ISOLATION AND CHARACTERIZATION OF UNIQUE FRUCTOPHILIC LACTIC ACID BACTERIA FROM DIFFERENT FLOWER SOURCES.

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ABSTRACT

This study was aimed to investigate Lactic acid bacteria (LAB) that contain an important group of bacterial strains such as fructophilic lactic acid bacteria that usually isolated from fructose rich niches. These groups of bacteria have been considered as probiotics, especially in animal applications. In this study these bacteria were isolated from 6 Plant sources (flowers) such as: *Convolvulus arvensis*, *Hibiscus rosa-sinensis* , *Nerium oleander*, *Rosa rugosa*, *Tagetes erecta* and *Zinnia elegans*. The bacterial isolates were identified by morphological and molecular methods through the detection of 16SrRNA gene. Carbohydrate fermentation profile as well as their antibacterial activity using the dual culture overlay assay was also detected. The results revealed that 5 types of different lactic acid bacteria were isolated included: *Fructobacillus fructosus*, *Lactobacillus kunkeei*, *Enterococcus durans*, *Enterococcus faecium* , and *Lactobacillus brevis*. These isolates showed variety in fermentation of carbohydrates. Also, all isolates exhibit inhibition effect against pathogenic bacteria (*Staphylococcus aures* و *Pseudomonas aeruginosa*) except *Enterococcus faecium* which showed no effect it.

Keywords: flower microbial flora, fructose rich niches, antibacterial activity.

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عزل وتشخيص بكتيريا حامض اللاكتيك المحبة للفركتوز من مصادر مختلفة للزهور

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المستخلص

تحتوي بكتيريا حامض اللاكتيك على مجموعة مهمة من الانواع البكتيرية والتي تعرف ببكتيريا حامض اللاكتيك المحبة للفركتوزوالتي تعزل من البيئات الغنية بسكر الفركتوز. تعتبر هذه المجاميع البكتيرية من المعززات الحيوية خاصة في التطبيقات الحيوانية. تم عزل هذه البكتيريا في هذه الدراسة من 6 مصادر نباتية (زهرية) شملت : *Convolvulus arvensis*, *Hibiscus rosa-sinensis*, *Nerium oleander*, *Rosa rugosa*, *Tagetes erecta* و *Zinnia elegans*شخصت العزلات البكتيرية مظهريا وجزيئيا من خلال التحري عن جين 16SrRNA . كذلك تم التحري على قابلية هذه الانواع البكتيرية في تخمير الكاربوهيدرات كما درست الفعالية الضد مايكروبية لها بوساطة تقنية dual culture overlay . أظهرت النتائج عزل 5 انواع من بكتيريا حامض اللاكتيك من ضمنها المحبة للفركتوز شملت : *Fructobacillus fructosus*, *Lactobacillus kunkeei*, *Enterococcus durans*, *Enterococcus faecium* و*Lactobacillus brevis* كذلك أظهرت هذه العزلات قابليات مختلفة في تخمير الكاربوهيدرات. أظهرت جميع العزلات فعالية مثبطة للبكتيريا الممرضة (*Staphylococcus aures* و*Pseudomonas aeruginosa*) عدا عزلات ال *Enterococcus faecium* التي لم تظهر اي تاثير ضد البكتيريا الممرضة.

الكلمات المفتاحية: النبيت المايكروبي للازهار, البيئات الغنية بسكر الفركتوز, الفعالية ضد المايكروبية.

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INTRODUCTION

Fructophilic lactic acid bacteria (FLAB) is a special group of Lactic acid bacteria (LAB) with unique characters. When an external electron receptor is presented in the growth environment they can ferment D-fructose, but when the receptor is absent they ferment D-glucose poorly (14). This unique character differentiated them from their main group of LAB. They are Gram –positive bacilli or cocci, non-spore formers and generally non-motile. They habitat environments rich in sugars, vitamins, amino acids, minerals and derivatives of nucleic acids (21). Some of them are commonly found in guts of healthy individuals and provide them with antimicrobial substances that are considered as protection mechanism, they are named usually beneficial microorganisms (35). The FLABs include mainly *Lactobacillus kunkeei* and *Fructobacillus sp*. The *Fructobacillus* species were previously classified as species of the genus *Leuconostoc* and its growth nutrient depend mainly on D-fructose (9). The FLAB group was further divided into obligate and facultative fructophil bacteria in which both *Lactobacillus kunkeei* and *Fructobacillus sp* show obligate character while *Lactobacillus florum* considered a facultative fructophil (15), they are all found in the *Firmicute*s phyla which is known to have a low G+C content compared to other LABs and *Bifidobacterium* genus that have a high G+C content (34). Honeybees represent the most abundant source of FLAB isolates as well as other rich fructose sources (fruits, fruit fly, flowers and others) (13). Flowers are considered as a good habitat for many diverse bacteria. The flower part of a plant determines the type and availability of nutrients which differ from other parts of the plant like leaves, stalk, fruits and roots (25,26). Previous studies mentioned that flowers were considered unsuitable for bacterial growth (31). Later studies confirmed diverse bacterial communities that differ according to species of plants (18). The idea of transmission of these FLABs between flowers and honeybees that visit them has raised an important question, whether the transmission is from the flowers to honeybees or vice versa. A hypothesis suggested that the FLAB can affect the chemical composition of floral nectar, and as a result attracting the honeybees toward the nectar (19). Furthermore, a high percent of these bacteria in the floral nectar are also found in the stomach of honeybees and honeybee hives (2). Seasons of the year may affect the diversity of such bacteria, it was shown that a shift from *Lactobacillus kunkeei* to *Fructobacillus fructosus* from May to late June of the year (24). The flowers as source of FLAB open a wide range of studies for detecting there diversity and there importance for honeybees health and humans as well. This study was aimed to shed light on the predominant FLAB and LAB species isolated from different flowers and detect their antimicrobial activity in an advantage to be a source of substances alternatives of antibiotics.

MATERIALS AND METHODS

Collection of flowers

To study the bacterial composition of flowers, 6 types of fresh flowers were collected from different areas of al-Jadria campus of university of baghdad /college of science, these flowers included: *Convolvulus arvensis*, *Hibiscus rosa-sinensis*, *Nerium oleander*, *Rosa rugosa*, *Tagetes erecta* and *Zinnia elegans*, their blooming time, size and colors are shown in, (Table 1), (Fig .1).

Table 1. Scientific names, common names, family, blooming time and sizes of flowers used in this study

Plant species (Common name)	Family	Blooming time	size
Convolvulus arvensis **(Silver bush)**	***Convolvulaceae***	**June-August**	**small**
Hibiscus rosa-sinensis **(Rose mallow)**	***Malvaceae***	**seasonal**	**large**
Nerium oleander **(Oleander)**	***Apocynaceae***	**seasonal**	**small**
Rosa rugosa **(Rugosa rose)**	***Rosaceae***	**May-June**	**small**
Tagetes erecta **(Marigold flower)**	***Asteraceae***	**May-October**	**large**
Zinnia elegans **(Elegant zinnia)**	***Asteraceae***	**May-August**	**large**

Figure 1. Flowers in its normal habitat used in this study. a) *Convolvulus arvensis*, b) *Nerium oleander*, c) *Tagetes erecta*, d) *Hibiscus rosa-sinensis*, e) *Rosa rugosa*, f) *Zinnia elegans*

Isolation of FLAB from the flowers

Procedure of isolation was done according to Endo, *etal* (16) with modification. Each group of fresh flowers (2-3 of large flowers and 10-15 of small size flowers) was picked and placed in sterile plastic bags (using sterile gloves to reduce contamination). An aliquot of 10 ml MRS broth was added to each bag sample, closed tightly and incubated at 30 Ċ for 24 hr. After incubation period the suspension in each bag was transferred to sterile plane tubes under sterile conditions and centrifuged at 5000g for 5 min., the supernatant was discarded and the pellet was resuspended in 4ml fresh MRS broth. Volume of 100µl of each sample was inoculated on fresh prepared MRS agar plate, and spread by a glass rod spreader, then incubated anaerobically at 30 Ċ for 72 hr. After incubation visible colonies were seen on plates and pure isolation was conducted by picking different colonies randomly by loop from different parts of the plate, each colony was then streaked on new MRS agar plates and MRS agar contain (5g/L) $CaCO_3$ plates, then all plates incubated anaerobically at 30 Ċ for 24 hr. Colonies were examined for their size, shape and hydrolysis of CaCO3 in which clear zones formed around colonies, as a result of lactic acid production. Each pure culture was regrown on MRS broth , after incubation 400 µl of 30% glycerol was added to 800 µl of MRS broth culture in a 2ml micro tube, the stock cultures were then stored for further experiments.

DNA extraction and PCR

The DNA of pure bacterial colonies was extracted by Genomic DNA mini extraction kit (Geneaid /Korea**)**, according to the manufacture protocol pure bacterial colonies were collected from the plates with a sterile inoculation loop into 1 ~ 2 ml tube of buffer, centrifugation for 1min at 16,000 rpm, the supernatant was discarded and 200 µl of Gram+ Buffer (lysozyme was added to G+ buffer) was added into the sample tube, then re-suspended the pellet by vortex. The lysate was incubated for 30 min at 37℃ (tubes were inverted every 10 min during incubation for complete break of cell wall). GT Buffer (200 µl) was added into the sample tube and re-suspended by vortex. Proteinase K solution (20 µl) was added into the sample tubes, then mixed for 10 sec. by vortex, and incubated for at least 10 min at 60℃ (during incubation tubes were inverted 3-4 times). GB Buffer (200 µl) was added into the sample tube, and mixed by vortex for 10 sec., then incubated at 70℃ for 10 min (For complete lysis tubes were inverted every 3 min). After lysis has completed, absolute ethanol (200 µl) was added into the sample lysate and mixed

immediately by vortex. A volume of 620 µl of mixture was transferred into GD column (in a 2 ml Collection Tube) without wetting the rim, cap closed tightly, and centrifuged at 16,000 rpm for 2 min. The GD column was placed in a new 2 ml Collection Tube after the filtrate was discarded. W1 Buffer (400 µl) was added to the spin column (without wetting the rim), then centrifuged for 30 sec at 16000 rpm. The flow-through was neglected and reused the collection tube. Wash Buffer (600 µl) was added to the GD Column, and then centrifuged for 30 sec at 16000 rpm. The flow-through was neglected and reused the collection tube, and centrifuged again for additionally 3 min at 16000 rpm to dry the column matrix. The spin column was placed into a new 1.5 ml small tube, and Pre-heated Elution Buffer (50-100 µl) directly added onto the membrane then incubated for 3-5 min at room temperature to allow complete absorption, and centrifuged for 30 sec at 16000 rpm to elute the purified DNA. To amplify the 16S rRNA gene, the primers used for amplification were 27F 5'-AGAGTTTGATCCTGGCTCAG-'3 (forward primer) and 1492R 5'-GGTTACCTTGTTACGACTT-'3 (reverse primer), the expected size of the amplicons was about 1450 bp. PCR was done by a Thermal Cycler in a 25 µl reaction volume containing 5 µl of Taq PCR premix, 10 picomoles/µl of the forward primer, 10 picomoles/µl of reverse primer, 1.5 µl of genomic DNA, and 16.5µl 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 45seconds, annealing at 52℃ for 1 min, and elongation at 72˚C for 1 min, followed by a final 7 minutes elongation step at 72˚C. (22,20). The PCR products were analyzed by gel electrophoresis on a 1.5% w/v agarose gel in 1x TBE buffer for 60 minutes and visualized by staining with red safe stain. PCR products from different bacterial isolates were sequenced, and then the sequences were compared to already published sequences from the NCBI nucleotide database by BLAST-N (http:// blast.ncbi.nlm.nih.gov/Blast.cgi).

Detection of suspected yeast isolates

Isolates that were suspected as yeasts were inoculated in 10ml MRS broth and incubated at 30 Ċ for 18 hr. The *Lactobacillus kunkeei* (LC278386.1) was inoculated in another 10ml MRS broth tube as control. A stock solution of Cycloheximide (CHX) was prepared by dissolving 0.2gm CHX in 6ml of sterile distilled water (in hot water bath) then filtrated by a 0.2 µm milipore filter. Working solution of CHX (2ml of stock solution diluted in 8ml of sterile double distilled water) to reach final concentration of 6.7 mg/ml. Culture tubes contained 1ml MRS broth, and CHX working solution was added in different concentrations, (Table 2). These contents were mixed well. Then added 50µl of an overnight culture of the suspected samples thereafter these tubes were incubated at 30 Ċ for 24hr.

Table 2. Dilution series and CHX working solution volumes that gives final concentration

	No. of dilution tubes , final volume (1ml)					
	1	**2**	**3**	**4**	**5**	**6**
CHX working solution (6.7 mg/ml) (µl)	**0**	**1**	**2**	**5**	**10**	**25**
CHX final con.(µg/ml)	**0**	**6.7**	**13.4**	**33.5**	**67.0**	**167.5**

Sugar fermentation profile

The medium used to detect the fermentation of the carbohydrates included the following ingredients: 5 gm yeast extract ,10 gm peptone, 1 ml Tween 80, 2 gm K_2HPO_4, 2 gm triammonium citrate, 200 mg $MgSO_4.7H_2O_2$, 50 mg $MnSO_4.4H_2O$, and 5 gm sodium acetate hydrate, all ingredients were dissolved in 900 ml of distilled water, pH was adjusted to 6.3 . Chlorophenol red (0.04 gm) was added as indicator. The volume was completed to 1000 ml with distilled water and autoclaved at 121℃ for 15 minutes. Solution of 11 carbohydrates (D-fructose, D-glucose, D-galactose , D-ribose, D- xylose, L-arabinose, D-mannose, D-mannitol, D-maltose, D-lactose, D- trehalose) were prepared by dissolving 10 gm of each sugar in 100 ml of distilled water and sterilized by filtration through 0.2 µm milipore filter. Carbohydrates solutions were added separately to the medium to give a final concentration 1% (15). The medium with sugar then was divided into plane tubes each contain 5ml and inoculated with the bacterial inoculum (100µl) previously grown over night. All tubes were incubated

anaerobically at 30 ℃ for different time intervals to detect the change in color of the indicator from red to yellow which elucidate the carbohydrate fermentation and acid product.

Detection of antibacterial activity of FLAB isolates

The LAB isolates were tested to detect the antibacterial activity by adapting the dual culture overlay assay described previously by (33), against pathogenic bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* previously identified and provided from Department of biology –College of Science – University of Baghdad. Bacteria were inoculated in broth for 18 h at 37℃. , the concentration of each bacterium was subjected to **$1.5*10^8$(cell/ml)** that confronts McFarland tube (0.5 turbidity). LAB isolates were placed into filter discs by adding 40µl of growing bacteria on the filter disc; the disc was then placed on MRS agar plates, and incubated anaerobically for 18 hr at 30℃. From the overnight pathogenic cultures $200\mu l$ of each was mixed with a 10 ml sterile soft nutrient agar (0.8 %), holding temperature of 42℃. Each pathogen mixture of soft agar was poured as an over layer on the top of the MRS incubated plates containing the LAB discs; the plates were incubated again aerobically at 37 ℃ for 24hr. Zone diameter of inhibition was then measured in millimiters.

RESULTS AND DISCUSSION

Growth characteristics of bacterial strains and hydrolysis of Calcium carbonate

All different flower groups showed the growth of many colonies of bacteria on MRS agar plates which were varied in size, look smooth, non translusent and with a creamy white color. Some plates showed growth of colonies in aerobic condition rather than anaerobic conditions, they were larger and sometimes had rough edges and produced pigments, these colonies were suspected to be yeasts and were submitted to the CHX test. The total number of isolates from all flower samples was 40 isolate , 4-8 for each flower group. All isolates showed the ability to hydrolyze Ca_2CO_3, as a result of acid production, Fig (2).

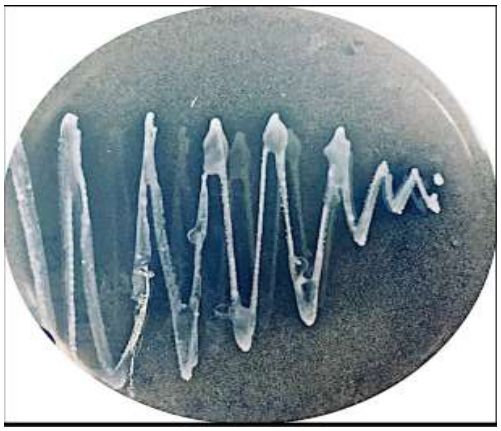

Figure 2. Hydrolysis of Ca_2CO_3 by LAB isolates

Phylogenic identification

About 4-8 bacterial isolates from each flower group (31 for all flower groups) were detected. *Convolvulus arvensis* and *Nerium oleander* gave *Staphylococcus epidermidis* and *Enterobateriaceae* with no presence of LAB, whearase *Tagetes erecta* gave *L.brevis* isolate; *Hibiscus rosa-sinensis* gave *L.kunkeei, E.durans* and *E.feacium* isolates ;*Rosa rugoso* gave *L.kunkeei, L.brevis* and *F.fructococus* isolates (previous three flower groups showed the presence of LAB), while *Zinnia elegans* have the most diverse bacterial component *Enterobateriaceae*, *Staphylococcus epidermidis* , *E.feacium* and *L.brevis*. Results revealed that 20 (64.5%) of the 31 isolated bacteria were from the lactic acid bacteria group, wherase *Staphylococcus epidermidis* and *Enterobateriaceae* isolates revealed 11(35.4%). The distribution of bacterial isolates from the flower groups shown in Fig (3).

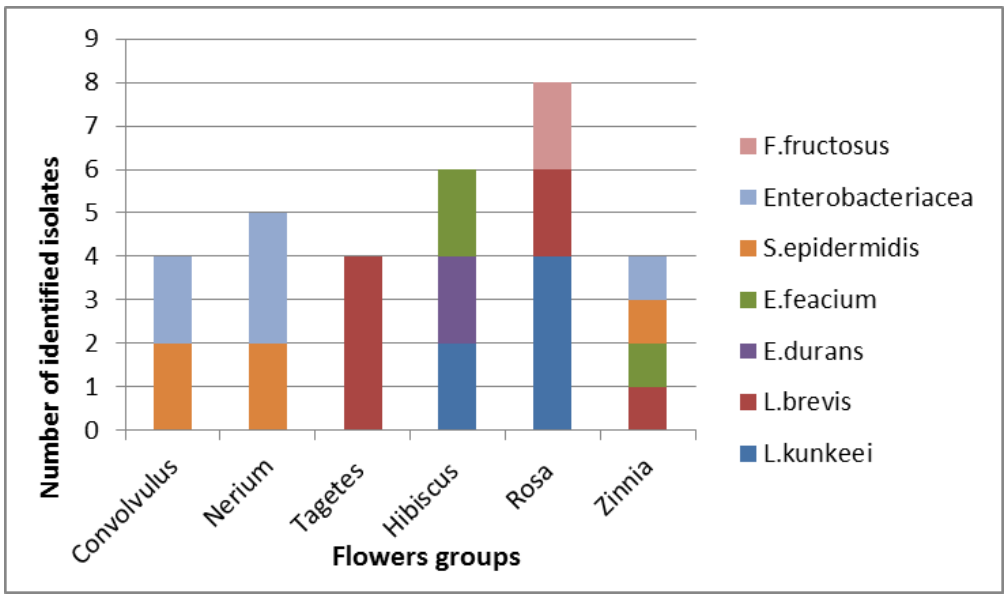

Figure 3. Distribution of all identified isolates among various flower groups. *S.epidermidis* 5(16.1%), Enterobateriaceae 6 (19.4%), *F.fructococus* 2(6.5%), *E.feacium* 3(9.7%), *E.durans* 2(6.5%), *L.brevis* 7(22.5%) *and L.kunkeei* 6(19.4%) .

The 20 lactic acid bacteria from all flowers groups were elected for DNA extraction using the Genomic DNA mini extraction kit (Geneaid /Korea). The DNA quality and integrity were estimated through remarking DNA bands by gel electrophoresis on agarose 0.8% for 30 minutes. The bands appeared single not diffused, and have no smear which may result from DNA degradation. Polymerase chain reaction technique (PCR) was performed for the LAB isolates by using universal primer targeting the gene 16SrRNA. The PCR product size was 1450 bp when compared to the DNA ladder as shown in Fig (4). Studies targeting the same 16SrRNA gene was used to detect the LAB isolates (22,33)

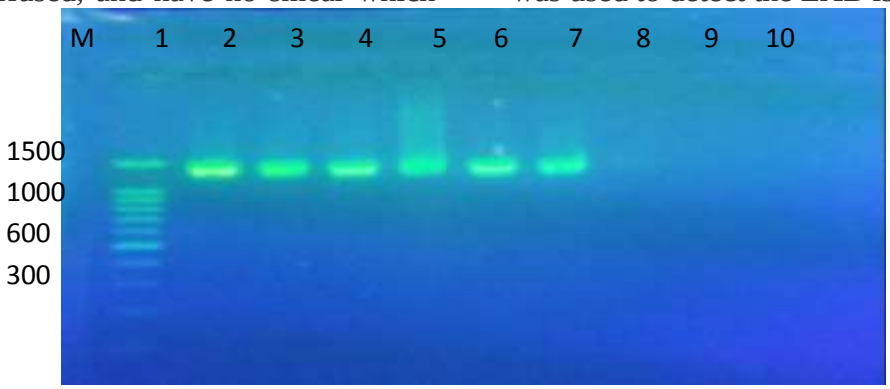

Figure 4. Gel of electrophoresis of PCR product of 16SrRNA gene, size of the bands 1450 bp. Electrophoresis was done on 1.5% agarose at 80 volt for 50 minute. DNA ladder (100), lane 1-5 referred to lactic acid bacteria Isolates, lane 6 referred to the control *L.kunkeei* (LC278386.1) **as positive PCR result while the lane 7-10 referred to non-bacterial isolates. M is a marker, PCR amplified product visualized by U.V.=**

Sequencing was preformed and data was analysed and compared with reference strains using the BLAST available in NCBI database. The results of sequence 20 isolates revealed, that *L.berevis* a highest percentage (22.5%), followed by *L.kunkeei* (19.4%) and *E.feacium* (9.7%), while *F.fructosus* and *E.durans* showed the lowest isolation percentage (6.5%).

Table 3. Sequencing ID in Gene bank, Score, Expect and compatibility of 16SrRNA for Fructophilic lactic acid bacteria (FLAB) isolates

Isolates	Sequence ID	Score	Expect	Identities	Position in Gene Bank
Hd1	KY962884.1	1897	0.0	98%	25-1058
Hd2	KY962884.1	1879	0.0	96%	71-1101
Zf1	KY962900.1	1598	0.0	97%	30-1254
Hf1	KY962900.1	980	0.0	97%	21-921
Hf2	KY962900.1	1387	0.0	96%	12-1021
Tb1	MF179529.1	2013	0.0	96%	8-986
Tb2	MF179529.1	1976	0.0	96%	54-1065
Tb3	MF179529.1	1032	0.0	98%	13-953
Tb4	MF179529.1	2021	0.0	95%	30-1943
Rb1	MF179529.1	1298	0.0	95%	32-1435
Rb2	MF179529.1	998	0.0	96%	2-976
Zb1	MF179529.1	1754	0.0	96%	36-899
Rf1	KF600363.1	1749	0.0	98%	24-1710
Rf2	KF600363.1	1847	0.0	95%	71-984
Rk1	JQ009336.1	845	0.0	95%	34-817
Rk2	JQ009336.1	897	0.0	95%	8-848
Rk3	JQ009336.1	1200	0.0	96%	41-877
Rk4	JQ009336.1	971	0.0	95%	31-870
Hk1	AB559822.1	1275	0.0	95%	80-1090
Hk2	AB559822.1	793	0.0	96%	11-765

Hd: ***Hibiscus/ E.durans*****; Hf:** ***Hibiscus/ E.feacium*** **; Hk:** ***Hibiscus / L.kunkii*****; Zf:** ***Zinnia / E.feacium*****; Zb:** ***Zinnia /L.brevis;*** **Tb:***Tagetes***/** ***L.brevis*****; Rb:** ***Rosa / L.brevis*****; Rk: Rosa /** ***L.kunkeei*****; Rf:** ***Rosa / F.fructosus*****.**

The results showed that the bacterial flora from different flower groups were varied. *Convovulus* and *Nerium* group showed no LAB but only *Staphlococci* and *Enterobacteriaceae*, which may be present as a result of the nature of these flowers, *Nerium* is known to be a toxic plant that may not attract butterflies or honeybees to transfer LAB , the *Tagetes* group showed only one type of isolate *L.brevis* , which was usually isolated from different other kinds of flowers , its bright color and fragrance that attract honeybees and fruit flies that visit this flower regularly (28), Other species like *Lactobacillus florum* was isolated with *L.brevis* from *Paeonia suffruticosa* and *Chrysanthemoides monilifera* flowers (16), the *Hibiscus* group showed three types of isolates *L.kunkeei*, *E.feacium* and *E.durans*, this flower has special shape, that expose its middle part far from the petals, it was also visited from honeybees which explain the presence of *L.kunkeei* isolate , but its shape also make it exposed to animals such as birds that transfer *Enterococcus* species presented in its feces and are also commonly found in the environment (7) , *Rosa* flower group known to produce rich nectar with different fragrances that attract large numbers of honeybees which explain the presence of *F.fructosus* and *L.kunkeei* . The *Fructbacillus sp*. has been isolated from *Tropaeolum majus* flower and other flower species (17). They are well known FLABs usually isolated from honeybee guts and beehives (12,3). The *Zinnia* group showed the most diverse isolates, it was a type of flowers usually used for decorations of gardens and may be in contact with animals such as birds as well as humans. The diversity of bacterial isolates in the flower groups may be as a result of different composition of the flower nectar , some may be rich in fructose for example while others do not, some do not produce nectar at all . The isolation of the bacterial species under the study was only from the flower part of the plant, that may differ from the spices of bacteria that habitat a specific part of the plant which means that the leaves or stalk may have different other varieties of bacterial isolates ,also this study showed the bacterial variation in the whole flower. The parts of the flower may be inhabited with specific isolates such as the petals , pollen and nectar are the most parts of the flower that are visited by honeybee and butterflies, this mechanism can be a key for bacterial transfer, although it was not clear in which way the transmission may occur. Several studies described microbial diversity on flower surface and showed that it was changed after a

number of visits of insects, suggesting the transfer of microbes from the insects to the flower surface (35). This theory was supported with a study which showed that the microbial flora of the honeybee more similar to the uncovered flowers compared to the covered ones, finally honeybees may introduce the microbes to the flower (4). Plasmid profile of bacterial isolates from honeybee gut showed high percentage with high diversity compared to plasmids from bacterial isolates that habitat flower (30).

Suspected yeast isolates

From plates which showed colonies with no amplicons that was suspected to be a eukaryote and not prokaryote they were conducted to the CHX eukaryote inhibitor, 9(22.5%) isolates from the 40 were tested; 4 from the *Convolvulus* flower group; 2 from the *Nerium* group and 3 from the *Zinnia* group, while the *Tagetes*, *Hibiscus*, and *Rosa* groups had no suspected isolates. All tubes with concentrations ranging from (6.7-167.5) µg/ml have no growth, while the control tube that contain the control *L.kunkeei* (LC278386.1) showed visible growth. Microscopic slides were prepared for all suspected samples and showed large cells with oval shape that confirm the cells were yeasts compared to the control slide that showed very small thin bacilli shaped bacteria.

Sugar fermentation

The fermentation of sugars was detected for elected isolates that were submitted to molecular detection. Isolates showed variable fermentation ability. All isolates fermented fructose and glucose in one day or more. The *L.kunkeei* and *F.fructosus* was able to ferment only 3 sugars (glucose, fructose and mannitol) whereas, *L.brevis* fermented all sugars except (mannitol and maltose). The *Enterococci* bacteria showed variable fermentation in between these sugars, as shown in Table (4). The fermentation results coincide rationally with documented studies of same species. The variability of some fermentation behavior may be regarded to the plasmid profile of the strains, it has been shown that sucrose metabolism for example is controlled by plasmid gene and for this reason may be strain dependent (11).

Table 4. Sugar fermentation of LAB isolates from different flower groups

Carbohydrates	*F.fructosus*	*L.kunkeei*	*E.durans*	*E.faecium*	*L.brevis*
L-arabinose	**-**	**-**	**-**	**-**	**1d**
D-ribose	**-**	**-**	**2d**	**2d**	**+**
D-xylose	**-**	**-**	**-**	**-**	**W**
D-galactose	**-**	**-**	**-**	**+**	**+**
D-Glucose	**1d**	**+**	**1d**	**2d**	**W**
D-fructose	**1d**	**1d**	**1d**	**1d**	**1d**
D-mannose	**-**	**-**	**W**	**1d**	**W**
D-mannitol	**2d**	**+**	**-**	**-**	**-**
D-maltose	**-**	**-**	**+**	**+**	**-**
D-lactose	**-**	**-**	**W**	**W**	**2d**
D-trehalose	**-**	**+**	**-**	**-**	**1d**

d": days to reach positive result, "w": weak, "-": negative (no color change), "+":positive (within 4-7days).

Antibacterial activity

Results revealed obvious inhibition of LAB isolates against pathogenic bacteria except for the *E.feacium* isolate that showed no antibacterial effect , the highest inhibition zone was detected for *F.fructosus* (20 and 22 mm) against *S.aureus* and *P.aeruginosa* respectively, whereas the lowest was for *E.durans* (15 and 17 mm), Fig.(4), Table(5)

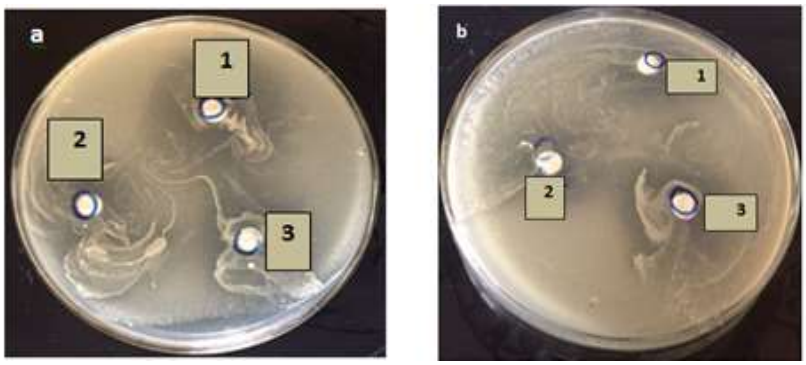

Figure 4. Inhibition zones of highest 3 study isolates against pathogenic bacteria, a) against *S.aureus*; b) against *P.aeruginosa*. 1: *Frutobacillus fructosus*; 2: *Lactobacillus kunkeei*; 3: *Lactobacillus brevis*

The antibacterial activity of LAB has been studied, and this character has also been confirmed in FLAB isolated from honeybee guts (33, 5), as fare as it comes to isolates from this group isolated from flowers there are very scarce information in literature. The results of this study showed a similar antibacterial activity against pathogenic bacteria.

Table 5. Inhibition zones in (mm) of LAB isolates showing antibacterial activity against pathogenic bacteria.

LAB isolates	Inhibition zone (mm) (Mean±SE)*	
	Staphylococcus aureus	*Pseudomonas aeruginosa*
Lactobacillus brevis	**16 ± 0.31**	**17 ± 0.10**
Enterococcus durans	**14 ± 0.97**	**16 ± 0.57**
Enterococcus feacium	**0.0**	**0.0**
Frutobacillus fructosus	**20 ± 0.62**	**22 ± 0.95**
Lactobacillus kunkeei	**17 ± 0.19**	**19 ± 0.45**

*** Mean ±SE (represent triplicate experiments)**

Studies showed that *L.brevis* bacteria isolated from dairy products produced an antibacterial heat-stabile substance which considered being a bacteriocin like product (1, 29). The *E.durans* bacterium previously classified as *Streptococcus durans* , the bacteria was isolated from vagina of healthy women and showed antibacterial activity against pathogenic *E.coli* and *S.aureus* (27), it is considered as a promising probiotic that is able to produce anti-inflammatory agents and stimulate the abundance of other friendly bacteria mostly *Faecalibacterium praunsnitzii* (a bacterium producing butyrate with anti-inflammatory effects) which suggest the use of *E.durans* in therapy for disorders of gut inflammations (8). On the other hand, *E.feacium* a species that should not be confused with *E.feacalis*, found as commensal organism in the gastrointestinal tract (1-10%), but it may be pathogenic and cause different diseases (10). As a matter of fact the FLAB *F.fructosus* and *L.kunkeei* isolated from honeybee gut and beehives showed properties of combating other pathogenic bacteria due to their ability to produce antibacterial compounds (33, 6, and 23). Since these honeybees are constantly visitors of flowers there is a possible chance that such insects transfer these FLABs to flowers during the season of pollination. Flowers are parts of plants that show a unique habitat for a diverse group of microorganisms. In this study these microorganisms were shown to be very big importance in nature as they belong to FLAB and LAB that have an important to honeybee's life cycle. Also considered a good defender against pathogens, which can be a benefit for humans as well.

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