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Identification of *Enterococcus* bacteria in gastrointestinal tract of dwarf honey bee, *Apis florea* Fabricius, 1973 (Hymenoptera: Apidae)

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Abstract
Apis species that engage in symbiotic association with Lactic Acid Bacteria (LAB), have diverse functions on their hosts. This study was intended to isolate and identify *aeoccus* bacteria living in the gastrointestinal tract of Asian dwarf honey bee, *Apis florea*, in Iran. One hundred isolates were Gram-stained and tested for catalase reaction. By using bacterial universal primers, the 16S rDNA gene of bacterial colonies was amplified. 16S rDNA genes from thirty bacteria were sequenced. Phylogenetic analysis showed that *Enterococcus* flora in the gastrointestinal tract of *A. florea*, contained five phenotypes which classified in the species *E. faecium*, *E. faecalis* and *E. hirae*. Based on the specific association between bacteria and *A. florea*, we divided the Asian dwarf honey bee populations into four categories.
Keywords: *Apis florea*, *Enterococcus* bacteria, LAB, symbiotic association

شناسایی باکتری‌های انتروکوکوس ازدستگاه گوارش زنبور عسل کوچک *Apis florea* Fabricius, 1973 (Hymenoptera: Apidae)

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چکیده
باکتری‌های لاکتیک اسید با گونه‌های مختلف جنس *Apis* روابط همزیستی دارند که می‌توانند اثرات زیادی بر روی میزبانشان داشته باشند. در این پژوهش ۱٤۰۰ زنبور عسل کارگر مربوط به ۱٤ کلنی از استانهای سیستان و بلوچستان، کرمان، هرمزگان، فارس، کهکیلویه و بویر احمد، بوشهر، خوزستان و ایلام جمع آوری گردید. نمونه‌ها در لوله‌های استریل حاوی نرمال سالین قرار داده شد، سپس جداسازی باکتری‌ها از دستگاه گوارش زنبور عسل کوچک با استفاده از محیط کشت‌های اختصاصی انجام گرفت. برای شناسایی، تست‌های بیوشیمیایی و سپس استخراج DNA کلنی‌های جدا شده انجام شد. تشخیص مولکولی کلنی‌ها با روش تعیین توالی ژن 16S rDNA و با استفاده از پرایمرهای اختصاصی انجام گرفت. پس از تعیین توالی و شناسایی نمونه‌ها، با به کار بردن نرم افزارهای مختلف ژنتیکی باکتری‌هایی که با هم از نظر ژنتیکی فاصله داشتند در بانک جهانی ژن (NCBI) ثبت گردید. در این آزمایش با تعیین توالی ۳۰ کلنی از باکتری‌های جداسازی شده از دستگاه گوارش زنبور عسل کوچک، در مجموع ۵ سویه مختلف متعلق به ۳ گونه *Enterococcus faecalis*، *Enterococcus faecium* و *Enterococcus hirae* شناسایی شدند. با توجه به نتایج بدست آمده زنبورهای عسل کوچک در ایران از لحاظ باکتری‌های انتروکوکوس موجود در دستگاه گوارش به ٤ گروه طبقه‌بندی شدند. به طور کلی جداسازی و شناسایی این باکتری‌ها می تواند قدم مثبتی در جهت افزایش درجه سلامت مواد غذایی مورد استفاده انسان‌ها و حیوانات باشد.
واژگان کلیدی: زنبور عسل کوچک، باکتری‌های انتروکوکوس، باکتری‌های لاکتیک اسید، رابطه همزیستی

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Introduction

Symbiotic bacteria undertake numerous roles such as pH adjustment, vitamin biosynthesis, plants material degradation, mineralization, organic compounds recycle, methane production, nitrogen fixation, pheromones production, chemical decomposition, lignin and cellulose degradation and preventing pathogen colonization and some rare cases induce disease (Engel and Moran, 2013; Hooper *et al*., 2012). Based on previous studies, endosymbiotic microorganisms associated with insects are classified into two groups: obligate endosymbionts (termed primary endosymbiont) and accessory endosymbionts (termed facultative or secondary endosymbionts). The main function of primary endosymbionts is to supply host insect with the essential nutrients such as amino acids, vitamin B which are lacking in their diets. Secondary endosymbionts enhance the resistance of host insect to heat stress and attacks from parasitoids and pathogens in addition to their nutritional functions (Kerry *et al*., 2010). Feeding plays a key role in the diversity of symbiotic bacteria in bees, which is also affected by climate conditions and flora (Waldan *et al*., 2016).

Probiotics are live micro-organisms that adjust microbial balance in the host's intestine and prevent pathogen growth and colonization (Castro *et al.*, 2016). Among the probiotic micro-organisms, LAB, which are the most common microbes hired as probiotics, are found in honey and another honey bee's product. Probiotic bacteria boost natural microflora in host's intestine and control pathogens' population so that reduce risk of food poisoning (Tajabadi *et. al.* 2011). *Enterococcus* is a major genus of LAB. The common occurrence of *Enterococcus* bacteria might be due to their resistance to growth inhibitor factors such as, acidity, salinity, drought, temperature and chemical disinfectant (Franz *et al*., 2011).

Iran, with different types of climates and ecosystems, encompasses 11 different climates out of 14 climates, is a suitable place for studying symbiotic bacteria (Tajabadi *et al*., 2010). Out of nine described honey bee species, the species *Apis florea* F. and *Apis mellifera* L. are known from Iran. *Apis florea* or dwarf honey bee is one of the most important pollinators in southern provinces of Khuzestan, Bushehr and Sistan and Balouchestan (Parichehreh, *et al*. 2013).

This study was conducted to isolate and identify *Enterococcus* bacteria from the dwarf honey bees' gastrointestinal tract and enrich the Bacterial Bank for further studiestowards improving the immune system through production of probiotic diets.

Material and methods

From 14 different southern regions of Iran (Fig. 1), 100 worker honeybees of *A. florea* (per each location), were collected during April to March 2015 and maintained in sterile

glass tubes containing 10 ml normal saline (Tajabadi *et al.* 2011). Thirty samples from each colony were individually dissected on a petri dish and whole gastrointestinal tract was collected using aseptic excision under luminal flow (Olofsson and Vasquéz 2008).

Fig. 1. Collection sites of workers of the honeybee species, *A. florea*(1: Iranshahr (N27º20'84", E60º68'76"), 2: Jiroft(N28º67'32" , E57º72'99"), 3: Kahnuj (N28º94'34" , E57º70'04"), 4: Rudan (N27º20'40" , E55º95'16"), 5: Bandar Abas (N27º26'09" , E56º41'75"), 6: Qeshm (N26º86'16", E55º99'30"), 7: Fasa (N28º93'78" , E53º63'12"), 8: Jahrom (N28º51'11" , E53º57'74"), 9: Bushehr (N28º90'73" , E50º83'75"), 10: Kangan (N27º20'84" , E50º68'76"), 11: Gachsaran (N30º35'78", E50º80'36"), 12:Behbahan (N30º60'58" , E50º21'78"), 13: Ahvaz (N31º59'64" , E48º83'77"), 14: Dehloran (N32º60'54" , E47º27'39")

Culture method and biochemical screening

Enterococcus culture and isolation followed the methods described by Tajabadi *et al*. (2011). Ten percent of honey stomach solutions was prepared in normal saline, and Enterococci was isolated from the honey stomachs in MRS (DE Man, Rogosa, and Sharpe) agar medium (Oxoid). The isolates were incubated for 3-4 days at 37 ºC under anaerobic conditions using anaerobic jars with anaerocult A gas packs (Merck, Darmastadt, Germany). To obtain pure bacterial isolates, we sub-cultured 100 colonies with different morphological features following the method by Olofsson and Vasquéz (2008).

DNA extraction

DNA extraction kit protocol (QIAGene) and the modified of Ward *et al*. (1994) were used to extract DNA. DNA samples were dissolved in 150 µl of double-distilled water and

stored at -24 ºC. The DNA quality was tested by running the samples on 1 % agarose gel and the DNA purity was quantized using spectrophotometer by recording absorbance ratios at 260 and 280 nm.

PCR and program

The 16SrDNA gene (1500 bp) was amplified using 27F and 1492R primers (5'AGAGTTTGATCCTGGCTCAG-3' and 5'GGTTACCTTGTTACGACTT-3', repectiely), (Lane, 1991) targeting the genus level of *Enterococcus*. Standard PCR reaction was conducted in a final 20 µl reaction volume which contained 0.1 µl Pfu DNA polymerase, 2 µl Pfu DNA polymerase buffer, 1.5 µl $MgCl_2$, 0.25 forward and Reverse primers and 1 µl DNA template. Deionized water (14.9 µl) was added to final volume of 20 µl (Tajabadi *et al.* 2013). To conduct PCR, initial DNA was denaturized for 3 min at 95°C for 1 cycle, 40 cycles of 95°C for 30s, 55°C for 30s, 72° C for 1min and a final extension cycle of 72°C for 10min (Tajabadi *et al.* 2013a). Five µl PCR products were electrophoresed on 1% agarose gel sustained with ethidium bromide (Fig. 2). Using QIA quick PCR purification kit (QIAGEN, Hilden, Germany), PCR products was purified. The purified PCR products were obtained from different isolates and sequenced using 27F and 1492R primers by Sequencing Company (Macrogen, South Korea). Determined sequences were compared directly with all 16S rDNA sequences registered in GenBank using BLASTN, at NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analysis was conducted by neighbor-joining method using the program Mega 4 (Tamura *et al.* 2007).

Reference sequences used in phylogenetic analysis

The following bacterial 16S rDNA gene sequences were tested as out groups in phylogenetic analysis: *Enterococcus faecalis* AY (653231), *Enterococcus faecium* DO (017960), *Enterococcus faecium* CA12 (GU122154), *Enterococcus faecium* AY (653231), *Enterococcus durans* WR2 (GQ421476), *Enterococcus* sp. TAJ-KS29 (HM027646), *Enterococcus hirae* SS1227 (GQ337029), *Enterococcus hirae* ATCC9790 (015845), *Enterococcus hirae* (Y17302), *Enterococcus* sp. Taj-KH5 (HM027646), *Enterococcus* sp. *Taj-KS29* (HM027647) (Cluster I in Fig.3), *Enterococcus faecalis* AB (154827), *Enterococcus faecalis* V583 (004686), *Enterococcus faecalis* (Y18293) (Cluster II in Fig. 3).

Results

In order to perform limited biochemical tests, 100 developed colonies were selected from MRS plates. Thirty colonies were sequenced and subjected to phylogenetic analysis. The isolated strains exhibited very high similarity (99%) to three closest database

sequences deposited in NCBI (Table 1). According to phylogenetic analysis, five different phenotypes, belonging to three different species including *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus hirae*, formed *Enterococcus* flora found in gastrointestinal tract of dwarf honey bee (*A. florea*). Three phenotypes were associated to *Enterococcus faecium*, (Clusters I in Fig. 3 and Table 1) and one phylotype related to *Enterococcus faecalis* (Cluster II in Fig. 3 and Table 1). Furthermore, *Enterococcus hirae* was clustered in cluster III with a sequence similarity level of 99% compared with *Enterococcus hirae* ATCC9790 (Fig. 3 and Table 1).

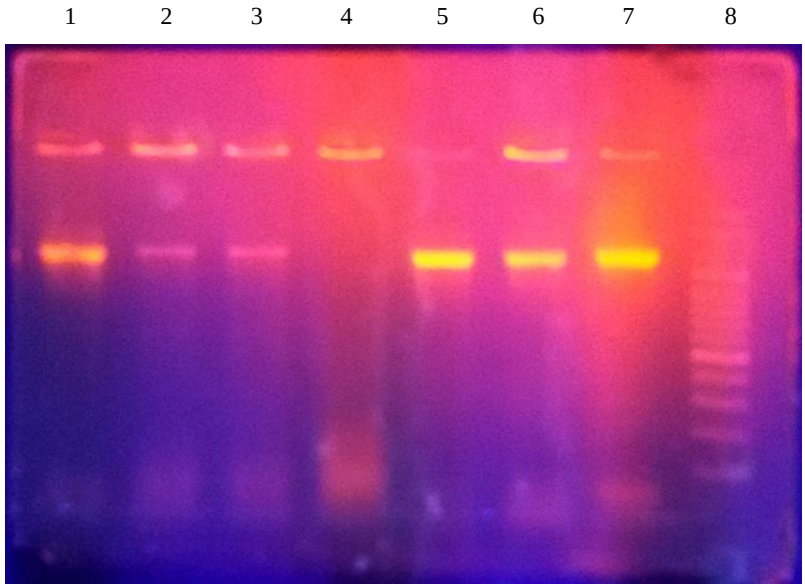

Fig 2. 16S spacer region of *Enterococcus* bacteria amplified by PCR from various locations using primer pair 27F and 1492R and separated in a 1% agarose gel. Lane 1- 7 PCR products from bacterial isolates; Lane 8 Molecular marker 100 bp

The results suggested that *Enterococcus faecium*, the most common bacteria in *Apis* species, was associated with *A. florea* populations of Roudan, Bandar-Abbas, Iranshahr and *Enterococcus faecalis* was found in the gastrointestines of bees of Gachsaran. *E. hirae* was isolated from Qeshm population. The bees in Behbahan, Dehloran, Jahrom, Bushehr, Kangan, Ahvaz, Jiroft, Kahnouj and Fasa had no *Enterococcus* bacteria.

The nucleotide sequence of *Enterococcus* 16S rDNA gene was deposited at NCBI website under the accession numbers of KU359948, KU359949, KU359950, KU5994351, and KU359952.

Discussion

The insect's intestinal tract is a rich source of nutrients that contains indigenous LAB populations which are also known as important symbiotic bacteria (Dillon and Dillon,

2004). *Lactobacilli, lactococci, leuconostocs, enterococci, streptococci* and *bifidobacteria* have been isolated from insects (Kacaniova *et al*., 2004; Pidiyar *et al*., 2004) and widely used in food industries (Dillon and Dillon, 2004) as probiotic bacteria for improving food quality and human health and wellbeing (FAO, 2002). LAB assists in decomposition and detoxification of non-digested food,protects insects from the invasion of intestinal pathogens (similar to probiotic strains in humans and animals) and produces vitamins or forms complex interactions with the immune system of the host (Dillon and Dillon, 2004; Kacaniova *et al*., 2004). Traditionally, culture-based approaches were used to isolate and identify these numerous microorganisms (Gilliam, 1997). Comparison between honey bees' microbial flora and mammals' microbial flora indicated that all symbiotic bacteria found in honey bee's gastrointestinal tract can be cultured in laboratory conditions (Olfosson *et al*., 2011; Engel *et al*., 2013; waldan *et al*., 2016). Therefore, culture method can be considered as a reliable method to identify all symbiotic bacteria in honey bees. Recently, culture independent 16S rDNA gene sequences analyses have been used to study the community based on single-strand conformation polymorphism (waldan *et al*., 2016). We employed both classical cultivation procedures and 16S rDNA sequencing to study the bacterial diversity and phylogenetic relationships of *Lactobacillus* housed in the gastrointestinal tract of *A. florea.*

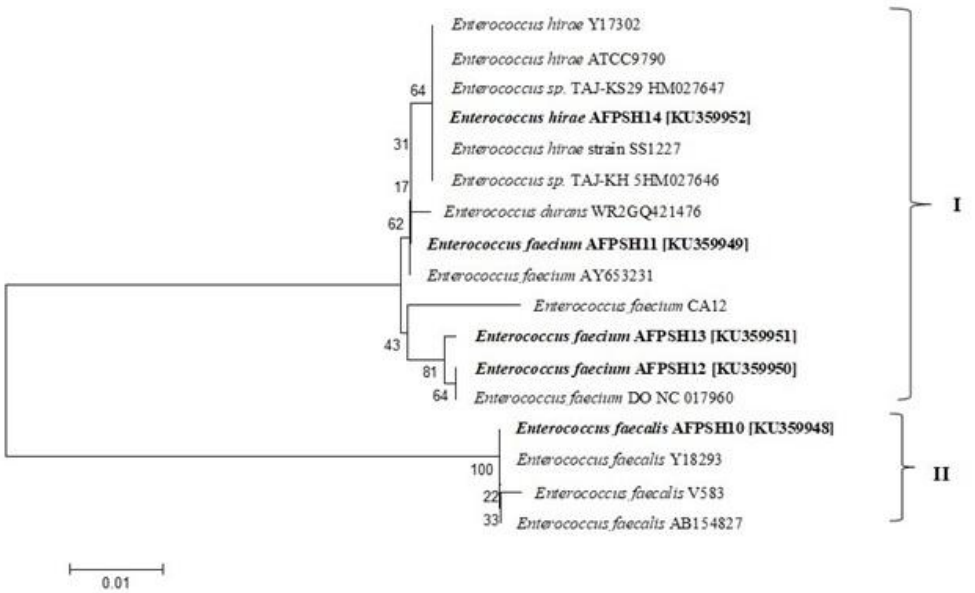

Fig 3. Phylogenetic analysis of *Enterococcus* bacteria housed in *Apis florea* distributed in Iran. Phylogenetic tree based on a distance matrix analysis of 1,275 positions in the 16S rDNA gene. The phylogenetic tree was constructed by ClustalW using the neighbor-joining method within the MEGA (4) package (Tamura *et al*. 2007).

Our phylogenetic analysis showed that the species *E. faecium, E. faecalis* and *E. hirae* which are associated with *A. florea*, occur in different parts of of Iran. We successfully isolated the species of *Enterococcus* associated with A. *florea* similar to *Lactobacillus* isolations from *A. florea* (Parichehreh *et al*., unpublished data). Six out of 14 populations harbored *Enterococcus* bacteria. *Enterococcus faecium* was present in three populations including Roudan, Bandar Abbas and Iranshahr and found to be the most common bacterium in dwarf honey bee. Bauer *et al*, (2000) and Hirose *et al*., (2006) isolated this bacterium from termites and true bugs respectively. *E. faecalis* was isolated only from bees in Gachsaran region.. The *E. hirae* was isolated from bees in Qeshm region. Phylogenetic analyses showed that *E. faecium* and *E. hirae* , belong in a clade and are closely related. Ibarguren and *et al*. (2010) who studied the bacteria flora of *Apis mellifera* from Argentina argued that there were four *Enterococcus* bacteria in honey combs and feral combs, although Olfosson and Vasquez (2008) were unable to detect these bacteria in honey stomach and fresh honey. Tajabadi *et al*., (2013) reported four strains of *Enterococcus* bacteria from giant honeybee *Apis dorsata*. Honey bee's microbial diversity in relation to geographical locations and temporal patterns, was investigated by Hroncova and *et al*. (2015), whom discovered that most of isolated microbiota were related to gram positive bacteria that is similar to our findings.

Table 1. Sequenced identified in *Apis florea* gut with accession number of the genes in NCBI

Accession number	Gene size and similarity %	The most similar bacteria	No. of isolated and identified bacteria	Isolated bacteria
KU359948	(1200) 99%	*Enterococcus faecalis* V583	(6)	*Enterococcus faecalis* AFPSH10
KU359949	(999) 99%	*Enterococcus faecium* DO chromosome	(8)	*Enterococcus faecium* AFPSH11
KU359950	(1048) 99%	*Enterococcus faecium* DO chromosome	(5)	*Enterococcus faecium* AFPSH12
KU359951	(1188) 99%	*Enterococcus faecium* DO chromosome	(5)	*Enterococcus faecium* AFPSH13
KU359952	(996) 99%	*Enterococcus hirae* ATCC9790	(6)	*Enterococcus hirae* AFPSH14

Based on these endosymbiotic patterns, we divided the populations into four groups 1) Roudan, Bandar Abbas and Iranshahr 2) Gachsaran 3) Qeshm and 4) Dehloran, Ahvaz, Jahrom, Fasa, Kahnouj, Jiroft, Bushehr and Kangan. These results showed that the *Enterococcus* has a modest fauna in *Apis florea*. Regions with lower latitude (Fig. 1) were classified in a same group (first group) and *E. faecium* was isolated from gastrointestinal of bees which were collected from these regions. Therefore, it can be concluded that there is a positive correlation between *E. faecium* distribution and latitude. Morphological characteristics of dwarf honey bees, collected from southern regions of Iran, were studied by Tahmasebi *et al*. (2002) who divided the bees into two different groups, western and

southwest bees distributed at higher latitudes and south and southeast bees distributed at lower latitudes. Our results agree with those reported by Tahmasebi *et al.* (2002). In addition, *E. hirae* was isolated only from isolated region of Qeshm due to its different climate conditions, specific vegetation and lower latitude. Overall, it can be concluded that vegetation, climate conditions and latitude have a considerable effect on *Enterococcus* bacteria diversity in dwarf honey bees' gastrointestinal. Based on this fact that each region has diverse flora, we could hypothesize that *Enterococcus* diversity might be due to differences in nutrient content of nectar and pollen or microbes found on the flowers of each regions. Temporary floral microbes may stimulate resident LAB micro-biota growth in bees and activate antimicrobial substances production.

Enterococcus faecium is mainly used as an animal probiotic and *E. faecalis* as a human probiotic. *Enterococcus faecium* differs from *E. faecalis* in its growth requirements and metabolism. It requires folic acid for growth and is unable to derive energy from pyruvate, citrate, malate, gluconate and serine (Aarestrup *et al*., 2011). Some strains of LAB may increase the safety and quality of fermented products due to production of different antimicrobial compounds, which can prevent the growth of pathogenic and spoilage bacteria. Antimicrobial metabolites of LAB include organic acids, hydrogen peroxide, diacetyl and additional metabolites called bacteriocins. Bacteriocins are ribosomally synthesized antimicrobial proteinaceous compounds doted of general bactericidal activities, often toward bacteria closely related to the bacteriocin producing strain (Cintas *et a*l., 2001).

Our study led to the isolation of *E. faecium, E. faecalis and E. hirae* from the stomach of honey of *A. florea* from in different areas of Iran. Further researches are required to identify new isolates of native LABs with commercial properties. These results can be documented in a bacteria bank for the future.

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