isolates and the most closely related type strains was performed using BRIG (version 0.95) (Alikhan, Petty, Ben Zakour, & Beatson, 2011). Assembled genomes of *B. velezensis* WRB-ZX-001 and WRB-ZX-002 were submitted to Sequence Read Archive (SRA) and GenBank under the BioProject ID PRJNA580475 and PRJNA596478. SRA accession numbers are SRR10397796 and SRR10729003.

Results

Four of 15 bacterial isolates from clover honey and 8 of 23 isolates from

orange blossom honey yielded an inhibition zone when spotted on at least one fungal indicator. The 16S rRNA gene sequence of these 12 isolated strains showed highest identity to that of several *Bacillus* spp. To evaluate the antifungal potential of honey isolates, food-isolated fungal strains were selected as indicators for antifungal assay (Table 1). Cross reactivity of the honey bacterial isolates against these fungal strains and BLAST identification results were summarized in Table 2. Isolates that showed antifungal activity against at least three fungal indicators were selected for antifungal production in liquid broth. The production conditions, including the medium type, incubation temperature, and shaking speed, were optimized. As the only two isolates showing the ability to excrete antifungal compounds, isolate Co-29 and Co-30 were selected and renamed as WRB-ZX-001 and WRB-ZX-002 for the following experiments. These two isolates were grown in BHI broth at 30 °C, 150 rpm for 24 hours and 48 hours, and cell-free supernatant showed clear inhibition zones against fungal indicators. The antifungal compounds produced by the isolates were further purified and the isolates were whole genome sequenced.

Table 3.1. Food-isolated fungal strains used in this study as indicators (adapted from Snyder, Churey, and Worobo (2019)).

Organism	Strain ID	Food source
Syncephalastrum	S11-0015	Raw sprouted almonds
Aspergillus	S11-0016	Nut mix
Aspergillus	S11-0033	Oatmeal
A. fumigatus	S11-0039	Kombucha
A. niger	S11-0054	Pomegranate juice
Rhodotorula	S11-0057	Red hot sauce
P. glabrum	S11-0071	Hard-boiled egg
Cladosporium	S11-0111	Juice beverage

Table 3.2. Summary of identity, source, and cross-reactivity against food-associated fungal indicators of honey bacterial isolates.

Isolates	BLAST ID [a]	Honey Source	Cross reactivity[b]							
			Syncephalastrum	*Aspergillus* S11-0016	*Aspergillus* S11-0033	*A. fumigatus*	*A. niger*	*Rhodotorula*	*P. glabrum*	*Cladosporium*
Co-1	*B. toyonensis*	Clover	-	-	-	+	-	-	-	-
Co-5	*B. toyonensis*	Clover	-	-	+	++	++	-	++	-
Co-6	*B. toyonensis*	Clover	+	-	+	++	++	+	++	-
Co-10	*B. aerius*	Clover	\	\	\	+	-	\	+	+
Co-17	*B. cereus*	Orange blossom	+	-	+	++	+	-	+	-
Co-18	*B. megaterium*	Orange blossom	\	\	\	-	-	\	-	-
Co-20	*B. amyloliquefaciens*	Orange blossom	+	\	-	+	+	+	-	++
Co-21	*B. cereus*	Orange blossom	+	-	+	+	-	-	-	-
Co-26	*B. amyloliquefaciens*	Orange blossom	+	\	\	+	-	-	+	+
Co-29	*B. amyloliquefaciens*	Orange blossom	+	\	\	+	+	-	-	++
Co-30	*B. amyloliquefaciens*	Orange blossom	+	\	\	+	+	-	-	++
Co-33	*B. aryabhattai*	Orange blossom	\	\	\	-	-	\	-	-

[a] BLAST ID was determined based on 16S rRNA gene homology search using NCBI Nucleotide BLAST tools. The species with the highest BLAST score were reported.

[b] Cross reactivity was determined using deferred overlay inhibition assay. The inhibition level against the fungal indicators was defined based on visual observation. "+": low inhibition level. "++": strong inhibition level. "-": no observed inhibition. "\": inconclusive result.

Table 3.3. Antifungal activity of purification products of *Bacillus velezensis* isolates against food-isolated *Aspergillus fumigatus*. Antifungal activity unit (AU/mL) is defined as the reciprocal of the highest dilution showing a clear inhibition zone.

Purification procedure	*Bacillus velezensis* WRB-ZX-001	*Bacillus velezensis* WRB-ZX-002
Cell-free filtrate	20 AU/mL	40 AU/mL
Ammonium sulfate precipitant	800 AU/mL	800 AU/mL
Solid phase extraction eluate	800 AU/mL	1600 AU/mL
HPLC fraction	200 AU/mL	200 AU/mL

Antifungal compounds produced by isolates WRB-ZX-001 and WRB-ZX-002 were first purified by ammonium sulfate precipitation of the cell-free culture supernatant. Precipitate from 60% ammonium sulfate showed the highest antifungal activity (Fig 1, Table 3). Ammonium sulfate precipitates were further purified by solid phase extraction with C18 columns and acetonitrile. The eluants for the optimal recovery of antifungal compounds were 50% and 60% acetonitrile. The SPE eluates were loaded onto HPLC, and fractions were collected to test for antifungal activity. Two major peaks were observed in the HPLC spectra and fractions with elution times between 28.5 min to 30 min for both isolates showed highest antifungal activity (200 AU/mL). These fractions were loaded once more onto HPLC to confirm their purity, and single peak was observed for both samples (Fig 2). SPE eluates (50% acetonitrile) and HPLC fraction collection samples (28.5 min to 30 min) for isolates WRB-ZX-001 and WRB-ZX-002 were analyzed with DIMS and LC-MS, respectively. The HPLC samples analyzed with LC-MS showed major peaks with *m/z* value of 1057.57 (Fig 3), which was also present in SPE sample WRB-ZX-002 (results not shown). Another compound with singly charged *m/z* value of 1043.55 and doubly charged *m/z* value of 522.28 was present in both SPE eluates and HPLC collected samples (S1 Fig.). Based on results from previous studies, we presumed that the ions with *m/z* value of 1043.55 and 1057.57 were C_{14} and C_{15} iturin A $[M+H]^+$, respectively (Pathak & Keharia, 2014; Price, Rooney, Swezey, Perry, & Cohan, 2007). The molecular formula of C_{14} and C_{15} iturin A is $C_{48}H_{74}N_{12}O_{14}$ and $C_{49}H_{76}N_{12}O_{14}$, respectively (Peypoux, Guinand, Michel, Delcambe, Das, & Lederer, 1978).

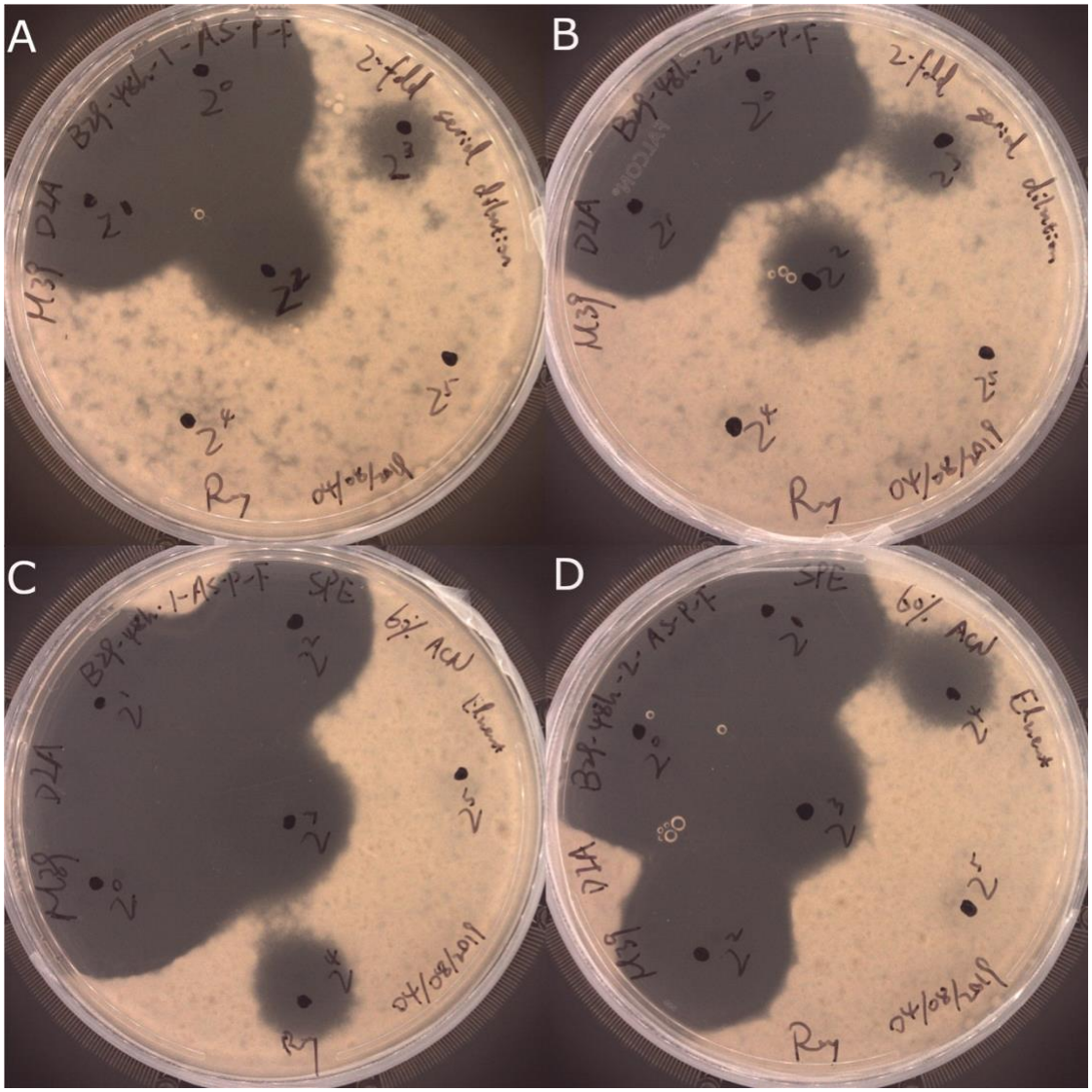

Figure 3.1. Deferred inhibition assay of purified products from *Bacillus velezensis* WRB-ZX-001 and WRB-ZX-002 against food-isolated *Aspergillus fumigatus*.

Precipitates of WRB-ZX-001 and WRB-ZX-002 from 60% ammonium sulfate were shown in A and B. Solid phase extraction eluates of 60% acetonitrile for WRB-ZX-001 and WRB-ZX-002 were shown in C and D. Two-fold serial dilution was performed for all samples to determine the antifungal activity units.

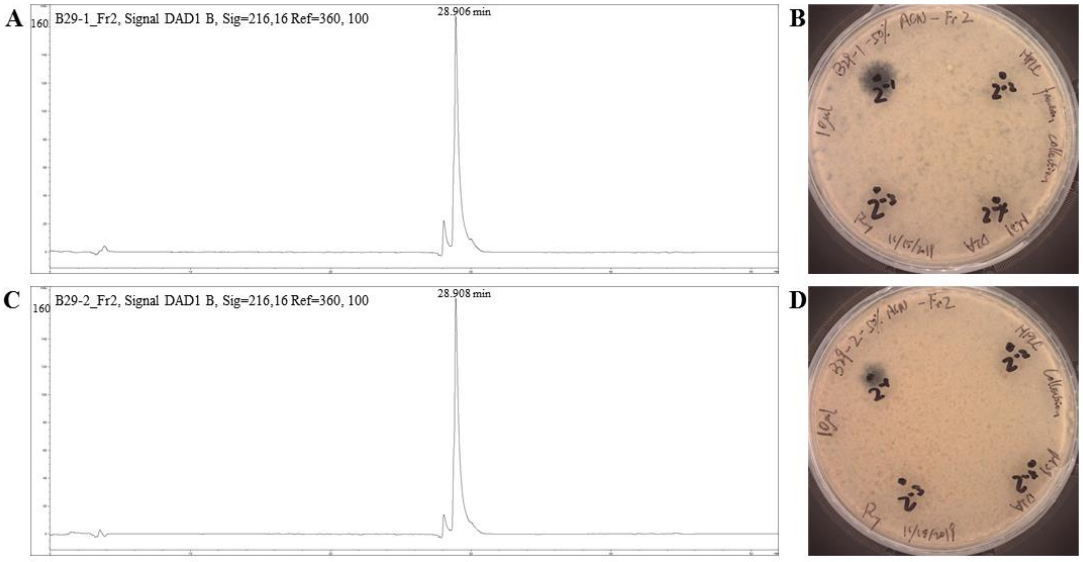

Figure 3.2. Reversed-phase HPLC of purified products of *Bacillus velezensis* WRB-ZX-001 and WRB-ZX-002. Purification process included ammonium sulfate precipitation, solid phase extraction, and HPLC fraction collection. Single peaks shown in A and C were from isolate WRB-ZX-001 and WRB-ZX-002, respectively, and both have shown inhibition against fungal indicator strain *Aspergillus fumigatus* as shown in B and D.

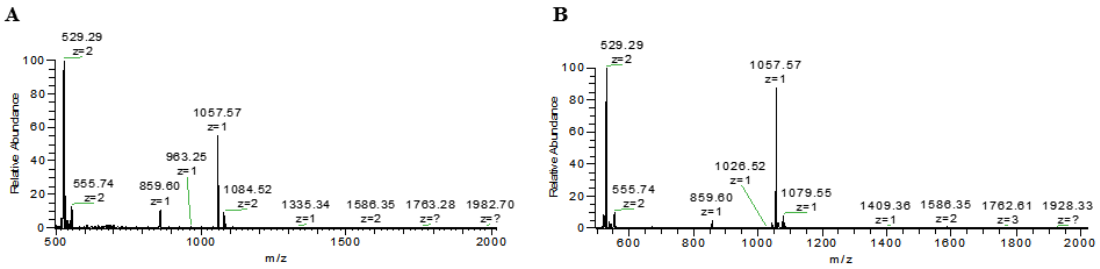

Figure 3.3. Mass spectra for purified antifungal compounds produced by *Bacillus velezensis* WRB-ZX-001 and WRB-ZX-002. A and B are LC-MS spectra for HPLC collected active fraction of WRB-ZX-001 and WRB-ZX-002. Ion with *m/z* value of 1057.57 was assigned to C_{15} iturin A $[M+H]^+$. Ion with *m/z* value of 1079.55 was assigned to C_{15} iturin A $[M+Na]^+$.

For isolate WRB-ZX-001, the 4,183,488 bp genome was assembled to 15 contigs with an average coverage of 104x and N50 of 685,546 bp. For isolate WRB-ZX-002, the genome size is 4,185,188 bp, and the genome was assembled to 15 contigs with an average coverage of 128x and N50 of 1,001,971 bp. Both isolates have the same GC content of 45.97%. Isolate WRB-ZX-001 contains an estimated 4,165 genes and 4,003 coding sequences (CDSs), while isolate WRB-ZX-002 contains an estimated 4,167 genes and 4,004 CDSs. To obtain functional labels, protein BLAST hits were mapped against the curated Gene Ontology (GO) database and GO terms were assigned to the query sequences, with 3,261 annotated sequences for isolate WRB-ZX-001 and 3,263 annotated sequences for isolate WRB-ZX-002. Based on BLAST2GO genome annotation results, the predicted CDSs were assigned to three

principal categories: biological process, cellular component, and molecular function. For isolates WRB-ZX-001 and WRB-ZX-002, the most abundant groups in the category of biological process were cellular process (38%), metabolic process (36%), and biological regulation (8%). In the category of cellular component, the most dominant terms were integral component of membrane (55%), cytoplasm (26%), and plasma membrane (16%). In the category of molecular function, the most representative terms were hydrolase activity (32%), oxidoreductase activity (17%), metal ion binding (13%), transmembrane transporter activity (11%), DNA binding (10%), and ATP binding (9%). Detailed GO annotation and node score distribution for *Bacillus velezensis* WRB-ZX-001 and WRB-ZX-002 was reported in S3 Table. To calculate average nucleotide identity (ANI) and classify the two isolates at species level, orthoANI analysis was performed. The type strain that isolates WRB-ZX-001 and WRB-ZX-002 were most closely related to was *B. velezensis* FZB42, with orthoANI values of 98.96% and 98.93% respectively. Based on the proposed species boundary of 95-96% orthoANI value, we concluded that both WRB-ZX-001 and WRB-ZX-002 should be classified as *B. velezensis* species (Goris, Konstantinidis, Klappenbach, Coenye, Vandamme, & Tiedje, 2007; I. Lee, Ouk Kim, Park, & Chun, 2016; Richter & Rossello-Mora, 2009). To elucidate the phylogenetic relationships between our two isolates and the closely related *B. amyloliquefaciens* group, a total of 42 reference genomes were obtained from the NCBI database. Forty-one *B. amyloliquefaciens* group isolates and one *B. subtilis* subsp. *subtilis* str. 168 were included in the phylogenetic analysis. The phylogenetic tree based on 4,035 core genome SNPs revealed close relatedness of the two isolates from this study with type

strains *B. velezensis* FZB42 and *B. velezensis* KACC18228 (Fig 4). Additional genome comparison of *B. velezensis* type strains FZB42 and CBMB205, *B. amyloliquefaciens* type strain DSM7, and isolates WRB-ZX-001 and WRB-ZX-002 was visualized with BRIG version 0.95 (Fig 5). Gaps in the circular chromosome represented regions with no homology to the reference strain *B. velezensis* FZB42. Gaps for the two isolates from our study were consistent due to high levels of nucleotide homology. Several gaps were present when comparing two isolates from this study with the closely related type strain *B. velezensis* FZB42, indicating the potential presence of novel gene products. To evaluate the secondary metabolite synthesis potential, genomes of WRB-ZX-001 and WRB-ZX-002 were annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) database. BAGEL4 was used to predict open reading frames (ORFs) for ribosomally synthesized proteins and peptides, including bacteriocins, ribosomally synthesized and post-translationally modified peptides (RiPPs). Five putative gene clusters of interest were identified by BAGEL4 in the genomes of *B. velezensis* WRB-ZX-001 and WRB-ZX-002. Both strains contained 3 contigs with genes related to the production of secondary metabolites, including antimicrobial peptide LCI and thiopeptide, bacteriocin amylocyclicin, linear azole/azoline-containing peptide (LAP), and lantibiotic cerecidin. Additionally, antiSMASH was used to identify secondary metabolite biosynthetic gene clusters (BGCs) including nonribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), RiPPs, and other antimicrobial synthases. A total of 16 putative BGCs were identified in both genomes, including 5 NRPSs for bacillibactin, fengycin, bacillomycin D, iturin and surfactin, three trans-acyl-

transferase polyketide synthases (transAT-PKS) for macrolactin H, bacillaene and difficidin, one type III PKS, three RiPP clusters for thiopeptide, lanthipeptide, amylocyclin, and others (Table 4). According to the results of antiSMASH analysis, both isolates contained a gene cluster with 88% similarity to iturin synthetase, and the predicted peptide sequence of the nonribosomal peptide is L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser. To further confirm the presence of iturin gene cluster, BLAST analysis was performed on both genomes. Four iturin genes (*ituD*, *ituA*, *ituB*, *ituC*) were detected in the genome of both isolates, with a similarity of 98.60% to *itu* operon complete CDS from the reference strains *B. subtilis* ZK0 (NCBI accession number: KT781920.1) and *B. subtilis* subsp. *krictiensis* str. ATCC 55079 (NCBI accession number: KU170613.1). The presence of iturin gene clusters in the genome further validated the MS data, indicating the production of C_{14}-iturin (*m/z* of $[M+H]^+$ 1043.55) and C_{15}-iturin (*m/z* of $[M+H]^+$ 1057.57).

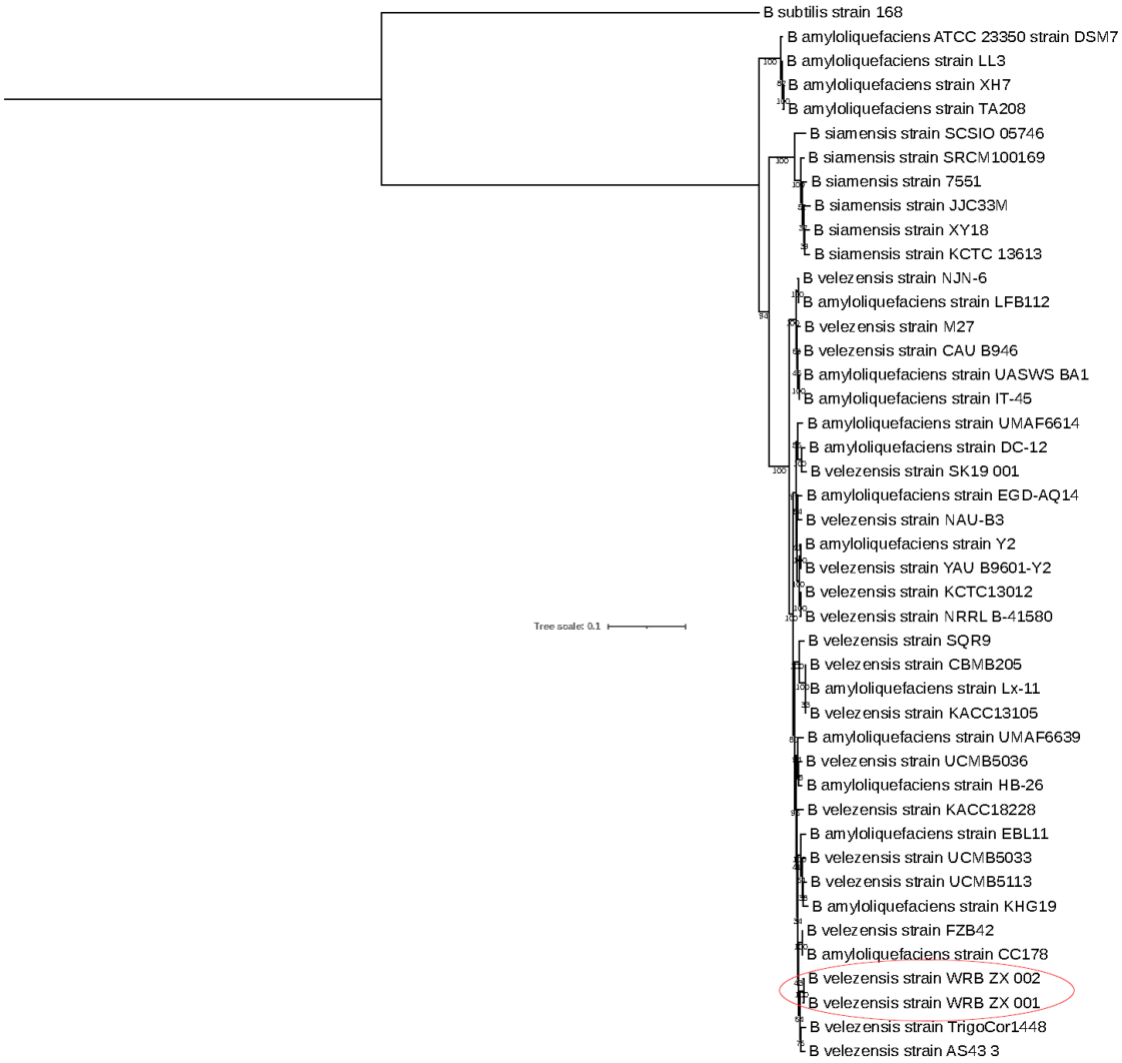

Figure 3.4. Core genome phylogeny of 43 *Bacillus amyloliquefaciens* group isolates. Maximum likelihood tree was constructed with core genome SNPs identified by kSNP. 41 reference genomes of *Bacillus amyloliquefaciens* group isolates were obtained from NCBI genome database. The core genome of *Bacillus subtilis* 168 was used as outgroup. Phylogeny was inferred by RAxML under time-reversible model with gamma distributed substitution sites and 1000 bootstrap repetitions. Bar represents 0.2 substitution per site. Isolates from this study were

labeled with red circle.

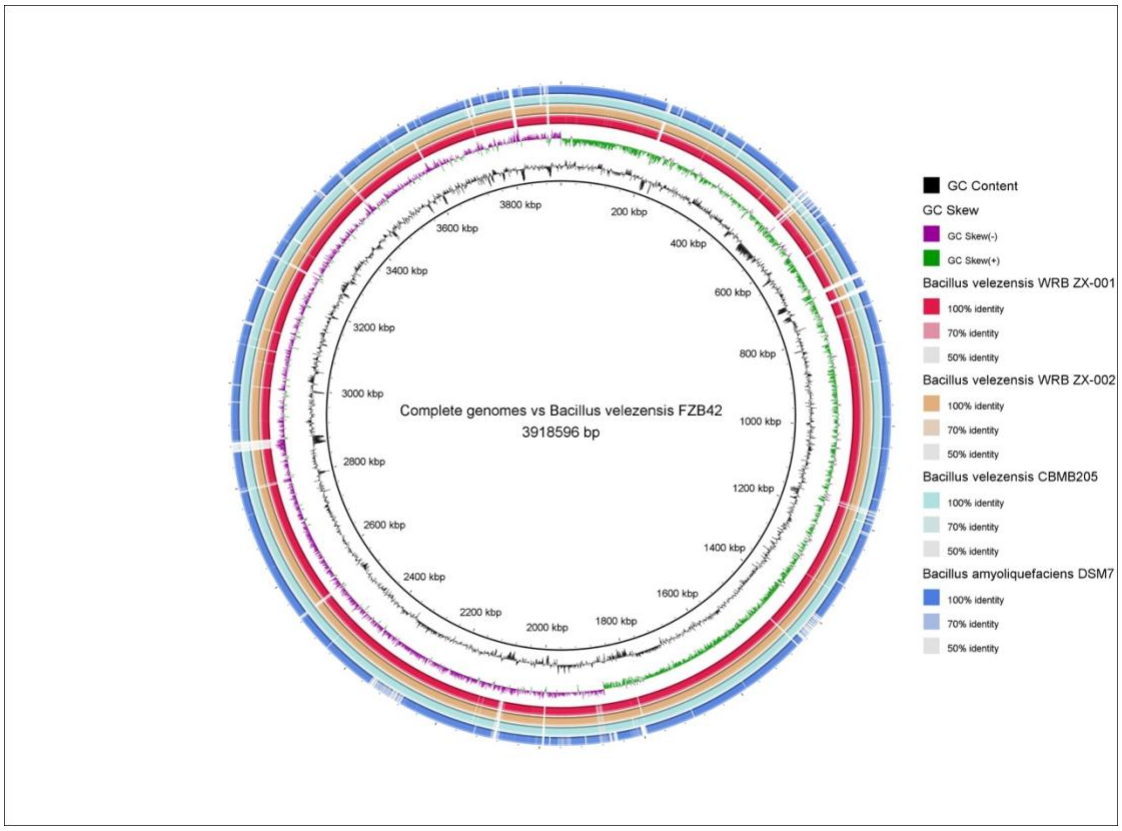

Figure 3.5. Genome comparison of *Bacillus velezensis* WRB-ZX-001 and WRB-ZX-002 against closely related *Bacillus* type strains. *Bacillus velezensis* FZB42 was used as the reference strain. The circular ring map was constructed by BLAST Ring Image Generator (BRIG, version 0.95). From inner to outer ring: 1) GC content; 2) *Bacillus velezensis* FZB42 nucleotide sequence; 3) GC Skew; 4) *Bacillus velezensis* WRB-ZX-001 nucleotide sequence; 5) *Bacillus velezensis* WRB-ZX-002 nucleotide sequence; 6) *Bacillus velezensis* CBMB205 nucleotide sequence; 7) *Bacillus amyloliquefaciens* DSM7 nucleotide sequence.

Table 3.4. Potential secondary metabolite synthesis gene clusters identified in *Bacillus velezensis* WRB-ZX-001 and WRB-ZX-002 by antiSMASH.

Strain	Cluster	Type	From[a]	To[a]	Secondary metabolite	Similarity[b] (%)
	1	Other	298857	354273	Bacilysin	100
	1	Other	500231	554177	Teichuronic acid	100
	1	NRPS	876498	928287	Bacillibactin	100
	1	RiPP	876498	928287	Amylocyclicin	100
	2	PKS-like	65404	106648	\	\
	2	Terpene	189448	210188	\	\
	2	TransAT-PKS	557837	646070	Macrolactin H	100
	3	T3PKS	212154	250873	\	\
	3	Terpene	314561	336444	\	\
	3	NRPS	360563	498152	Fengycin/Plipastatin	100
	3	NRPS	360563	498152	Bacillomycin D	100
	3	NRPS	360563	498152	Iturin	88
	3	TransAT-PKS	560507	670621	Bacillaene	100
	4	TransAT-PKS	85797	191987	Difficidin	100
	5	Thiopeptide/LAP	114108	143862	\	\
	5	NRPS	154585	219992	Surfactin	91
Bacillus velezensis WRB-ZX-001	8	Class II lanthipeptide	47789	66288	\	\
Bacillus velezensis WRB-ZX-002	1	TransAT-PKS	550815	656586	Difficidin	100

	1	T3PKS	954998	993717	\	\
	1	Terpene	1057405	1079288	\	\
	1	NRPS	1103407	1240996	Fengycin/Plipastatin	100
	1	NRPS	1103407	1240996	Bacillomycin D	100
	1	NRPS	1103407	1240996	Iturin	88
	1	TransAT-PKS	1303351	1413465	Bacillaene	100
	2	NRPS	73679	125468	Bacillibactin	100
	2	Other	447789	501741	Teichuronic acid	100
	2	Other	647699	703115	Bacilysin	100
	3	PKS-like	65404	106648	\	\
	3	Terpene	189448	210188	\	\
	3	TransAT-PKS	557837	646070	Macrolactin H	100
	4	Thiopeptide/LAP	114001	143734	\	\
	4	NRPS	154457	219864	Surfactin	91
	6	Class II lanthipeptide	1	18500	\	\

[a] Location of gene clusters in the *Bacillus velezensis* genome.

[b] Similarity based on BLAST analysis against known gene clusters.

Absorption at OD 600nm was used to plot the growth curve for isolate WRB-ZX-001 and WRB-ZX-002. Antifungal activity against fungal indicator *A. fumigatus* was calculated and plotted with the growth curve (Fig 6). The antifungal production started at 24 hours and 18 hours for WRB-ZX-001 and WRB-ZX-002, respectively. The highest antifungal production occurred after cells reached late stationary phase. The antifungal activity was quantified by serial dilution, which was the reason why the antifungal activity fluctuated before reaching maximum production. To optimize the production of antifungal compounds, bacterial cells were collected at 48 hours for the following experiments. The heat stability and protease stability for the antifungal compounds produced by WRB-ZX-001 and WRB-ZX-002 were tested and results were summarized in Table 5. After heat treatment using a 15 min, 121 °C cycle in the autoclave, a 2-fold decrease in antifungal activity for WRB-ZX-001 was observed while sample WRB-ZX-002 had no decrease. For the protease stability test, antifungal compounds produced by WRB-ZX-001 and WRB-ZX-002 showed resistance to pronase E, α-chymotrypsin, and trypsin, with no change in their antifungal activity compared to control. Only sample WRB-ZX-002 showed a 2-fold decrease in antifungal activity after treatment with pepsin. Based on these results, we concluded that antifungal compounds produced by WRB-ZX-001 and WRB-ZX-002 were heat-resistant and protease-resistant.

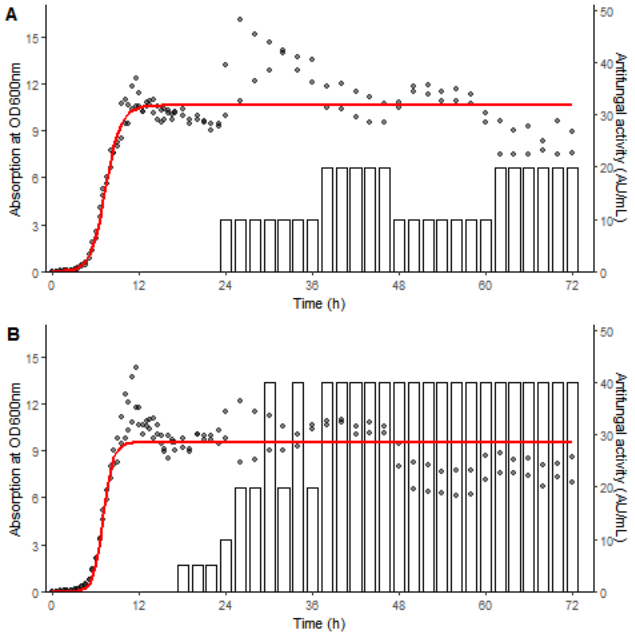

Figure 3.6. Growth curve and antifungal activity curve for *Bacillus velezensis* WRB-ZX-001 (A) and WRB-ZX-002 (B). Growth curve was plotted by measuring absorption at OD_{600nm} every 30 min and a standard form of logistic equation was used to fit the absorption data (red line). Antifungal activity was measured by well diffusion overlay inhibition assay of serially diluted cell-free supernatant every two hours against fungal indicator strain *Aspergillus fumigatus* and data were shown in bar plots.

Table 3.5. Antifungal activity of heat-treated and protease-treated purified products of *Bacillus velezensis* isolates against food-isolated *Aspergillus fumigatus.*

Treatment	*Bacillus velezensis* WRB-ZX-001	*Bacillus velezensis* WRB-ZX-002
Control	800 AU/mL	800 AU/mL
121 °C, 15 min	400 AU/mL	800 AU/mL
Pronase E	800 AU/mL	800 AU/mL
Chymotrypsin	800 AU/mL	800 AU/mL
Pepsin	800 AU/mL	400 AU/mL
Trypsin	800 AU/mL	800 AU/mL

Discussion

In general, bacterial spores are abundant in raw honey, many of which have the potential to exhibit antifungal properties (Bulgasem, Lani, Hassan, Wan Yusoff, & Fnaish, 2016). Previous studies have isolated *Bacillus* spp., *Clostridium* spp., *Lactobacillus* spp. and other lactic acid bacteria (LAB) from raw honey (Grabowski & Klein, 2017). Many members from LAB and *Bacillus* species have been shown to be antifungal, including *Lactobacillus casei*, *Lactobacillus plantarum*, *B. subtilis* and *B. velezensis* (Schnürer & Magnusson, 2005). Bioactive compounds, like ribosomally synthesized bacteriocins and non-ribosomally synthesized small peptides, can be produced by these bacteria, which could potentially be exploited for industrial and medical applications. In this study, our two antifungal *B. velezensis* isolates from raw honey are inhibitory against various food-isolated fungi (Table 2). *Bacillus* species devote a large portion of their genome to secondary metabolism, potentially due to competition they face in the environment (Chen, Koumoutsi, Scholz, Eisenreich, Schneider, Heinemeyer, Morgenstern, Voss, Hess, Reva, Junge, Voigt, Jungblut, Vater, Sussmuth, Liesegang, Strittmatter, Gottschalk, & Borriss, 2007). *Bacillus* species are ubiquitous in soil and the ocean, which often have complex microbial communities. By producing secondary metabolites that can inhibit closely related species and other microorganisms in the ecological niche, *Bacillus* species have gained significant survival advantages (Harwood, Mouillon, Pohl, & Arnau, 2018). Previous researchers have isolated a variety of secondary metabolites with antibacterial and antifungal properties from *Bacillus* species, some of which are nonribosomal peptides (NRPs) (Harwood, Mouillon, Pohl, & Arnau, 2018). NRPs are

synthesized by nonribosomal peptide synthetases (NRPSs) and independent of messenger RNA. NRPs usually go through extensive modifications, including glycosylation, acylation, and hydroxylation. Due to these modifications, some NRPs are amphiphilic and able to insert into cell membrane to form pores, like gramicidin, surfactin, fengycin, iturin, and other lipopeptides. Pore formation in cell membrane will lead to ion leakage and cell death (Maget-Dana, Harnois, & Ptak, 1989; Maget-Dana, Ptak, Peypoux, & Michel, 1985). Some NRPs target closely related cells while others have broad spectrum. Taking account of the results from LC-MS (Fig 3) and secondary metabolite genome mining pipeline (BAGEL4 and antiSMASH) (Table 4), we determined that the major broad-spectrum antifungal compound produced by our *B. velezensis* isolates was a nonribosomal lipopeptide, iturin A.

The iturin A operon was demonstrated to contain four open reading frames (ORFs): *itu*D, *itu*A, *itu*B, and *itu*C. *itu*D encodes a putative malomyl coenzyme A transacylase, while *itu*A, *itu*B, and *itu*C encode iturin synthetases (Tsuge, Akiyama, & Shoda, 2001). Iturin A production is regulated by the promoter on the upstream of *itu*D (Tsuge, Akiyama, & Shoda, 2001). All four ORFs as well as the promoter P_{itu} were present in the genome of our two *B. velezensis* isolates based on BLAST search, with an identity of 98.6% to *itu* operon complete CDS. The iturin family is a group of cyclic lipopeptides with hydrophilic C-terminal heptapeptides and characteristic hydrophobic N-terminal β-amino fatty acids. The aliphatic chain of iturin contains between 14 to 17 carbons and the peptide chain has a chiral sequence of LDDLLDL (Penha, Vandenberghe, Faulds, Soccol, & Soccol, 2020). The iturin family primarily has broad-spectrum antifungal activity, with limited antibacterial activity (Cochrane &

Vederas, 2016). In our study, iturin-producing *B. velezensis* strains showed broad spectrum antifungal activities, with antagonistic ability against *Aspergillus*, *Cladosporium*, *Syncephalastrum* (Table 2), and *Candida albicans* (results not shown). The proposed antifungal mechanism for the iturin family is that they can interact with sterol components on the surface of fungal membrane and increase potassium permeability (Maget-Dana & Peypoux, 1994). Previous studies showed that iturin A can form ion-conducting pores on bimolecular lipid membranes and cholesterol can facilitate the pore-formation by expanding the open-state lifespan (Grau, Ortiz, de Godos, & Gomez-Fernandez, 2000; Maget-Dana, Harnois, & Ptak, 1989; Maget-Dana, Ptak, Peypoux, & Michel, 1985). Additionally, iturin is able to self-associate and interact with lipid membranes by forming a stoichiometric complex with cholesterol on the membrane surface (Maget-Dana & Peypoux, 1994). Furthermore, iturins with longer acyl chains have stronger antifungal properties due to their ability to form oligomers and insert deeply into target membranes to form ion-conducting pores (Malina & Shai, 2005). The pore-forming and membrane permeabilizing abilities of iturin A is concentration dependent. At high concentrations, iturin A showed higher antagonistic activity against fungal cells and higher hemolytic activity (Ines & Dhouha, 2015).

In previous studies, *Bacillus* species have been demonstrated to be able to produce antifungal lipopeptides including members from iturin family. In a study by Pathak and Keharia (2014), iturin isomers and surfactin families were isolated from crude extract of *B. subtilis*. Iturin A2 and Iturin A3/A4/A5 were found to have broad spectrum antifungal activities against *Aspergillus*, *Fusarium*, *Chrysosporium*, *Candida*

albicans, *Trichosporium*, *Alternaria*, and *Cladosporium* (Pathak & Keharia, 2014). One of the iturin A homologues in their study had a mass of 1057.5, the same as the iturin isolated from our study. Similar to the results from our research, Gong et al. (2006) identified antifungal lipopeptides from *B. subtilis* strain PY-1 that was temperature stable and protease resistant. By using ESI-TOF MS, FAB-MS/MS CID spectrometry and NMR, they identified the antifungal compounds as iturin A isomers and determined that the $(M+H)^+$ ions at *m/z* 1057 were iturin A3 and A4 (C17 aliphatic chain) (M. Gong, Wang, Zhang, Yang, Lu, Pei, & Cheng, 2006). Moreover, another group of researchers isolated *B. amyloliquefaciens* S76-3 from wheat spikes, which produced antifungal lipopeptides active against *Fusarium graminearum*. These lipopeptides were identified through RP-HPLC and ESI-MS, with iturin A and plipastatin A being the most abundant molecules. The *m/z* value of iturin A with C-14 acyl acid chain was 1043.35. Fluorescence microscopy analyses and transmission electron microscopy (TEM) analyses of lipopeptide-treated *Fusarium graminearum* conidia and hyphae showed damages to cell wall and plasma membrane, which was consistent with the proposed antifungal mechanism of iturin family (A. D. Gong, Li, Yuan, Song, Yao, He, Zhang, & Liao, 2015). Overall, *itu operon* is common in *B. subtilis* group and *B. amyloliquefaciens* group, and our *B. velezensis* isolates were demonstrated to possess *itu* operon and produce $C_{14\text{-}15}$ iturin A.

The production of iturin and other lipopeptides by *Bacillus* species is dependent on the environment factors, including temperature, pH, carbon source, and oxygen availability. Iturin is mainly produced at temperature between 25 °C and 37 °C under aerobic conditions (Jacques, 2011). In our study, the optimum temperature for

the production of iturin A by *B. velezensis* strains was 30 °C. A neutral pH is generally favorable for the production of lipopeptides (Ines & Dhouha, 2015). In our study, iturin production was optimized by adjusting the pH of BHI broth to 7.4. In a recent study by Dang et al (2019), the optimal condition for the iturin A production by *B. amyloliquefaciens* LL3 derivative strain was thoroughly investigated using single factor optimization and response surface methodology. It was determined that inulin was the best carbon source and L-sodium glutamate was the best nitrogen source. The optimal production condition was determined to be pH 7.0 and 27 °C with 7, 15 and 0.5 g/L of inulin, L-sodium glutamate and $MgSO_4$ (Dang, Zhao, Liu, Fan, Huang, Gao, Wang, & Yang, 2019). In our future studies, this condition will be validated to optimize the production of iturin by our *B. velezensis* isolates.

Compared to conventional synthetic fungicides, which raise concerns regarding chemical residues and antibiotic resistance, biocontrol agents synthesized by living organisms are relatively more environmentally friendly for agricultural applications (Meena & Kanwar, 2015; Ongena & Jacques, 2008). Lipopeptides, like iturin, are considered safe, biodegradable, and eco-friendly. Some previous studies have demonstrated their potential application. Lipopeptides produced by *B. subtilis* RB14, containing iturin A and surfactin, were effective at suppressing the damping-off of tomato seedings cause by *Rhizoctonia solani*. A mutant of *B. subtilis* RB14 that cannot produce iturin A or surfactin failed to inhibit *R. solani*. Restoration of the gene successfully reinstated the suppressibility toward the fungal disease (Asaka & Shoda, 1996). Another study constructed a mutant of *B. subtilis* ATCC6633 by replacing native promoter with constitutive promoter to increase the production of mycosubtilin.

The mutant strain was able to reduce *Pythium* infection in tomato seedlings and increase germination rate (Leclere, Bechet, Adam, Guez, Wathelet, Ongena, Thonart, Gancel, Chollet-Imbert, & Jacques, 2005). Romero et al (2007) showed in their study that direct application of lipopeptide-producing *B. subtilis* cells or cell-free filtrate to leaf surface can prevent powdery mildew caused by *Podosphaera fusca*. Furthermore, by using site-directed mutagenesis, they demonstrated that bacterial mutants that lost the ability to produce bacillomycin, fengycin or iturin A were not able to control the powdery mildew disease (Romero, de Vicente, Rakotoaly, Dufour, Veening, Arrebola, Cazorla, Kuipers, Paquot, & Perez-Garcia, 2007). Antifungal lipopeptides produced by *Bacillus* species could have additional applications. These lipopeptides possess the ability to change biofilm formation, motility, and virulence gene expression of various microorganisms. It is also associated with plant root colonization, plant defense, and plant growth promotion (Raaijmakers, De Bruijn, Nybroe, & Ongena, 2010). With the increased need for biopesticides that have high specificity, low environmental persistence, and low toxicity, industrial exploitation of these chemicals or compounds derived from natural products as food preservatives and crop protection agents is continuously expanding (Seiber, Coats, Duke, & Gross, 2014). Iturin A, a biopesticide produced by food-isolated *Bacillus spp.* and naturally present in food systems, can be exploited for industrial applications.

The safety of the producer strains and their products need to be evaluated to achieve broader application of iturin A produced by *Bacillus* species. One of the major producer strains for iturin A, *B. subtilis*, has Qualified Presumption of Safety (QPS) status according to European Food Standards Authority (EFSA), which indicates that

this strain does not harbor acquired antimicrobial resistance (AMR) genes or exhibit toxigenic activity (Harwood, Mouillon, Pohl, & Arnau, 2018). *B. velezensis* FZB42 type strain, which is closely related to our isolates, was also evaluated by US Environmental Protection Agency (EPA) and considered not toxic, pathogenic, or infective. Therefore, a tolerance exemption for residues of *B. velezensis* FZB42 in food commodities was established. As for the surface-active agents produced by these strains, including surfactin, iturin and other detergents, they can penetrate cell membrane but are not necessarily cytotoxic. Toxicity assays need to be developed to determine their cytotoxicity specifically. For now, current safety measures taken by the industry, including historical safety data and routine testing of the strains and products, are sufficient to ensure the safe usage of strains from the *B. subtilis* and *B. amyloliquefaciens* group as enzyme production workhorse (Harwood, Mouillon, Pohl, & Arnau, 2018). Regarding the safety of iturin A, the acute and subacute toxicity was previously evaluated in mouse models. Preliminary toxicology study showed that iturin A can induce hepatotoxicity and was deposited in liver, lung, and spleen. However, organ-specific toxicity of iturin A was reversible after discontinuation of treatment, which indicated that medical application is still possible (Dey, Bharti, Banerjee, Das, Das, Das, Jena, Misra, Sen, & Mandal, 2016). On the other hand, in a study by Zhao et al (2018), iturin produced by *B. subtilis* was intragastrically administrated to mouse models. In acute (7-day) and subacute (28-day) toxicity tests under concentration of 5000 mg/kg and 2000 mg/kg, respectively, iturin was deemed safe and non-toxic, with no significant damage to liver, kidney, or small intestines (Zhao, Li, Zhang, Lei, Zhao, Shao, Jiang, Shi, & Sun, 2018). Overall, rigorous and

large scale *in vivo* and clinical studies are still needed to fully understand the potential toxicity of iturin A.

A large portion of recent publications on antifungal lipopeptides produced by *Bacillus* species and other gram-positive bacteria focused on partially purified mixtures with varying antifungal activities. The chemical identities of these semi-purified compounds remain uncharacterized, and the biological implications of these studies remain unclear, which poses barriers to future studies. Moreover, with the increased availability and popularity of next-generation sequencing (NGS) and genome mining tools, more recent studies are using these tools to evaluate the secondary metabolites produced by *Bacillus* species. However, chemical confirmation of those potential metabolites is falling behind. Future studies need to combine genetic and genomic methods with traditional chemical identification methods to properly identify, classify, and characterize these potential secondary metabolites. As for the future of iturin family, additional studies are necessary to improve the production efficiency and evaluate the resistance development in fungal model systems.

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CHAPTER 4

LOOKING AHEAD: UNLOCK THE FULL POTENTIAL OF RAW HONEY

Honey is a valuable food with antimicrobial properties. It is widely used in folk medicines since ancient times for wound and burn care. We attributed part of the antimicrobial activities of raw honey to its microbiota and antimicrobial metabolites in our studies. The honey microbiome is a great reservoir of natural antimicrobials with potential industrial applications.

Environmental factors influence the physicochemical properties as well as the microbiome composition of raw honey. Honeybee foraging behavior is affected by several factors, including bee morphology, geographic origin, season, temperature, time of the day, and floral phenology, which in turn affects the collected pollen and honey production (Alqarni, 2006; Joshi & Joshi, 2010). Honeybees with longer wingspan and body size have longer foraging distances and prefer certain types of flowers, influencing wildflower honey microbiota (Mostajeran et al., 2006; Oldroyd et al., 1992). Nectar and pollen collected from different flowers have different physicochemical properties (pH, acidity), chemical and microbial composition, contributing different properties to beehives and honey (Egorova, 1971; Lenaerts et al., 2016; Loper et al., 1980).

Traditional culture methods to profile and analyze food microbiota are well-established and robust. But these methods are semi-quantitative, time-consuming, and labor-intensive. The need for identifying microorganisms in low concentration or in persister states, and the need for information on metabolites and genomic potentials of these microorganisms further validate the necessity of using genomic methods to

analyze food microbiome accurately and efficiently (Andjelković et al., 2017; Giacometti et al., 2013; Senoh et al., 2012). In our study, we chose to use high-throughput amplicon sequencing methods to characterize the microbial composition of raw honey. Additionally, we combined the genomic approach with traditional culture isolation methods to identify two bacterial strains from honey with antifungal potentials. High-throughput analysis of honey microbiomes can facilitate traditional culture-based screening methods to discover novel antimicrobials. Genome mining of microorganisms in the food of interest can reveal the vast repertoire of antimicrobials that are encoded in the genome, revealing those putative metabolites that may be overlooked by traditional culture-based methods, which are limited by physicochemical and environmental conditions. Future application of the broad range of antimicrobials in honey and other natural foods is unlimited.

The next step of investigating the honey microbiome is using foodomic approaches. Shotgun metagenomic sequencing will provide vast amounts of data on all fragmented DNA in the sample without amplification. Shotgun sequences can be assembled and used for functional characterization, providing prediction of potential metabolites. Additionally, metatranscriptomic and metabolomic approaches will provide gene expression profiles and functional information, contributing to our understanding of microbial diversity in honey and improvement of food safety and quality.

However, there are still some potential problems for these genomic, transcriptomic, proteomic, and metabolomic techniques. One issue is false-positive identification and lack of reproducibility, especially for detection of low-abundance

components (Gallo & Ferranti, 2016; Martinović et al., 2016). The complex food matrix may interfere with sequencing sample preparation, yielding low quality results and inaccurate interpretation (Andjelković et al., 2017). Metal ions, lipids, fat, and proteins are likely to inhibit DNA purification and PCR reactions (Bickley et al., 1996; Rossen et al., 1992). One solution is to use proper sample treatment to remove any components in the food matrix that may interact with target molecules, which should be validated during experimental design. Some other common issues encountered in microbiome data interpretation include limitations of the sequencing platform (limited read length, high error rate), and limitations of selected primers (primer biases) (Claesson et al., 2010). Better platforms with high-quality, longer reads can provide more coverage to elucidate complex, diverse microbial systems in food. Additionally, sampling and storage methods, DNA extraction and amplification methods, and sequence analysis pipelines are all variables that may compromise the reproducibility and comparability of the sequencing results, leading to dubious diversity analysis (Gihring et al., 2012; Salonen et al., 2010; Sinclair et al., 2015). For example, improper conditions of sample transportation and storage may alter the microbial composition, leading to unrepresentative amplified sequences and thwarting the correct interpretation of foodomic sequencing data. Similarly, biases can originate from the different sensitivities of microorganisms to cell lysis agents and DNA extraction methods, different levels of DNA amplification for selected primers, and reference databases selected for sequence analysis, all of which can contribute to unrepresentative microbial abundance and gene expression profiles (Engelbrektson et al., 2010). Moreover, proper internal controls should be included in foodomic studies.

For the positive controls, DNA sequences from known bacterial mock communities should be sequenced in parallel to provide an estimate of the sequencing errors for downstream analysis (Kozich et al., 2013). This is especially important for sequencing error evaluation of 16S rRNA amplicon studies. Negative controls should also be included to evaluate any trace amount of contamination that may be present in sequencing reagents (Salter et al., 2014). Contamination may be unavoidable, considering the sensitivity of the sequencing platform, but downstream analysis can be performed to remove background noise from contamination.

Honey microbiome research, like other microbiome studies, should be driven by hypotheses and concepts, not methods (Brüssow, 2020). Due to the rapid development of highly sophisticated sequencing technologies and enormous amount of complex data, researchers may easily lose sight of research hypotheses and pursue studies only based on technical developments. For future food microbiome studies, appropriate experimental, methodological, and statistical design is necessary to perform high quality research and obtain spatial, temporal, and community dynamic information (Berg et al., 2020). Ideally, for a dynamic system like honey, the microbial interaction in a space-time continuum is more meaningful for the interpretation of microbiome function and evolutionary dynamics than a snapshot of a particular time and space. Future studies of honey microbiome should evaluate and compare honey samples over time during different stages, like honeybee collection, maturation in hive, human processing, storage and transportation.

In terms of food applications of antimicrobial secondary metabolites from

natural sources, these natural compounds are highly desirable because of their stability and broad inhibition spectrum. However, considering the complexity of food matrices, these natural compounds may not function properly when used as food additives due to the presence of proteins, lipids, and other inhibitory components in food. Other challenges include the undesirable taste or smell, processing conditions that may reduce or inactivate the antimicrobial properties of these compounds. Using processing and packaging technologies to safely deliver these antimicrobials and preserve their antimicrobial properties at appropriate concentrations while not changing the sensory characteristics of food products is the essential issue we have to solve for industrial application of these food biopreservatives.

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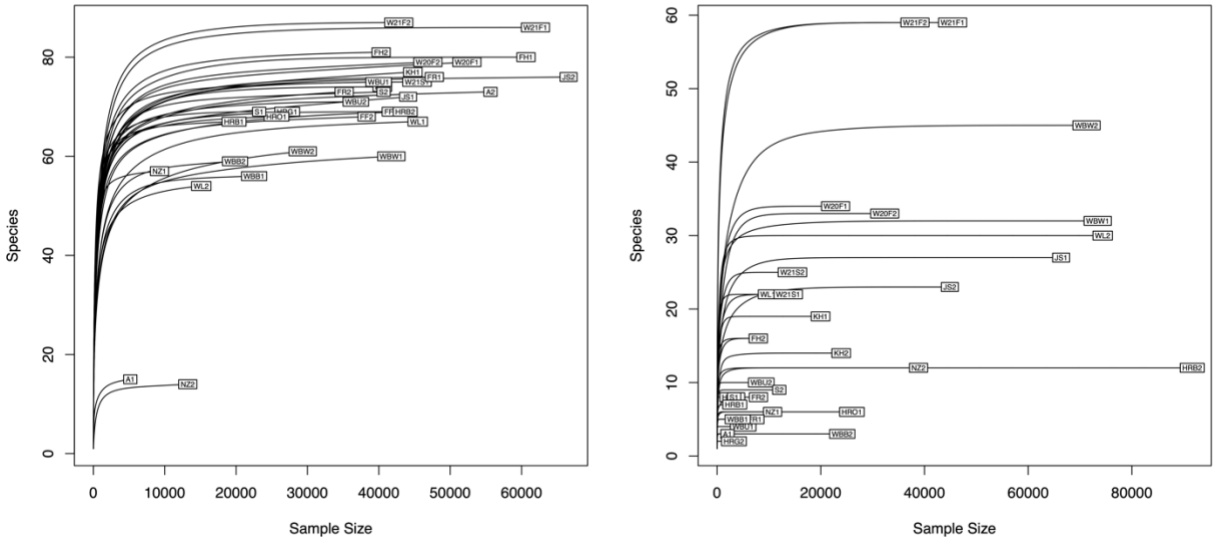
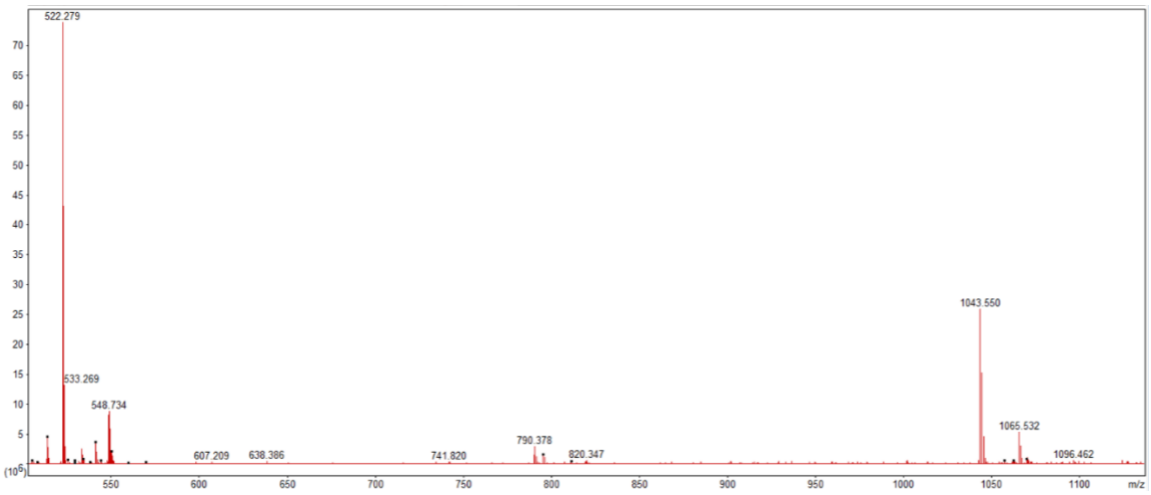


Supplemental Figure 2.1. Rarefaction curves of bacterial and fungal ASV diversity for each honey sample based on the number of obtained reads in the sequencing libraries and identified species.



Supplemental Figure 3.1. LC-MS spectrum for singly charged m/z 1043.5 and doubly charged 522.3 of C_{14} iturin A. Spectrum was extracted from LC-MS for purified antifungal compounds produced by *Bacillus velezensis* WRB-ZX-001.

Supplemental Table 3.1. List of publicly available *Bacillus* spp. genome assembly included in this study.

Isolate	Assembly Accession Number
B_amyloliquefaciens_ATCC_23350_strain_DSM7	GCA_000196735.1
B_amyloliquefaciens_strain_CC178	GCA_000494835.1
B_amyloliquefaciens_strain_DC-12	GCA_000330805.1
B_amyloliquefaciens_strain_EBL11	GCA_000559145.1
B_amyloliquefaciens_strain_EGD-AQ14	GCA_000465655.1
B_amyloliquefaciens_strain_HB-26	GCA_000784675.1
B_amyloliquefaciens_strain_IT-45	GCA_000242855.2
B_amyloliquefaciens_strain_KHG19	GCA_000835145.1
B_amyloliquefaciens_strain_LFB112	GCA_000508265.1
B_amyloliquefaciens_strain_LL3	GCA_000204275.1
B_amyloliquefaciens_strain_Lx-11	GCA_001077735.1
B_amyloliquefaciens_strain_TA208	GCA_000195515.1
B_amyloliquefaciens_strain_UASWS_BA1	GCA_000469015.2
B_amyloliquefaciens_strain_UMAF6614	GCA_001593785.1
B_amyloliquefaciens_strain_UMAF6639	GCA_001593765.1
B_amyloliquefaciens_strain_XH7	GCA_000221645.1
B_amyloliquefaciens_strain_Y2	GCA_000262385.1
B_siamensis_strain_7551	GCA_002271775.1
B_siamensis_strain_JJC33M	GCA_000798615.1
B_siamensis_strain_KCTC_13613	GCA_000262045.1
B_siamensis_strain_SCSIO_05746	GCA_002850535.1
B_siamensis_strain_SRCM100169	GCA_001662915.1
B_siamensis_strain_XY18	GCA_000966575.1
B_subtilis_strain_168	GCA_000009045.1
B_velezensis_strain_AS43_3	GCA_000319475.1
B_velezensis_strain_CAU_B946	GCA_000283695.1
B_velezensis_strain_CBMB205	GCA_002117165.1
B_velezensis_strain_FZB42	GCA_000015785.2
B_velezensis_strain_KACC13105	GCA_000960265.2
B_velezensis_strain_KACC18228	GCA_001461835.1
B_velezensis_strain_KCTC13012	GCA_001267695.1
B_velezensis_strain_M27	GCA_000299615.1
B_velezensis_strain_NAU-B3	GCA_000493375.1
B_velezensis_strain_NJN-6	GCA_000973585.1
B_velezensis_strain_NRRL_B-41580	GCA_001461825.1
B_velezensis_strain_SK19_001	GCA_000513755.1
B_velezensis_strain_SQR9	GCA_000685725.1
B_velezensis_strain_TrigoCor1448	GCA_000583065.1
B_velezensis_strain_UCMB5033	GCA_000455565.1
B_velezensis_strain_UCMB5036	GCA_000341875.1
B_velezensis_strain_UCMB5113	GCA_000455585.1

B_velezensis_strain_YAU_B9601-Y2	GCA_000284395.1

Supplemental Table 3.2. Gene Ontology (GO) annotation and node score distribution for *Bacillus velezensis* WRB-ZX-001 and WRB-ZX-002.

GO term	*Bacillus velezensis* WRB-ZX-001	*Bacillus velezensis* WRB-ZX-002
Biological Process (BP) Number of Sequences	1953	1955
Transport	11%	11%
Carbohydrate derivative metabolic process	10%	10%
Regulation of gene expression	9%	9%
Transcription, DNA-templated	9%	9%
Carbohydrate metabolic process	8%	8%
Alpha-amino acid metabolic process	8%	8%
Organonitrogen compound biosynthetic process	7%	7%
Nucleobase-containing small molecule metabolic process	5%	5%
Phosphate-containing compound metabolic process	5%	5%
Organophosphate metabolic process	4%	4%
Monocarboxylic acid metabolic process	4%	4%
Regulation of cellular macromolecule biosynthetic process	4%	4%
Cellular Component (CC) Number of Sequences	1870	1870
Integral component of membrane	55%	55%
Cytoplasm	26%	26%
Plasma membrane	16%	16%
Catalytic complex	3%	3%
Molecular Function (MF) Number of Sequences	2660	2662
Hydrolase activity	32%	32%
Oxidoreductase activity	17%	17%
Metal ion binding	13%	13%
Transmembrane transporter activity	11%	11%
DNA binding	10%	10%
ATP binding	9%	9%
Transferase activity; transferring phosphorus-containing groups	8%	8%