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Detection and Identification of Novel Bacterial Strains Isolated from Fresh Clover Bee Honey

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ABSTRACT

Bee honey have a highly intensity and specialized microbiota which plays an important role in metabolism, immunity, growth as well as development an antagonistic effect against pathogens. Thus, the aim of work was isolation genotype characterization of some strains of lactic acid bacteria (LAB) from fresh clover bee honey. Five samples of fresh clover honey were analyzed to detect the microbiome contents. Dilution method and cultivation in selective media was used for counting of LAB strains, and the isolates were identified by their morphological characteristics that measured using Gram staining method and screened by catalase activity. Furthermore, the morphological characteristics of bacterial cells were examined using Scanning Electron Microscope (SEM). The genotype of isolated strains was carried out by Polymerase Chain Reaction (PCR) method. The results showed that, two novel bacterial strains of LAB were isolated from fresh clover bee honey. The strains of LAB which identified were; Lactobacillus brevis MK250013 and Lactobacillus casei MK250003.

Keywords: Bee honey, Lactic acid bacteria, Clover honey, SEM, PCR.

INTRODUCTION

Bee honey is the best primary product of bee colonies (Gallai *et al.,* 2009) and many culture in whole world use of honey as a source of food also as a magic therapeutic material for many diseases for human health (Aronstein and Murray, 2010; Forsgren *et al.,* 2010 and Fries, 2010). The chemical composition of honey mainly composed of sugars (mono saccharides) and water with minor constituent of amino acids, organic acids, minerals, vitamins, flavonoids and other phenolic as well as aromatic substances. The minor constituents of honey were related with botanical and geographical origin and there were affected by time and conditions of storage (De Graaf *et al.,* 2006 and Yoshiyama *et al.,* 2013).

The microflora in bee honey studied by Snowdon and Cliver (1996). The lactic acid bacteria (LAB) usually found in a rich food which containing carbohydrate substances such as bee honey (Eva *et al.,* 2009 and Audisio *et al.,* 2011). LAB play an important roles not only for fermented food production but also for the benefit of health (Naidu *et al.,* 1999). It is believed that naturally occurring LAB from different sources are known as a key for producing different compounds that have antibacterial effect against both bacteria and fungi, furthermore LAB strains are considered safe food-grade microorganisms or Probiotic and used in human healthy nutrition (Aplevicz *et al.,* 2014).

The microflora associated with the honeybee *Apis mellifera L.* is complex. Which consist of yeasts, Gram- positive bacteria and Gram-negative bacteria (Endo and Salminen, 2013). These bacteria are endemic in the honey sac of forager workers maybe depending on seasons. Furthermore, this worker bee's bacteria was comes from gathering of pollen grains or through contact with older worker bees.

Lactic Acid Bacteria was found in humans and other animals which play an important role by producing antimicrobial metabolites and encourage the immunity system. LAB microbiota works in a synergistic manner. They produce proteins, peptides, enzymes, and bacteriocins as antimicrobial agents (Aween *et al.,* 2012; Gomaa and Rushdy, 2014 and Douglas *et al.,* 2015).

Thus, the present work was carried out to isolate and identify of novel bacterial strains from fresh clover bee honey.

MATERIALS AND METHODS

This work was carried out at the Faculty of Agriculture apiary, Cairo University during summer season of 2017. Five samples of pure natural clover honey were collected, and there were stored at room temperature till analysis.

Isolation and characterization of LAB strains.

Ten gram of honey samples were suspended in 90 ml peptone water (0.1% w/v), then 1 ml of suspension was added to 10 ml of MRS broth and incubated at 37 °C for 48 h under microaerophilc conditions followed by serial dilutions in sterile physiological saline solution (0.85 w/v Nacl, 0.1% w/v peptone) then 1 mL of the appropriate dilution was mixed with 10 ml of melted MRS agar to enumerate the total LAB with the pour plate method. Cycloheximide at a concentration of 0.01% (v/v) was added to the MRS plates in order to prevent the growth of fungi. Meanwhile, the appropriate dilution was evenly spread onto the MRS agar (De Man *et al.,* 1960) (Difco Laboratories, India).

Colonies with distinct morphological differences based on (color, shape, size, rough or smooth surface) were selected and then purified using another agar plate of the same culture medium.

Physiological characteristics of isolates.

Isolates were examined using Gram staining and catalase activity as a tools for identification (Hammes *et al.,* 1992). Gram staining procedure was applied then under light microscopy gram positive of isolates were determined (Devriese *et al.,* 1992). For catalase reaction fresh liquid cultures were used by dropping hydrogen peroxide solution onto 1 ml of cultures. Negative, which didn't give gas bubbles, were choose.

Molecular identification

Bacterial identification by polymerase chain reaction (PCR) and DNA Sequencing of 16S rRNA gene. DNA was extracted according to DNA extraction

kit protocol with some modifications, as described by (Ward and Downie, 2005). While PCR amplification conducting by two universal primers 28F 5'AGAGTTTGATCCTGGCTCAG- 3' (positions 8-28 in *E. coli* numbering) and 1512 R 5'ACGGCTACCTTGTTACGACT-3' (positions 1512-1493 in *E. coli* numbering) were used as described before (Hassan *et al.*, 2014).

PCR amplified DNA segments were separated by electrophoresis in 0.8% agarose gel and visualized using ethidium bromide. Product size was determined by comparison with a DNA 1 KB ladder. Products were purified using QIA quick PCR Purification Kit according to the manufacturer's instructions. The purified products were then run on 0.8% agarose gel to verify the purification.

Sequencing and database search

DNA sequencing was done by faculty of pharmacy (a commercial service provider, Egypt). The Gen Bank database (NCBI, USA) was then used to search for 16S rRNA sequence similarities.

Morphological examination

Cellular morphology of isolated LAB strains were identified using SEM at Cairo University, Faculty of agriculture. Bacterial cells cultivated overnight in MRS media broth according to (Elzeini *et al.,* 2017). Samples were examined at 10 - 25 KV through Scanning Electron Microscope.

RESULTS AND DISCUSSION

Data illustrated in Table (1) showed that, Gram positive stain and negative catalase activity of isolated bacterial strains. It was mentioned that after 48 h under microaerophiles incubation on MRS agar, the colonies of strain 1 was generally grayish-white or beige in coloration and circular with smooth surface, while strain 2 appeared characteristically milky white and rounded with smooth colonies.

Table 1. Characterization of LAB isolated from fresh clover honey.

Bacterial strains	Strain 1	Strain 2
Gram staining	+	+
Catalase test	-	-
Colony morphology	Beige color, circular with smooth Surface	Milky white colonies, rounded and smooth
Characters	slender rods to short non motile and non sporulated	rod shaped non motile and non sporulated

Phylogenetic identification

Two isolates of LABs, designated as strain 1 and 2 were successfully isolated from fresh clover honey. Genomic DNA isolated from the isolates was amplified using 16S rRNA gene. The size of amplified product of PCR for all the isolates was approximately 1500 bp. After removing the vector backbone and identification of forward and reverse primer sequences, the data showed 1221 and 1349 base pairs for strains 2 and strain5, respectively.

The Gen Bank database (NCBI, USA) was then applied to search for 16S rRNA sequence

As shown in Table (2), the Isolate No. 1 was identified molecularly as *Lactobacillus brevis* and the gen bank number is MK250013, whereas isolate No. 2 was identified as *Lactobacillus casei* and the gen bank number is MK250003. Furthermore, the percentage of maximum identification of *Lactobacillus brevis* and *Lactobacillus casei* were 96 and 92 %, respectively.

Table 2. Identification 16S rRNA gene sequences generated from isolates.

Isolated strains	Gen bank number	Sequence lengths (bp)	Genus/species from Gen Bank database	% of Max. Ident
Strain 1	MK250013	1221	*Lactobacillus brevis*	96%
Strain 2	MK250003	1349	*Lactobacillus casei*	92%

The results obtained in fig. 1. illustrated the partial sequences for 16S rRNA gene of each isolate, the similarities of the closest type of isolates are observe as percentage. Taxonomic relationship was recognized by comparing the sequence in the National Center for Biotechnology Information (NCBI). *L. brevis* strain MK250013 gave a 96% gene sequence similarity, whereas second strain was predominated by the formerly described *Enterococcus* sp. this species of *Enterococcus faecalis* were reportedly with 85% - 93% gene sequence similarities, and *L. casei* MK250003 with 92%.

Topographical images obtained using (SEM) analysis the cell of the isolated strains of LAB, as shown in Fig. (2A). with *L. brevis* was aligned like bricks in a wall and observed in aggregation cells, while in Fig. (2B). with *L. casei* the cell was oval shape in random separated.

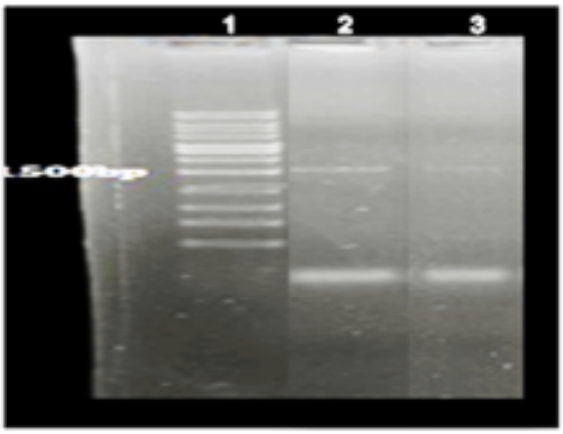

Fig. 1. A Photo showing the detected PCR products on gel electrophoresis for amplified segments of 16S rRNA gene. Lane 1: 1Kbp ladder, lane 2 and 3: amplicons of 16S rRNA gene by isolated strains 1 and 2, respectively.

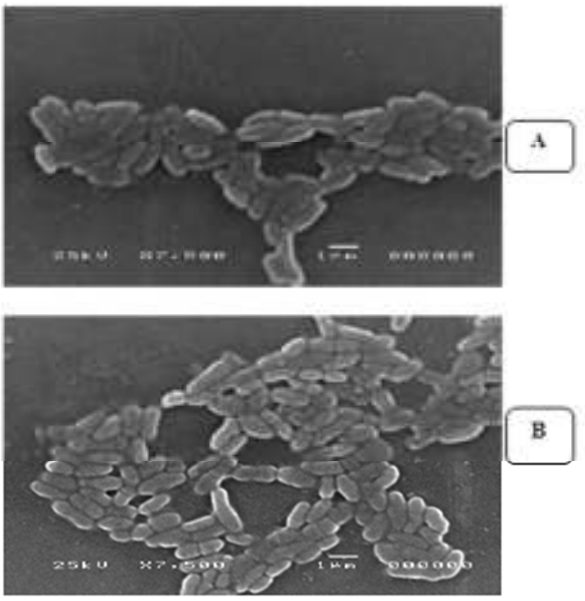

Fig. 2. The scanning electron microscope (SEM) for isolated strains from fresh clover honey, (A) is the Lactobacillus brevis, (B) is the Lactobacillus casei.

The results of the present research have demonstrated that, the honeybee and beehives are rich and effective sources for novel strains of LAB, and the floral within the honeybee showed one or perhaps two LAB phylotypes, differed in numbers compared to the honey stomach LAB flora found in honeybees in which eight different *Lactobacillus* and four different *Bifidobacterium* phylotypes were isolated. The results are in agreement with those reported by Sobrun, *et al.,* 2012 and Olofsson and Alejandra, 2015. However, Olofsson and Vásquez, 2008 and Vásquez *et al.,* 2009 reported that, the newly discovered lactic acid bacteria living in the honey stomach of honeybee is probably honeybee symbionts that have evolved together with the honey bee which protects the production of honey from spoilage microorganisms during its transformation from nectar to honey, this process that can take days to reduce the water content from 80 % in the nectar to below 20% in the ripened honey. Rabadjiev *et al.* (2015) mentioned that, there were 14 lactic acid bacterial strains isolated from all intestinal tracts of honeybee. According to Mrazek *et al.* (2008) the influence of geographic location, season, age, and part of the digestive tract on the bacterial diversity of the intestinal microflora of honeybees. As well as the nutrition habits were the main factor affecting the honeybee microflora.

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استكشاف وتعريف بعض سلالات بكتريا حامض اللاكتيك المعزولة من عسل نحل البرسيم الطازج

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يحتوي عسل النحل على مجموعه من البكتريا المتخصصة والتي تلعب دورًا مهمًا في عملية التمثيل الغذائي ، المناعة ، النمو والتطور ، والحماية من المسببات المرضية. لذا ، فإن الهدف من تلك الدراسة هو استكشاف وتحديد بعض السلالات الجديدة من بكتيريا حمض اللاكتيك المعزولة من عسل البرسيم الطازج. حيث قد تم تحليل عدد خمس عينات من عسل البرسيم الطازج للكشف عن محتواها البكتيري. وقد تم استخدام طريقة التخفيف والاستزراع في الوسائط الانتقائية لحساب سلالات LAB ، وتم تحديد تلك العزلات من خلال خصائصها المورفولوجية التي تم قياسها باستخدام طريقة الصبغ بجرام وكذلك اختبار نشاط إنزيم الكتاليز. وعلاوة على ذلك، فقد تم تحديد المورفولوجيا الخلوية لكل سلالة بكتيرية معزولة باستخدام المجهر الإلكتروني الماسح (SEM). كما تم تنفيذ النمط الجيني للسلالات المعزولة من خلال طريقة تفاعل (PCR). وأظهرت النتائج أن نوعين من السلالات البكتيرية الجديدة من LAB تم عزلها من عسل نحل البرسيم الطازج. وكانت السلالات المعزولة كالتالي؛ السلالة رقم 1 هي *Lactobacillus brevis* MK250013 والسلالة رقم 2 هي *Lactobacillus casei* MK250003.