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Fructophilic lactic acid bacteria symbionts in honeybees – a key role to antimicrobial activities.

Shahad Mansour Salman* and GhadahmohammedSaleh*
**Baghdad University/College of Science/Biology Department – Iraq/Baghdad*
Corresponding Author: Shahad Mansour Salman

Abstract: *Antibiotic resistance has emerged as an intensive subject for research to find an alternative tool against infections caused by pathogens with antibiotic resistance, Hence, a unique lactic acid bacteria (LAB) microbiota which is in symbiosis with honeybees was isolated in this study and was detected and identified by biochemical and molecular manners and its antibacterial activity was detected against pathogenic bacteria using the dual culture overlay assay. Results reveled isolation and identification of two genera: Fructobacillussp. (92.3%) and Lactobacillus sp. (7.7%) from a total of 38 isolate, these isolates were known as Fructophilic lactic acid bacteria (FLAB). The highest inhibition zone (37mm) produced by Fructobacillus fructosus(SHGH-1,SHGH-4, andSHGH-14) against Pseudomonas aeruginosaand (33mm) forFructobacillus fructosus(SHGH-11) against Staphylococcus aureus (MRSA). This study shows that FLABs are unique sources of antibacterial substances which is effective against pathogenic Gram positive and Gram negative bacteria.*

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I. Introduction

Fructophilic lactic acid bacteria (FLAB)is a groupthat differs from Lactic acid bacteria (LAB) by its ability to prefer the fermentation of fructose instead of glucose, it also produce trace amounts of ethanol because it lacks acetaldehyde dehydrogenase (1). They are usually found in fructose-rich niches, e.g. flowers, fruits, fermented foods and in the stomach of some insects that feed on nectar of flowers which is rich with fructose, like honeybees (2). Studies showed that only two *lactobacillus sp*. included in the fructophilic group, i.e. *Lactobacillusflorum* and *Lactobacillus kunkeei*(3), while all other fructophilic LAB have been categorized in the genus *Fructobacillus*, which contains *Fructobacillus durionis*, *F. ficulneus, F. fructosus*, *F. pseudoficulneus*and *F. tropaeoli* (4), based on their phylogenetic positions, morphological and biochemical characteristics that arephylogenetically closely related to the genera *Leuconostoc*, *Oenococcus* and *Weissella* (5).

Studies have shown that honeybees carry specific microbiota very different than other animals, including humans.Most of the gut bacterial community of the adulthoney bee (*Apis.mellifera*) is related to the phyla Firmicutes, Actinobacteria, and Proteobacteria (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria) and are known to be the core gut bacteria (6).LAB has been considered as probiotics that contribute to health of humans and animals (7), they also are important players in the gastrointestinal tract of the honeybees, which support the intestinal barrier mechanism (8).These LAB symbionts contribute in the production of honey and are found in fresh honey in large numbers.Therefore, LAB, especially the FLAB group, may be related with bee health as well as human health (9).

It has been shown that proteins found in variety honey types can be traced back to LAB species with a wide spectrum of proteins and enzymes, The LAB symbionts from the honeybee (*A.mellifera)*are important candidates in the antimicrobial action of honey by forming a large group of antimicrobial metabolites and peptides, thereforetreatment of wounds and other infections could be gainedby its combination with heather honey as an ecological and effective alternative management (10).

It was indicated thatsignificant bee pathogens *Melisococcusplutonius* and *Paenibacilluslarvae* that cause American and European Foul brood Disease are effectively inhibited by such LAB symbionts as well as its antimicrobial activity against yeasts (11,12). The harmful consequences of antibiotic use and the high rate of resistance of bacterial pathogens that may put human lives in risk are important reasons to search for alternatives for the treatment and prevention of infectious diseases in humans as well as of honeybee infectious diseases.The aim of this study was to rediscover alternative inhibitors from friendly bacteria of the FLAB group against infectious pathogens.

II. Material and methods

1. Sample collection:

Healthy adult incoming worker bees (107) of the species *Apismillifera* were collected between October and December 2016, from four hives from different geographic regions of Baghdad city (45 , 9, 37, 16 bee) respectively and were collected in separated sterile plane tubes , they were immediately transferred to the laboratories of BiologyDepartmentatUniversity of Baghdad for further experiments.

2. Honeybees (*Apis spp.*) dissection:

Prior to gut dissection, bees were submerged in 94% alcohol solutionto remove external microbes on the surface of the bee body. Each honeybee was immersed for 2 minutes and then rinsed three times in sterile distilled water (13). The honeybees were aseptically dissected with sterile forceps under sterile conditions, Fig (1). The nectar stomach of each bee was separated from other parts of the gut and transformed to sterile tube containing 10 ml of MRS broth supplemented with (2% fructose and 0.1% cysteine). A sterile wooden applicator was used for homogenization.

Figure (1): The honeybee with its full nectar stomach from feeding on flower nectar, (a) is the nectar stomach which is separated from the rest of the digestive tract at the (b) proventriculus.

3. Isolation and identification of FLAB:-

The homogenized mixture(200μl) was spread on MRS agar plates with an L-shaped glass spreader for isolation of FLAB and incubated anaerobically at 30℃ for 48 hr. Bacterial colonies grown on plates were studied and differentiated according to size, color and morphology. Colonies then were repeatedly cultured on new MRS agar plates for pure bacterial growth.Further tests were performed for identification, including Gram stain, Catalase test as well as sugar fermentation tests and confirmed by molecular technique with 16SrRNA detection.

4. DNA extraction:

Bacterial pure colonies DNA were extracted with G- spin DNA extraction kit , (intron biotechnology) , cat.no. 17045, according to the manufacture protocol:

Bacteria were collected from the plates with a sterile inoculation loop into 1 ~ 2 ml tube of buffer. Pellet bacteria were collected by centrifugation for 1 min at 13,000 rpm, and the supernatant(except 50µl) was discarded. The cell pellet was resuspended completelyinto remnant supernatant by vigorously vortexing. 100 µl Buffer MP and 3 µl lysozyme solution was added into sample tube and then resuspended for 30 sec by vortexing, the lysate was then incubated for 15 min at 37℃ (tubes were inverted 5-6 times during incubation for complete break of cell wall), pre-lysate was centrifuged for 1 min at 13000 rpm at room temperature , supernatant discarded (liquid completely removed) and then cell pellet resuspended by vortexing to pre-lysis perfectly, (200 µl Buffer MG, 20 µl Proteinase K and 5 µlRNase A) Solution was added into sample tube and mixed by vortexing vigorously. Lysate was incubated at 65℃water bath for 15min.(tubes were inverted 5-6 times during incubation for complete lysis). After lysis completely, 250 µl of Buffer MB was addedto the lysate and mixedby pipetting then centrifuged to remove drops from inside the lid. 250 µl of 80% ethanol was added

into the lysate, and mixed well by gently inverting 5 - 6 times. After mixing, the 1.5 ml tube was briefly centrifuged to remove drops from inside of the lid. 750µl was carefully applied to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, the cap closed, and centrifuged at 13,000 rpm for 1 min. The filtrate was discarded and the Spin Column was placed in a 2 ml Collection Tube. 700 µl of Buffer MW was added to the Spin Column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and reused the Collection Tube. Then again centrifuged for additionally 1 min to dry the membrane.The Spin Column was placed into a new 1.5 ml tube, and 50 - 100 µl of Buffer ME directly added onto the membrane. Incubated for 1 min at room temperature and then centrifuged for 1 min at 13,000 rpm to elute.

5. PCR and DNA sequencing

To amplify the 16S rRNA gene, the primers used were shown in table (1), PCR was done by a (MultiGeneOptiMax Gradient Thermal Cycler, Labnet) in a 25µl reaction volumecontaining 5µl of Taq PCR premix (Intron,Korea), 10 picomoles/µ of the forward primer, 10 picomoles/µ of reverse primer, 1.5µl of genomic DNA, and 16.5 Distilled water. The PCR conditions consisted of an initial denaturation step at 94℃ for 3 minutes, followed by 35 cycles of denaturation at 94℃ for 45sec, annealing at 52C for 1 min, and elongation at 72℃ for 1 min, followed by a final 7-minute elongation step at 72℃.

Table (1): The specific primer of gene 16SrRNA

Primer	***Sequence***	***Tm (°C)***	***GC (%)***	***Product size***
Forward	27F 5'-AGAGTTTGATCCTGGCTCAG-'3	60.0	68.25%	1450
Reverse	1492R 5'-GGTTACCTTGTTACGACTT-'3	62.00	67.59%	base pair

The PCR products were analyzed by gel electrophoresis (CBS,Scientific) on a 2% w/v agaros gel in 1x TBE buffer for 90 minutes and visualized by staining with red safe stain.PCR products from different bacterial isolates were sequenced, Then, the sequences were compared to already published sequences from the NCBI nucleotidedatabase by BLAST-N (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

6. Dual culture overlay assay:

Antibacterial activity of FLAB isolates was measured by adapting the dual culture overlay assay described previously by (14),FLAB isolateswere placed into a filter disk (after preparing an overnight growth of the bacteria in MRS broth to reach an 0.2 at O.D 600) by adding 40µlof the growth on the filter disk, the disk was then placed on supplemented MRS agar plates, and incubated anaerobically for 18 hr at 30℃.Two wound bacterial pathogens (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) were cultured in nutrient broth and incubated for 18hr at 37℃. 200*µl*of each pathogen culture was mixed with a 10 ml sterile soft nutrient agar (0.8 %), holding temperature of 42℃. Each pathogen mixture of soft agar was poured asan over layer on the top of the MRS incubated plates containing the FLAB disk; the plates were incubated aerobically at 37 ℃ for 24hr. Zone diameter of inhibition was then measured in millimiters.

III. Results and Discussion:

1. Isolation and Identification:

Results of isolation revealed 38 bacterial isolate from the total of107 honeybee nectar stomach. The isolates were all Gram positive bacilli, negative to catalase test; they also fermented fructose, glucose, mannitol,sucrose and raffinose sugars. The isolates were identified by molecular technique using 16SrRNA genes, only 13 isolates were amplified and confirmed byagarose gel electrophoresis after PCR. Twelve (92.3%) isolates were identified as *Fructobacillus* genus and one (7.7%) isolate belong to *Lactobaccilus* genus as shown in Table (2). These isolates were isolated from the nectar stomach of worker honeybees (*A.millifera*) in Baghdad city, three of these isolates were registered for the first time to Iraq in the NCBI gene bank, the first two *Fructobacillus fructosus* LC278384.1 and LC278385.1, the third *Lactobacillus kunkeei* LC278386.1 , Table (2).

Fructophilic lactic acid bacteria are considered members of the normal microbiota of the digestive system of many living species and vary in its distribution and prevalence according to many factors including the species,the environment as well as the type of nutrition.

Culture-independent studies based on 16SrRNA sequences and metagenomic surveys of adult honey bees (*A. mellifera*) demonstrated the existence of distinct gut microbial communities comprising core bacterial phylotypes belonging to the Acetobacteraceae, Betaproteobacteria, Gammaproteobacteria, and Firmicutes (15).In this study*Fructobacillus* and*Lactobacillus* both belong to the Firmicutes, as shown in other studies (16,17)that shows the presence of FLAB in necter stomach of adult worker honeybees.

In addition to the core bacteria, highly diverse, transient microorganisms are also found in honey bees and within the hive environment, which may be transmitted by environmental sources such as yeasts and other

bacterial families. Gut bacteria play significant roles in host survival and fitness and can be transmitted either vertically or horizontally (18).

2. Antibacterial Activity of FLAB:

The bacterial isolates of FLAB were tested for their antibacterial activity against pathogenic bacteria (*P.aeruginosa and S.aureus*)by using the dual culture overlay assay. The diameter of inhibition zones shown in Table (2).

Table (2): Antibacterial activity of FLAB isolates against pathogenic isolates.

FLAB local isolates	Inhibition zone (mm) Mean±SE	
	P.aeruginosa	***S.aureus***
Fructobacillus fructosus SHGH-1	37±2.5	30±0.5
Fructobacillus fructosus SHGH-2	35±9.0	30±0.5
Fructobacillus fructosus SHGH-4	37±9.0	32±0.5
Fructobacillus fructosus SHGH-6	33±2.5	29±1.4
Fructobacillus fructosus SHGH-8**(LC278384.1)***	31±0.5	31±4.0
Fructobacillus fructosus SHGH-9	32±3.4	32±3.4
Fructobacillus fructosus SHGH-10	33±0.5	29±2.0
Fructobacillus fructosus SHGH-11	29±3.4	33±2.9
Fructobacillus fructosus SHGH-12	33±0.5	27±2.0
Fructobacillus fructosus SHGH-13	31±2.5	28±10.4
Fructobacillus fructosus SHGH-14	37±1.4	29±4.5
Fructobacillus fructosus SHGH-15**(LC278385.1)***	28±10.9	26±3.4
Lactobacillus kunkeei SHGH-7**(LC278386.1)***	28±8.4	28±4.5

***FLAB isolates registered in NCBI gene bank.

The highest inhibition zone against *P.aeruginosa* was (37mm) by *Fructobacillus fructosus* (SHGH-1, SHGH-4 and SHGH-14);while the highest inhibition zone against *S.aureus* was (33mm) by *Fructobacillus fructosus*(SHGH-11). The lowest inhibition zone against *P.aeruginosa*were (28mm) by *Fructobacillus fructosus* (SHGH-7 andSHGH-15); *S.aureus* were(26mm) by *Fructobacillus fructosus* (SHGH-15).

A recent exciting finding showed that these FLAB symbionts separately and together have strong antimicrobial activity against a wide variety of human pathogens including antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) (19).It has been recently observed that these symbionts produce antimicrobial substances such as formic acid, hydrogen peroxide and free fatty acids as well as different extracellular proteins such as enzymes, bacteriocins and lysozymes (20), such products have antibacterial effect against various Gram-positive and Gram-negative bacteria, and this is an important character for choosing an organism for probiotic uses (21).

In this study FLAB isolates displayed a high antagonistic activity against *P.aeruginosa* and *S.aureus*. Many studies have referred to the isolation of FLAB, but little information was found about its antibacterial activity against pathogenic bacteria, moreover these FLABs were shown to have other antimicrobial substances than bacteriocins which raise its importance as a good source of new alternative antibacterial drugs (22).

It can be realized from this study that the healthy state of honeybees harboring such potential bacteria suggests that they are responsible for controlling the pathogen growth and existence through antagonism and competition upon the pathogens inside the nectar stomach of the honeybees.

IV. Conclusion:

Multidrug resistance of pathogenic bacteria is arising and is considered as an important medical problem as a result of overuse and irresponsible use of antibiotics around the world, such pathogens may find their way to humans through processed foods, drinks and others. The antibacterial activity of FLAB can be suggested to be used as a source of safe substances for treatment or as additives in food processing to achieve the advantage of safe alternative without enhancing the antibiotic resistance of pathogenic bacteria.

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