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Identification, characterization and phylogenetic analysis of gut microbiota of the honey bee, *Apis mellifera* L., from Saudi Arabia

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The honey bee, *Apis mellifera* is regarded one of the most beneficial creatures on our planet. The impact of gut microbiota on insect host is lasting beyond the symbiotic interaction. Therefore, the current work was designed to identify and characterize the gut microbiota of *A. mellifera* collected from beekeeping farm at Sakaka city, Aljouf, Saudi Arabia. Bees were sampled from five distinct colonies (20 bees/ colony), disinfected, the whole guts were homogenized under sterile conditions. Homogenates were cultured on different media, incubated and morphologically differentiated. A single colony of each morphotype was propagated and purified. Phenotypic and biochemical and molecular characterization of all purified colonies were carried out on the bases of 16S rRNA and 18S rRNA genes. Sequence and phylogeny were analyzed. Combining morphological, biochemical and molecular data, species composition of the gut microbiota was determined. Out of 12 bacilli bacteria, 6 aerobic and 6 facultative anaerobic, 7 non-sporulating and 5 endospore-forming bacteria were identified. One yeast-like facultative anaerobic, endospore-forming fungus was recognized. Biochemical tests confirmed that the gut bacteria are capable of producing commercial enzymes and biologically active substances, as well as they have ability to ferment carbohydrates and acidify the gut media. Some on these bacteria are revealed to be waterborne or foodborne. We identified 12 bacterial species belonging to 8 genera. Out of the 8 genera, 6 genera belong to the phylum Proteobacteria (75.0%), and 2 genera belong to the phylum Firmicutes (25.0%). In addition, one fungal species belonging to the phylum Ascomycota and family Saccharomycetaceae. Eleven bacteria and one fungus were, successfully, identified at species level. One bacteria was identified at its genus level. Four species were indicated to be novel strains or subspecies. Phylogentic analyses revealed both overlap and divergence of genera. Identification of the species was methodology-dependent. Species constituting gut microbiota community may function to generate bio-products which ultimately enable the honey bees to tolerate climatic and infectious stresses. Our findings provide a basic step for investigating the specific impacts of the gut microbiota on *A. mellifera*.

Keywords: Honey bee, *Apis mellifera*, gut microbiota, bioactive compounds, symbiosis.

INTRODUCTION

Intense research was published on gut microbiota of *A. mellifera* owing to its crucial function in insect health. This close symbiotic relationship benefits both partners as gut microbes protect honey bees from pathogens and parasites (Engel and Moran, 2013), contribute to host development and nutrient acquisition (Hung et al. 2018) and aids in food digestion, provides essential nutrients, and detoxifies harmful chemicals (Engel and Moran, 2013; Dong et al. 2020). Factors like host physiology, diet, and immunity influence the gut microbiota composition (Babendreier et al. 2007). Additionally, interactions with other hive inhabitants and environmental factors like pollen source shape the microbiota (Flint et al. 2012; Powell et al. 2014).

Honey bees (*Apis mellifera*) serve as a valuable model organism for gut microbiota studies. Molecular identification techniques reveal a core community of around nine bacterial species (Martinson et al. 2011; Sabree et al. 2012). *Lactobacillus spp*. are dominant, while *Bifidobacterium spp*. are less abundant (Babendreier et al. 2007; Bottacini et al. 2012a). Other members like *Bartonella apis*, *Frischella perrara*, and *Parasaccharibacter apium* exhibit fluctuating abundance (Jeyaprakash et al. 2003; Philipp et al. 2013; Vanessa et al. 2014). The diverse microbiota isolated from the *A. millefera* gut encompassed gram-negative, gram-positive bacteria, and yeasts (Endo and Salminen, 2013).

Interestingly, gut communities differ between queens and worker bees of varying ages (Anderson et al. 2018; Dong et al. 2020).

Dominant phyla include Proteobacteria, Actinobacteria, and Firmicutes (Martinson et al. 2011). Core bacterial groups belong to the Acetobacteraceae, Betaproteobacteria, Gammaproteobacteria, and Firmicutes (Martinson et al. 2011; Moran et al. 2012; Kwong and Moran, 2016). These gut bacteria play a vital role in fitness and survival of the host. Additionally, these microbes can be transmitted vertically (from parents) or horizontally (between individuals) (Engel et al. 2012).

Honey bees (*A. mellifera*) are economically vital insects, generating billions of dollars annually through honey, wax, propolis, and pollination services (Southwick and Southwick, 1992; van Engelsdorp and Meixner, 2010; Liu et al. 2011). However, beekeeping faces challenges like climate change, pesticide overuse, antibiotic misuse, and infectious diseases (Langowska et al. 2017; Anderson et al. 2011; Colin et al. 2019; Owen, 2017; Cameron et al. 2011). In Saudi Arabia, beekeeping is a traditional practice contributing significantly to rural income. Two bee races are prevalent: the native *Apis mellifera jemenitica* and the imported *Apis mellifera carnica* (Al-Ghamdi et al. 2012). Native bees are better adapted to the harsh climate, possibly due to their distinct gut microbiota (Khan et al. 2017). Understanding the natural gut microbiota could lead to development of probiotics for promoting bee health, replacing antibiotics and offering a sustainable solution. It's also believed that gut microbiota composition influences the honey bee's metabolic capacity and nutrition (Zheng et al. 2019).

Therefore, the current study aims to discover and characterize gut microbial communities (bacteria and fungi) of honey bees from the Al-Jouf region, Saudi Arabia.

MATERIALS AND METHODS

Sample Collection:

Worker bees of *A. mellifera* were sampled from five separate colonies of a private beekeeping farm at Sakaka city, Aljouf, Saudi Arabia. Using sterile forceps, twenty bees were collected from each colony, and kept in sterile 50 mL tubes containing 35 mL of sterile physiological saline (0.9% [wt/vol] NaCl, 0.1% [wt/vol] Tween 80, and 0.1% [wt/vol] Peptone) (Engel et al. 2013). To minimize contamination, bees were disinfected by immersion in sodium hypochlorite (1%) for 2 minutes, then followed by three rinses in sterile water (Engel et al. 2013). Then, the entire guts were demonstrated and handled under sterile conditions.

Isolation of gut microbiota:

For bacterial isolation, 10 mL of sterile phosphate-buffered saline (PBS) were added to 10 guts from the same colony. Guts were well-homogenized, and the homogenates were spread over nutrient agar (NA) and four selective agar media (Brain Heart Infusion agar (BHI), Tryptic Soy Agar Blood Base (TSA), Lactobacilli MRS agar (LMA), and BSM agar (BSM)). Five plates of each medium were used. All plates were incubated at 30 °C under aerobic conditions for 2 days, and then studied for bacterial identification. Based on colony morphology, isolates were differentiated. A single colony of each morphotype was separately propagated, purified, and then stored in 5% glycerol at -80 °C until further studies (Galal and Seufi, 2020).

For fungal isolation, the homogenates were spread over five Potato Dextrose agar (PDA) plates, supplemented with the antibacterial Rose Bengal. Plates were incubated at 28 °C under aerobic conditions for 7-15 days, and then examined for fungal identification. A single pure colony was separately propagated on a new sterile PDA plate, and pure colonies were used for further investigation. These colonies were stained with lactophenol blue and examined under a light microscope (Galal et al. 2017).

Biochemical Characterization of Bacterial Isolates:

Biochemical tests were done for bacterial characterization following the methods described by Galal et al. (2013), and Varghese and Joy (2014). Catalase test, Oxidase test, Urease test, Tryptophan deaminase test, Indole test, Methyl red test (MR), Citrate test, Sugar fermentation tests (Glucose, Sucrose, and Lactose), Triple Sugar Iron (TSI) test, Carbon dioxide (CO2) production test, Hydrogen sulfide (H2S) production test, and Voges-Proskaeur's test (VP) were employed (Galal et al. 2013; Varghese and Joy, 2014).

Phenotypic Characterization of Bacterial and Fungal Isolates:

Colony and cell characteristics were employed to characterize bacteria. The obtained data were compared to the corresponding bacteria identified by Bergey's Manual of Systematic Bacteriology (Holt, 1984). Gram's staining was performed according to the method described by Varghese and Joy (2014).

Fungal colonies were characterized based on their macroscopic morphology as previously described (Ellis et al. 2010; Varghese and Joy, 2014).

Molecular Characterization of Bacterial and Fungal Isolates

Genomic DNA Purification

Two replicates from each monomorphic colonies were selected for further molecular investigations. QIAprep DNA Bacteria Kit (QIAGEN GmbH, Germany) was used for bacterial DNA purification. In parallel, Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co. Ltd., Hangzhou, China) was used for fungal DNA purification.

Purified DNAs from bacteria and fungi were assayed for integrity, concentration, and purity as previously described (Galal and Seufi, 2020). DNA was stored at -20 °C until further studies.

Oligonucleotides and PCR amplification of 16S rRNA and 18S rRNA genes

The primers utilized for PCR amplification and sequencing of the present work are shown in Table (1). PCR master mix for all reactions was developed in a sterile biosafety cabinet. This master mix consists of 1.5mM $MgCl_2$, 2.5 *µ*l PCR buffer, 200 *µ*M dNTPs, 1 U Taq DNA polymerase (Ampli-Taq, Perkin-Elmer). All the following components were mixed in a PCR tube: 7.5 *µ*l of master mix, 25 pmol of each primer (forward and reverse), 40 ng of template DNA and distilled water up to a final volume of 25 *µ*l. PCR reaction with no template was considered negative control. A thermocycler ABI GeneAmp 9700 (Applied Biosystems, USA) was run for a cycle at 94 °C for 3 min (initial denaturation). Consequent 40 cycles were programmed as: denaturation step for 1 min at 94 °C, annealing step for 1 min at (54 °C for 16S rRNA and 55 °C for 18S rRNA), extension step for 1 min at 72 °C. A final extension step for 10 min at 72 °C was run and finally reaction tube was held at 4 °C. PCR amplicons were visualized on 1.5% agarose gel and molecular size of the amplicon was ensured by 1 Kb ladder (MBI, Fermentas, USA). Amplicon bands were cut and DNAs were purified using QIAGEN gel extraction kit (QIAGEN, GmbH, Germany) as illustrated by the manufacturer's protocol.

DNA sequencing of 16S rRNA and 18S rRNA genes

For each microbial isolate, two PCR amplicons were submitted to sequencing (for certain exclusion of errors) using specific sequencing primer pairs (Table 1). Big Dye terminator sequencing kit (Version 3.1, Applied Biosystems, USA) and ABI PRISMTM 3100 DNA sequencer (Applied Biosystems) were employed for sequencing.

Sequence analysis and phylogeny:

Sequencing data were analyzed to identify potential bacterial and fungal genera using the Ribosomal Database Project (RDP) Classifier. Phylogenetic trees were constructed to determine the closest related species for each isolate. Sequences were aligned, compared to databases (NCBI), and used to build phylogenetic trees (MrBayes) to confirm identifications. A combination of morphological, biochemical, and molecular data was used to definitively identify the isolated bacteria and fungi.

Table 1: Sequences of the primers used in PCR amplification and Sequencing of the gut microbiota of the honey bee, *A. mellifera*.

Primer Name	Sequence 5'→3'	Purpose
16S rRNA 27F	AGAGTTTGATCMTGGCTCAG	PCR amplification for **bacteria**
16S rRNA **1492R**	TACGGYTACCTTGTTACGACTT	
16S rRNA **785F**	GGATTAGATACCCTGGTA	Sequencing of PCR amplicon
16S rRNA **907R**	CCGTCAATTCMTTTRAGTTT	
18S rRNA F	TCCGTAGGTGAACCTGCGG	PCR amplification for **fungi**
18S rRNA R	TCCTCCGCTTATTGATATGC	
18S rRNA F	TCCGTAGGTGAACCTGCGG	Sequencing of PCR amplicon
18S rRNA R	TCCTCCGCTTATTGATATGC	

RESULTS

Herein, we analyzed colony and cell characteristics of the isolated microbiota. Alongside with that, molecular and morphological characteristics were consolidated to get a clear and correct inference of species identification (Table 2).

Morphological characteristics of gut microbiota

Eight opaque, three iridescent and one translucent bacterial colonies were observed. Nine convex, one flat, one concave and one raised colonies were recognized. Out of the twelve circular bacterial colonies, nine with entire margins and three with smooth margins were distinguished. Six small-sized, five medium-sized and one relatively large-sized colonies were described. In addition, two Gram positive and ten Gram negative stain bacterial cells were differentiated. All of the 12 bacterial isolates were realized as rod-shaped bacilli (Table 2). The isolate 13, *Meyerozyma athensensis,* is yeast-like fungus showing hair-free, down smooth, glabrous and round colonies with white to creamy-color. Microscopic examination clarified subspherical budding cells with well-branched pseudohyphae carrying dense blastoconidia (Table 2).

Oxygen tolerance and sporulation

Oxygen tolerance and sporulation of the identified gut microbiota are summarized (Table 3). Six aerobic and six facultative anaerobic bacteria were reported. Out of the 12 bacterial species, 7 are non-spore forming, however, 5 are endospore-forming species (Table 3). Meantime, the yeast-like fungus, *M. athensensis,* is characterized as facultative anaerobic endospore-forming species (Table 3).

Table 2: Colony and cell characteristics of the isolated gut microbiota of the honey bee, *A. mellifera*.

Colony Characteristic						Cell Characteristics	
Bacterial Species	Shape	Size	Elevation	Opacity	Margin	Gram Staining	Morphology
Bacillus proteolyticus	Circular	Medium	Flat	Opaque	Smooth	+ve	Bacilli
Providencia vermicola	Circular	Relatively large	Convex	Opaque	Entire	-ve	Bacilli
Serratia marcescens	Circular	Small	Convex	Opaque	Entire	-ve	Bacilli
Serratia nematodiphila	Circular	Small	Convex	Opaque	Entire	-ve	Bacilli
Citrobacter werkmanii	Circular	Medium	Convex	Iridescent	Entire	-ve	Bacilli
Citrobacter freundii	Circular	Medium	Convex	Iridescent	Entire	-ve	Bacilli
Serratia surfactantfaciens	Circular	Medium	Convex	Translucent	Entire	-ve	Bacilli
Leclercia adecarboxylata	Circular	Small	Raised	Opaque	Entire	-ve	Bacilli
Stenotrophomonas tumulicola	Circular	Small	Convex	Opaque	Smooth	-ve	Bacilli
Serratia sp.	Circular	Small	Convex	Opaque	Entire	-ve	Bacilli
Priestia megaterium	Circular	Medium	Concave	Opaque	Smooth	+ve	Bacilli
Klebsiella grimontii	Circular	Small	Convex	Iridescent	Entire	-ve	Bacilli
*Meyerozyma athensensis**	Circular	Medium	Convex	Opaque	Entire	N/A	Subspherical

*Yeast-like fungus

Table 3: Oxygen tolerance and sporulation of the identified bacterial and fungal* species.

Cell parameters	Oxygen tolerance	Spore formation		
Bacterial species		Sporulation	Spore shape	Spore type
Bacillus proteolyticus	Facultative anaerobic	Yes	Central elliptical	Endospore
Providencia vermicola	Facultative anaerobic	No		
Serratia marcescens	Facultative anaerobic	Yes	Central elliptical	Endospore
Serratia nematodiphila	Aerobic	No		
Citrobacter werkmanii	Aerobic	No		
Citrobacter freundii	Aerobic	No		
Serratia surfactantfaciens	Facultative anaerobic	Yes	Central elliptical	Endospore
Leclercia adecarboxylata	Aerobic	No		
Stenotrophomonas tumulicola	Aerobic	No		
Serratia nematodiphila	Facultative anaerobic	Yes	Central elliptical	Endospore
Priestia megaterium	Aerobic	Yes	Central elliptical	Endospore
Klebsiella grimontii	Facultative anaerobic	No		
*Meyerozyma athensensis**	Facultative anaerobic	Yes	Hat-shaped ascospores	Endospore

*Yeast-like fungus

Biochemical characterization of gut bacteria

Biochemical experimentation was performed to investigate commercial and economic merits of the species. It was discernable that the 12 bacterial species are catalase- secreting, only one species (*Providencia vermicola*) is urease-secreting, and no oxidase-secreting bacteria was reported (Table 4).

IMViC assays elucidated that five bacterial species (*Providencia vermicola, Serratia marcescens, S. nematodiphila, S. surfactantfaciens* and *Leclercia adecarboxylata*) are tryptophan deaminase-secreting; three bacterial species (*P. vermicola, L.* and *Klebsiella grimontii*) are indole-secreting; only four species were MR negative (*C. werkmanii, C. freundii, L. adecarboxylata* and *K. grimontii*); all bacterial species except two species (*L. adecarboxylata* and *S. tumulicola*) were citrate-positive; and all bacterial species except three species (*C. freundii, L. adecarboxylata* and *S. tumulicola*) were VP-positive (Table 4).

Sugar fermentation tests demonstrated that the twelve bacterial species were glucose-, sucrose- and lactose-fermenters except *Citrobacter werkmanii* which was non-sucrose-fermenter. In addition, TSI and H_2S tests showed that all bacterial species except two species (*Citrobacter werkmanii* and *C. freundii*) were trisugar-iron-fermenters; and only *C. werkmanii* and *C. freundii* were H_2S gas-producers. Finally, all bacterial species except *C. werkmanii* were CO_2 gas-producers (Table 4).

The fungus *M. athensensis* was tested as positive glucose-, sucrose-, maltose and trehalose-fermenter.

Molecular characteristics of gut microbiota

Twelve pure colonies of bacteria and one pure fungal colony were identified using 16S rRNA and 18S rRNA genes (Tables 5 and 6). Molecular data were integrated with morphological and biochemical data resulting in recognition of 12 bacterial species belonging to 8 genera, 5 families (Bacillaceae, Morganellaceae, Yersiniaceae, Enterobacteriaceae and Xanthomonadaceae), 3 orders (Bacillales, Enterobacterales and Xanthomonadales), 2 classes (Bacilli and Gammaproteobacteria) and 2 phyla (Firmicutes and Proteobacteria). Out of the 8 genera, 5 genera belong to the phylum Proteobacteria, class Gammaproteobacteria and order Enterobacterales (62.5%), 2 genera belong to the phylum Firmicutes, class Bacilli and order Bacillales (25.0%) and one genus belongs to the phylum Proteobacteria, class Gammaproteobacteria and order Xanthomonadales (12.5%).

In addition, one fungal species belonging to the phylum Ascomycota, class Saccharomycetes, order Saccharomycetales and family Saccharomycetaceae. Taxonomic scanning of the bacterial and fungal species characterized in the current work is illustrated in Table (5). We encompassed the closest species of the assured genera (Table 5).

PCR identification and sequence alignment

The primer sets of 16S rRNA and 18S rRNA genes were planned to amplify ≈1350 and ≈1600 bp fragments, respectively. Specific bands of corresponding sizes were eluted and sequenced. For satisfactory similarity measures, raw sequences were trimmed and modified to be illegible for further appropriate analyses. The product sizes after trimming ranged from 696 bp (*C. freundii*) to 1433 bp (*S. nematodiphila*). A satisfactory query coverages (95-100%) were shown for all studied sequences (Table 6). Initially, sequences resulting from two amplicons of the same colony were compared to validate the sequence. Meantime, the identity between the resulting sequences was tested to confirm the number of different sequences. Sequences exhibiting identity 99% or more were considered the same sequence. Sequence analyses clarified that gut microbiota of *A. mellifera* comprised 12 bacterial species and one yeast-like fungus. RDP Classifier succeeded to differentiate all of our isolates at genus level (8 bacterial and one fungal genera).

To attribute each genus to the closest species, 16S rRNA and 18S rRNA genes of the type species and other corresponding genes of the genera were downloaded, genera were aligned to the related sequences, a distance-based phylogenetic trees were constructed. Genera were attributed to the closest sensible species relying on the illation of phylogenetic relatedness and similarity measures of the sequences (Tables 5 and 6). The best metrics to get a decision for to the closest species were maximum percentage similarity, query coverage, maximum score and E-value (Table 6). Twelve bacterial and one fungal sequences were separately searched by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?) and FASTA (https://www.genome.jp/tools/fasta/) to disclose percentage of similarity with published sequences. Significant similarities of 93.0% to 99.85%, query coverages of 96% to 100%, maximum scores of 1218-2591 and E-values of 0.0 were reported (Table 6). Out of the 13 species, 10 sequences created more than 98% identity with related species.

Molecular phylogeny

Phylogenetic tree was built from 64 sequences including our bacterial sequences. Neighbor Joining (NJ) method was employed using MrBayes 3.1.2 software. Phylogenetic tree presented the sequences in two main divisions. Division I comprises eleven sequences in two subdivisions and three sister clades. However, division II covers fifty three sequences in two subdivisions.

Table 4: Biochemical characteristics of the identified bacterial species from the gut of the honey bee, *A. mellifera*.

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Biochemical test / Bacterial species	Catalase	Oxidase	Urease	Tryptophan deaminase	Glucose fermentation	Sucrose fermentation	Lactose fermentation	Voges-Proskauer-Test	Indole	MR	Citrate	TSI- test	CO_2 production	H_2S production
Bacillus proteolyticus	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
Providencia vermicola	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
Serratia marcescens	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
Serratia nematodiphila	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
Citrobacter werkmanii	+ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve
Citrobacter freundii	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
Serratia surfactantfaciens	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
Leclercia adecarboxylata	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve
Stenotrophomonas tumulicola	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve
Serratia spp.	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
Priestia megaterium	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
Klebsiella grimontii	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve

Table 5: A key table showing taxonomical analysis of the gut microbiota of the honey bee, *A. mellifera*.

Suggested genus	Suggested species	Phylum	Class	Order	Family
Bacillus	*B. proteolyticus*	Firmicutes	Bacilli	Bacillales	Bacillaceae
Priestia	*P. megaterium*				
Stenotrophomonas	*St. tumulicola*	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae
Providencia	*P. vermicola*			Enterobacterales	Morganellaceae
Serratia	*S. surfactantfaciens*				Yersiniaceae
Serratia	*S. marcescens*				
Serratia	*S. nematodiphila*				
Serratia	*Serratia* sp.				
Citrobacter	*C. werkmanii*				Enterobacteriaceae
Citrobacter	*C. freundii*				
Leclercia	*L. adecarboxylata*				
Klebsiella	*K. grimontii*				
*Meyerozyma**	*M. athensensis*	Ascomycota, Saccharomycotina	Saccharomycetes	Saccharomycetales	Saccharomycetaceae

*Yeast-like fungus

Table 6: Summarized similarity measures of the gut microbiota of the honey bee, *A. mellifera* using 16S rRNA and 18S rRNA genes.

Isolate No.	Suggested name	Product size in bp	GenBank Acc#	Max. score	Total score	Query coverage	E-value	Max. % Identity	Ref. Accession
1	*Bacillus proteolyticus*	709	MZ496502	1249	1249	96%	0.0	99.85%	NR_157735.1
2	*Providencia vermicola*	933	MZ496503	1347	1347	99%	0.0	93.00%	NR_042415.1
3	*Serratia marcescens*	701	MZ496504	1218	1218	98%	0.0	98.41%	NR_036886.1
4	*Serratia nematodiphila*	1433	MZ496505	2591	2591	99%	0.0	99.58%	NR_044385.1
5	*Citrobacter werkmanii*	1119	MZ496506	2001	2001	99%	0.0	99.19%	NR_024862.1
6	*Citrobacter freundii*	696	MZ496507	1240	1240	97%	0.0	99.56%	NR_117752.1
7	*Serratia surfactantfaciens*	703	MZ496508	1247	1247	97%	0.0	99.42%	NR_169468.1
8	*Leclercia adecarboxylata*	1242	MZ496509	2246	2246	99%	0.0	99.44%	NR_104933.1
9	*Stenotrophomonas tumulicola*	1255	MZ496510	2237	2237	99%	0.0	99.12%	NR_148818.1
10	*Serratia* spp.	1192	MZ496511	2039	2039	99%	0.0	97.80%	NR_044385.1
11	*Priestia megaterium*	1165	MZ496512	2108	2108	98%	0.0	99.74%	NR_117473.1
12	*Klebsiella grimontii*	1190	MZ496513	2111	2111	98%	0.0	99.23%	NR_159317.1
13	*Meyerozyma athensensis**	921	MZ645943	1543	1543	100%	0.0	97.29%	NG_064893.1

*Yeast-like fungus

The first subdivision encompasses four sub-collective sister clades. Meanwhile, the second subdivision includes multiple sub-collective sister and sub-sister clades. All of our genera were embraced by their related sequences except in the cases of *P. vermicola* and the four *Serratia* spp. which were diverged in unique separate sub-sister clades. Both divergence and overlap of genera were demonstrated in the phylogenetic tree. Overlap of genera was shown in the case of *Citrobacter* and *Klebsiella*. Meanwhile, the divergence was shown in the genus *Providencia* (Figure 1).

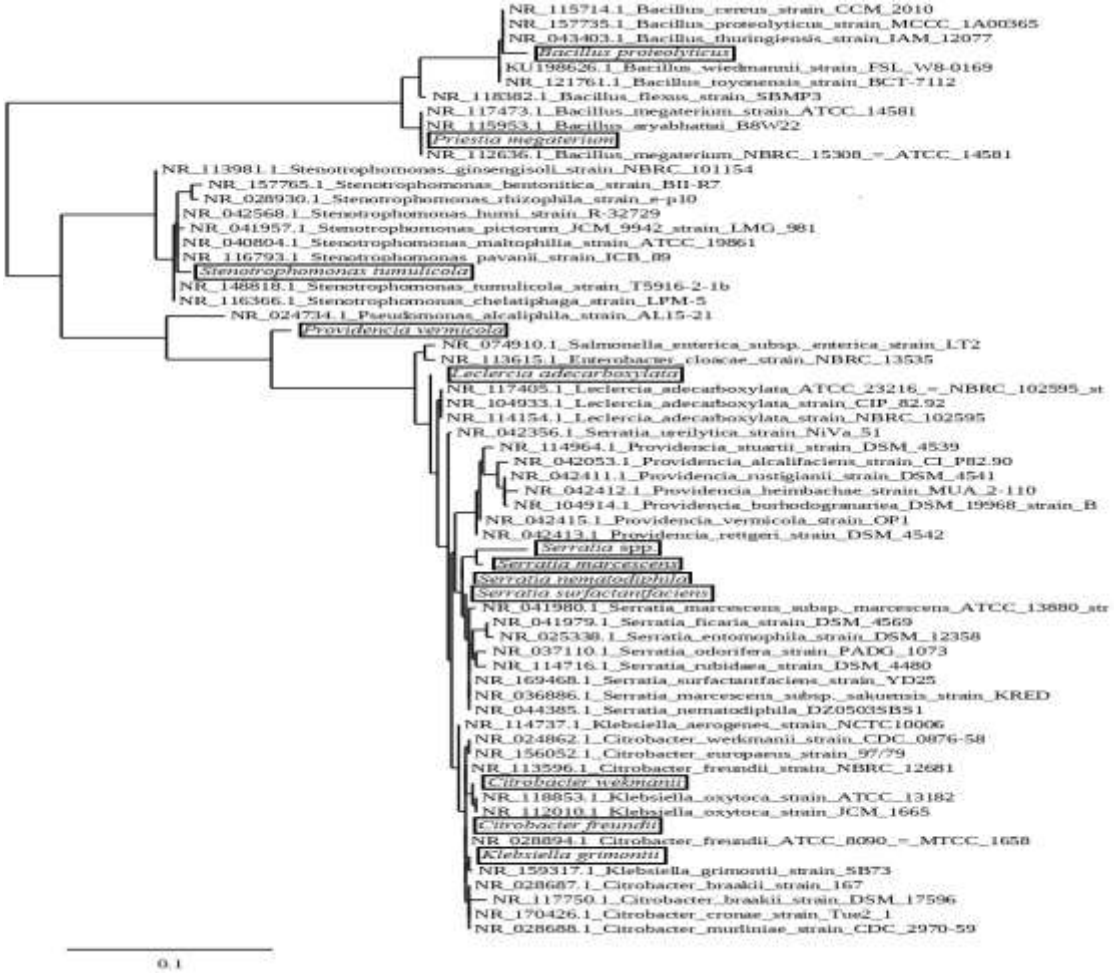

Figure 1: Phylogenetic analysis of the 12 isolated bacterial species with the closely related species on the genbank. The phylogenetic tree is constructed using 63 bacterial species using neghbour-joining method and 0.1 cut-off value.

DISCUSSION

Significance of gut microbiota research counts on its great impact on the physiology and productivity of insect host (Kwong and Moran, 2016). Meantime, insect and gut microbiota demonstrated close symbiotic relationship. The honey bee, *A. mellifera* is one of the most important economic insects. Therefore, this work sheds the light on the gut microbiota of the honey bee, *A. mellifera* from Aljouf, Saudi Arabia.

Out of 12 bacteria, 11 genera (91.7%) were identified at species level and one at genus level. In addition, one yeast-like fungus was identified to its species level. Ten bacterial species (83.3%) belong to the phylum Proteobacteria, four to the family Enterobacteriaceae, four to the family Yersiniaceae, one to the family Morganellaceae, and one to the family Xanthomonadaceae, and two species (16.7%) belong to the phylum Firmicutes, family Bacillaceae. In the same context, 60%, 26% and 14% of the isolated species from gut bacteria from *A. mellifera* were reported to belong to three phyla: Firmicutes, Proteobacteria and Actinobacteria, respectively. Generally, isolates were identified as: *Bacillus, Staphylococcus, Enterococcus, Sphingomonas, Ochrobactrum, Ralstonia, Corynebacterium, Micrococcineae* and *Enterobacteriaceae* (Angum et al. 2018); other researchers demonstrated that gut bacteria of *A. mellifera* were prevailed by 6 families: Acetobacteriaceae, Lactobacillaceae, Rhizobiaceae, Neisseriaceae, Orbaceae and Bifidobacteriaceae (Moran, 2015; Anderson and Ricigliano, 2017; Romero et al. 2019; Bleau et al. 2020); and recently, five lactic acid bacterial isolates from *A. mellifera* gut were identified as *Enterococcus faecalis* KX073783, *Enterococcus faecalis* MG890204, *Lactobacillus brevis* MH191230, *Lactobacillus casei* KT273339 and *Enterococcus faecalis* EU594564 (Elzeini et al. 2020). Gut microbiome of other honey bees were investigated; for example: fourteen species of bacteria: 2 *Corynebacterium,* 3 *Lactobacillus,* 2 *Micrococcus,* 1 *Staphylococcus,* 1 *Aeromonas,* 2 *Citrobacter,* 2 *Klebsiella* and 1 *Pseudomonas* were identified from guts of *A. mellifera adansonii* (Fasasi, 2018); and gut of *Apis nigrocincta* was reported to be dominated by Proteobacteria (58%), Firmicutes (29%) and Actinobacteria (8%). *Lactococcus garvieae* (13.45%), *Lactobacillus* spp. (8.19%), and *Enterococcus* spp. (4.47%), *Enterobacteriaceae* (28.2%), *Erwinia* (6.43%), *Klebsiella* (4.90%) and *Bifidobacterium* spp. (7.96%) (Lombogia et al. 2020). The variation in community structure of gut microbiota of *A. mellifera* reported in previous studies and ours might be due to pesticide exposure of the hive (Kakumanu et al. 2016; Motta et al. 2018), probiotics exposure of the hive (Alberoni et al. 2018), supplementary floral forage (Rothman et al. 2018), collection during the course of a honey production season (Subotic et al. 2019), the age of collected individuals (Dong et al. 2020), winter and summer seasonal changes (Kešnerová et al. 2020), climatic and nutritional changes (Bleau et al. 2020), social and labor group changes (Vernier et al. 2020), and seasonal vegetation and climatic changes (Papp et al. 2021). It was verified that worker *A. mellifera* acquired the core Gram-negative species of bacteria, *Snodgrassella alvi, Gilliamella apicola* and *Frischella perrara* from the nurse worker bees or their fecal material. Whereas, the Gram-positive species might be acquired from the hive components (Powell et al. 2014).

Much studies have been published to differentiate between methodologies of microbial identification. Especially in health affair issues, the quick, simple, thorough and precise identification methodology is very considerable (Wolcott et al. 2010; Dowd et al. 2011; Rhoads et al. 2012, Rajapaksha et al. 2019). In the current research, we combined morphology, biochemistry and molecular data to classify the gut microbiota to their species. Morphological and biochemical characteristics of bacterial colony and cells could group similar colonies and suggest potential taxa. Use of sequencing data of 16S rRNA and 18S rRNA genes and RDP Classifier was effective in assigning the most probable genera. BLAST, FASTA searches, multiple alignments and phylogenetic analyses were profitable to place genera into the most conceivable species in 8 out of 12 bacterial genera and one yeast-like fungus. Meantime, combination of morphological, biochemical and molecular analyses could be successful in assigning species of three unassigned genera (*Serratia marcescens, S. nematodiphila* and *Citrobacter freundii*). Only one bacterium was recognized to its genus level (*Serratia* sp.). Recently, negatives and positives of identification of microorganisms by different technologies have been reviewed (Bajinka and Secka, 2017; Rajapaksha et al. 2019). Out of our 13 sequences, 12 sequences generated similarities greater than 97% when aligned to published sequences in GenBank. General rule of thumb illustrated that the identity of new species with the closest published species should be lesser than 97% (Rosselló-Mora and Kampfer, 2004). Touching this rule, only one novel species of the genus *Providencia* was reported in this study as *Providencia vermicola* (93%). Lately, updates of the 97% rule have been published (Edgar, 2018; 2018a). According to these updates, precise prediction is minimized to 50% when sequence similarity lowered to 97% (Edgar, 2018). In addition to that, sequencing of more than one hypervariable locus was suggested to improve prediction accuracy (Edgar, 2018a). Other researchers proposed 98.65% identity as a threshold for allegation of two distinct species of bacteria using full-length of 16S rRNA sequence (Kim et al. 2014). Pearson chose comparing protein or translated DNA rather than DNA: DNA in similarity search tools to increase accuracy of prediction.

Furthermore, other measures were recommended for homology deduction (Pearson, 2013). Regarding the above-stated updates, four of the described isolates were indicated to be novel strains or subspecies showing similarity less than the 98.65% threshold (*Providencia vermicola* (93%), *Serratia marcescens* (98.41%), *Serratia* spp. (97.80%), and *Meyerozyma athensensis* (97.29%)). Combination of our results with previous ones clarified that the identification quality and quantity of genera and species is methodology-dependent. Notwithstanding the fact that culture-independent sequence-based methodologies identified more genera and species, some genera could be only identified by using culture-dependent methodologies (Yang et al. 2018).

Although the collected bees were seemingly healthy, some of the identified microbiota are known to be pathogens or contaminants. So it is worthy to realize their source and role in the gut microbiota. For instance, *Serratia marcescens* and *Citrobacter freundii, C. werkmanii, Leclercia adecarboxylata* and *Klebsiella girmontii* are counted as common pathogens showing antibiotic resistance (Zhang et al. 2008; Jones, 2010; Cheong et al. 2012; Liu et al. 2018; Peter et al. 2018; Spiegelhauer et al. 2018; Passet and Brisse, 2018). Recently, *K. grimontii* was observed to be lethal (73.3%) to worker *Apis mellifera caucasia* (Çil et al. 2021). In addition, some species of *Meyerozyma* are well-recognized pathogens (Cebeci et al. 2017). On contrary, several strains of these species were reported to have multiple beneficial uses. For example: *S. marcescens* were proved to produce various enzymes like proteases, chitinases, cellulases, and lipases (Dhar et al. 2018). In addition, some strains of *S. marcescens* produce molecules which promote plant growth (Indole Acetic Acid (IAA) and siderophore) and trigger secretion of plant defense antifungal molecules. That is why this bacterium could suppress root rots disease of plants (Dhar et al. 2018). *Citrobacter freundii* and *C. werkmanii* produce phosphatase and selenocysteine beta-lyase enzymes which may have significant role in Biotech industry and bioremediation (Chocat et al. 1985). Food and water are potential sources of *Citrobacter* species. Thus, the *Citrobacter* species could be food or water-borne pathogens (Liu et al. 2017). In the same context, a strain of *L. adecarboxylata* producing IAA and ACC (1-Aminocyclopropane-1-Carboxylate) deaminase could promote the growth of *Solanum lycopersicum* and enhance its tolerance to salinity stress by inducing plant to modulate the concentration of endogenous secondary metabolites (Kang et al. 2019). Although well-known pathogen, *K. grimontii* is considered a part of the normal gut microbiota of many animals (Passet and Brisse, 2018). It may be a contaminant acquired through the dynamic changes occurred by the contact between social groups of bees and colonial activities (Vernier et al. 2020). The gut microbiota have an essential role in the health of bees (Raymann and Moran, 2018), meantime, the pathogenic bacteria like *Klebsiella* in the guts of honey bees could be a cause for the collapse of the colony (Galatiuk et al. 2019). Some species of *Meyerozyma* can be used for preservation of vegetables and fruits in agricultural and food industries (Yan et al. 2018). Many species of *Meyerozyma* (*M. athensensis* SB18) exhibited higher ability to digest xylose of ligno-cellulosic hydrolysate, and to produce high-yield of xylitol which is a sugar substitute having many medical uses (Martini et al. 2016; Vaz de Arruda et al. 2017), suggesting great potential in bioconversion of hemi-cellulosic hydrolysate (Zhang et al. 2012; Felipe et al. 2019; Zahoor et al. 2021). Meanwhile, both *Bacillus proteolyticus, Providencia vermicola* and *Serratia surfactantfaciens* demonstrated antimicrobial, antifungal activities and be used for antimicrobial and probiotic preparations (Hussain et al. 2015; Su et al. 2016; Aish et al. 2019; Chamedjeu et al. 2019; Zeng et al. 2021). In addition, *Priestia megaterium* was reported to produce bioproducts which have multiple bioactivities and multiple uses in biotechnology (Freedman et al. 2018; Biedendieck et al. 2021). The isolation of *Serratia nematodiphila* might be an indication for infection with the entomopathogenic nematode, *Heterorhabditidoides chongmingensis* (Kwak et al. 2015). *S. nematodiphila* genome was proved to have a full chitinase operon and other chitin digesting enzymes (Kwak et al. 2015). Lately, the antibiosis of *S. nematodiphila* against both plant pathogenic fungi and insects was reported (Vahedi-Shahandashti et al. 2017; Wu et al. 2021). Additionally, many strains of *P. vermicola, S. marcescens,* and *C. werkmanii* were reported to produce lactic acid and other bioactive substances (Hussain et al. 2015; Kurbanoglu et al. 2015; Zhou et al. 2017). Finally, *Stenotrophomonas tumulicola* was major contaminant of stones (Handa et al. 2016), and it could be acquired by the contact with contaminated surfaces through foraging activities of the worker bees.

Biochemical characterization of the identified species showed that several species were enzyme-secreting (12 are catalase- secreting, 5 are tryptophan deaminase-secreting, and one is urease-secreting). Enzyme-secreting bacteria might be bioprocessed to produce these enzymes for marketing. In addition, IMViC tests are used to differentiate members of Enterobacteriaceae (enteric bacteria). It clarified whether the organisms can metabolize tryptophan when detecting indole, and to use citrate as a unique carbon source. This is an indication for waterborne or foodborne microbiota. Furthermore, the carbohydrate-, trisugar-iron-fermenters, and 11 CO_2 gas-producers could acidify the gut medium. The biochemical results confirmed that the gut bacteria are capable of producing commercial enzymes and biologically active substances. They have the ability to ferment carbohydrates and acidify the gut

media. Some on these bacteria are revealed to be waterborne or foodborne. These results are consistent with the results obtained previously by other researchers.

In the phylogenetic tree, the overlap of species might be due to common ancestor of the overlapping species (*Citrobacter* and *Klebsiella*). Meanwhile, the divergence of our *P. vermicola* (93% similarity) in a separate sister clade, distant from the other *Providencia* species, may be an indication for a novel divergent species.

Conclusively, most of the identified species have been reported to produce bioactive products which promote tolerance to climatic, and infectious stresses. Some of these species were reported to be pathogenic or contaminants. These species are believed to be food or water-borne and could be acquired through feeding, foraging, and colonial activities and/ or through direct contact with contaminant surfaces.

CONCLUSIONS

The current study identified 12 bacterial species (4 species were novel) and one yeast-like fungus from the gut of *A. mellifera*. It was clarified that the community structure of gut microbiota embraced many species predominated by species of Gammaproteobacteria, Enterobacterales (4 species of Enterobacteriaceae, 4 species of Yersiniaceae and one species of Morganellaceae). The identification quality and quantity of genera and species was reported to be methodology-dependent. The combination of methods was found the most efficient in identification of species. Many of the identified species were enzyme-producing and bioactive-secreting species which might be bioprocessed to produce these substance at commercial scale. Other gut bacterial species were revealed to be waterborne or foodborne. Overall the species that constitute the community of the gut microbiota may cooperate to produce bioactive substances which ultimately enhance the tolerance of honey bees to climatic and infectious stresses. Our findings provide a basic step for investigating the specific impacts of the gut microbiota on *A. mellifera*.

Supplementary materials

Not applicable.

Author contributions

RAA conducted experimental work, both SSA, FHG and AMS contributed this work equally. They designed the study, shared experimental work, performed data analyses, discussed the results and wrote the manuscript.

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Conflict of interest

The authors declared that they have no conflict of interest.

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