

CONTENTS

<i>Title</i>	<i>Page</i>
Introduction	1
Aim of Work	3
Literature Review	4
1. Beekeeping and Honey bee races in Egypt.	5
2. Honey bee and their castes.	6
3. Pathogens threaten Honey bee health:	8
3.1. American foulbrood disease.	9
3.1.1. Pathogen of American foulbrood disease.	10
3.1.2. Symptoms and diagnosis of American foulbrood disease.	11
3.1.3. Mode of <i>P. larvae</i> pathogenicity.	12
3.1.4. Availability of American foulbrood pathogen.	15
3.1.5. Controlling of American foulbrood disease.	15
3.2. Chalkbrood disease.	19
3.2.1. Pathogen of chalkbrood disease.	20
3.2.2. Symptoms and diagnosis chalkbrood disease.	21
3.2.3. Mode of <i>Ascospaera apis</i> pathogenicity.	23
3.2.4. Availability of chalkbrood pathogen.	25
3.2.5. Controlling of chalkbrood disease.	26
4. Microbial flora of Honey bee alimentary tract.	29
5. Incidence and role of Lactic acid bacteria as probiotic in Honey bee.	35
Material and Methods	41
1. Isolation and purification of bacterial and fungal isolates:	41

1.1. Media for isolation and purification of LAB.	42
1.2. Media for isolation and purification of <i>Enterobacteriaceae</i>	42
1.3. Media for Isolation and purification of fungal isolates.	43
2. Screening antagonistic activity of bacterial and fungal isolates.	44
2.1. Medium for screening antagonistic activity against <i>P. larvae</i> .	44
2.2. Medium for screening antagonistic activity against <i>Ascospaera apis</i> .	45
3. Identification of bacterial and fungal isolates:	45
3.1. Identification of bacterial isolates.	45
3.1.1. Morphological Identification.	45
3.1.2. Biochemical tests used for identification of bacterial isolates.	47
3.2. Identification of fungal isolates.	54
4. Evaluation of Antagonistic activities of bacterial and fungal isolates:	54
5. Determination of Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations:	55
5.1. Preparation of Cell-Free Supernatant (CFS).	55
5.1.2. Determination of Minimum Inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC).	56
6. Characterization of antibacterial and antifungal bioactive	

metabolites.	56
6.1. Extraction, isolation, purification and identification of antimicrobial metabolites of isolate 90:	56
6.1.1. Extraction of bioactive compounds.	56
6.1.1.1. Preparation of Cell-Free Supernatant.	56
6.1.1.2. Preparation of Cell-Free Supernatant (CFS).	57
6.1.1.3. Liquid- liquid phase extraction of bioactive metabolites from CFSs.	57
6.1.2. Antimicrobial assay of extracted fractions:	58
6.1.3. Purification of bioactive compounds.	58
6.1.4. Antimicrobial assay of bioactive compounds.	59
6.1.5. Identification of the antibacterial compounds.	59
6.2. Extraction, and identification of antimicrobial metabolites of isolate No. 60:	60
6.2.1. Extraction of bioactive metabolites:	60
6.2.1.1. Preparation of Cell-Free Supernatant (CFS).	60
6.2.1.2. Liquid- liquid phase extraction of bioactive metabolites from CFSs.	60
6.2.2. Identification of the antifungal compounds.	60
7. DNA manipulation and 16s rDNA gene sequencing fingerprint of isolates No. 90 and 60.	61
7.1. DNA extraction of the most potent antagonistic bacteria (isolates No. 90 and 60):	61
7.2. PCR procedures by using Maxima Hot Start PCR Master Mix (Thermo K1051).	62
7.3. PCR clean up and PCR product using GeneJET™ PCR Purification Kit (Thermo K0701)	64
8. In vivo therapeutic effect of <i>Fructobacillus fructosus</i>	

and <i>Lactobacillus plantarum</i> CFSs.	66
9. Statistical analysis.	69
Results.	70
1. Gut Microbial count of 5th instar larvae and forager honey bees.	70
1.1. Microbial count of apiary A samples (Zagazig).	70
1.2. Gut Microbial count of Apiary B samples (San El Hager).	75
1.3. Gut Microbial counts of Apiary C samples (El Tal El kabier).	79
2. Effect and Correlation of Different variables honey bee gut microbial loads.	83
3. Distribution of Antagonistic activities of endogenous microbial isolates against Pathogens of American foulbrood and Chalkbrood Diseases.	87
4. Identification and Frequency of occurrence of different bacterial and fungal isolates among total microbial isolates.	94
4.1. Frequency of occurrence of microbial isolates.	94
4.2. Identification of Bacterial isolates.	97
4.3. Identification of fungal isolates.	100
5. Molecular identification of the most potent antagonistic bacteria.	109

6. Identification of active metabolites of <i>Fructobacillus Fructosus</i> and <i>Lactobacillus plantarum</i> :	112
6.1. Identification of active metabolites of <i>Fructobacillus Fructosus</i>	112
6.1.1. Detection of bioactive fractions.	112
6.2. Identification of active metabolites of <i>Lactobacillus plantarum</i> CFS.	118
7. Determination of minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC):	123
8. In vivo therapeutic effect of <i>Fructobacillus fructosus</i> and <i>Lactobacillus plantarum</i> CFSs.	125
8.1. Therapeutic effect of <i>Fructobacillus fructosus</i> against <i>P. larvae</i> .	125
8.2. Therapeutic effect of <i>Lactobacillus plantarum</i> against <i>Ascospaera apis</i> .	128
Discussion	132
Summary and Conclusion	152
Reference	158
Arabic Summary	1

List of Tables

No.	<i>Title</i>	Page No.
1	Maxima® Hot Start PCR Master components.	63
2	PCR thermal cycling conditions.	64
3	Daily amounts and composition for artificial feeding according to the age of worker larvae in percentage of weight.	69
4	Gut Microbial count of honey bee 5 th instar larvae and Foragers of Apiary A during different blooming seasons.	73
5	Gut Microbial count of honey bee 5 th instar larvae and Foragers of Apiary B during different blooming seasons.	77
6	Gut Microbial count of honey bee 5 th instar larvae and Foragers of Apiary C during different blooming seasons.	81
7	Effect of Correlation between cultural medium, geographical and blooming seasons on gut microbial counts.	85
8	Distribution of Antagonistic activities of endogenous microbial isolates against honey bee Pathogens of American foulbrood and Chalkbrood Diseases.	90
9	Frequency of occurrence of different bacterial and fungal isolates among total isolates	95

No.	<i>Title</i>	Page No.
10	Morphological and biochemical characters of bacterial isolates recovered on Nut. and Mac	98
11	Distribution percentage of fungal isolates	103
12	Antagonistic activities of identified fungi against honey bee pathogens; <i>P. larvae</i> and <i>Ascosphaera apis</i> .	104
13	Common name and chemical structure of bioactive constituents of EA extract of <i>Fructobacillus fructosus</i> CFS.	115
14	Common name and chemical structure of bioactive constituents of EA extract of <i>Lactobacillus plantarum</i> CFS.	119
15	MIC and MBC of <i>Fructobacillus fructosus</i> and <i>Lactobacillus plantarum</i> against honey bee pathogens.	124
16	Effect of CFS of <i>Fructobacillus fructosus</i> on survival rates percentage of larvae infected with <i>P. Larvae</i> spores.	126
17	Effect of CFS of <i>Lactobacillus plantarum</i> on survival rates percentage of larvae infected with <i>Ascosphaera apis</i> spores.	129

List of Figures

No.	Title	Page No.
1	Gut Microbial count of honey bee 5 th instar larvae and Foragers of Apiary A (Zagazig).	74
2	Gut Microbial count of honey bee 5 th instar larvae and Foragers of Apiary B (San El Hager).	78
3	Gut Microbial count of honey bee 5 th instar larvae and Foragers of Apiary C (El Tal El kabier).	82
4	Effect of cultural medium on both larval and foragers gut microbial count.	86
5	Effect of geographical location on both larval and foragers gut microbial count.	86
6	Effect of honey flow season on both larval and foragers gut microbial count.	87
7	Distribution of Antagonistic activities of endogenous lactic acid bacterial isolates against Pathogens, <i>Paenibacillus larvae</i> and <i>Ascosphaera apis</i> .	91
8	Distribution of Antagonistic activities of endogenous honey bee fungal isolates against Pathogens; <i>Paenibacillus larvae</i> and <i>Ascosphaera apis</i> .	91
9	Antagonistic activities of some fungal and bacterial isolates; Photo (A:C) against <i>P. larvae</i> ; (A) <i>A. nidulans</i> ; (B,1) <i>P. corylophilum</i> , (C,1): <i>A. terreus</i> , and (D): <i>A. terreus</i> against <i>Ascosphaera apis</i> , (E & F) bacterial isolates 95, 34, 20 and 65 against <i>Ascosphaera apis</i> .	92

List of Figures

No.	<i>Title</i>	Page No.
10	Photo (A); antagonistic activities of two LAB isolates No.90 & isolate No. 34 against <i>P. larvae</i> . Photo (B); antagonistic activity of isolate No. 60 against <i>Ascospaera apis</i> .	93
11	Distribution of different microbial isolates.	96
12	Photo A; two different colonies of LAB isolates, B; <i>Staph</i> isolate on blood agar, C; green pigment (flourrschien) of <i>Pseudomonas</i> , D, E, F, G, H, and I gram stain of LAB isolates.	99
13	Identification report of fungal isolates.	102
14	Distribution percentage of fungal isolates.	103
15	Morphological characters of <i>Pochonia suchlasporia</i> : Photo (A); colony on PDA, Photo (B); reverse colony on PDA, Photo (C); colony on CZA medium, Photo (D); reverse colony on CZA, C; light microscopic pictures of conidiophores and conidia.	105
16	Morphological characters of <i>Aspergillus terrus</i> : Photo (A); colony on CZA, Photo (B); reverse colony on CZA, Photo (C); colony on PDA, Photo (D); reverse colony on PDA and E; under light microscope.	106
17	Morphological characters of <i>Penicillium corylophilum</i> : Photo (A); colony on CZA, Photo (B); reverse colony on CZA, C; colony on PDA, Photo (D); under light microscopic.	107

List of Figures

No.	<i>Title</i>	Page No.
18	Morphological characters of <i>Aspergillus nidulans</i> : Photo (A); colony on CZA, Photo (B); reverse colony on CZA, Photo (C); colony on PDA.	108
19	Morphological characters of <i>Penicillium chrysogenum</i> : Photo (A); colony on CZA, Photo (B); reverse colony on CZA, Photo (C); colony on PDA medium.	108
20	(A)Agarose gel electrophoresis showing amplification of 16s rDNA gene; Lane 1: 1 kb Plus DNA Ladder; 2: <i>Fructobacillus fructosus</i> HI-1; 3: <i>Lactobacillus plantarum</i> HI-2, (B); partial sequence of 16S rRNA gene of DNA of <i>Fructobacillus fructosus</i> HI-2, (C); partial sequence of 16S rRNA gene of DNA of <i>L. plantarum</i> HI-2.	110
21	Phylogenetic tree of <i>Fructobacillus fructosus</i> starin HI-1.	111
22	Phylogenetic tree of <i>Lactobacillus plantarum</i> starin HI-2.	111
23	TLC of bioactive purified fractions of <i>Fructobacillus fructosus</i> metabolites extracted by ethyl acetate	113
24	Antibacterial activity of bioactive fractions of <i>Fructobacillus fructosus</i> metabolic EA extract against <i>P. larvae</i> .	113

List of Figures

No.	<i>Title</i>	Page No.
25	GC-MS analysis of fraction No. 1, hepta -2-methyl butyrate, Di-isobutyl and D-Turanose,heptakis (trimethylsilyl).	116
26	GC-MS analysis of fraction No. 5, 1,2-Benzenedicarboxylic acid; diisooctyl. ester	116
27	Mass spectrum analysis of compound No. 6, Hyodeoxicholic acid	117
28	GC-MS analysis of metabolites of <i>Lactobacillus Plantarum</i> EA. extract.	120
29	Mass spectrum of compound No. 1 disopropyl phenylacetate.	120
30	Mass spectra of compound No. 2; 3,4-dihydro-6,7 dimethoxyisoquinoline 2-oxide.	121
31	Mass spectrum of compound No. 3 pentyl acetate.	121
32	Mass spectrum of compound No. 4 Ethyl isopropoxy acetate.	122
33	Mass spectrum of compound No. 5; à-Hydroxyisocaproic acid.	122
34	Mass spectrum of compound No. 6; di-(2-ethylhexyl) phthalate.	123
35	Effect of CFS of <i>Fructobacillus fructosus</i> on survival rates percentage of larvae infected with <i>P. Larvae</i> spores.	129
36	Effect of CFS of <i>Lactobacillus plantarum</i> on survival rates percentage of larvae infected with	130

List of Figures

No.	<i>Title</i>	Page No.
	<i>Ascospaera apis</i> spores.	
37	<i>In vivo</i> therapeutic effect of <i>Fructobacillus fructosus</i> and <i>Lactobacillus plantarum</i> CFSs. Photo; H, I larvae infected with <i>P. larvae</i> ; Photo; J , larvae infected with <i>Ascospaera apis</i> .	131

List of Abbreviations

A/A	Acid/acid
AAB	acetic acid bacteria
A/Ag	Acid/acid,gas
AFB	American foulbrood
°C	Celsius
CCD	Colony collapse disorder
CFS	Cell Free Supernatant
CFU	Colony forming unit
cm	centimeters
DEHP	Di-(2-ethylhexyl) phthalate
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
FAO	Food and Agriculture Organization
EFB	European foulbrood
EMB	Eosin-methylene blue agar
FLAB	Fructophilic lactic acid bacteria
GC	Gas chromatography
GC/MS	Gas chromatography/ mass spectroscopy
hr	Hour
Kg	Kilogram
Km	Kilometer
LAB	Lactic acid bacteria
LD₅₀	Lethal dose

L.S.D	Least Significant Difference
MAC	MacConkey`s medium
MBC	Minimum Bactericidal concentration
MIC	Minimum inhibitory concentration
mg	milligram
min	Minute
MR	Methyl red test
MRS	de Man, Rogosa and Sharpe medium
MYPGP	Muller Hinton, yeast, phosphate, glucose & pyruvic acid medium
NCBI	National Center for Biotechnology Information
ND	Not Detected
OIE	Office International des Epizooties
ONPG	O- Nitrophenyl- β - D- galactopyranoside
OTCR	OTC-resistant
P	Probability
PAA	Phenyl Acetic Acid
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar medium

pH	potential of hydrogen
ppm	part per million
rDNA	ribosomal DNA
rRNA	ribosomal Ribonucleic acid
rpm	Round per minute
SD	Standard deviation
SE	Standard Error
Sp	Species
TCA	trichloroisocyanuric acid
TcR	tetracycline- resistant
TSI	Triple Sugar Iron Agar
UV	Ultra violet
µl	microliter
µm	micrometer
VOC	Volatile organic compound
VP	Vogas- Poskauer test
w/v	Weight / volume
YGPSA	yeast, glucose, phosphate, starch agar medium

ABSTRACT

The current study was investigating the variation in microbial loads of honey bee gut of 5th instar larvae and adult foragers in addition to documented the effect and correlation of different variables; type of microorganism, blooming season, and apiary location on these microbial loads. Different bacterial and fungal isolates were isolated and purified from samples.

Antibacterial and antifungal activities of all isolated microflora were screened against two honey bee pathogens; *Paenibacillus larvae* and *Ascosphaera apis* (pathogens of American foulbrood and chalkbrood diseases, respectively). Identification of isolated bacteria and fungi was carried out, also distribution of antagonisms among isolated and identified microflora was summarized.

Two new bacterial strains were identified as most potent isolates against *Paenibacillus larvae* and *Ascosphaera apis* using 16S rDNA and Blast analysis; *Fructobacillus fructosus* HI-1 and *Lactobacillus plantarum* HI-2, respectively. Also, isolation and identification of active metabolites of ethyl acetate extract of *Fructobacillus fructosus* HI-1 and *Lactobacillus plantarum* HI-2 CFSs were conducted. In addition, MICs and MBCs of *Fructobacillus fructosus* HI-1 CFS and *Lactobacillus plantarum* HI-2 CFS were determined.

The therapeutic effect of *Fructobacillus fructosus* HI-1 and *Lactobacillus plantarum* HI-2 was revealed in laboratory on honey bee larvae artificially infected with *Paenibacillus larvae* and *Ascosphaera apis*, respectively.