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Research Article
Isolation, Identification and Antimicrobial Activity of Unprecedented Lactic Acid Bacterial Isolates from Honeybees

[1,2]A.A. El-Sohaimy, [3,4]S.H.D. Masry, [2]M.G. Shehata, [5]S.N. Al-Kahtani, [6]T.E. Abdelwahab, [3]Y.A.T. Abdelmotaleb and [7]M.E. Nour

[1]Department of Technology and Organization of Public Catering, Institute of Sport, Tourism and Service, South Ural State University, 454080 Chelyabinsk, Russia
[2]Department of Food Technology, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, 21934 Alexandria, Egypt
[3]Department of Plant Protection and Molecular Diagnoses, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, 21934 Alexandria, Egypt
[4]Abu Dhabi Agriculture and Food Safety Authority, Al Ain, United Arab Emirates
[5]Department of Arid Lands Agriculture, Faculty of Agricultural Science and Food, King Faisal University, Al-Ehsaa, Saudi Arabia
[6]Department of Pests and Plant Protection, National Research Center, Dokki, Giza, Egypt
[7]Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University, Egypt

Abstract

Background and Objective: Lactic acid bacteria are generally recognized as safe that could be beneficial for several uses in food industry to get their health benefits. The present study was focused on the isolation and identification of some new lactic acid bacteria that might be naturally occurred in the honeybees stomach and tried to explore their benefits. **Materials and Methods:** Twenty five isolates of lactic acid bacteria were isolated from the stomach of three different types of Egyptian bees (*Apis mellifera lamarckii*), Carniolan bees (*A.m. carnica*) and hybrid Carniolan bees. Identification of isolates was carried out based on phenotypical tests and carbohydrate assimilation using API50 CHL and 16S rDNA sequencing. **Results:** In the present study, the results emphasized *Lactobacillus plantarum* to be the predominant species (62.5%), other strains were identified as *Pediococcus pentosaceus* (12.5%), *Lactobacillus pentosus* (12.5%) and *Lactobacillus sakei* (12.5%). Eight of 25 isolates showed a potential antibacterial activity especially against *Salmonella senftenberg* strain. The novel isolates (HBMSS1, HBMSS3, HBMSS4, HBMSS5, HBMSS6 and HBMSS8) showed a significant antimicrobial activity against *C. botulinum*, *E. coli*, *S. Senftenberg* and *S. epidermidis* as food borne pathogens and *P. larvae* and *M. plutonius* as honeybee pathogens. **Conclusion:** These promising findings might be beneficial for discovering novel preservatives in food industry and substitution of antibiotic drugs used in the treatment of honeybees' infection.

Key words: Lactic acid bacteria, isolation and characterization, honeybees, antimicrobial activity

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Corresponding Author: A.A. El-Sohaimy, Department of Technology and Organization of Public Catering, Institute of Sport, Tourism and Service, South Ural State University, 454080 Chelyabinsk, Russia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Honeybees (*Apis mellifera*) perform a crucial function as pollinators of horticultural land and agricultural crops in USA, pollination of horticulture crops by honeybees was around 15 billion dollars per year in agricultural sector production[1]. However, pathogenic bacteria, fungi, viruses and protozoa could infect honeybee colonies. American foulbrood (AFB) and European foulbrood (EFB) are considered dangerous honeybee infections caused by the Gram-positive bacteria *Paenibacillus larvae* and *Melissococcus plutonius*[2,3]. Overall, the microbiota of the honeybee *A. mellifera* L. is convoluted and difficult to be resolved; nonetheless, it has been reported to be principally consists of yeasts and Gram-positive bacteria (such as *Lactobacillus* spp., *Lactococcus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Bacillus* spp. and *Streptococcus* spp.) and Gram negative bacteria[4,5]. Corby-Harris *et al*.[6] described that these bacteria as being capable of residing in the bees' digestive tract and not dependent on seasonal or food factors. In addition, Bastos *et al*.[7] has reported that the natural microorganisms of bees derive from pollen or direct contact with forager worker bees. In hibernating honeybees, Lyapunov *et al*.[8] emphasized the presence of gut *Enterobacteria*. Interestingly, there are a handful studies focusing on the isolation of lactic acid bacteria (LAB) from the honeybee microbiota[1,4,5,9-11]. In few studies, *Lactobacillus* bacteria might be able to produce catalase and reduce nitrate, even though sometimes these bacteria might be not inline with these properties[12]. Thus, only the subsistence of culturable bee-gut (*Lactobacillus*, *Enterococcus* or *Bifidobacterium)* strains associated with the intestinal tract of the honeybee has been described. Although, it has always been the most powerful isolation of microorganisms from native sources to obtain useful and genetically stable strains. Olofsson and Vasquez[10] were disclosed a remarkable growth in the number of LAB in honeybees from healthy honeybee colonies. Moreover, honeybees infected with *P. larvae* showed fewer *Lactobacillus* spp. content[13]. LAB are fundamental in food and dairy technology due to their capability to generate the organic acids, diacetyl compounds, hydrogen peroxide and bacteriocins which may act as natural preservatives that can play a decisive function in the extension of the shelf-life of foods. Indeed, LAB are becoming increasingly accepted as probiotics that stimulating the immune response, inhibiting bacterial infection and preventing diarrhoea[14-17]. There are different microorganisms seriously affecting honeybees, such as *P. larvae* and *M. plutonius*, as gram-positive *bacilli* causing AFB and EFB in bee larvae[18,19]. Moreover, random antibiotic use against these pathogens has not only resulted in chemo-resistant strains but also causing honey contamination[20]. So, LAB isolated from honeybees might perform a substantial role in conserving the adult bees and larvae against pathogens, with an *in vitro* inhibitory effect on the causative agents of AFB and EFB[21,22]. Hence, the present work was focused on screening, isolation and phenotypic and genotypic characterization of LAB from honeybees' stomach and midgut honeybees. Furthermore, determine their *in vitro* antimicrobial potential against different Foulbrood and foodborne pathogens, which lead us to explore novel natural food preservatives and feasible biological control for honeybees' infection.

MATERIAL AND METHODS

The present investigation was carried out in the Department of Food Technology, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt from June 2017, to December, 2018.

Sample collection: During the foraging season from April to June, 2017, 180 forager worker bees were collected from colonies of three races and hybrids in Egypt. Sixty forager worker bees from Egyptian honeybee (*Apis mellifera lamarckii*) colonies, 60 forager worker bees from Carniolan honeybee (*A.m. carnica*) colonies and 60 forager worker bees from hybrid Carniolan honeybee colonies were collected. The colonies of *A.m. lamarckii* were located at Upper Egypt (Assiut Governorate); those of *A.m. carnica* and hybrid Carniolan honeybees were located at New Borg El-Arab City, northwestern Egypt, at the apiary of City of Scientific Research and Technological Applications (SRTA-City). The samples of foraging worker bees were collected alive in wooden cages and immediately transported to the laboratory of Department of Food Technology (SRTA-City). Selected foraging worker bees were surface-sterilized with 70% ethanol for 60 sec, followed by 5% NaCl for 60 sec and then washed with sterile distilled water to isolate the honey stomach and gut bacteria. In a laminar flow hood, the worker bees were dissected and the honey stomach and mid-gut were separated from the alimentary canal[23].

Table 1: Indicator strains and their growth conditions

Pathogenic microorganisms	Medium and growth	Temperature (°C)
Foodborne pathogens		
Bacillus subtilis DB 100	Nutrient Broth	37
Candida albicans ATCCMYA-2876	YPD Broth	37
Clostridium botulinum ATCC 3584 T	TPGY Broth	37
Escherichia coli BA 12296	LB Broth	37
Salmonella senftenberg ATCC 8400	Nutrient Broth	37
Staphylococcus aureus NCTC 10788	Nutrient Broth	37
Staphylococcus epidermidis	Nutrient Broth	37
Streptococcus pyogenes	Nutrient Broth	37
Foulbrood pathogens		
Paenibacillus larvae (AFB)	Nutrient Broth	37
Melissococcus plutonius (EFB)	Nutrient Broth	37

YPD broth: Yeast peptone dextrose, TPGY broth: Tryptone-peptone-glucose-yeast extract, LB broth: Luria-Bertani medium, AFB: American foulbrood, EFB: European foulbrood

Isolation of honeybee stomach and mid-gut bacteria: In 100 µL of phosphate buffered saline (PBS), the honey stomach was homogenized with a plastic pestle. On Brain Heart Infusion (BHI) agar medium (Difco), the stomach and mid-gut homogenates were aerobically incubated at 35°C for 2 days. The size, color and morphology selected the colonies that grew on the plates. On solid agar, isolated colonies have been grown repeatedly.

Antimicrobial activity: The antimicrobial activity of the isolates against pathogenic bacteria was determined (Table 1) using the spot-on-lawn method[24,25]. LAB was grown in MRS for 16-18 h with 1% inoculum and cells were extracted from the medium by centrifugation (6,500 g for 10 min, 4°C) in order to obtain a supernatant free of cells. Indicator strain lawns have been prepared by adding 0.125 mL (2×10^7 cell mL^{-1}) of 10×-diluted overnight culture to 5 mL of corresponding soft agar (Table 1). The tubes contents are gently mixed and poured over pre-poured MRS agar plate's surfaces. Ten microliters of each cell-free supernatant were spotted on the agar's surface and the plates were tested for an inhibition area after 24 h of incubation. Clear zones around spots show the isolated bacteria's antibacterial activity.

Identification of promising LAB isolates

Phenotypic characterization and biochemical analysis: Promising LAB isolates have been phenotypically identified using Bergey's Systematic Bacteriology Manual[26,27]. Microscopic examination has been used for phenotypic identification after gram staining. Furthermore, according to the manufacturer's instructions, fermentation patterns are determined using API 50 CHL and API 20 kits (Biomerieux SA, France).

Molecular identification

DNA extraction: Microbial DNA was extracted from LAB using the Fermentas DNA extraction kit (FDEK), according to the protocol described by the manufacturers. The DNA extracts are examined by 1% agarose gel electrophoresis and stained with ethidium bromide.

Polymerase chain reaction amplification: PCR reaction was carried out using universal 16S rDNA primers for invariant region that purchased from Sigma Scientific Company, Germany[28]. The PCR amplification was performed in a thermocycler (Santa Clara, California, USA) with the following program: Initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 40 sec, 55°C for 40 sec and 72°C for 1 min, with a final extension at 72°C for 10 min. PCR product was identified by loading on 1% agarose gel containing 1 µg mL^{-1} ethidium bromide. A 100-1500 bp DNA marker (TAKARA BIO INC., Shiga, Japan) was used as a DNA marker.

DNA sequencing: An automated DNA sequencer was used to perform DNA sequencing reactions. The National Center for Biotechnology Information (NCBI) website (http:/www.ncbi. nlm.nih.gov/BLAST[29]) used the latest release of the non-redundant DNA sequence database.

Nucleotide sequence accession numbers: The sequences from the 8 amplicon samples representing different combinations of extracted DNA and PCR amplification were uploaded to GenBank (NCBI) under the following accession numbers: MF662586, MF662587, MF662588, MF662589, MF662590, MF662591, MF662592, MF662593.

Phylogenetic analysis: BLAST-N searches (http:/blast.ncbi. nlm.nih.gov/) retrieved closely related 16S rRNA sequences from the GenBank/EMBL database. Several sequence alignments with ClustalW (http:/www.ebi.ac.uk/ Tools/msa/clustalo/) were performed and used to reconstruct phylogenetic relationships using neighbour-joining (NJ) algorithms with bootstrapping of 1000 times in MEGA7.

Statistical analysis: All assays were carried out in triplicate and the results are expressed as the mean with the standard deviation (mean±SD) using SPSS16 software. Values were compared by ANOVA with a general linear model followed by Duncan's *post hoc* test and $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Isolation and phenotypic characterization of LAB: Twenty-five LAB isolates have been isolated from the honeybee stomach of 3 different types of Egyptian bees (*A. mellifera lamarckii*), Carniolan bees (*A.m. carnica*) and hybrid Carniolan bees (Table 2). All 25 isolates obtained from the honey stomach were found to be catalase negative and gram positive. Eight isolates exhibiting antimicrobial activity potential against food-borne and foulbrood pathogenic microorganisms were chosen for further characterization (Table 3). Morphologically, the cells of 7 isolates were rod shaped and arranged either in pairs or chains, colonies on MRS agar were orbicular and entirely low convex in shape and cream coloured. The remaining isolate was coccus shaped and arranged in pairs and tetrads. The phenotypic features of the isolates suggest their close resemblance to *Lactobacilli* spp. and *Pediococcus* spp.[30,31]. Previous studies on the association of LAB with the honeybee gut and honey stomach are limited. For instance, Mohr and Tebbe[32] assured the existence of *Lactobacillus* in the bees gut over molecular analysis without isolation and the detected strains were uncultured. Mrazek *et al.*[33] have examined a number of insect intestinal microbiota, only mentioning the possible presence of *Bifidobacterium* sp. without isolation in honeybees. Additionally, *Lactobacillus* sp., *Enterococcus* and some *Bifidobacterium* spp. have been isolated by Nora and Amel[34] and the honeybee digestive tract microbiota has been investigated.

Antimicrobial activity against food born pathogens: The antimicrobial activity of the LAB isolates and their power to restrain the outgrowth of diverse pathogens lead to food deterioration have been examined by using nutrient agar media and a spot-on-lawn assay (Table 3). In a preliminary assay, indicator organisms were tested for antimicrobial activity by LAB under aerobic conditions: indicator organisms (*Escherichia coli* BA 12296, *Candida albicans* ATCCMYA-2876, *Bacillus subtilis* DB 100, *S. senftenberg* ATCC 8400, *Staphylococcus aureus* NCTC 10788, *Clostridium botulinum* ATCC 3584, *Staphylococcus epidermidis* and *Streptococcus pyogenes*) were inoculated individually and overlaid with a plain nutrient agar plate (control) or over plates with the supernatant of LAB grown in MRS broth. Ehb8 isolate was exhibited the highest antimicrobial activity against *S. senftenberg* ATCC 8400 (Fig. 1). Additionally, an anti microbial activity with considerable zone of inhibition against *Salmonella senftenberg* ATCC 8400 was noted with Ehb (2, 5, 8 and 9) isolates. However, negative results were found for other isolates, with no inhibitory zones against *B. subtilis* DB 100, *C. albicans* ATCCMYA-2876 or *S. pyogenes* (Table 3). Thus, current experimental data showed that some of the isolated strains of LAB inhibited the growth of various pathogenic bacteria and yeast especially those causing honeybee diseases. These findings might be due to the differences in the essence of the bioactive secondary metabolites produced by LAB (organic acids, hydrogen peroxide, acetaldehyde, diacetyl compounds and bacteriocins) that inhibit pathogenic and spoilage microorganisms in food[25,35]. Experimental data in the present work were proven by the literature in relation to the different susceptibility of *Salmonella* spp., *Clostridium* spp. and *E. coli* to various LAB[16,36,37].

Antimicrobial activity against AFB and EFB: The antimicrobial potency of LAB isolates against *P. larvae* (causing AFB) and *M. plutonius* (causing EFB) have been investigated as shown in (Fig. 2). Five isolates out of 25 (Ehb2, Ehb8, Ehb9, Hhb11 and Chb19) showed inhibitory halo zones against *P. larvae* and *M. plutonius* (Fig. 1). This finding for Egyptian honeybees was as opposed to that for European honeybees that showed activity against a wide range of bacterial species. This may point out to variations in the gut's physiological conditions, such as differences in pH and antimicrobial substances in the bees diet. European

Table 2: Isolation sources of lactic acid bacteria

Isolation sources	Number of examined samples	Number of isolates	Location
Egyptian honeybees (*Apis mellifera lamarckii*)	60	10	Assiut governorate
Hybrid honeybees	60	7	New Borg El-Arab City (SRTA City)
Carniolan honeybee (*Apis mellifera carnica*)	60	8	New Borg El-Arab City (SRTA City)
Total	180	25	

Table 3: Antimicrobial activities of cell-free supernatants of 25 LAB isolates against various pathogens

Isolate number	Symbol	Sources of isolation	Incubation temperature (°C)	Antimicrobial activity against pathogens (mm)									
				Bacillus subtilis	*Candida albicans*	*Clostridium botulinum*	*Escherichia coli*	*Melissococcus plutonius*	*Paenibacillus larvae*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Streptococcus pyogenes*	*Salmonella senftenberg*
1	Ehb1	HS	30	0	0	0	0	0	0	0	0	0	0
2	Ehb2	HS	30	0	0	0	30	0	11	0	0	0	20
3	Ehb3	HS	30	0	0	0	0	0	0	0	0	0	18
4	Ehb4	HS	30	0	0	0	0	0	0	0	0	0	0
5	Ehb5	HS	30	0	0	0	22	0	0	0	0	0	20
6	Ehb6	HS	30	0	0	0	0	0	0	0	0	0	0
7	Ehb7	HS	37	0	0	0	0	0	0	0	0	0	0
8	Ehb8	HS	37	0	0	0	0	0	13	0	20	0	22
9	Ehb9	HS	37	0	0	0	0	18	0	0	16	0	20
10	Ehb10	HS	37	0	0	0	0	0	0	0	0	0	0
11	Hhb11	HbM	30	0	0	10	0	0	20	0	0	0	0
12	Hhb12	HbM	30	0	0	15	0	0	0	0	0	0	0
13	Hhb13	HbM	30	0	0	16	0	0	0	0	0	0	0
14	Hhb14	HbM	37	0	0	0	0	0	0	0	0	0	0
15	Hhb15	HbM	37	0	0	14	0	0	0	0	0	0	0
16	Hhb16	HS	37	0	0	0	0	0	0	0	0	0	0
17	Hhb17	HS	37	0	0	0	0	0	0	0	0	0	0
18	Chb18	HS	30	0	0	0	0	0	0	0	0	0	0
19	Chb19	HS	30	0	0	13	0	0	18	0	0	0	0
20	Chb20	HS	30	0	0	0	0	0	0	0	0	0	0
21	Chb21	HS	30	0	0	10	0	0	0	0	0	0	0
22	Chb22	HS	37	0	0	12	0	0	0	0	0	0	0
23	Chb23	HS	37	0	0	0	0	0	0	0	0	0	0
24	Chb24	HS	37	0	0	0	0	0	0	0	0	0	0
25	Chb25	HS	37	0	0	19	0	0	0	0	0	0	0

Ehb: Egyptian honeybees, Hhb: Hybrid honeybees, Chb: Carniolan honeybees, HS: Honey stomach, HbM: Honey bee midgut, diameter of the zone of inhibition (mm) using the agar well diffusion assay method

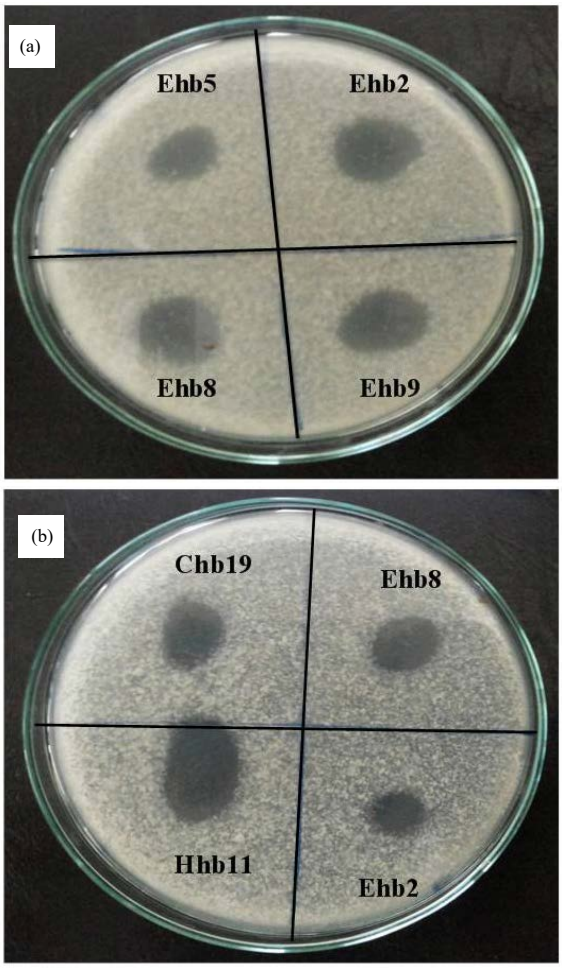

Fig. 1(a-b): Inhibitory activity of the cell-free supernatant of isolates against (a) *Salmonella senftenberg* ATCC 8400 and (b) *P. larvae*

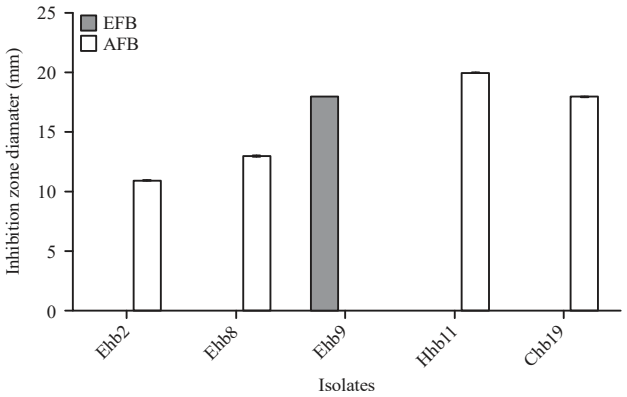

Fig. 2: Inhibitory activities of antagonistic bacteria isolated from the honey stomach against honeybee pathogens
AFB: *Paenibacillus larvae*, EFB: *Melissococcus plutonius*

honeybees *A. mellifera*, accommodate numerous species of gut bacteria[9], including antagonistic bacteria to young larvae pathogens[38]. The existence of *Lactobacilli* in *A. mellifera* and *A.m. scutellata* have been reported[6,32] and a novel bacterial flora of the *A. mellifera* honey stomach composed of LAB of the genera *Lactobacillus* and *Bifidobacterium* has recently been reported[10]. On the other hand, Evans and Armstrong[38] failed to isolate of *Lactobacillus* species from *A. mellifera*, suggested that the bacterial gut population is not even constant in the same species. In the current investigation, the honey stomach of *A. mellifera* harboured *Lactobacillus* and *Pediococcus* species and we also detected several stomach LAB that had not previously been detected in the genus *Apis*. Honeybees visit several types of flowers that vary seasonally and geographically; in addition, honeybees of different species tend to visit similar species of flowers. Consequently, changes in feed source may be correlated with the lack of a characteristic honey stomach bacterial profile in *Apis*. Antibiotics such as oxytetracycline hydrochloride (OTC) and sulfathiazole are commonly used for controlling AFB and EFB worldwide, though *P. larvae* and *M. plutonius* have developed antibiotic resistance properties. Because the LAB strains isolated in the present work displayed defence activity against honeybee pathogens, a promising management strategy might be developed to prevent honeybee bacterial diseases to avoid antibiotic treatment.

Morphological and biochemical identification: Morphological classification of LAB isolates was carried out according to the schemes described by Systematic Bacteriology Manual of Bergey[26,27]. The existence of *Lactobacillus* was confirmed by tiny yellow colonies of similar sizes that appeared on MRS agar using the streak plate process, as previously reported[30]. Furthermore, biochemical analysis of LAB was conducted by the API50 strip kit and selected strains were identified as Gram-positive rods or coccus-shaped isolates most closely associated with *Lactobacilli* and *Pediococcus* spp., respectively (Table 4). The API web software confirmed that lactobacilli strains are typically able to utilize carbohydrates such as galactose, glucose, fructose, glycerol, D-xylose, mannose, mannitol, ribose, N-acetyl-glucosamine, amygdalin, α-methyl-D-glucoside, esculin, arbutin, cellobiose, salicin, maltose, sucrose, lactose, melibiose, melezitose, raffinose, trehalose, gentiobiose, D-arabitol, D-tagatose and gluconate. Moreover, the *Pediococcus* strain was able to ferment carbohydrates that included L-arabinose, glycerol, D-xylose, galactose, glucose, ribose, mannose, fructose, mannitol, gentiobiose,

Table 4: Biochemical characterizations of lactic acid bacteria based on carbohydrate interpretation using the API 50 CHL kit

LAB isolates	Ehb2	Ehb3	Ehb5	Ehb8	Ehb9	Hhb11	Hhb15	Chb19
Control	-	-	-	-	-	-	-	-
Glycerol	+	+	-	-	+	-	-	+
Erythritol	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-
L-arabinose	-	+	-	-	-	-	+	-
Ribose	+	+	+	+	+	+	+	+
D-xylose	-	+	+	-	+	-	+	+
L-xylose	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-
β-methyl-D-xyloside	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+
Sorbose	-	-	-	-	-	-	-	-
Rhamnose	-	-	+	-	+	-	+	+
Dulcitol	-	-	-	-	-	-	-	-
Inositol	-	-	+	-	+	-	-	+
Mannitol	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	+	-
α-methyl-D-mannoside	-	-	-	-	-	-	-	-
α-methyl-D-glucoside	+	-	-	+	+	-	-	-
N-acetyl-glucosamine	+	+	+	+	+	+	+	+
Amygdalin	+	-	+	+	+	+	+	+
Arbutin	+	-	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	-	+
Salicin	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+
Maltose	+	-	+	+	+	+	+	+
Lactose	+	-	+	+	+	+	+	+
Melibiose	+	-	+	+	+	+	+	+
Sucrose	+	-	+	+	+	+	+	+
Trehalose	+	-	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-
Melezitose	+	-	+	+	+	+	+	+
Raffinose	+	-	+	+	+	+	-	+
Starch	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
Gentiobiose	+	+	+	+	+	+	+	+
Turanose	-	-	-	-	-	-	+	-
D-lyxose	-	-	-	-	-	-	-	-
D-tagatose	+	+	-	+	-	-	+	+
D-fucose	-	-	-	-	-	-	-	-
L-fucose	-	-	-	+	-	+	-	+
D-arabitol	+	-	+	+	+	+	-	+
L-arabitol	-	-	-	-	-	-	-	-
Gluconate	+	-	+	+	+	+	+	+
2-Keto-Gluconate	-	-	-	-	-	-	-	-
5-Keto-Gluconate	-	-	-	-	-	-	-	-

-: Bacterium does not use this carbohydrate, +: Bacterium uses this carbohydrate, Ehb2*: Lactobacillus plantarum*, Ehb3*: Pediococcus acidilactici*, Ehb5*: Lactobacillus plantarum*, Ehb8*: Lactobacillus plantarum*, Ehb9*: Lactobacillus plantarum*, Hhb11*: Lactobacillus plantarum*, Hhb15: *Lactobacillus sakei*, Chb19: *Lactobacillus plantarum*

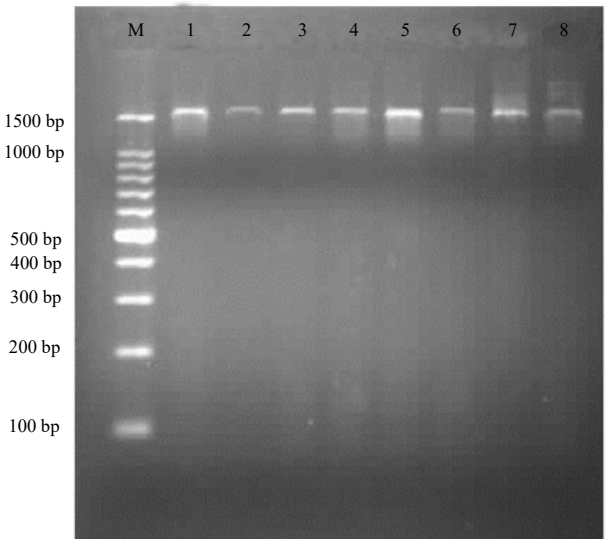

Fig. 3: Agarose gel electrophoresis of PCR products amplified using 16S rDNA primers
M: GeneRuler DNA ladder, Lane 1: HBMSS1, 2: HBMSS2, 3: HBMSS3, 4: HBMSS4, 5: HBMSS5, 6: HBMSS6, 7: HBMSS7, 8: HBMSS8

Table 5: Identification of isolates based on 16S rRNA sequences

Isolates	Accession number	Closest affiliation in GenBank	Similarity (%)
Ehb2	MF662586	*Lactobacillus plantarum* strain YK-9 16S (HBMSS1)	92
Ehb3	MF662587	*Pediococcus pentosaceus* strain LAB6 (HBMSS2)	90
Ehb5	MF662588	*Lactobacillus plantarum* strain BSR4 (HBMSS3)	81
Ehb8	MF662589	*Lactobacillus pentosus* strain b52 (HBMSS4)	96
Ehb9	MF662590	*Lactobacillus plantarum* strain 0825 (HBMSS5)	98
Hhb11	MF662591	*Lactobacillus plantarum* strain L415 (HBMSS6)	94
Hhb15	MF662592	*Lactobacillus sakei* strain C-11 (HBMSS7)	93
Chb19	MF662593	*Lactobacillus paraplantarum* strain R5 (HBMSS8)	94

esculin, cellobiose, salicin, N-acetyl-glucosamine and D-tagatose (Table 4). The color change in the strip capsule from violet to yellow was indicated complete *Pediococcus pentosaceus* 4I1 fermentation[39]. *Lactobacillus* species isolated from fermented sausage using an API 50 kit have recently been described phenotypically and biochemically by Casaburi *et al*.[40].

16S rRNA sequencing and phylogenetic analysis: Eight strains were chosen because of their antimicrobial potential and 16S rRNA sequence analysis was used to classify them (Fig. 3). Strains HBMSS1, HBMSS2, HBMSS3, HBMSS4, HBMSS5, HBMSS6, HBMSS7 and HBMSS8 exhibited 92, 90, 81, 96, 98, 94, 93 and 94% DNA sequence identity of known species *Lactobacilli* and *Pediococcus* registry entries (Fig. 4). In addition, the similarity of the BLAST 16S rRNA sequence and biochemical tests showed that these strains in GenBank were closely linked to LAB. The HBMSS1, HBMSS2, HBMSS3, HBMSS4, HBMSS5, HBMSS6, HBMSS7 and HBMSS8 nucleotide sequence data were deposited in GenBank (accession numbers MF662586, MF662587, MF662588, MF662589, MF662590, MF662591, MF662592 and MF662593, respectively) (http://www.ncbi.nlm.nih.gov). Using the 16S rRNA universal primers[28], nearly full-length 16S rRNA gene regions were successfully amplified and sequenced and according to sequence similarity, these isolates were identified as LAB (*Lactobacilli* and *Pediococcus*) (Table 5). A phylogenetic tree representing different reference species of LAB with the isolates was generated using the NJ method (Fig. 4). *Lactobacillus plantarum*, *Lactobacillus sakei* and *Pediococcus pentosaceus* were clearly separated from different *Lactobacilli* and *Pediococcus* strains (Fig. 4-c). *Lactobacillus plantarum* isolates HBMSS1, -3, -4, -5, -6 and -8 clustered in the *Lactobacilli* linage, close to *L. plantarum* reference strains. The *P. pentosaceus* isolate grouped with the *Pediococcus* sp. lineage, with a high degree of genetic resemblance to *Pediococcus acidilactici*. In addition, *Lactobacillus sakei* grouped within the *Lactobacillus* spp. cluster.

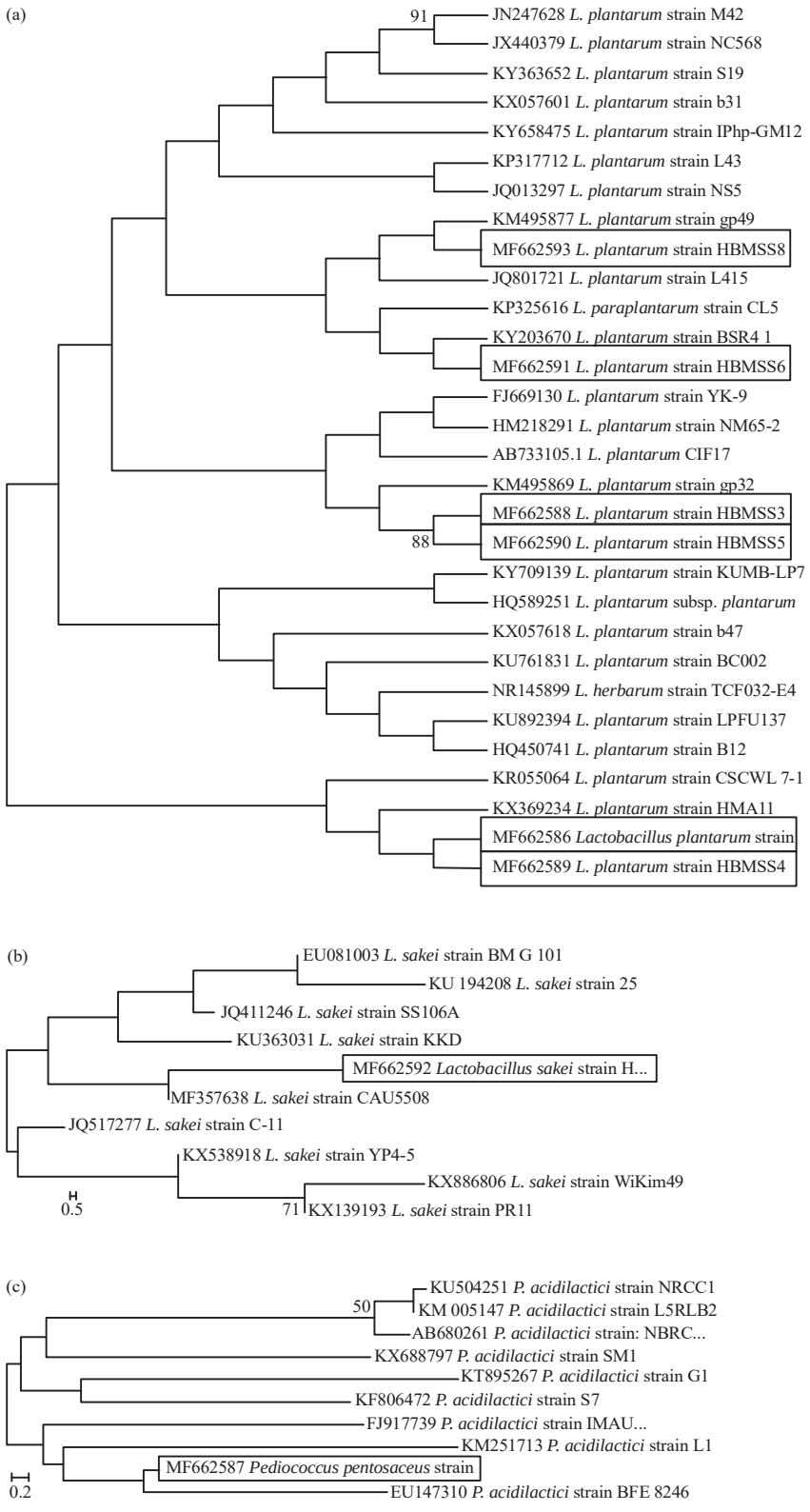

Fig. 4(a-c): A phylogenetic relationship based on 16S rDNA sequences of honeybee stomach isolates, (a) *Lactobacillus plantarum*, (b) *Lactobacillus sakei* and (c) *Pediococcus pentosaceu*

CONCLUSION

The current study aimed to isolate and identify lactic acid bacteria from the stomach of Egyptian honeybees *(Carniolan* and hybrid honeybees). Furthermore, define the *in vitro* antibacterial activity of novel isolates against several pathogenic bacteria. The novel isolates (HBMSS1, HBMSS3, HBMSS4, HBMSS5, HBMSS6 and HBMSS8) showed a significant antimicrobial activity against *C. botulinum*, *E. coli*, *S. senftenberg* and *S. epidermidis* as foodborne pathogens and *P. larvae* and *M. plutonius* as honeybee pathogens. Accordingly, due to their good antimicrobial activity, these promising new strains might be used as natural preservatives in food industry to prevent or delay the deterioration of food products. Moreover, these promising LAB isolates might be used against honeybee pathogens to prevent honeybee bacterial diseases as natural and save antibiotic alternatives.

SIGNIFICANCE STATEMENT

This study discover the novel lactic acid bacteria isolates from honeybees stomach with good antimicrobial activity that can be beneficial for food industry and food safety as a natural preservatives instead of synthetic ones. In the same time, these isolates could be used for treating the honeybees' infection with *P. larvae* and *M. plutonius* as honeybee pathogens. This study will help the researcher to uncover the critical areas of food safety and Honeybees infection that many researchers were not able to explore.

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