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Original Article

Metabolic profiles of *Weissella* spp. postbiotics with anti-microbial and anti-oxidant effects

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Abstract

Introduction: This present study aimed to isolate beneficial bacteria from honey bee pollen microbiota and to investigate the metabolite profiles of postbiotics exhibiting anti-microbial and anti-oxidant properties.

Methodology: Pour plate technique was used to isolate bacteria from honey bee (*Apis mellifera* L.) pollen samples. Different colonies grown on agar plates were selected and screened for their anti-microbial activity against important pathogens using agar well diffusion assay. The isolates that exhibited remarkable inhibitory effects against all tested pathogens were identified by 16S rRNA sequence analysis. DPPH (2, 2-diphenyl-1-picrylhydrazil) free radical scavenging assays were used to assess the antioxidant capacity of their postbiotics. Besides, the total phenolic and total flavonoid compounds in postbiotics were determined as gallic acid and quercetin equivalents, respectively. The valuable metabolites in postbiotics were also profiled using chromatographic tools and Mass Spectrophotometry (MS) analysis.

Results: Twenty-seven strains were isolated from different honey bee pollen samples. 16 out of the 27 strains exhibited antagonistic activity against at least one reference strain of pathogens, tested. The most effective strains belonging to the genus *Weissella* were identified as *W. cibaria* and *W. confusa.* Postbiotics above 10 mg/mL exhibited higher radical scavenging activity and high total phenolic and total flavonoid contents. MS analysis demonstrated that metabolites in postbiotics derived from *Weissella* spp. were found very similar to the metabolites found in honeybee pollen.

Conclusions: The outcomes of this study revealed that honey bee pollen could be considered a potential source for the bacteria which produce anti-microbial and anti-oxidant agents. The similarity with the nutritional dynamics of honey bee pollen also indicated that postbiotics could also be used as novel and sustainable food supplements.

Key words: Honey bee; pollen; microbiota; *Weissella*; postbiotics; bio-activity.

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Introduction

The World Health Organization has endorsed the improvement of human nutrition to support the health status of the world population as one of the global goals for 2025 [1]. The increase in the incidence of diet-related diseases has led to increased consumer awareness about healthy lifestyles and well-being in today's society. This awareness has also led consumers to prefer environmentally friendly and natural products. Therefore, the demand for organic superfoods with their positive effects on health has emerged [2]. Recently, "Superfoods" which is used as an umbrella term for various foods and ingredients with valuable nutritional and health benefits has started to gain attention [3]. Considering the human health implications, the global superfood market size is expected to reach $ 209.1 billion by 2026, with a market growth of 7.3% CAGR over the forecast period [4].

Honey bee pollen defined as a subgroup of superfoods and considered an important and natural product for human health, includes a mixture of flower pollen, nectar, and bee secretions [5]. It is categorized as a superfood due to its valuable and nutrient-rich profile especially containing amino acids, essential fatty acids, vitamins, phenolic compounds, bio-elements, enzymes, and co-enzymes [5,6]. To date, scientific and current evidence shows that honey bee pollen exhibits potential anti-oxidant, anti-allergenic, anti-microbial, anti-viral, anti-carcinogenic, and anti-inflammatory effects [7,8]. However, its nutritional and biological properties can be variable according to the botanical diversity, geographical origin, ecological habitat, and climate conditions where bee pollen is collected [9]. Besides, some risk factors should be considered such as serious allergic reactions [10], and the possibility of honey bee pollen contaminations

including heavy metals, pesticide and herbicide residues, and mycotoxin-producing molds [11]. Considering these disadvantages, more research is needed to develop alternative products from honey bee pollen with sustainable effects to reduce concerns about the safety of pollen consumption in the human diet.

Recent studies have shown that the microbiological quality of honey bee pollen is associated with the presence of beneficial bacteria and that the pollen microbiota influences the nutritional profile and biological activity of honey bee pollen [12]. Most of the dominant bacteria in the pollen microbiota originate primarily from the flowers visited by honey bees [13] and also from the bees themselves [14]. These bacteria are considered safe and play key roles in the health benefits of honey bee pollen [15]. This present study aimed to isolate beneficial bacteria from honey bee pollen microbiota and to investigate the metabolite profiles of postbiotics exhibiting anti-microbial and anti-oxidant properties. The valuable metabolites in postbiotics were also profiled using chromatographic tools and Mass Spectrophotometry (MS) analysis. In conclusion, postbiotics that might have the potential to be used as natural and sustainable food supplements were investigated.

Methodology

Isolation of bacteria from honey bee pollen

Honey bee (*Apis mellifera* L.) pollen samples (n = 3) were collected from various honey bee farms in different provinces in Turkey (Corum, Amasya, and Tokat), and immediately transferred to the laboratory under cold chain and aseptic conditions to perform microbiological analysis. Briefly, 1 g pollen of each sample was suspended in 9 mL MRS (De Man, Rogosa, and Sharpe) broth medium (Merck, Germany) separately, and then homogenized. Following the incubation period at 37 ºC for 48 hours, the pre-enriched culture medium was diluted in saline solution and 100 µL aliquot of each sample was spread onto MRS agar plates. The plates were incubated aerobically at 37 ºC for approximately 1-3 days. Colonies displayed different morphologies were picked up and then purified by streak plating. Pure isolates with Gram-positive and catalase-negative phenotypes were selected and then stored in sterile glycerol/MRS broth mixture (50:50%; v/v) at -80 ºC, for further studies [16].

Molecular identification of the isolates

The selected isolates were identified by sequencing of the 16S ribosomal-RNA (16S rRNA) subunit gene. Briefly, genomic DNA was isolated from overnight culture using a commercial kit (Qiagen, Germany) according to the manufacturer's instructions. 16S rRNA region was amplified by polymerase chain reaction (PCR, Thermal Cycler, Bio-Rad, USA) using the universal primers of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [17]. Each reaction including 100 ng of genomic DNA as a template, 25 µL of 2 × PCR Taq Master Mix (Applied Biological Materials Inc., Canada), and 0.5 µM of each primer was performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA) with an initial denaturation at 95 ºC for 6 minutes, 40 cycles of denaturation at 95 ºC for 30 seconds, annealing at 50 ºC for 1 minute, and extension at 72 ºC for 1 minute. A final extension was performed at 72 ºC for 10 minutes. Amplification products were analyzed using 2% (w/v) agarose gel electrophoresis and the amplicons were purified from agarose gel using a purification kit (Qiagen, Germany). 16S rRNA gene was then sequenced by the commercial services of BM Labosis Inc (Ankara, Turkey). The obtained sequences were compared with the sequences in the National Centre for Biotechnology Information (NCBI) database using the BLASTn program (https://www.ncbi.nlm.nih.gov/). Identification was defined based on the highest nucleotide sequence similarity.

Extraction of postbiotics derived from pollen-originated isolates

To obtain postbiotics secreted into the culture medium, each isolate was inoculated in MRS broth and incubated for 24 hours at 37 ºC. Following the incubation period, the cultures were centrifuged (6,000 rpm, 20 minutes, 4 ºC), and the supernatants were passed through a sterile membrane filter with a diameter of 0.22 µm. After filtration, the supernatant was lyophilized for 24 hours (freezing conditions of -20 ºC, the vacuum pressure of 0.120 mB, and a condenser temperature of -58 ºC; Christ freeze dryer, Germany), and dissolved in ddH_2O.

Screening of the isolates for their anti-microbial activity

To determine the anti-microbial activity, postbiotics as metabolites of randomly selected isolates were tested against different pathogens using agar well diffusion assay, in triplicates [18]. Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* NCTC 5348, *Bacillus licheniformis* DSM-13, *Staphylococcus epidermidis* ATCC 35984,

Klebsiella pneumaniae ATCC 700603, *Salmonella enterica* ATCC 13311, and *Shigella flexneri* ATCC 24570 were used as indicator strains and grown in BHI (Brain Heart Infusion, Merck, Germany) medium at 37 ºC for 24 hours. Briefly, each pathogen adjusted to 0.5 McFarland standard (Biomeireux, France) was transferred into BHI soft agar and then poured onto BHI agar plates. Wells with six mm diameters were formed on agar surfaces using the back of a sterile one-ml tip and then filled with postbiotics (100 mg/mL). The postbiotics were left to dry, and the plates were incubated at 37 ºC for 24 hours. Chloramphenicol (30 µg; Sigma-Aldrich, Germany) with its broad range anti-bacterial activity was used as a positive control. Following the incubation period, plates were examined for the inhibition zones and the diameters were measured with calipers as mm. Isolate exhibiting the maximum inhibition zone [ranked as high (> 15 mm)], was selected for further analysis.

Assessment of anti-oxidant activity

The phenolic content of postbiotics was determined using the spectrophotometric method of Singleton and Rossi [19]. Briefly, 100 µL of Folin-Ciocalteu (%50, Sigma-Aldrich, Germany) reagent was mixed with 100 µL postbiotics and then incubated in darkness at room temperature for 3 minutes. Following the incubation period, 2 mL of 2% sodium carbonate (Merck, Germany) solution was added into the tubes and incubated for 30 minutes in similar conditions. Different gallic acid (Sigma-Aldrich, Germany) concentrations (0.05, 0.1, 0.15, 0.2, 0.4 mg/mL) were used to establish a phenolic standard, while MRS was used as a blank. The color change due to the reaction was determined by spectrophotometric measurements at 750_{nm} wavelength. Results were analyzed using the gallic acid calibration curve and expressed as gallic acid equivalents (GAE) per gram weight of dry postbiotics.

The total amount of flavonoid substance in postbiotics was determined using the aluminum chloride colorimetric method [20]. Briefly, the reaction was prepared by mixing 300 µL of ethyl alcohol, 20 µL of aluminum chloride (10%, Merck, Germany), 20 µL of 1M sodium acetate (Merck, Germany), 560 µL of distilled water, and 100 µL of postbiotics. Different quercetin concentrations (0.025, 0.05, 0.1, 0.15, 0.2 mg/mL) were used to establish a flavonoid standard, while MRS was used as a blank. Following the incubation in darkness at room temperature for 30 minutes, their absorbances at 415_{nm} were measured using a spectrophotometer and the concentration of flavonoids was calculated as quercetin equivalents (QE) per gram weight of dry postbiotics.

2,2-Diphenyl-1-picrylhydrazyl (DPPH Sigma-Aldrich, Germany) radical scavenging activity (%) of postbiotics was determined by the method of Blois [21]. 200 µL of postbiotics in different concentrations (1-50 mg/mL) was mixed with 500 µL 120 µM DPPH in ethanol. Following the incubation period in darkness at room temperature for 30 minutes, the color change was analyzed by spectrophotometric measurements at 517_{nm}. DPPH free radical scavenging capability was calculated as % by the equation of $[(A_{control} - A_{sample})/A_{control}] \times 100$ (A: absorbance; Acontrol: absorbance of the control containing DPPH in ethanol; Asample: the absorbance in the presence of postbiotics). MRS was used as a negative control. IC50 value of each postbiotics was estimated from the percentage inhibition versus concentration plot, using a non-linear regression algorithm.

Metabolic characterization of postbiotics

Organic acids, fatty acids, multi-vitamins, amino acids, and sugars in postbiotics were determined by chromatographic tools and MS analysis using LabSolution software systems (Shimadzu, Kyoto, Japan) according to Omeroglou *et al.* [22]. To identify the organic acids (lactic acid, oxalic acid, tartaric acid, malic acid, maleic acid, succinic acid, formic acid, acetic acid, citric acid, and butyric acid) in postbiotics, High-Performance Liquid Chromatography (HPLC, Shimadzu Prominence Series-LC-20A, Kyoto, Japan) was applied using 87H-3 column (250 mm × 4.6 mm, 5 µm; Transgenomic; USA). 0.08 N H_2SO_4 solution was used as a mobile phase with an isocratic pump program. The column oven temperature was set at 37 ºC and the flow rate was set at 0.6 mL/minute. Substances were detected with a UV-visible detector (210_{nm}) and the linear calibration curves obtained from reference standards (Merck, Germany) were used for the normalization, calibration, and quantification of organic acids in postbiotics.

For the analysis of fatty acids in the postbiotics, Gas Chromatography-Mass Spectrometry (GC-MS, Shimadzu QP2020, Kyoto, Japan) was performed following the preparation of Fatty Acid Methyl Esters (FAMEs) [23]. The FAMEs were analyzed on a capillary column (TRCN 100; 100 m × 0.25 mm × 0.25 µm; Teknokroma, Spain) with the operating conditions as follows; SPL injection port set at 225 ºC, Interface at 200 ºC, Ion Source at 250 ºC with 70 eV Filament current, 99.999% Helium as carrier gas; flow rate at 0.75 mL/minute, Split ratio as 20:1 and 1µL of injection

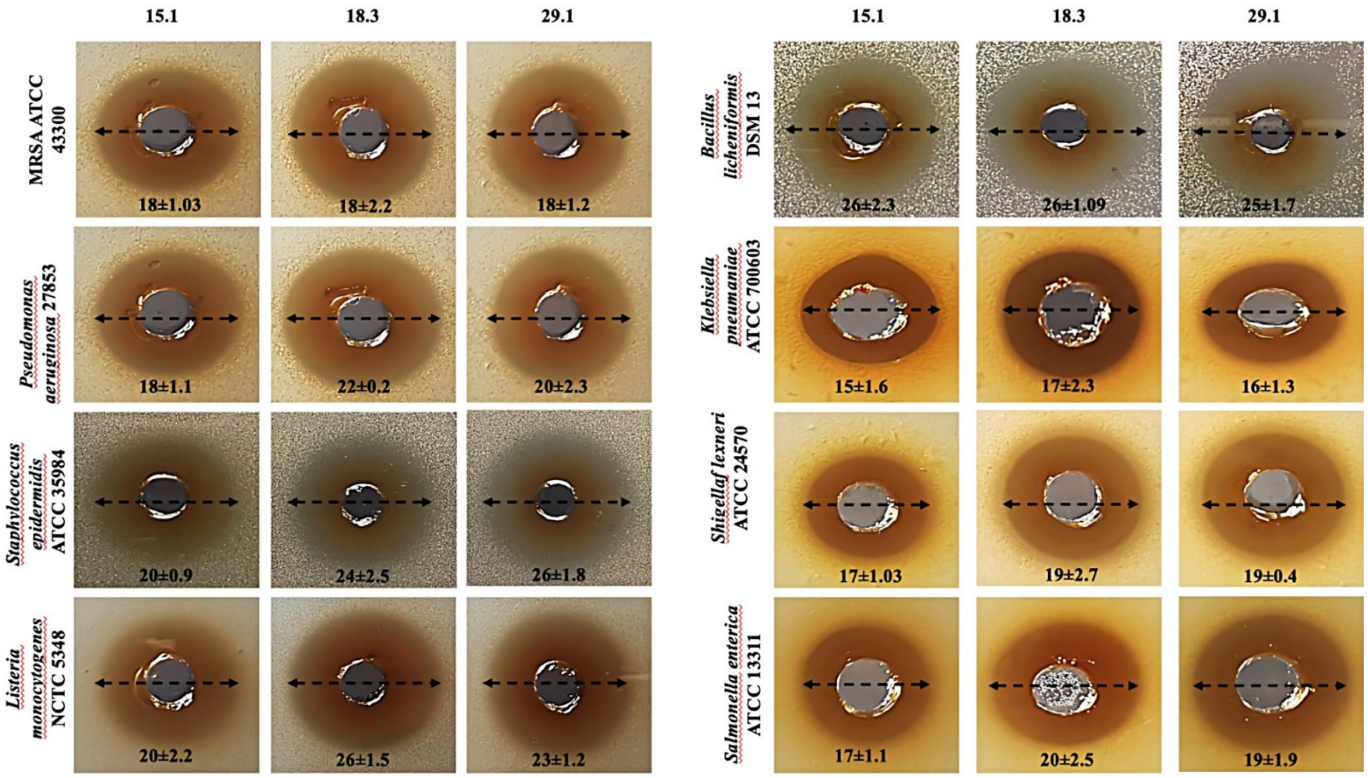
volume. Calibration curves with area normalization obtained from the reference standards of FAMEs (Restek 35077, Food Industry FAME Mix, Restek, USA) were used for the normalization, calibration, and quantification of FAMEs in postbiotics.

Multi-vitamin analysis including water-soluble and water-insoluble vitamins was also carried out using a High-Performance Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) system (Shimadzu LC-MS 8045, Kyoto, Japan) with a biphenyl featured column (50 mm × 2.1 mm, 1.8 µm, Restek, USA) at a flow rate of 0.3 mL/minute. A multistep linear solvent gradient system consisting of (A) 0.1% formic acid in water and (B) methanol was used as a mobile phase. Postbiotics and standards were prepared in methanol, filtered with a 0.22 µm Teflon filter (GLScience, Japan), and injected as 1 µL volume. Positive ESI (Electron Spray Ionization) technique was used for LC-MS/MS analysis and MRMs (Multi Reaction Monitoring) was applied for determining the water-soluble and water-insoluble vitamins in postbiotics compared to the reference standards of vitamins (Merck, Germany).

A pre-column derivatization HPLC method with a dual-channel RF-20AXS fluorescence detector (Shimadzu Prominence Series, LC-20A, Kyoto, Japan) was used for the analysis of the amino acid content of postbiotics. One gram of lyophilized postbiotics was dissolved in 100 mL of 0.1 N HCl. Derivatization was carried out by mixing the solutions with 90 µL of 3-mercaptopropionic acid, 44 µL of ortho-phthalaldehyde, and 20 µL of 9H-fluorenyl methyloxycarbonyl. The separation was performed in a mobile phase [potassium-phosphate buffer (20 mM, pH: 6.9), and acetonitrile/methanol/water (45:45:10%), respectively] with a gradient pump program using an InertSustain C18 analytical liquid column (150 × 4.0 mm, 3.5 µm; GLScience, Japan). The flow ratio of the mobile phase was set as 1 mL/minute under the controlled temperature of 35 ºC. Primarily amino acids were detected using 350_{nm} for excitation and 450 nm for emission, while secondary amino acids were detected using 266_{nm} for excitation and 305_{nm} for emission. Data were analyzed using the calibration curve of the reference standards (Sigma-Aldrich, Germany).

The mono and disaccharide compositions of postbiotics were analyzed by HPLC (Shimadzu, Prominence 20A Series) using a Shodex SP0810 Carbohydrate 300mm × 8.0 mm ID (Kyoto, Japan) column with a RI detector. LC-MS Grade Water (Merck, Germany) was applied as a mobile phase with a flow rate of 0.6 mL/minute. The injection volume was used as 20 µL, and the column oven temperature was set as 80 ºC. Quantitative measurements were

Figure 1. Inhibition zones of postbiotics on agar plates.



determined using a linear calibration curve prepared with the different concentrations of standard sugars such as fructose, glucose, saccharose, and maltose (Merck, Germany).

Statistical analysis

All assays were performed with three independent experiments (biological replicates) and each measurement was carried out in triplicate (technical replicates). Data were analyzed using SPSS version 22.0 (IBM, New York, NY, USA), by one-way analysis of variance (ANOVA) followed by Dunnett's test and unpaired *t*-test (GraphPad Prism v.3.0, GraphPad Software, San Diego, CA, USA). All results were presented as a mean ± standard deviation and $p < 0.05$ was used to indicate a significant difference.

Results

Isolation, identification, and selection of bacteria with anti-microbial activity

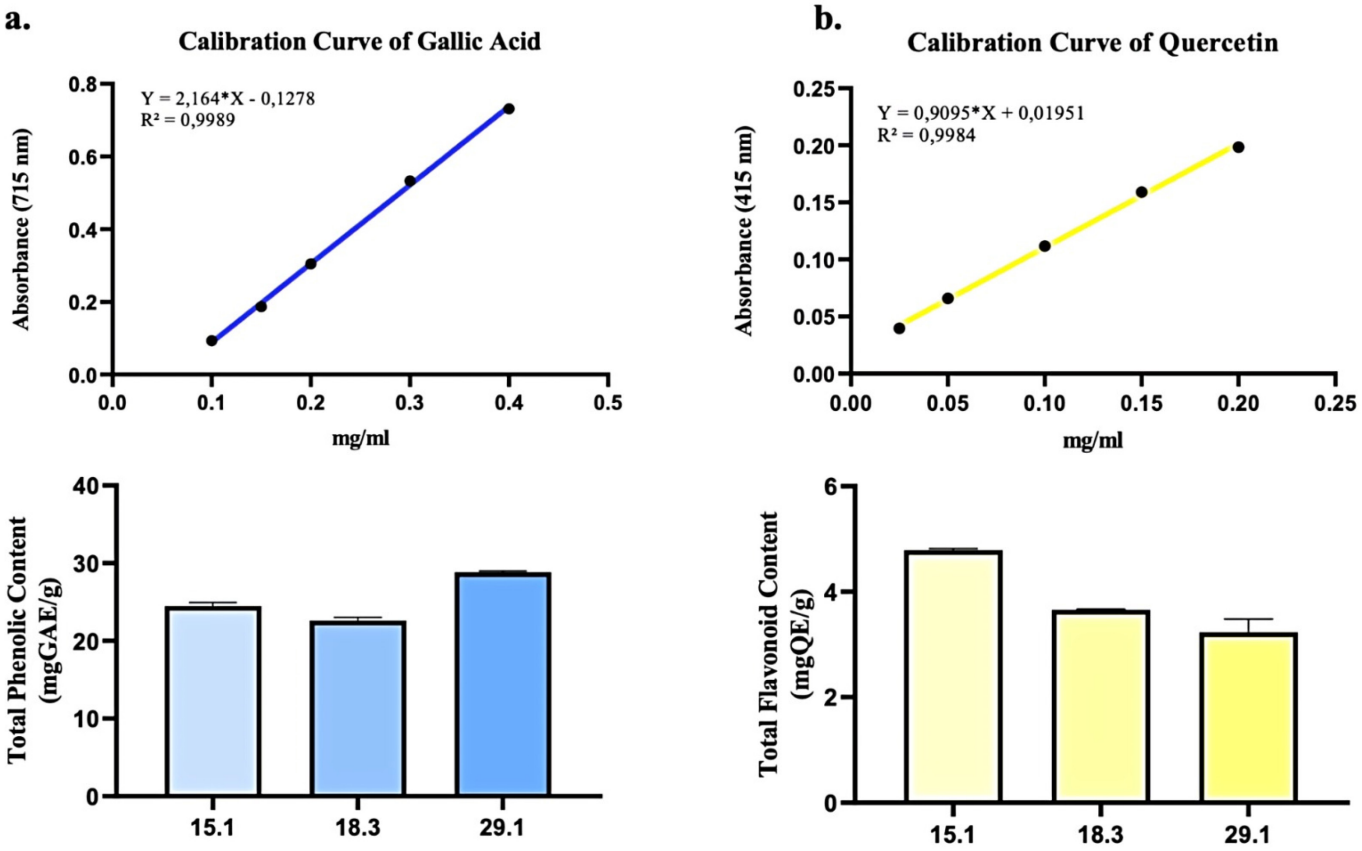
In the present study, three colonies for each honey bee pollen sample (27 colonies in total) which displayed Gram-positive reaction and no catalase activity were selected for further analysis. Postbiotics extracted from these isolates were tested for their anti-microbial activity against different pathogens. According to the results, eleven of them displayed no inhibition against the tested pathogens, while seven isolates were found inhibitory to at least one or two pathogenic strains (Supplementary Table 1). Among the isolates, three of them named as 15.1, 18.3, and 29.1 exhibited a remarkable inhibitory spectrum against all tested bacteria (Figure 1). Postbiotics of these isolates displayed strong inhibition against all clinical isolates with inhibition zones between 15-26 mm. The highest anti-microbial activity was observed against *Bacillus licheniformis* DSM-13.

The selected isolates were identified by sequencing of their 16S rRNA gene region (1.492 bp) followed by BLAST search against GenBank Bacteria and Archaea 16S ribosomal RNA sequence database. According to the results obtained, isolates 15.1 most closely matched with *Weissella confusa*, while isolates 18.3 and 29.1 matched with *Weissela cibaria* with the similarity of 99%.

Anti-oxidant activity of postbiotics

The anti-oxidant activity was evaluated in terms of the phenolic and flavonoid contents of the postbiotics, and their DPPH radical scavenging activity. The total

Figure 2. Total (**a**) phenolic and (**b**) flavonoid content of postbiotics.

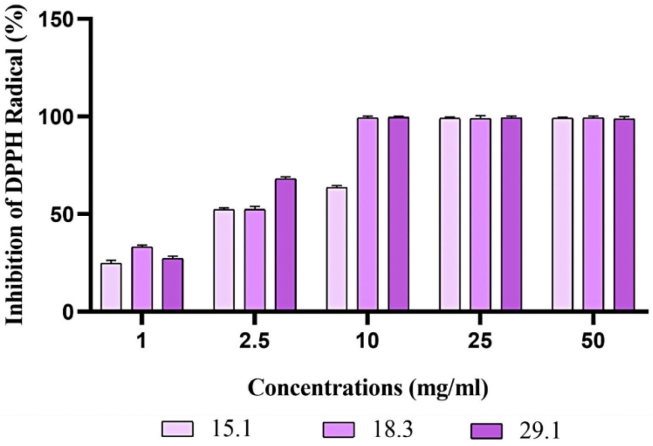


phenolic content of postbiotics was determined according to the Folin Ciocalteu method and the results were expressed as GAE using the standard curve (Figure 2a). Among the isolates, postbiotics derived from *W. cibaria* 29.1 displayed the highest phenolic content at 28.23 mg GAE/g. Our results also showed that postbiotics derived from *W. confusa* 15.1 had the highest amount of flavonoid content at 4.8 mg QE/g when compared with the other strains (Figure 2b). According to the results of total anti-oxidant activity, postbiotics above 10 mg/mL exhibited strong DPPH radical scavenging activity (Figure 3). IC50s were also determined as 5.28 mg/mL, 2.33 mg/mL, and 1.73 mg/mL for *W. confusa* 15.1, *W. cibaria* 18.3, and *W. cibaria* 29.1, respectively.

Table 1. Metabolites identified in postbiotics of Weissella spp.

	15.1	18.3	29.1
FAMEs (%)			
C4:0 Methyl butyrate	0.12 ± 0.2	0.11 ± 0.2	0.11 ± 0.4
C6:0 Methyl caproate	0.22 ± 0.1	0.18 ± 0.2	0.15 ± 0.1
C8:0 Methyl caprylate	0.09 ± 0.03	0.07 ± 0.01	0.06 ± 0.04
C13:0 Methyl tridecanoate	0.00	0.00	0.08 ± 0.001
C18:0 Methyl stearate	0.19 ± 0.02	0.25 ± 0.1	0.30 ± 0.3
C20:1 cis-11 Methyl eicosenoate	0.00	0.13 ± 0.3	0.00
C20:2 cis-11,14 Methyl eicosadienoate	0.18 ± 0.2	0.00	0.00
Amino Acids (%)			
Aspartic acid	0.48 ± 0.01	0.60 ± 0.2	0.50 ± 0.03
Glutamic acid	1.11 ± 0.3	1.26 ± 0.1	1.15 ± 0.01
Glutamine	0.30 ± 0.1	0.45 ± 0.4	0.42 ± 0.02
Histidine	0.19 ± 0.02	0.22 ± 0.03	0.21 ± 0.01
Glycine	0.15 ± 0.3	0.18 ± 0.03	0.18 ± 0.5
Threonine	0.45 ± 0.04	0.55 ± 0.4	0.51 ± 0.1
Citrulline	0.02 ± 0.1	0.03 ± 0.2	0.03 ± 0.2
Arginine	0.60 ± 0.02	0.86 ± 0.3	0.81 ± 0.1
Alanine	0.58 ± 0.3	0.56 ± 0.01	0.54 ± 0.1
Tyrosine	0.14 ± 0.09	0.23 ± 0.2	0.22 ± 0.02
Cystine	0.73 ± 0.6	0.91 ± 0.4	0.85 ± 0.3
Valine	0.22 ± 0.8	0.30 ± 0.03	0.26 ± 0.05
Methionine	0.33 ± 0.02	0.44 ± 0.7	0.40 ± 0.1
Trytophan	0.28 ± 0.3	0.45 ± 0.08	0.43 ± 0.6
Phenylalanine	0.45 ± 0.1	0.73 ± 0.3	0.69 ± 0.2
Isoleucine	1.17 ± 0.2	1.58 ± 0.2	1.48 ± 0.03
Leucine	0.61 ± 0.06	1.21 ± 0.05	1.15 ± 0.02
Lysine	1.58 ± 0.1	2.49 ± 0.1	2.19 ± 0.1
Hydorxy proline	0.21 ± 0.01	0.36 ± 0.3	0.37 ± 0.3
Proline	0.50 ± 0.5	0.44 ± 0.6	0.40 ± 0.04
Vitamins (µg/L)			
Vitamin B1	78.1 ± 0.3	48.18 ± 0.2	50.13 ± 0.3
Vitamin B2	20.19 ± 0.1	27.62 ± 0.5	25.98 ± 0.3
Vitamin B3	1.89 ± 0.3	2.01 ± 0.2	2.55 ± 0.1
Vitamin B5	50.46 ± 0.06	23.95 ± 0.05	12.65 ± 0.03
Vitamin B12	300.15 ± 0.07	351.62 ± 0.3	321.01 ± 0.4
Vitamin D3	0.25 ± 0.2	0.34 ± 0.01	1.45 ± 0.05
Vitamin E1	59.96 ± 0.2	40.04 ± 0.3	75.34 ± 0.4
Vitamin K3	7.83 ± 0.06	7.15 ± 0.08	6.99 ± 0.03
Carbohydrates (%)			
Saccharose	0.56 ± 0.2	0.78 ± 0.4	0.50 ± 0.1
Glucose	11.63 ± 0.06	1.86 ± 0.02	2.79 ± 0.01
Maltose	0.02 ± 0.03	0.09 ± 0.01	0.16 ± 0.07
Fructose	0.01 ± 0.01	0.52 ± 0.01	0.39 ± 0.03
Organic Acids (mg/mL)			
Maleic Acid	1.15 ± 0.02	2.89 ± 0.03	2.74 ± 0.04
Citric Acid	0.00	0.10 ± 0.2	0.00
Tartaric Acid	0.90 ± 0.3	1.39 ± 0.5	1.25 ± 0.2
Lactic Acid	21.45 ± 0.6	22.54 ± 0.3	21.38 ± 0.3
Succinic Acid	0.00	0.68 ± 0.01	0.00
Formic Acid	3.07 ± 0.2	4.08 ± 0.1	3.75 ± 0.4
Acetic Acid	1.30 ± 0.02	1.00 ± 0.08	0.98 ± 0.0
Butyric Acid	0.15 ± 0.1	0.09 ± 0.2	0.15 ± 0.1

Figure 3. DPPH (2,2-diphenyl-1-picrylhydrazil) scavenging activity of postbiotics.



Metabolites in postbiotics derived from Weissella spp.

Metabolites in postbiotics were identified by chromatographic analysis and listed in Table 1. When the results were evaluated in terms of organic acids, HPLC chromatograms (Figure 4) were shown that lactic acid was the highest (21.38- 22.54 mg/mL) organic acid in postbiotics. Meanwhile, succinic acid and citric acid were only found in postbiotics derived from the strain of 18.3 at 0.68 mg/mL and 0.10 mg/mL, respectively. Overall, all the organic acids tested were found in higher amounts in postbiotics derived from the strain of 18.3 compared with the others. From the point of 37 FAMEs examined in postbiotics by GC-MS (Figure 5), our results were shown that stearic acid (C18:0), caprylic acid (C8:0), caproic acid (C6:0) and butyric acid (C4:0) were found in all postbiotics between the range of 0.06 to 0.30 % concentrations. Besides, tridecanoic acid (C13:0), eicosenoic acid (C20:1, cis-11), and eicosadienoic acid (C20:2, cis-11,14) were only found in postbiotics obtained from the strains of 29.1, 18.3 and 15.1, respectively. Among the vitamins identified by LC-MS/MS and defined by MRM (Multi Reaction Monitoring) transitions, water-soluble B-complex vitamins (B1 thiamine, B2 riboflavin, B5 pantothenic acid, and B12 cobalamin) were highly observed in postbiotics between the range of 20.19 to 351.62 µg/L. In addition, some fat-soluble vitamins (E1 and K3) were detected in postbiotics between the range of 6.99 to 75.34 µg/L. Vitamin B12 was identified as the most abundant vitamin among the vitamins in the postbiotics. According to the amino acid analysis performed by post-column derivatization HPLC with dual-channel fluorescence detection, it was shown that amino acids were detected in all postbiotics in different concentrations between the range of 0.02 and 2.49% (Figure 6).

Figure 4. HPLC chromatograms of organic acids in postbiotics derived from (**a**) Weissella confusa 15.1, (**b**) Weissela cibaria 18.3, and (**c**) Weissella cibaria 29.1.

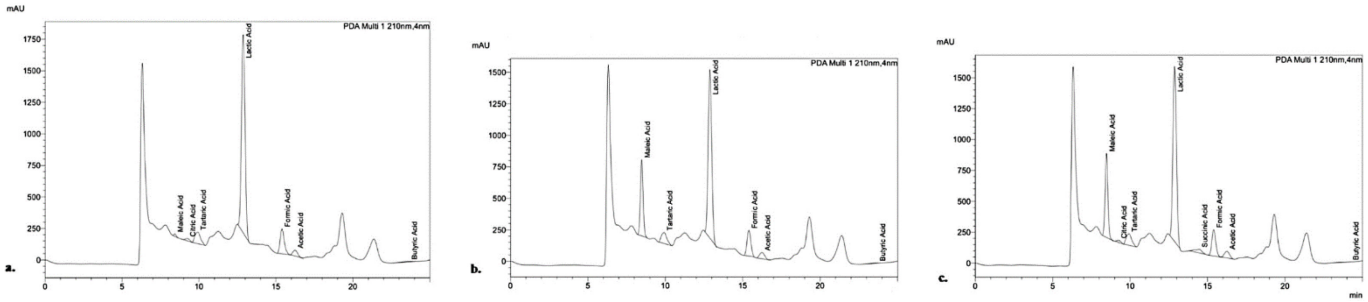


Figure 5. MS chromatograms of fatty acid methyl esters in postbiotics derived from (**a**) Weissella confusa 15.1, (**b**) Weissela cibaria 18.3, and (**c**) Weissella cibaria 29.1.

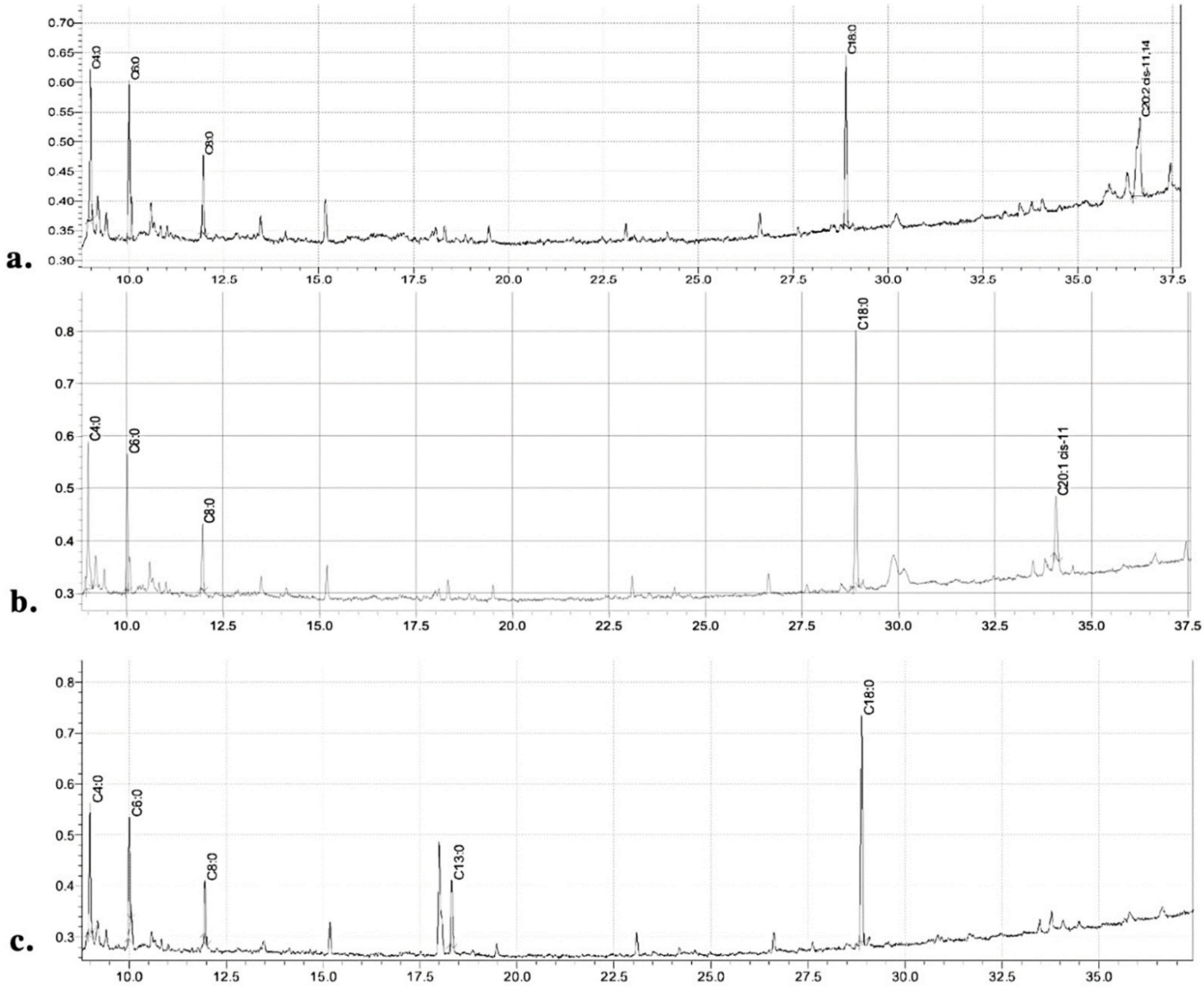


Figure 6. HPLC chromatograms of amino acids in postbiotics derived from (**a**) Weissella confusa 15.1, (**b**) Weissela cibaria 18.3, and (**c**) Weissella cibaria 29.1.

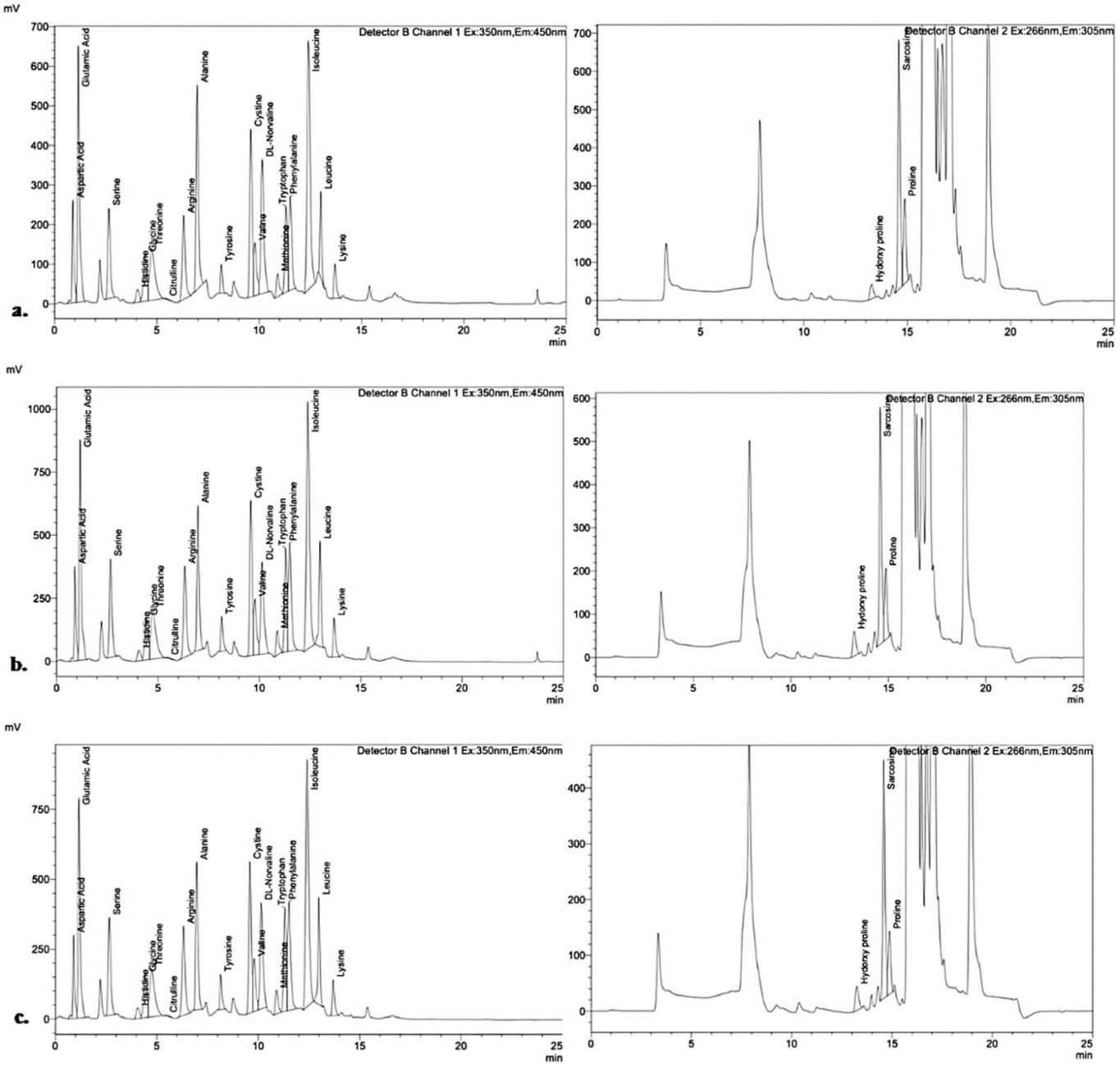
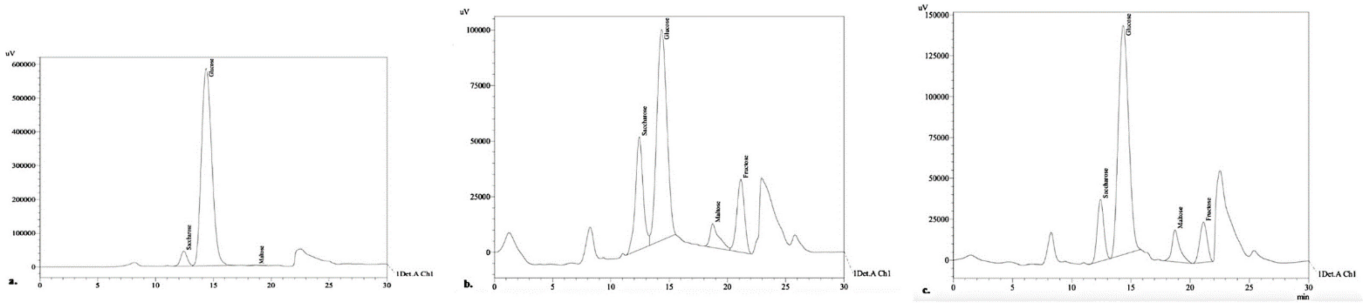


Figure 7. HPLC-RID (Refractive Index Detector) chromatograms of mono and disaccharide compositions in postbiotics derived from (**a**) Weissella confusa 15.1, (**b**) Weissela cibaria 18.3, and (**c**) Weissella cibaria 29.1.



Lysine was identified as the most abundant amino acid in postbiotics. From the point of carbohydrate analysis carried out by HPLC, it was determined that glucose was the highest sugar in postbiotics when compared to others (Figure 7). The highest glucose amount was determined in the postbiotics obtained from the strain of 15.1 at 11.63 ± 0.06 %.

Discussion

With the increasing consumer interest in alternative medicine products and wellbeing supplements, honey bee pollen is gaining attention as an important apitherapeutic agent with its significant nutritional properties and functional values [24]. Recent studies have proven the potential contribution of honey bee pollen microbiota to the nutritional dynamics of pollen as well as to the regulation of its mechanism that exert health benefits [12]. Although, little is known about which bacterial strains in the microbiota of this apitherapeutic product contribute its high nutritional value and health benefits, the outcomes of recent investigations have suggested that lactic acid bacteria are of primary importance [15]. The main purpose of this study is the isolation and identification of bacteria found in honey bee pollen microbiota. The obtained isolates were first screened for their potential to inhibit selected human pathogens. Twenty-seven strains were isolated from different honey bee pollen samples. 16 out of the 27 strains were exhibited an antagonistic activity against at least one reference strain of pathogens, tested. Interestingly, sequence analysis of the 16S rRNA genes of the isolates revealed that the most effective strains belonged to the genus *Weissella* spp., with two different species identified as *W. cibaria* and *W. confusa.*

To date, different *Weissella* spp. strains have been isolated from a wide range of habitats such as saliva, milk, and feces of animals and humans as well as foods, plants, and vegetables [25]. Similar to our results, scientists have also reported the isolation of *Weissella* spp. from honey bee pollen [26] and honey [27]. However, Pelka *et al.* [15] identified *Bacillus* spp. as the most common type of bacteria in honey bee pollen that exhibited high antagonistic activity against important clinical human pathogens. The main difference between this study and our study is that the researchers used dried honey bee pollen samples in their studies which might have led to the isolation of highly resistant bacteria such as spore-forming *Bacillus* spp. Similar to our results, *Weissella* strains were screened for their anti-microbial activity in several studies [15,28-30]. *W. cibaria* isolates from children's saliva were shown to inhibit bacterial pathogens in dental caries [31]. Hydrogen peroxide-producing *W. cibaria* species were found capable of inhibiting the halitosis indicators [32]. Six bacteriocins have so far been reported for *Weissella* strains belonging to the *W. cibaria*, *W. paramesenteroides*, and *W. hellenica* species which have shown a broad inhibitory activity against human and food-borne pathogens [33-36].

Apart from the technical role of certain *Weissella* spp. species involved in the traditional fermentation process, specifically *Weissella* spp. strains are also receiving attention for their potential anti-oxidant activities. Our results showed that postbiotics obtained from each strain highly exhibited phenolic and flavonoid content, and DPPH inhibition activity. Similarly, *W. cibaria* FB-069 isolated from traditional Korean salted squid [37], *W. cibaria* JW15 [30], *W. confusa* (KR780676) isolated from an Indian traditional fermented food [38], and *W. cibaria* DMA 18 isolated from tender coconut water [39] displayed significant radical scavenging effects. Recent studies confirmed that the higher anti-oxidant activity could be related to the production of bioactive compounds such as flavonoids, and phenolics which are also considered the efficient anti-oxidant compounds of honey bee pollen [5,7,8].

In recent years, postbiotics secreted by live bacteria during the fermentation process have gained attention. Regarding the importance of the postbiotics with their bioactive metabolites involved in the beneficial effects of *Weissella* spp. strains, metabolite profiles of the postbiotics were also determined using chromatographic tools. Similar to the nutritional dynamics of honey bee pollen, our results showed that amino acids, sugars, lipids, and vitamins were found as the main components of postbiotics derived from *Weissella* spp. strains [8]. Lysine, threonine, and isoleucine amino acids which cannot be synthesized in our bodies but also play important roles in optimal growth and health were found in higher amounts in postbiotics and honey bee pollen [40]. It is well known that glucose and fructose exist in honey bee pollen at 30.8% as a source of energy [41]. Similar to honey bee pollen, glucose was found the most abundant carbohydrate in postbiotics. With respect to the lipid content of honey bee pollen, 5.1% of lipids are found as essential fatty acids such as linoleic acids [42]. In our study, stearic acid was found the most common fatty acid in postbiotics. In addition to these important metabolites, honey bee pollen is a potential source of water-soluble vitamins such as vitamins B1, B2, B6, and C; and fat-soluble vitamins such as vitamin E, A,

and D [5]. Among the vitamins that we identified in postbiotics, B-complex vitamins were found as the most common vitamins which might act as important catalysts for enzymes in energy metabolism [43] and influence the host mechanisms [44]. Overall, the metabolites of postbiotics were generally found similar compared to the metabolites of honey bee pollen that contribute to their therapeutic potential.

Conclusions

Important gaps in our knowledge still remain regarding the microbial composition of honey bee pollen and the key roles of these microorganisms in the product and humans who consume it [15]. However, recent evidences draw attention to the axis between the honey bee pollen and its microbial composition that is involved in the production of different metabolites related to its nutritional dynamics and health benefits. The outcomes of this study revealed that honey bee pollen could be considered natural sources for bacteria with anti-microbial and anti-oxidant activities. Besides, their postbiotics with their valuable ingredients such as vitamins, amino acids, sugars, and fatty acids could be a potential source for their particular industrial importance. However, it is necessary to conduct further studies to determine the critical mechanisms related to the pharmacological activities of the postbiotics obtained from honey bee pollen microbiota.

Authors' Contributions

FK and HKD provided the initial idea for the study. FK developed the protocol. All authors analyzed and interpreted the data. FK wrote the first draft of the manuscript. All authors contributed to the preparation of subsequent versions of the manuscript and approved the final version for publication. FK is the guarantor.

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Annex – Supplementary Items

Supplementary Table 1. Inhibition zones (mm) of postbiotics derived from honey bee pollen microbiota against different pathogens.

Isolate No	MRSA ATCC 43300	*Pseudomonas aeruginosa* ATCC 27853	*Staphylococcus epidermidis* ATCC 35984	*Listeria monocytogenes* NCTC 5348	*Bacillus licheniformis* DSM 13	*Klebsiella pneumaniae* ATCC 700603	*Salmonella enterica* ATCC 13311	*Shigella flexneri* ATCC 24570
1	6 ± 0.1	0	8 ± 0.1	0	0	0	0	0
2 (Strain:15.1)	**18 ± 1.03**	**18 ± 1.1**	**20 ± 0.9**	**20 ± 2.2**	**26 ± 2.3**	**15 ± 1.6**	**17 ± 1.03**	**17 ± 1.1**
3	0	0	0	11 ± 0.3	14 ± 1.2	0	0	0
4	0	0	0	0	0	0	0	0
5	16 ± 0.6	0	0	0	19 ± 0.4	0	0	0
6	0	0	0	0	0	0	0	0
7	12 ± 0.2	9 ± 0.6	14 ± 1.2	11 ± 0.1	18 ± 1.1	7 ± 0.2	8 ± 0.1	7 ± 0.1
8	0	0	0	0	0	0	0	0
9 (Strain:18.3)	**18 ± 2.2**	**22 ± 0.2**	**24 ± 2.5**	**26 ± 1.5**	**26 ± 1.09**	**17 ± 2.3**	**19 ± 2.7**	**20 ± 2.5**
10	11 ± 0.2	13 ± 0.1	16 ± 1.2	14 ± 0.6	21 ± 2.2	7 ± 0.2	8 ± 0.1	7 ± 0.1
11	14 ± 0.4	8 ± 0.1	12 ± 0.6	13 ± 0.4	18 ± 2.1	9 ± 0.2	8 ± 0.1	6 ± 0.1
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	11 ± 0.1	16 ± 0.2	0	0	0
15	14 ± 1.6	11 ± 1.6	18 ± 1.2	16 ± 0.4	19 ± 1.8	11 ± 0.1	9 ± 0.1	6 ± 0.1
16	0	0	0	0	0	0	0	0
17	12 ± 0.6	9 ± 1.1	21 ± 2.6	14 ± 0.4	12 ± 1.8	9 ± 0.1	7 ± 0.1	6 ± 0.1
18	0	0	0	0	0	0	0	0
19	8 ± 0.2	0	11 ± 0.8	0	0	0	0	0
20	11 ± 1.1	14 ± 1.2	21 ± 2.6	9 ± 0.6	13 ± 1.4	10 ± 0.4	7 ± 0.1	8 ± 0.2
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	9 ± 0.4	0	0	0	17 ± 0.2	0	0	0
24	13 ± 0.1	0	0	0	21 ± 0.8	0	0	0
25	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0
27 (Strain:29.1)	**18 ± 1.2**	**20 ± 2.3**	**26 ± 1.8**	**23 ± 1.2**	**25 ± 1.7**	**16 ± 1.3**	**19 ± 0.4**	**19 ± 1.9**