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ORIGINAL ARTICLE

Isolation and characterization of the lactobacillus strain from honey and its probiotic properties

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ABSTRACT

Background and Objectives: The lactobacilli are abundant in honey, helping protect against pathogens and providing antimicrobial properties. This study aimed to isolate lactobacillus species from different honey regions and evaluate their potential probiotic properties.

Materials and Methods: Eighty-eight samples were collected from different regions, including the northern, central, and southern areas, and obtained through retail stores. All samples were independently examined for the presence of *Lactobacillus* using both culture and real-time PCR methods. Probiotic tests were performed on the isolated *Lactobacillus* strains, including hemolytic activity, bile, acid, and pepsin resistance. Additionally, the antibiotic resistance of the obtained strains was investigated using seven different antibiotics.

Results: Thirteen *Lactobacillus* isolates were obtained from 7 (8.0%) honey samples. Of these, eight isolates were identified as *L. plantarum* (61.54%), four isolates as *L. rhamnosus* (30.77%), and one isolate as *L. acidophilus* (7.69%). All strains were devoid of hemolytic activity, and three isolates (23.07%) were found to be resistant to acid, while 2 (15.38%) showed resistance to bile and pepsin. All isolates were resistant to vancomycin (100%). Additionally, only one strain exhibited resistance to all tested antibiotics. Furthermore, the present study demonstrates a significant association (p-value<0.05) between the presence of *Lactobacillus* in various regions of Iran.

Conclusion: Various factors, such as climatic conditions and geographical location, can influence honey's composition and microbial diversity. Identifying and isolating potential probiotic species in honey could significantly expand their use in the food and pharmaceutical industries, offering numerous health benefits and potential therapeutic applications.

Keywords: Honey; Probiotics; *Lactobacillus*; Real-time polymerase chain reaction; Antibiotics

INTRODUCTION

Honey is a valuable food valued as a delicious and therapeutic ingredient since ancient times. Bees collect nectar from different types of flowers and convert it into a dense, high-energy, and delightful product. The composition and type of honey depend on the diet of the honeybees, the types of flowers used, and the regional climate. Due to the different plant sources, it has different colors and tastes. Honey contains various sugars, especially fructose, glucose, proteins, amino acids, organic acids, enzyme values, phenolic acids, flavonoids, antioxidants, and high osmotic pressure (1-3).

The unique compositions of honey make it helpful in treating diseases such as wounds and burns

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and improving breathing (4-6). Most bacteria cannot grow in honey and only have a small growth or survival capacity. These bacteria obtain their origin from two primary and secondary sources. Honeybees' digestive system is the primary origin of the microflora in honey, while nectar, pollen, propolis, floral sources, and the hive's internal and external milieu are secondary origins (7).

The microorganisms found in small numbers in honey include fungi, yeast, *Bacillus* species, *Clostridium* species, and lactic acid bacteria (1, 8). Among the critical bacteria in honey, lactic acid bacteria are obtained by consuming nectar and pollen and in contact with mature honeybees (8, 9). These bacteria are especially abundant in environments rich in carbohydrates. There is a hypothesis that lactic acid bacteria play a crucial role in converting nectar into honey and pollen into bee bread due to their fermentative properties (10, 11).

The lactic acid microbiota of honey is of great importance for the health of honeybee colonies, helps protect against pathogens, and has antimicrobial properties for honey (12-14). One of the most important genera of lactic acid bacteria is the lactobacilli, that can produce lactic acid as the final product of fermentation. *Lactobacilli* possess the unique ability to generate bacteriocins. These substances inhibit competing microorganisms' proliferation and diminish competition for vital nutrients, creating inhospitable conditions for the growth of other bacteria (15).

The employment of lactobacillus strains as probiotics in various foodstuffs, particularly dairy products, has garnered significant attention. Numerous investigations have been undertaken to discover and introduce probiotic bacteria. Probiotics have beneficial effects on the host and can be used as a complementary treatment in conjunction with drug therapies and as effective preservatives. Considering that Iran has a unique climate, is a rich source of various plant species, and is a country with great potential to produce various types of honey, this study was carried out to isolate lactobacillus species from different honey regions and evaluate their potential probiotic properties.

MATERIALS AND METHODS

Collecting honey samples. A prospective study was conducted to analyze 88 samples from various geographical locations in Iran. Samples were collected from different northern, central, and southern regions of Iran and obtained through retail channels. The samples were stored under ambient conditions before laboratory analysis.

Bacteria isolation. Approximately 45 grams of honey sample were gently mixed with 180 milliliters of peptone water (0.1% weight/volume) in a container with a wide mouth opening. A quarter of this mixture was then subjected to centrifugation, and the resulting sediment was inoculated into 10 milliliters of MRS broth, which was subsequently incubated at 37°C for 24-48 hours. Later, the MRS broth was subcultured on MRS agar and incubated again at 37°C for 48 hours. Multiple colonies exhibiting varying phenotypes were isolated and subjected to morphological and catalase tests. Pure colonies displaying the characteristics of Gram-positive bacilli, non-spore-forming, catalase-negative, and nonmotile were identified and preserved in MRS broth containing 15% glycerol at -20°C for further analysis.

Identification of *Lactobacillus* using real-time PCR. DNA was isolated using a DNA extraction kit (Karmania pars Gene, Iran) following the manufacturer's instructions for the qualitative real-time PCR analysis. The real-time PCR reactions were conducted using the ExcelTaq™ 2× Q-PCR Master Mix (SMOBIO, Taiwan) and were carried out on a Light-Cycler 96® (Roche Diagnostics, Mannheim, Germany), following the manufacturer protocol. The initial denaturation step was performed at 95°C/10 minutes, followed by 40 cycles of denaturation at 95°C/25 seconds, annealing at a temperature range of 59 to 62°C/30 seconds, and extension at 72°C/30 seconds. The primers used are listed in Table 1.

Probiotic characterizations

Hemolytic activity. Sheep red blood cell lysis by microorganisms was investigated using a blood agar medium. The isolated organisms were inoculated in spots on the blood agar medium (a base medium containing 7% defibrinated sheep blood). The plates were then incubated at 37°C for 24 hours, and a clear zone around the colonies was examined. The absence of hemolytic activity is a characteristic of probiotics.

Resistance to bile salts. 100 µL of recently cultured

Table 1. Primer used in this study.

Primer Name	Sequence	Primer Tm	Amplicon Size	References
Lplan-F	AAAATCATGCGTGCGGGTAC	58.4	210	(16)
Lplan-R	ATGTTGCGTTGGCTTCGTCT	58.4		
Lbrevis-F	GCAGTTGCCGAGGTCCAA	58.4	64	(17)
Lbrevis-R	CCAACGCATTTTCAGCATCA	56.4		
Lreu-F	CAGGATCGGTAATTGATG	51.4	171	(18)
Lreu-R	TGGATATGGAAGTTCGTC	51.4		
Lfer-F	ACTAACTTGACTGATCTACGA	55.5	191	(19)
Lfer-R	TTCACTGCTCAAGTAATCATC	55.5		
Lcas-F	CAGTCGTACATGCAGATACC	58.4	139	(20)
Lcas-R	TGCCAAGCTCCTAAGTCTGA	58.4		
Lrham-F	GGACAGGTAGAAAGTCAAACGA	60.1	186	(21)
Lrham-R	GCTGACCGTAAACGCAATCTTAG	62.9		
Sakei-F	AGGCGCTTCAATGTTATCGG	58.4	161	(22)
Sakei-R	TCGCTGGTTGCTTGATGCTA	58.4		
Pento-F	CAAGCCCGGTTAATGTCACA	58.4	70	(23)
Pento-R	GTGGGATGGTCTTTGTCTTGTTC	62.9		
Acido-F	GTAATCGTGTTCTACATATACATAG	59.2	152	(24)
Acido-R	GGTTATAAAGTTAACAGCATTGTTC	59.2		

bacteria was inoculated into MRS broth containing 0.3% sterile bile salts. Bacterial growth was monitored after 8 hours by quantifying the absorbance at a wavelength of 620 nm. The extent of growth inhibition was calculated using the previously described formula (25). The inhibition coefficient (Cinh) should be equal to or less than 4.0, indicating the resistance of the bacteria to bile salts. The experiment was conducted in triplicate, and the entire procedure was replicated at two different time points.

Acid resistance test. 50 µL of bacterial suspension, corresponding to an optical density of 0.5 McFarland, was introduced into 5 ml MRS broth with pH values of 2.5 and 4.0. After 3-4 hours of incubation at 37°C, a solution loop was streaked onto MRS agar plates. MRS agar plates were then incubated for 24-48 hours, and the number of colonies counted should not be less than 10^6 cfu/ml, indicating the resistance of the bacteria to acid.

Pepsin resistance test. The sediment derived from the bacterial suspension investigated was washed two times with PBS solution and subsequently adjusted to a turbidity level equivalent to 0.5 McFarland. 400 µL of this suspension was then introduced to 2 ml of pre-prepared pepsin solution (pH=2.5). A volume of 50 µL of the previous solution was added to 4.95 mL of PBS to obtain a 10^6 cfu/mL concentration. Next, 10 µL and 100 µL of the previous solution were introduced into MRS agar at zero, 2, and 6 hours of incubation, and the resulting colonies were assessed for growth. If the bacterial count exceeds 10^6, the sample is considered positive.

Antibiotic sensitivity test. The Kirby-Bauer disk diffusion method was used for the antibiotic sensitivity test (26). A bacterial suspension adjusted to a 0.5 McFarland standard was cultured on Mueller-Hinton agar containing 20% MRS. The isolates were subjected to antibiotic disks (PadtanTeb, Iran), including vancomycin (30 µg), cefixime (5 µg), sulfamethoxazole (25 µg), chloramphenicol (30 µg), erythromycin (15 µg), tetracycline (30 µg), and gentamicin (10 µg) to evaluate resistance. The diameter of the inhibition zones was measured and compared following the guidelines provided by the Institute of Clinical and Laboratory Standards (26).

Statical analysis. Given the descriptive nature of the data, frequency tables and percentages were utilized to present the results. The chi-square test was used to compare between groups. All tests were conducted using SPSS software version 19 (Chicago,

USA). P-values less than 0.05 were considered statistically significant.

Ethical considerations. The Ethics Committee of the Iran University of Medical Sciences, Tehran, Iran, approved this study (Code: IR.IUMS.REC.1400.540).

RESULTS

This empirical investigation analyzed 88 honey samples obtained from distinct geographic regions of Iran. Specifically, 67.0% (n=59) originated from the northern, 19.3% (n=17) from the central, and 13.6% (n=12) from the southern regions of Iran (Table 2).

Among the honey samples analyzed, 36 (40.9%) were found to be sterile, while 52 (59.1%) were found to harbor various microorganisms such as *Lactobacillus*, *Bacillus*, fungi, and cocci (Table 2).

In this study, seven honey samples (8.0%) were determined to harbor *Lactobacillus*, while 81 samples (92%) were confirmed to be free of *Lactobacillus*. Among *Lactobacillus* positive samples, 13 *Lactobacillus* strains were identified using real-time PCR analysis (Fig. 1). Regarding other microorganisms, such as cocci, bacillus, and fungi, 34 (38.6%) of the honey samples investigated were confirmed to contain at least one of these microorganisms. Additionally, with regards to contamination of the honey samples, 18 (20.5%) samples tested positive for cocci, 20 (22.7%) samples tested positive for bacillus, and 17 (19.3%) samples tested positive for fungi (Table 2).

Table 2. Evaluation of the honey studied according to their geographical origin, level of contamination, and the presence of microorganisms such as *Lactobacillus* and other microbial species

Variable	Groups	N (%)
Regions	North	59 (67.0)
	Center	17 (19.3)
	South	12 (13.6)
Contamination	Positive	52 (59.1)
	Negative	36 (40.9)
Lactobacillus	Positive	7 (8.0)
	Negative	81 (92.0)
Other microorganisms	- Cocci	34 (38.60)
	Bacillus	18 (20.5)
	Fungus	20 (22.7)
		17 (19.3)

The results of the real-time PCR analysis showed that eight of the *Lactobacillus* strains were identified as *Lactobacillus plantarum* (61.54%), four samples were identified as *Lactobacillus rhamnosus* (30.77%), and one sample was identified as *Lactobacillus acidophilus* (7.69%) (Fig. 1).

Table 3 displays the microorganisms isolated from Iran's northern, central, and southern regions. As indicated, the respective areas yielded 39 (44.3%), 7 (8.0%), and 6 (6.8%) microorganisms. In this study, *Lactobacillus* has been isolated only from the central (n=4, 4.5%) and the southern region (n=3, 3.4%) of Iran. Based on the Chi-square analysis, a significant association has been observed between the isolation

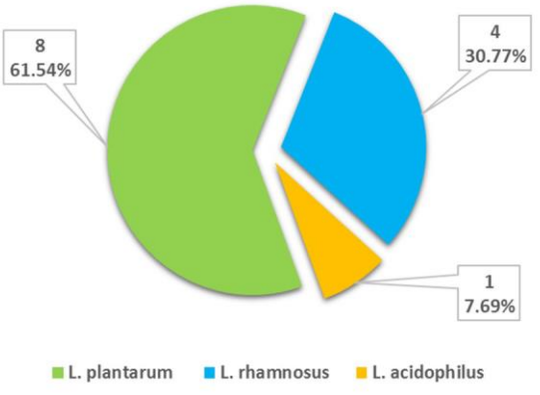

Fig. 1. Results of the identification of *Lactobacillus* species using the real-time PCR method

regions and the presence of *Lactobacillus* (p-value = 0.001). Therefore, more *Lactobacillus* has been isolated from Iran's central and southern regions (Table 3). Furthermore, in terms of the isolation rate of other microorganisms from different areas, 20 samples (22.7%) from the north, nine samples (10.2%) from the central region, and five samples (5.7%) from the south were found to contain other microorganisms investigated (Table 3).

The correlation between the simultaneous presence of *Lactobacillus* and other microorganisms investigated was evaluated. The results revealed a significant association between the presence of *Lactobacillus* and cocci (p-value=0.030) (Table 4). However, no statistically significant association was observed between the presence of *Lactobacillus* and *Bacillus* or fungi (p-value>0.05) (Table 4).

All isolated *Lactobacillus* strains exhibited negative hemolysis (non-beta hemolysis) in this study (n=13,

Table 3. Investigating the relationship between collection areas and the presence of microorganisms

Variable	North (%)	Center (%)	South (%)	p-value*
Contamination	39 (44.3)	7 (8.0)	6 (6.8)	0.145
Lactobacillus	0 (0)	4 (4.5)	3 (3.4)	0.001
Other Microorganisms	20 (22.7)	9 (10.2)	5 (5.7)	0.355
Cocci	10 (11.4)	4 (4.5)	4 (4.5)	0.413
Bacillus	14 (15.9)	4 (4.5)	2 (2.3)	0.865
Fungus	14 (15.9)	2 (2.3)	1 (1.1)	0.319

* Chi-square test

100%). Additionally, 15.38% (n=2) of *Lactobacillus* isolates were resistant to bile and pepsin, and 23.07% (n=3) of lactobacilli were resistant to pH 2.5 and 4 (Table 5).

The results of the microbial sensitivity test demonstrated that all honey-derived strains were resistant to vancomycin, while 46.15% (n=6) of isolates were resistant to cefixime, 30.77% (n=4) were resistant to sulfamethoxazole, 23.08% (n=3) were resistant to erythromycin, and 15.38% (n=2) were resistant to gentamicin and tetracycline. Moreover, 7.69% (n=1) of the strains resisted to chloramphenicol antibiotics (Table 6).

Out of all the strains, one was found to be resistant to all antibiotics, two were resistant to four antibiotics, one was resistant to three antibiotics, four were resistant to two antibiotics, and five were resistant to one antibiotic (Fig. 2).

DISCUSSION

Honey is one of the most valuable natural products used for centuries as a food and an essential substance with many properties in traditional medicine. The microorganisms in honey have a wide variety and are associated with bee pollen, nectar, and bee gut (9, 27, 28). The bacteria that produce lactic acid are diverse in carbohydrate-rich environments such as honey, and the primary source of these bacteria is bee pollen, nectar, and the gut (8). The present study studied 88 honey samples from various geographical regions of Iran, including northern, central, and southern areas. Approximately 70 microorganisms were identified using bacteriological methods, including Gram-positive bacilli, Gram-positive cocci, and molds. Thirteen samples representing 18.5% were identified as *Lactobacilli* using the Real-Time

Table 4. Investigating the relationship between the presence of lactobacilli and other microorganisms isolated from honey.

Variable		*Lactobacillus*		p-value*
		Positive (%)	Negative (%)	
Other Microorganisms	Positive (%)	5 (5.7)	29 (33)	0.103
	Negative (%)	2 (2.3)	52 (59.1)	
Cocci	Positive (%)	4 (4.5)	14 (15.9)	0. 030
	Negative (%)	3 (3.4)	67 (76.1)	
Bacillus	Positive (%)	1 (1.1)	19 (21.6)	1.000
	Negative (%)	6 (6.8)	62 (70.5)	
Fungus	Positive (%)	1 (1.1)	16 (18.2)	1.000
	Negative (%)	6 (6.8)	65 (73.9)	

* Fisher's exact test

Table 5. Probiotic properties of isolated lactobacilli

Probiotic Test	N (%)
Negative hemolysis	13 (100)
Acid Resistance	3 (23.07)
Bile Resistance	2 (15.38)
Pepsin Resistance	2 (15.38)

Table 6. The level of resistance to the investigated antibiotics

Antibiotic	Resistance (%)
Vancomycin	13 (100)
Cefixime	6 (46.15)
Sulfamethoxazole	4 (30.77)
Erythromycin	3 (23.08)
Gentamicin	2 (15.38)
Tetracycline	2 (15.38)
Chloramphenicol	1 (7.69)

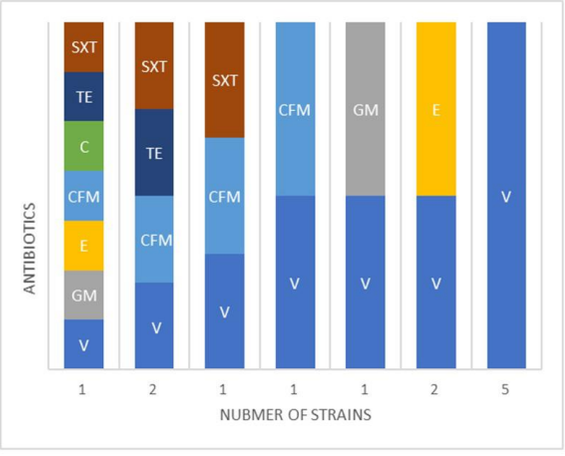

Fig. 2. Multi-antibiotic resistance of isolates. (V: vancomycin, CFM: cefixime, SXT: sulfamethoxazole, E: erythromycin, G: gentamicin, TE: tetracycline, C: chloramphenicol)

PCR method. Of the 13 strains identified, nine strains (61.54%) were related to *L. plantarum*, three strains (33.77%) were *L. rhamnosus*, and one strain (7.69%) was *L. acidophilus*.

Regarding the separation of honey microorganisms, some studies have been carried out, and the focus of most studies has been on the gut microbiota and the honey obtained from the bee gut. In the study by Mathialagan et al., 42 strains related to six genera were isolated, including *Enterococcus* (23.8%), *Micrococcus* (18.8%), *Streptococcus* (13.8%), *Pediococcus* (13.8%), *Lactococcus* (10.0%) and *Lactobacillus* (10%), which is similar to our results (29). In the study by Hosny et al., of 25 total honey samples, 25% of the isolates were from the genus *Lactobacillus*, including *L. plantarum* (24%), *L. Kazie* (28%), and *L. acidophilus* (48%), which has a higher level of *Lactobacillus* than our study. In that study, the other isolates belong to *Bacillus*, *Enterococcus*, *Lactococcus*, micrococcus, fungi, and yeast (30).

In a study conducted in 2020, among 88 honey samples, 27 strains of *L. kunkeei* were identified. Furthermore, four strains of *L. plantarum*, two strains of *L. paracasei*, one isolate of *L. brevis*, *L. rhamnosus*, *L. casei*, and *L. fermentum* were also identified (25). Using a molecular method, identify seven types of lactic acid bacteria (LAB) species, including *L. acidophilus*, *L. delbrueckii*, *L. kazacii*, *L. gasseri*, *L. plantarum*, *L. reuteri*, and *L. rhamnosus*, was achieved with 93.6% accuracy (31). Owen et al. and Rezmagah et al. from the honey sample have separated the *L. acidophilus* (32, 33). The findings of the studies mentioned above agree with the present study regarding the species obtained.

In several studies, honey stored in bee guts has been separated into various *Lactobacillus* species. For example, in a Faehgheh Feizabadia et al. survey of 40 strains, including *Enterococcus* and *Lactobacillus*, *L. plantarum* represented 25% and *L. pentosus* 5% (34). The study by Naser Tajabadi and colleagues also showed that *L. plantarum*, *L. pentosus*, and *L. fermentum* are the most common *Lactobacillus* species in the honey gut of the honeybee Apis (35). The difference in the reported separation of *Lactobacillus* species and the predominant species of *Lactobacillus* separated in studies may be due to differences in the climate and geographical region of honey production in terms of the diversity of plant coverage, water and weather, honey production season, nectar source, diversity and difference in sample (honey or honey gut or honey products), type of bee species, and methods used for identification in the studies (11, 36).
Although *L. kunkeei* is reported to be the most common species in the studies carried out (25), the separation of species such as *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* in this study, which was also separated in the studies mentioned above, indicates the ability and sustainability of these species in honey with different sugar content (9).

In examining the relationship between the collection areas and *Lactobacillus*, it was found that *Lactobacillus* was separated from the central and southern regions (4.5% and 3.4%, respectively). Additionally, samples from the northern region did not contain *Lactobacillus*. According to statistical analysis, a significant correlation was observed between areas and the presence of *Lactobacillus* (p-value=0.001). Therefore, the most considerable number of lactobacilli was separated from Iran's central and southern regions. The difference in separating *Lactobacillus* from honey produced in different areas of Iran may be related to the region's climate, water, and vegetation cover. The central and southern area has a dry and semi-arid climate, including desert and mountain regions.

The diversity of lactobacilli species in the southern region is higher and is mainly represented by the honey sample from the "Kunar" region. The "Kunar" honey is produced from the nectar of the "Kunar" tree located in warm and humid areas of the southern part of the country, such as Hormozgan, Bushehr, and Khuzestan, and has a higher saccharide content compared to the standard.

This study showed that of the 88 honey samples studied, 52 (59.1%) samples contained various microbiological agents. No microorganisms were isolated from 36 (40.9%) of the samples. Like other studies, in the current research, in addition to 13 isolates of *Lactobacillus*, other microorganisms such as bacillus (22.7%), thermophilic cocci (20.5%), and fungi (yeast and mold) (19.3%) were isolated. Similar studies have also reported the separation of various microorganisms, including bacillus, cocci, and mold (30, 34, 37).

Typically, the sources of bacteria and fungi in honey are environmental factors. Some bacteria also survive in honey, but due to the high osmolarity of honey, these microorganisms do not reproduce (38). In this study, a strain of *Staphylococcus aureus* was isolated from a sample of honey, which does not appear to be a naturally occurring inhabitant of honey and is more likely an environmental contamination.

Investigating the relationship between honey collection areas (North, Center, South) and other honey-isolated bacteria, including bacillus, cocci, and fungi, did not reveal any significant relationship (p-value>0.05). However, a significant association was observed between the simultaneous presence of *Lactobacillus* and cocci based on statistical analysis (p-value= 0.030). This issue suggests the presence of *Lactobacillus* and cocci that produce lactic acid (LAB), which may come from plant or insect sources (32). However, no significant relationship was found between the presence of *Lactobacillus* and other isolated bacteria (including spore-forming bacillus, fungi, and yeasts).

Honey is a nutritious substance with therapeutic applications for various diseases due to its composition (39-41). Additionally, microorganisms such as lactobacilli in honey can have beneficial therapeutic effects. The *Lactobacillus* genus, an important LAB group, is commonly used as a probiotic in humans and animals (35). This study further investigated the isolation of lactobacilli from honey and evaluated the probiotic properties of lactobacillus strains based on hemolytic activity loss, acid resistance, and bile resistance indicators. This research showed that all isolates (100%) were devoid of hemolytic activity. In addition, all isolates have at least one indicator of probiotic properties.

The results obtained from two acid resistance tests at pH 4 and 2.5 showed that 84.62% of the strains were resistant to pH 4, and 23.8% of the isolates also showed resistance to both pH values. In the case of the bile resistance test, only two strains, 4 and 11 (15.38%), had this property. These two strains also showed resistance to gastric juice or pepsin. Based on probiotic indices, strains 4 and 11 had probiotic properties and belonged to *L. plantarum*. In many studies on various food samples, including honey, strains of *L. plantarum* with probiotic potential have been introduced (42, 29). Since probiotics are generally administered orally, they must have survival ability during passage through the stomach and intestinal tract (43). Therefore, the above indices are essential for selecting probiotics, and the two mentioned strains meet these conditions. In addition, they can have good resistance to acidity in acidic foods. The characteristics of different climate honey probably affect the probiotic properties of *Lactobacillus* species.

Regarding the evaluation of antibiotic resistance of *Lactobacillus* isolates, all were resistant to vancomycin, and most were sensitive to antibiotics such as clindamycin, gentamicin, tetracycline, erythromycin, sulfamethoxazole, and cefixime. Also, two isolates had high resistance. Similar studies regarding vancomycin resistance are consistent and different results have been reported for the remaining antibiotics (33, 44, 45). Isolate 4 was sensitive to antibiotics clindamycin, gentamicin, and tetracycline that can be used for food processing purposes. However, isolate 11 showed resistance to these three antibiotics, and the remaining antibiotics were tested with the disk diffusion method. However, a more advanced technique must precisely evaluate its sensitivity to these antibiotics. Other *Lactobacillus* isolates obtained from honey had few probiotic indices, but they can be considered for non-food uses, such as the antibacterial effect, because of their other properties.

CONCLUSION

Honey is widely regarded as a healthy and nutritious food, and its composition and microbial content can vary depending on climatic conditions, flower source, and geographical location. Identifying probiotic species in honey enhances its nutritional value for consumption and expands its potential applications in the food and pharmaceutical industries.

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REFERENCES

1. Vit P, Vargas O, Lopez T, Valle F M. Meliponini biodiversity and medicinal uses of pot-honey from El Oro province in Ecuador. *Emir J Food Agric* 2015; 27: 502-506.
2. Oryan A, Alemzadeh E, Moshiri A. Potential role of propolis in wound healing: Biological properties and therapeutic activities. *Biomed Pharmacother* 2018; 98: 469-483.
3. Almasaudi S. The antibacterial activities of honey. *Saudi J Biol Sci* 2021; 28: 2188-2196.
4. Carnwath R, Graham E, Reynolds K, Pollock P. The antimicrobial activity of honey against common equine wound bacterial isolates. *Vet J* 2014; 199: 110-114.
5. Olaitan P B, Adeleke O E, Iyabo O. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. *Afr Health Sci* 2007; 7: 159-165.
6. Pećanac M, Janjić Z, Komarčević A, Pajić M, Dobanovački D, Mišković-Skeledžija S. Burns treatment in ancient times. *Med Pregl* 2013; 66: 263-267.
7. Olofsson T C, Vásquez A. Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee Apis mellifera. *Curr Microbiol* 2008; 57: 356-363.
8. Neveling D P, Endo A, Dicks L M. Fructophilic *Lactobacillus kunkeei* and *Lactobacillus brevis* isolated from fresh flowers, bees and bee-hives. *Curr Microbiol* 2012; 65: 507-515.
9. Endo A, Futagawa-Endo Y, Dicks L M. Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. *Syst Appl Microbiol* 2009; 32: 593-600.
10. Vásquez A, Olofsson TC, Sammataro D. A scientific note on the lactic acid bacterial flora in honeybees in the USA — A comparison with bees from Sweden. *Apidologie* 2009; 40: 26-28.
11. Olofsson T C, Alsterfjord M, Nilson B, Butler È, Vásquez A. *Lactobacillus apinorum* sp. nov., *Lactobacillus mellifer* sp. nov., *Lactobacillus mellis* sp. nov., *Lactobacillus melliventris* sp. nov., *Lactobacillus kimbladii* sp. nov., *Lactobacillus helsingborgensis* sp. nov. and *Lactobacillus kullabergensis* sp. nov., isolated from the honey stomach of the honeybee Apis mellifera. *Int J Syst Evol Microbiol* 2014; 64: 3109-3119.
12. Vásquez A, Forsgren E, Fries I, Paxton R J, Flaberg E, Szekely L, et al. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. *PLoS One* 2012; 7(3): e33188.
13. Forsgren E, Olofsson T C, Vásquez A, Fries I. Novel lactic acid bacteria inhibiting Paenibacillus larvae in honey bee larvae. *Apidologie* 2010; 41: 99-108.
14. Syed Yaacob S N, Huyop F, Kamarulzaman Raja Ibrahim R, Wahab RA. Identification of *Lactobacillus* spp. and *Fructobacillus* spp. isolated from fresh Heterotrigona itama honey and their antagonistic activities against clinical pathogenic bacteria. *J Apic Res* 2018; 57: 395-405.
15. Cotter P D, Ross R P, Hill C. Bacteriocins—a viable alternative to antibiotics? *Nat Rev Microbiol* 2013; 11: 95-105.
16. Yu J, Wang H, Zha M, Qing Y, Bai N, Ren Y, et al. Molecular identification and quantification of lactic acid bacteria in traditional fermented dairy foods of Russia. *J Dairy Sci* 2015; 98: 5143-5154.
17. Xu Y, Xie M, Xue J, Xiang L, Li Y, Xiao J, et al. EGCG ameliorates neuronal and behavioral defects by remodeling gut microbiota and TotM expression in Drosophila models of Parkinson's disease. *FASEB J* 2020; 34: 5931-5950.
18. Markazi A, Luoma A, Shanmugasundaram R, Mohnl M, Murugesan G R, Selvaraj R. Effects of drinking water synbiotic supplementation in laying hens challenged with *Salmonella*. *Poult Sci* 2018; 97: 3510-3518.
19. Jomehzadeh N, Javaherizadeh H, Amin M, Rashno M, Teimoori A. Quantification of intestinal *Lactobacillus* species in children with functional constipation by quantitative real-time PCR. *Clin Exp Gastroenterol* 2020; 13: 141-150.
20. Kim E, Yang S-M, Cho E-J, Kim H-Y. Novel real-time PCR assay for *Lactobacillus casei* group species using comparative genomics. *Food Microbiol* 2020; 90: 103485.
21. Huang C-H and Huang L. Rapid species-and subspecies-specific level classification and identification of *Lactobacillus casei* group members using MALDI Biotyper combined with ClinProTools. *J Dairy Sci* 2018; 101: 979-991.
22. Kim E, Yang S-M, Kim D, and Kim H-Y. Real-time PCR method for qualitative and quantitative detection of *Lactobacillus sakei* group species targeting novel markers based on bioinformatics analysis. *Int J Food Microbiol* 2021; 355: 109335.
23. Muthaiyan A, Ricke SC. Current perspectives on detection of microbial contamination in bioethanol fermentors. *Bioresour Technol* 2010; 101: 5033-5042.
24. Ramiah K, Van Reenen C, Dicks L. Expression of the mucus adhesion gene mub, surface layer protein slp and adhesion-like factor EF-TU of *Lactobacillus*

acidophilus ATCC 4356 under digestive stress conditions, as monitored with real-time PCR. *Probiotics Antimicrob Proteins* 2009; 1: 91.
25. Lashani E, Davoodabadi A, Dallal MMS. Some probiotic properties of *Lactobacillus* species isolated from honey and their antimicrobial activity against foodborne pathogens. *Vet Res Forum* 2020; 11: 121-126.
26. Humphries R, Bobenchik AM, Hindler JA, Schuetz AN. Overview of changes to the clinical and laboratory standards institute performance standards for antimicrobial susceptibility testing, M100, 31st Edition. *J Clin Microbiol* 2021; 59(12): e00213-21.
27. Anderson KE, Sheehan TH, Mott BM, Maes P, Snyder L, Schwan MR, et al. Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apis mellifera). *PLoS One* 2013; 8(12): e83125.
28. Ambika Manirajan B, Ratering S, Rusch V, Schwiertz A, Geissler-Plaum R, Cardinale M, et al. Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. *Environ Microbiol* 2016; 18: 5161-5174.
29. Mustar S, Ibrahim N. A sweeter pill to swallow: A review of honey bees and honey as a source of probiotic and prebiotic products. *Foods* 2022; 11: 2102.
30. Seraglio S K T, Schulz M, Brugnerotto P, Silva B, Gonzaga L V, Fett R, et al. Quality, composition and health-protective properties of citrus honey: A review. *Food Res Int* 2021; 143: 110268.
31. Kwon H-S, Yang E-H, Yeon S-W, Kang B-H, Kim T-Y. Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. *FEMS Microbiol Lett* 2004; 239: 267-275.
32. Aween MM, Hassan Z, Muhialdin BJ, Noor HM, Eljamel YA. Evaluation on antibacterial activity of *Lactobacillus* acidophilus strains isolated from honey. *Am J Appl Sci* 2012; 9: 807-817.
33. Razmgah N, Mojgani N, Torshizi M. Probiotic potential and virulence traits of *Bacillus* and *Lactobacillus* species isolated from local honey sample in Iran. *IOSR J Pharm Biol Sci* 2016; 11: 87-95.
34. Feizabadi F, Sharifan A, Tajabadi N. Isolation and identification of lactic acid bacteria from stored Apis mellifera honey. *J Apic Res* 2021; 60: 421-426.
35. Tajabadi N, Mardan M, Saari N, Mustafa S, Bahreini R, Manap MYA. Identification of *Lactobacillus plantarum, Lactobacillus pentosus* and *Lactobacillus fermentum* from honey stomach of honeybee. *Braz J Microbiol* 2014; 44: 717-722.
36. Khan K A, Ansari M J, Al-Ghamdi A, Nuru A, Harakeh S, and Iqbal J. Investigation of gut microbial communities associated with indigenous honey bee (Apis mellifera jemenitica) from two different eco-regions of Saudi Arabia. *Saudi J Biol Sci* 2017; 24: 1061-1068.
37. López AC, Alippi AM. Phenotypic and genotypic diversity of *Bacillus cereus* isolates recovered from honey. *Int J Food Microbiol* 2007; 117: 175-184.
38. Saccà M, Lodesani M. Isolation of bacterial microbiota associated to honey bees and evaluation of potential biocontrol agents of Varroa destructor. *Benef Microbes* 2020; 11: 641-654.
39. Cianciosi D, Forbes-Hernández TY, Afrin S, Gasparrini M, Reboredo-Rodriguez P, Manna PP, et al. Phenolic compounds in honey and their associated health benefits: A review. *Molecules* 2018; 23: 2322.
40. Escuredo O, Míguez M, Fernández-González M, Seijo MC. Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *Food Chem* 2013; 138: 851-856.
41. Alvarez-Suarez J M, Tulipani S, Romandini S, Bertoli E, Battino M. Contribution of honey in nutrition and human health: a review. *Med J Nutrition Metab* 2010; 3: 15-23.
42. C HC, T R K. Probiotic potency of *Lactobacillus plantarum* KX519413 and KX519414 isolated from honey bee gut. *FEMS Microbiol Lett* 2018; 365: 10.1093/femsle/fnx285.
43. Stojančević M, Bojić G, Al Salami H, Mikov M. The influence of intestinal tract and probiotics on the fate of orally administered drugs. *Curr Issues Mol Biol* 2014; 16: 55-68.
44. Anisimova EA, Yarullina DR. Antibiotic resistance of *Lactobacillus* strains. *Curr Microbiol* 2019; 76: 1407-1416.
45. Campedelli I, Mathur H, Salvetti E, Clarke S, Rea M C, Torriani S, et al. Genus-wide assessment of antibiotic resistance in *Lactobacillus* spp. *Appl Environ Microbiol* 2019; 85(1): e01738-18.