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Examining the effect of lactic acid producing bacteria on honey quality and quantity

Muna Barakat[1*], Shaymaa B. Abdulrazzaq[2], Ahmad Sinan Badwan[3], Sanad Naser El-Banna[3], Anfal Al-Dalaeen[4], Sawsan Abu Jamma'ah[3], Mahmoud Jaber[5], Samar Sabri Qaddoumi[6], Mohammad A.A. Al-Najjar[2].

[1]Department of Clinical Pharmacy and Therapeutics, Faculty of Pharmacy, Applied Science Private University, Amman, Jordan.
[2]Department of Pharmaceutics and Pharmaceutical Sciences, Faculty of Pharmacy, Applied Science Private University, Amman, Jordan.
[3]Research unit, Jubilee School, Amman, Jordan.
[4]Department of Clinical Nutrition and Dietetics, Faculty of Allied Medical Sciences, Applied Science Private University, Amman, Jordan.
[5]Apitherapy Expert, Bee Way company, Amman, Jordan.
[6]Immuno lab, Amman, Jordan

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***Corresponding author:**
Department of Clinical Pharmacy and Therapeutics, Faculty of Pharmacy, Applied Science Private University, Amman, Jordan.

Email: M_barakat@asu.edu. Jo

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ABSTRACT

Background: Honeybees play a pivotal role in the sustainability of ecosystems and biodiversity. Various environmental problems have affected the most significant pollinators—honeybees. Currently, many challenges are facing honeybee health, and lactic acid-producing bacteria, naturally found in honeybees' gut microbiota, could be used to enhance honey production and quality. This study aimed to examine the effect of using lactic acid-producing bacterial probiotics as a supplement in honeybee food on honey quality and quantity compared to unsupplemented honeybees.

Methods: Probiotic supplements (Lactobacillus reuteri, Lactobacillus helveticus, Lactobacillus bulgaricus, Lactobacillus acidophilus, and Bifidobacterium bifidum) for honeybees were prepared in three different forms (supernatant, pellet, and direct feeding probiotic) with a control group consuming only standard honeybee food (water with sugar). After the feeding process, honey samples were collected and analyzed for production rate (amount), proximate analysis (including HMF, ash, moisture, mineral content), and antioxidant content (flavonoid and phenolic levels).

Results: Our study showed that supplementing honeybee food resulted in an overall increase in honey production ($p < 0.0001$), especially in the supernatant group, which had a 147% increase. Phenolic content showed higher values overall, with particularly elevated mineral content in honeybees supplemented with the supernatant of probiotics only.

Conclusion: These results are expected to positively influence honeybee health and increase stress tolerance and disease resistance in the honeybee population. Additionally, an enhanced quality of honey produced could potentially be used as a supplemental food in the form of nutraceuticals to address nutrient deficiencies in humans.

Introduction

Western honeybees (*Apis mellifera*) are the invertebrate pollinators of a wide variety of agricultural crops (1). It also holds the distinction of being the most commonly occurring species of pollinator for crops on a global scale (2). The long history of domestication and transportation resulted in the cosmopolitan distribution as we know it today. Many factors, which include, land-use change, habitat loss, fragmentation, degradation, pesticide use, and resource diversity have affected the number of honeybee species (3, 4). Furthermore, climate change caused changes in floral abundance, timing and other environmental factors like drought (5), alongside the introduction of alien species, can cause genetic dilution by interbreeding (6), increasing the likelihood of pathogen spread in honeybee population (7). The cosmopolitan distribution also caused the invasion of ectoparasitic mite *Varroa destructor*, which has been responsible for the losses of millions of bee colonies in Europe throughout the last 5 decades (25% loss of colonies in central Europe between 1985 and 2005) (8). *Nosema ceranae* which is another parasite that affect honeybees in the United States with 59% loss of colonies between 1947 and 2005, which also led to the decline in honey production (9).

Honey is a natural substance that is usually defined as a sweet, flavorful liquid substance, packed with numerous beneficial components (10). Scientific research has demonstrated the therapeutic potential of using honey to promote various health benefits and optimize body functions (11). This versatile and nutrient-dense substance have been shown to possess a range of medicinal properties that can help in improving overall wellness, including antioxidant, antibacterial, and anti-inflammatory effects(12). Honey was proven to be crucial to humans throughout early stages of evolution (13). Thus, the decline in honey production is a significant problem tied to the pollinator decline crisis.

Many solutions has been proposed, one approach focused on trying to reduce land and habitat degradation, pesticide use, and other problems that lead to the pollinator and honey decline (14-22). The decline in honeybee health, generally, affects the quality and quantity of honey production rates (9). For instance, honeybee population might decline rapidly once the immunity is vulnerable to a certain disease, as mentioned by the International bee research association that the most common pathogens that attach honeybees are American foul brood (*Bacillus larvae*), European foul brood (*Streptococcus pluton),* sac brood (virus), chalk brood (*Ascosphaera apis*); adult bee diseases and parasites: nosema (*Nosema apis*), amoeba (*Malpighamoeba mellifica*), acarine (*Acarapis woodi*), bee louse (*Braula spp.*) (23). Scientists tried to enhance the immune system of bees against specific pathogens by studying the immune system rigorously and applying several bacteria present as probiotics and examining changes in its resistance to pathogens and different ectoparasites. The findings of previous studies examining the use of various forms of *Lactobacillus* bacteria were mostly lucrative (14-22). Lactic-acid producing bacteria are found as commensals within humans, insects and animals (24). Strains within lactic-acid producing bacteria are also generally recognized as safe (GRAS) food grade microorganisms and employed as probiotics benefiting human health (25). It was found that lactic acid producing microbiota are within the honey crop of the Western honeybee *Apis mellifera* (26, 27). The crop is a central organ in the honeybee's food production between the esophagus and ventriculus, used for collecting and transporting of nectar to hive. The crop microbiota of *A. mellifera* contain 13 bacterial species within the genera

Lactobacillus and *Bifidobacterium* (27-29) and it plays a key role in the production of honey (27) and bee-bread (30).

The purpose of this study is to examine the effectiveness of using commercially available lactic acid producing bacteria probiotics, namely *Lactobacillus Reuteri, Lactobacillus Helveticus, Lactobacillus Bulgaricus, Lactobacillus Acidophilus*, and *Bifidobacterium Bifidum* as a supplement for honeybees with three different ways of feeding (supernatant, pellet, direct feeding), on honey quality and quantity compared to a control sample produced.

Methodology

Preparation of honeybee supplemented food

The commercially available probiotics (Super Multi-Dophilusã) used in this study was obtained from community pharmacy in Jordan/Amman during 2022. Each sachet provides not less than 1 billion viable "good" bacteria including *Lactobacillus helveticus, Lactobacillus acidophilus*, *Lactobacillus* bulgaricus, *Streptococcus thermophilus*, *Bifidobacterium longum*, *Bifidobacterium bifidum, Bifidobacterium infants, Lactobacillus retutri, and oligofructose*, a natural prebiotic, which promotes bacterial growth.

A number of 12 beehives were divided to 3 for each group as following: for the control group, a solution of water and sugar, water to sugar ratio was 2:1. The second group, was the probiotic direct feeding group where the probiotics (Super Multi-Dophilus) sachet was mixed directly with the solution of water : sugar 2:1 rigorously. The third group was the supernatant group and the fourth was the pellet group, both had to be prepared previously. Firstly, four probiotic sachets were dispensed in 1 liter of MRS Broth (HiMedia, USA) for 18-24 hours in 37°C incubation conditions. Aliquots of 15mL of probiotic-cultured broth was prepared using 15mL sterile falcon tubes (Falcon, USA) and was centrifuged for 10 minutes at 6000rpm using high speed centrifuge (Hettich eba 20 centrifuge Tuttlingen, Germany), pellets of viable bacteria produced at bottom of falcon tubes were used in pellets group. Supernatant was collected carefully from each falcon tube into 50mL sterile containers, closed with lid and parafilm, to be used for supernatant group. Samples were stored as aliquots at the fridge to be used for further experiments, Figure 1. All procedures were conducted under laminar flow cabinet and repeated through-out the entire feeding process at Applied Science Private University.

Feeding process of Honeybee and arrangement of hives

Beehives (no. 12) were selected at Da'our Farm for Beehives – Jordan, Figure 2. Hives were divided into 4 groups, Direct Probiotic group taking the instant probiotic-supplemented food, supernatant group taking only supernatant added to the original food, pellet group taking pellet suspended into original food, and control group taking only original bee food (water with sugar), Table 1. Each 3 beehives received 1 liter of supplemented liquid, mixed with sugar/water bee food leveled to 2 liters in total. This procedure was repeated 5 times in 15 days, with a 3-day gap between each time. Honey samples were collected in containers after each feeding time and proceeded to food analysis lab for proximate analysis.

Table 1. Groups of 12 bee hives divided into four each with 3 beehives being treated with different treatment types.

Groups	Description	No. of hives
Control Group	Only water/sugar food	3
Pellet Group	Supplement Added (Pellet form dispensed in water/sugar food)	3
Supernatant Group	Supplement Added (Supernatant Form dispensed in water/sugar food)	3
Direct probiotic Feeding Group	Supplement Added (Direct feeding group)	3

Food analysis procedure

Proximate analysis was carried out on obtained honey samples. The proximate content of protein, fat, sugar, Hydroxymethylfurfural (HMF) and ash were determined based on the official analysis methods from Association of Official Analytical Chemists (AOAC). Briefly, the protein content was determined by Kjeldahl method, based on the total nitrogen content from the AOAC Official Method 991.20, 2005. The fat content was determined by using acid hydrolysis method based on the AOAC Official Method 14.019, 1984. The moisture content was measured by placing 5 g honey samples in an oven set at 105°C for 18 hours, according to the AOAC Official Method 925.10, 2002. The same samples were further analyzed for the ash content by calcinating them in a furnace at 550°C until constant weight. For HMF, analysis of 5 grams of honey dissolved in 25 ml of water, transferred quantitatively into a 50 ml volumetric flask, added by 0.5 ml of Carrez solution I and 0.5 ml of Carrez II and made up to 50 ml with water. The solution was filtered through paper rejecting the first 10 ml of the filtrate. Aliquots of 5 ml were put in two test tubes; to one tube was added 5 ml of distilled water (sample solution); to the second was added 5 ml of sodium bisulphite solution 0.2% (reference solution). The absorbance of the solutions at 284 and 336 nm was determined using a Shimadzu UV-1900 spectrophotometer. The quantitative value of HMF was determined both by the external standard method and by using the proposed formula for the method reported by IHC (IHC, Stefan Bogdanov, 1999, pp. 1–54).

The sugar content in honey was determined using 3 standard solutions. They were prepared with concentration of 5000ppm respectively in distilled water and diluted to 1000, 2000, 3000 and 4000 ppm as standards. Diluted honey solutions were prepared by dissolving

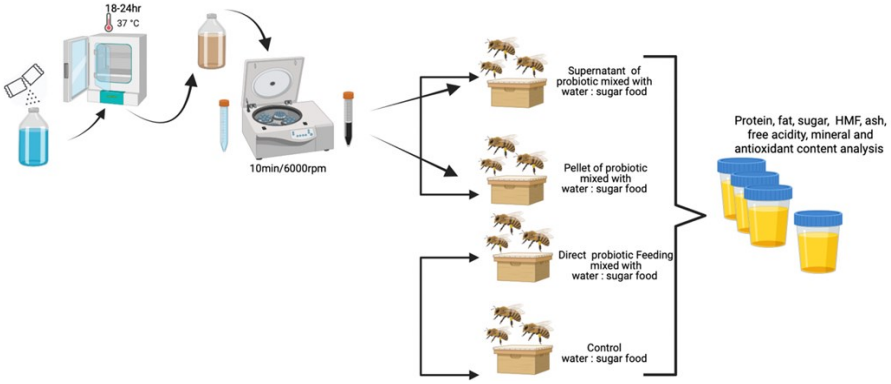


Figure 1. Schematic presentation of the used method.



Figure 2. Beehives used in the study at Da'our Farm for Beehives – Jordan

0.1 g of honey in 50 mL, 12 mL and 10 mL for sucrose, glucose, and fructose analysis respectively. By taking sucrose as example, 2 mL of each standard solution and samples were pipetted into different test tubes. The same amount of deionized (DI) water was used as blank. Then, 2 mL of 6 M hydrochloric acid (HCl) solution was added to each test tube and placed in boiling water for 10 minutes. Next, 8 mL of 2.5 M sodium hydroxide (NaOH) solution and 2 mL of 3,5-Dinitrosalicylic acid (DNSA) solution were introduced before the tubes were covered by parafilm and shook to mix. The mixtures were then placed in boiling water for another 5 minutes followed by 10 minutes in ice water. The absorbance of standards, blank and samples were measured at 580 nm and the concentrations were obtained from the standard calibration curve. For glucose and fructose, the steps of adding HCl solution and 10 minutes staying in boiling water were skipped because they can react readily with DNSA reagent (31). The absorbance of glucose and fructose were measured at 540nm and 490nm respectively.

The amount of ash was determined by calcining the material in a furnace for an entire night at 550°C. phosphorus, sodium, potassium, calcium, and iron were measured using an atomic absorption spectrophotometer (Model AA670 Shimadzu, Japan) following a wet digestion with sulphuric and nitric acids. This procedure involved heating a 5 g sample of honey in a Kjeldahl flask with a concentrated solution of nitric acid and sulfuric acid to oxidize carbonaceous materials. A blank was made for each sample at the same time using the same quantity of a mixture of sulfuric and nitric acids. The heating process

was carefully controlled to prevent excessive foaming. Small amounts of concentrated nitric acid were applied until all of the organic material had been oxidized. This stage was attained when a clear solution was obtained and there was no longer any discoloration of the solution after continuous heating. After cooling, it was moved to a 100 ml volumetric flask, with distilled water added to make up the capacity. By aspirating the solution into the oxygen-acetylene flame, the concentration of different metals was measured using an atomic absorption spectrophotometer (Model AA670 ShimadzuJapan). Determination of the total polyphenols content in the honey samples was done according to the Folin-Ciocalteu method (32), which is a colorimetric assay for measuring the total reducing capacity of a sample. An accurately weighed 5 g sample of each honey was put in a 50 mL volumetric flask, which was completed with Milli-Q water and filtered through Whatman No. 1 paper. 0.5 mL of this solution was then added with 5.0 mL Folin-Ciocalteu reagent (0.2 N) and mixed for 5 min followed by the addition of 4 mL of sodium carbonate (75 mg/L). Then the mixture solution was allowed for incubation at room temperature for 2 h and the absorbance was measured at 765 nm using spectrophotometer (SL 150, ELICO, India) at University of Jordan, while methanol was used as blank. Since this assay measures all phenolics, gallic acid is considered as the best standard, due to its availability and stability. In addition, the response to gallic acid has been shown to be equivalent to most other phenolic compounds. A 250 mg/L stock solution was prepared by dissolving 25 mg of dry gallic acid in 100 mL of 70% methanol, using a volumetric flask. A series of gallic acid standard solutions with concentrations of 0, 5, 10, 20, 50 and 100 mg/L were prepared for constructing the standard calibration curve. The mean of three absorbance measurements was used for the calibration plot and the total phenolic content of the real samples was stated in mg of gallic acid equivalents/100 g of honey. Standard calibration curve for total Phenolic Standard Absorbance (100-500 ppm) and Gallic Acid Standard Curve were demonstrated in the supplementary material (S1).

Moreover, the total flavonoid content of the target samples was obtained following the reported methods (33). In brief, 5 mL of 2% AlCl3 in methanol was mixed with the same volume of a honey solution, and the absorbance was measured at 415 nm after 10 min using spectrophotometer (SL 150, ELICO, India) at University of Jordan; the blank solution was prepared by mixing 5 mL honey solution with 5 mL methanol without the addition of AlCl3. The total flavonoid content was expressed as Quercetin equivalent.

Statistical analysis

All statistical analysis and data representation was made by using GraphPad Prism (version), by applying One-Way ANOVA and Dunnett's multiple comparisons test. Significance is annotated onto graphs and written in tables through p-value < 0.05.

Results

Production rate of honey

The production rates of honey were measured and reported as percentage value. Control group had the 100% reference value where all other groups compared to. All other three groups (supernatant, pellet, direct probiotic feeding) had a higher level of honey production rate when compared to the control group, all three groups showed significance of *p*- value < 0.0001, Figure 3.

Proximate analysis

It's shown in Table 3, the proximate analysis of protein, fat, sugar, Hydroxymethylfurfural (HMF), ash, free acidity, and mineral content per 100g of sample of honey produced showed different results for each group.

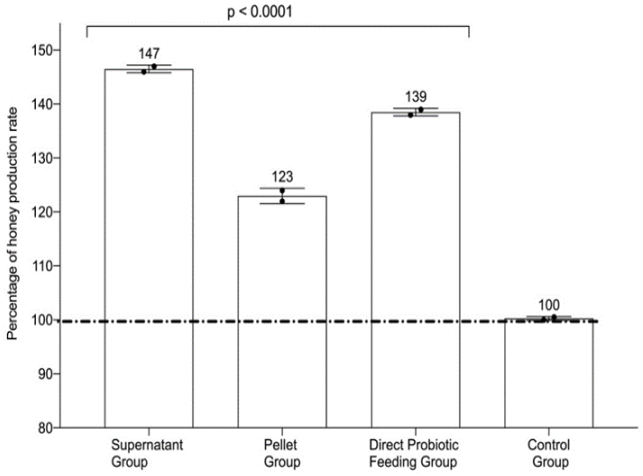


Figure 3. Percentage of production rates of honey from beehives for four groups represented as 100% for the control group. All other three groups (supernatant, pellet, direct probiotic feeding) are compared to the control group.

Moisture, ash, fat, protein, carbohydrates and three types of sugar (fructose, glucose, and sucrose) showed comparable numbers to control group with a *p*-value of <0.0001. On the other hand, free acidity analysis showed a drop in numbers when compared to control group. It was noticed that pellet group had the lowest free acidity with *p*-value of <0.0001. Moreover, Mineral's analysis showed phosphorus having greater values than control group, with most significant value is from the direct probiotic feeing group amounting to 4 mg. Meanwhile, potassium showed a very low value for the pellet group, while all other groups had a higher value when compared to control group. Sodium and iron exhibited closer value to control group, but calcium had surprisingly higher value in supernatant group when compared to control group.

Total phenolic and flavonoid concentration

The phenolic content was analyzed for all four groups and showed an increase in values when compared to control group, with supernatant group having the highest value of 30 mg GAE/100g. Meanwhile, flavonoid content was also analyzed and exhibited a similar trend to phenolic content with the highest value from the supernatant group 12 mg QUE/100 g when compared to control group, Table 4.

Discussion

Lactic acid producing bacteria are widely found in human and animals and, due to their beneficial effects on the host, sometimes used as probiotics. Honeybees possess an abundant, diverse lactic acid producing bacteria species in the gut and honey-making microbiota with direct contribution as a beneficial effect for bee health and defending them against microbial threats (34, 35). Prophylactic practices that enhance lactic acid producing bacteria species, or supplementary feeding of lactic acid producing bacteria species, can serve in integrated approaches to sustainable pollinator endurance (34, 36, 37). It was noticed in a study that was conducted by supplying certain species of probiotic to the normal food intake of honeybees including *L. acidophilus*, *L. casei*, *L. plantarum* and *L. rhamnosus showing* enhanced honeybee immunity and higher levels for abaecin and defensin (antimicrobial peptide cDNA gene families) in honeybee larvae (38-40). Another study showed that supplementing *B. bifidum*, *E. faecium*, *L. acidophilus*, *P. acidilactici* had an advantages of better bee survival and higher dry mass with crude fat level (34).Lactic acid producing bacteria as a supplemented food (probiotic enriched) was clearly increasing honey quantity by 147% for the supernatant group as the highest one, followed by direct probiotic feeding 139% and pellet group 126%. This can be related to the fact that probiotic use

Table 3. Proximate analysis of protein, fat, sugar, Hydroxymethylfurfural (HMF), ash, free acidity, and mineral content per 100g of sample of honey produced from four different groups (supernatant, pellet, direct probiotic feeding and control) with statistical significance for each group compared to control group.

Contents per100g	Supernatant Group	Statistical significance (p-value)	Pellets Group	Statistical significance (p-value)	Direct Probiotic Feeding Group	Statistical significance (p-value)	Control Group
Moisture (%)	13.93%	ns	13.92%	ns	15.48%	ns	14.1%
Ash (%)	0	ns	0	ns	0	ns	0
Fat (%)	0	ns	0	ns	0	ns	0
Protein (g)	0.55g	<0.0001	0.41g	ns	0.39g	ns	0.34g
Carbohydrates (total)	81.11g	<0.0001	81.53g	<0.0001	82g	<0.0001	82.53g
Fructose (g)	(38.95g)	<0.0001	(39.2g)	<0.0001	(39.32g)	<0.0001	(38.4g)
Glucose (g)	(30.19g)	<0.0001	(29.73g)	<0.0001	(30.27g)	<0.0001	(31.05g)
Sucrose (g)	(0.97g)	<0.0001	(1.16g)	ns	(0.91g)	<0.0001	(1.18g)
Free Acidity (Meq/Kg)	22.45	<0.0001	16.05	<0.0001	21.03	<0.0001	27.1
HMF (ppm)	4.19	<0.0001	5.12	<0.0001	5.81	<0.0001	8.1
Phosphorus (mg)	3.5	<0.0001	2.3	<0.0001	4.1	<0.0001	2.2
Potassium(mg)	3.5	<0.0001	3	<0.0001	3.2	<0.0001	2.5
Sodium (mg)	3.6	<0.0001	1.6	<0.0001	1.7	<0.0001	1.2
Calcium (mg)	6.6	<0.0001	3.4	<0.0001	6.1	<0.0001	3.1
Iron ((mg)	0.43	<0.0001	0.5	<0.0001	0.57	<0.0001	0.34

*ns: non-significant

Table 4. Phenolic and flavonoid content of honey samples of sample in all 12 beehives divided into 4 groups. *GAE: Gallic acid: QUE: Quercetin.

Sample	Phenolic (mg GAE/100 g)	Statistical significance (p-value)	Flavonoid (mg QUE/100 g)	Statistical significance (p-value)
Control Group	13.1±0.61	-	3.7±0.21	-
Supernatant Group	30±1.67	<0.0001	12±0.43	<0.0001
Pellet Group	18.32±0.76	<0.0001	4.5±0.23	0.6064
Direct Probiotic Feeding Group	23±1.54	<0.0001	8±0.79	0.0001

can enhance the bee's bodily functions leading to more production of honey (35, 37). One of the most important indicators of honey toxicity parameter and may display the heat and storage change effect on honey quality is the HMF value. Moreover, HMF also gives a clue about the levels of reducing sugars present in honey and the overall bee health. (41). HMF is absorbed from food through the gastrointestinal tract and, upon being metabolized into different derivatives, may exert some detrimental effects like mutagenic, genotoxic, organo-toxic and enzyme inhibitory effects (41). In previous studies, HMF has been reported to have negative consequences on human health, such as cytotoxicity toward mucous membranes, the skin and the upper respiratory tract; chromosomal aberrations; and carcinogenicity towards both humans and animals (42-45). HMF was seen to be lower in all three groups when compared to control group and thus it indicates the sustainability of honey shelf life and probable higher safety for consumers.

Meanwhile, an evident increase in the supernatant group in terms of phenolic content and flavonoids content as the number of bacterial cells introduced was relatively lower than pellet and direct probiotic feeding and contain residual bacterial exudates. Such exudates and/or probiotic use may have had an enhancing effect on the quantity of phenolic and flavonoid content in honey production process in terms of their ability to improve the antioxidant system and to decrease radical generation (46-49).

Moreover, the mineral content (phosphorus, sodium, potassium, calcium, and iron) was seen to be higher in the supernatant group when compared to control group and other groups. Knowing that supernatant group were fed only bacterial supernatant obtained after 18–24-hour culture, centrifuged and collected with minimal residual bacterial count and, thus, exudate of bacteria was at higher concentration in such media, the metabolites are hence utilized in the production of higher quantity and quality of honey, contributing to the overall health of honeybees (26, 35, 36, 49). Mineral content in honey has been broadly considered in several producers including USA, Germany, Spain, Poland, Italy, India, Chili, and Argentina (50-53). Knowing the elemental content of honey is medically helpful for treating patients with element deficiencies. For instance, using honey with relatively high level of calcium makes it an important food supplement for children and older people to build and support bones. Moreover, using honey with higher sodium content is an important aspect in treating electrolyte imbalances, and such vital ion existing in the extra-cellular fluids plays a crucial role in enzyme regulation and muscle contractions (54-56). This concept is potentially could be classified under the umbrella of nutraceuticals of obtaining more health benefits from fortified food products in addition to their original health benefits (57-59) yet, more research is required to obtain better understanding of using such potential nutraceuticals on human and/or animal health.

It is suggested that such type of research could be backed-up with sampling of oral region of honeybees to test the microbiome change in case of probiotics intake and how it may correlate to the proximate analysis results of the different studied groups. Metagenomics analysis can be used to identify a rich diversity of microbes within honeybees coupled with functional characterization of the endogenous crop microbiota to provide insights for the understanding of its role for bee health and disease in the different studied groups. Instrumental analysis of bacterial exudate in supernatant can be an effective tool by LC/MS to identify potential metabolites and corelate to current results.

Clinical implications

This study on lactic acid-producing bacteria's impact on honeybee health and honey production unveils promising clinical implications with ramifications for both apiculture practices and potential human health applications. Employing these bacteria as probiotics for honeybees emerges as a proactive measure, demonstrating notable benefits in enhancing bee immunity and overall survival. The supplementation of specific probiotic species, such *as L. acidophilus, L. casei, L. plantarum, L. rhamnosus, B. bifidum, E. faecium, P. acidilactici*, showcases the potential for fortifying bee health, thereby contributing to sustainable pollinator endurance. The consequential increase in honey quantity and quality, particularly through probiotic-enriched supplementation, signifies economic advantages for beekeepers. Furthermore, the evaluation of toxicity parameters, notably the Hydroxymethylfurfural (HMF) value, suggests that probiotic use may enhance honey shelf life and ensure a higher level of safety for consumers. The observed rise in phenolic and flavonoid content in honey, particularly in the supernatant group, implies an antioxidant augmentation attributed to residual bacterial exudates or the use of probiotics during honey production. Moreover, the heightened mineral content, encompassing phosphorus, sodium, potassium, calcium, and iron in the supernatant group, highlights the potential of honey as a nutritional supplement, offering remedies for specific nutrient deficiencies. This nutraceutical concept extends to considerations of honey with elevated calcium levels supporting bone health and sodium-rich honey addressing electrolyte imbalances. Importantly, the discussion raises the prospect of applying these findings in human health, though emphasizing the necessity for further research to comprehensively understand the implications and establish guidelines for incorporating such potential nutraceuticals into human diets.

The proposed future research directions, including studying the microbiome changes in honeybees with probiotic intake, metagenomics analysis, and instrumental analysis of bacterial exudate through LC/MS, underscore the evolving nature of this field and the depth of exploration required to harness its full potential.

Conclusion

It is perceived that the use of probiotics is a beneficial supplement for bees. Utilizing lactic-acid producing bacteria in regular bee food increase honey production rate and honey quality in general. Our research supports further investigation of honeybee community members and suggests that hive environments, including the food prepared by the bees, might impact development of the gut microbiome and could play a role in the improve of bee health.

Author contributions

Muna Barakat, Ahmad Sinan Badwan, Sanad Naser El-Banna, Shaymaa B. Abdulrazzaq Anfal Al-Dalaeen, Sawsan Abu Jama`ah, Mahmoud Jaber, Mohammad A.A. Al-Najjar: conceptualization. Ahmad Sinan Badwan, Sanad Naser El-Banna: investigation, Muna Barakat, Ahmad Sinan Badwan, Sanad Naser El-Banna, Shaymaa B. Abdulrazzaq Anfal Al-Dalaeen: methodology. Ahmad Sinan Badwan, Sanad Naser El-Banna: project administration. Muna Barakat, Mohammad A.A. Al-Najjar, Sawsan Abu Jama`ah: supervision. Muna Barakat, Ahmad Sinan Badwan, Sanad Naser El-Banna, Shaymaa B. Abdulrazzaq Anfal Al-Dalaeen,: writing— original draft preparation. Muna Barakat, Ahmad Sinan Badwan, Sanad Naser El-Banna, Shaymaa B. Abdulrazzaq Anfal Al-Dalaeen, Sawsan Abu Jama`ah, Mahmoud Jaber, Mohammad A.A. Al-Najjar: writing—reviewing and editing. All authors read and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Material

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