Journal of Entomological Research
Volume 12, Issue 3, pages: 13-28

Islamic Azad University, Arak Branch
ISSN 2008-4668
http://jer.iau-arak.ac.ir

Identification of the bacterial microflora of fresh edible yellow mealworm larvae (*Tenebrio molitor* L.)

S. Mohammadsalim[1], S. Imani[2], M. Abdigoudazi[3], N. Hasanzadeh[2], V. Zarrinnia[2]*

1- Ph. D. Student of Entomology, Department of Plant Pathology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
2- Respectively Assistant Professor and Associate Professor, Department of Plant Pathology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
3- Associate Professor, Department of Parasitology, Razi Vaccine and Serum Reasearch Institute, Agricultural Reasearch, Education and Extension Organization-AREEO, Alborz,Iran.

Abstract

The edible insects are consumed as an alternative animal protein source by most Asian countries. Since there is a limited evidence for their safety particularly from microbiological aspects, an attempt was made to assess the microbial population of *Tenebrio molitor* L. samples in laboratory conditions. Primary stocks were purchased from a local market, in Sari- a city in north of Iran. Microbial samples were prepared from both body surfaces and guts of insect. Samples were individually and cultured on nutrient agar (NA), incubated at taken 27^0C for 24-72h. Distinguished colonies were isolated and purified. Based on phenotypic characteristics, hypersensitive *response (HR)* on geranium leaves, as well as 16S rRNA gene sequencing analysis, the isolates were categorized into two groups. The pathogenic isolates were identified as *Bacillus cereus, Staphylococcus* sp, *Pseudomonas aeruginosa, Enterobacter asburiae*, *Bacillus firmus* and *Serratia marcescens*. Whereas, the nonpathogenic bacteria were assigned as *Enterobacter cloacae* and *Bacillus thuringiensis*. Undoubtedly, the presence of pathogenic microbes in the microflora of mealworm larvae by direct and indirect consumption of insect may pose a threat to human and animal health. These findings suggest an implementation of certain processing methods in order to decrease or eradicate risks of microbial contamination of diets using natural insects.

Keywords: Edible insect, Food, *Tenebrio molitor,* bacterial diversity,16S rRNA gene.

* Corresponding Author, E-mail: *Drsohrabimani@gmail.com*
Received: 8 July. 2020 – Accepted: 23 Sep. 2020

Introduction

Insects have played an important role in human and animal nutrition, particularly in poor countries (MacEvilly, 2000; Van Hius, 2013; Dobermann *et al.*, 2017). Recently, using edible insects as a source of protein has been suggested by the FAO. Edible insects can be a valuable alternative source of conventional animal proteins (Van Huis *et al.*, 2013). Many of the edible insect species use for as a food by human and animals like grasshoppers, caterpillars, beetles, locusts, grubs, termites, bees, wasps, crickets and others (Yen, 2009). In recent years many studies have foccused on the nutrient composition of edible insects (Rumpold & Schlüter, 2013; Van Huis *et al.*, 2013). Reports indicated that edible insects contain a valuable source of proteins, lipids, carbohydrates, fibers, vitamins and minerals (Mlcek *et al.*, 2014).

The larvae of the yellow mealworm, *Tenebrio molitor* L. (Coleoptea: Tenebrionidae) have been used widely as food in Asia (especially in Iran), Africa, United States, and Europe. Fresh larvae of the mealworm contains 56% water, 18% protein, 22% fat and 1.55% ash (Siemianowska *et al.*, 2013). However, in western countries the consumption of insects as food is still very disgusting and many influence of consumers health (Van Huis, 2013; Yen, 2009). Both insects in nature and rearing places may be infected with many pathogenic groups of microoranism, including bacteria, virus, fungi, yeast and others (Vega & Kaya, 2012).

There are few documented food safety cautions known for edible insects. These are nutritional composition, microbial diversity and safety and toxicological with respect to pesticide residue and heavy metals. Consumer health is a serious concern and aspects the consumer may have, allergic, toxic and other anti-nutrient symptoms (Klunder *et al*., 2012; Belluco *et al.*, 2013; Van der Spiegel *et al.*, 2013; Rumpold *et al*., 2014; Van Huis *et al.,* 2015, Dobermann *et al*., 2017). However this can be distinct for insects can be eaten raw or other forms of consumption including cooking, boiling, roasted or fried (Ogbalu, 2015).

Edible insects as high nutritional food sources provide favorable conditions for microbial growth and survival (Klunder *et al.*, 2012). Depending on processing methods and storage conditions (Belluco *et al.*, 2013). Some endospore-forming bacteria, Gram-positive and negative bacteria as well as many yeasts, fungi and molds can be survived even during industrial processing. Hence, fresh or processed insects may contain many microorganisms on the body surface and inside the intestinal tract, such as bacteria and fungi. In conclusion, proper processing methods should be devised to food safety (Grabowski *et al*., 2014).

Since, there is no published report on microbial aspects of edible insects in Iran. Mealworm (*T. molitor*) is currently present in our country as storage pest and can be reared on low-nutritive products and mealworm larvae have been introduced as livestock and human food. The aim of this research was to determine the microbial flora of gut and body surface of fresh larvae of mealworm.

Material and Methods

Insect Samples

The yellow mealworm were purchased from a local market, in Sari- North of Iran. The insects were kept in darkness at 27ºC and 55± 5% RH in plastic containers containing wheat bran and pieces of carrot as a source of food and water. Ten grams of fresh living larvae were taken and the whole body were surface-sterilized with 70% ethanol for approximately 30 seconds to remove any surface contaminants. The samples were taken out from the ethanol solution and washed three times with sterile distilled water.

Then, guts of larvae were pulled out with two sterile forceps under laminar airflow hood. The guts were transferred and homogenized aseptically into a sterile distilled water. Each larval suspension was streaked on nutrient agar (NA) (Merck, Germany) medium. Meanwhile, sterilized and non-sterilized intact bodies of mealworm were also placed on petri plates containing NA medium to isolate the bacteria on their surface. Culture plates in triplicates were incubated at 27ºC and incubated for 24-72h. The dominant colonies with different colony characteristic were purified and sub-cultured on NA slants for further characteristic analysis (Banjo *et al*., 2006; Saidi *et al*., 2016).

Characterization and Colony Counts of the Bacterial Isolates

Bacterial isolates were further characterized by microscopic and key morphological, physiological and biochemical tests (Cheesbrough, 2000). These were included: gram and spore staining (Baker,1967), motility test (Humphries, 1974), fluorescent pigmentation on King's B medium (King et al., 1954), NaCl tolerance (Acharya, 2014), oxidative/fermentation glucose, catalase, methyl red (MR), Voges Proskauer (VP), nitrate reduction, oxidase (Olutiola *et al*., 1991; Murinda *et al*., 2002), starch, lignin and cellulose hydrolyses tests, citrate utilization (Harrigan and McCance, 1976; Ijong, 2003; André *et al*.,2013), levan production (Sangiliyandi *et al*., 1999) and arabinose fermentation test (Dickey, 1979; Stock *et al*.,2009).

Determination of microbial counts

0.1 ml of dilutions obtained from the samples were aseptically inoculated onto freshly prepared NA plates medium. The experiment was done in a triplicate and NA plates were incubated at 27ºC for 48 h. The number of bacteria colonies on each plate was counted using a hand lens. The total counts from the plates were obtained for all bacteria. The total viable cells of the sample expressed as colony-forming units per milliliter (CFU/ml) (Harrigan & McCance, 1990; Cheesbrough, 2000).

Pathogenicity tests

Pathogenicity tests were performed on geranium plants in triplicate. A fresh bacterial suspension with optical density corresponding to 1×10^7 CFU/ml were injected underside of the leaves. Sterile distilled water was used as a control. Plants were maintained in the greenhouse at 22-25°C until HR symptoms (necrotic lesions) were developed.

Antibiogram test

The **disk-diffusion method was used for antibiotics test.** A colony from each bacteria was suspended into 5 mL of distilled water. The bacterial suspension was uniformly distributed on agar plates by sterile swab sticks in triplicates. The disks utilized were Chloramphenicol (30 µg), Penicillins (10 µg), Amoxicillin (25 µg), Tetracycline (30 µg) and Azithromycin (15 µg) (Padtan Teb company). An antibiotic disk were applied to the surface of an agar plate containing the organism with sterile forceps. The seeded agar plates were allowed to absorb and incubated at room temperature for 24-48 h. The diameter inhibition zones (mm) were measured and recorded as resistant (R), intermediate (I) or sensitive(S) (Clinical and Laboratory Standards Institute, 2015).

Genomic DNA extraction and 16S rRNA gene PCR amplification

Bacterial DNAs of 14 strains were extracted by alkaline lysis method (Elboutahiri *et al.*, 2009). In breif, bacteria were grown in agar media at 28°C for 2 days. A loopfull amount of bacterial suspension was added freshly prepared lysis buffer containing 0.1 N NaOH and 0.5% SDS. The mixture was boiled in a water bath for 15 min and then subjected to centrifugation for 15 min at 13,000 rpm. The supernatant formed by the aqueous phase that contains clear and suspended DNA was transferred to new sterile Eppendorf tube and stored at 4°C. The bacterial 16S rRNAgene was amplified using the P1, forward (5′ATATATAAGCGGCCGCAG AAAGGAGGTGATCC-3′) and P6, reverse (5′-ATATATAAGCGGCCGCAGAGTTTGATCATGCC TC-3′) primers (Wenzel *et al.*, 2002; Ramin *et al.*, 2008).

The polymerase chain reactions (PCR) were performed in a total volume of 25 µl, using master mix, distilled water, primers, and isolated DNA. The PCR amplification was carried out in the PCR thermal cycler (Bio-Rad My cycler) using hot-start procedure. The PCR protocol utilized included 4 min at 94°C, followed by 35 cycles of 60 sec at 94°C, 45 sec at 60°C and 60 sec at 72°C. A last extension step was performed at 72°C for 10 min. PCR products were analyzed using 0.8 % agarose gel in 1x TBE gel buffer electrophoresis.

DNA sequencing and phylogenetic analysis

PCR products with one sharp bands were sent to Topaz Gene Research Company (Microsynth "The Swiss DNA Company", Switzerland) for sequencing. The sequences were then trimmed with Chromas V 2.6.6 and DNA Baser Assembler V5.15.0 and assembled with DNA Baser Assembler V 5.15.0. and compared with sequences deposited in GenBank database (http://www.ncbi.nlm.nih.

gov). Phylogenetic analysis was performed using the MEGA 6.0 program. The tree topologies were evaluated using bootstrap analysis based on 1000 replicates and phylogenetic trees were inferred using the maximum likehood method based on the Tamura-Nei model (Felsenstein, 1981).

Results

Characterization and Colony Counts of the Bacterial Isolates

The results obtained from the microbiological analysis of the larvae of *T. molitor* show that different bacterial hava existed both in body surface and gut of the fresh samples. The bacteria were identified based on certain morphological, biochemical tests and molecular analysis. The members of two bacterial phyla i.e. the Firmicutes (54.5% all sequences) and the Proteobacteria (45.4%) were most dominant group. Six genera of bacteria, namely, *Staphylococcus*, *Bacillus*, *Pseudomonas, Serratia*, *Cronobacter* and *Enterobacter* were identified (Table 1 and 2). These were not varied by seasonal sample collection and characterizations.

It was observed that the bacterial isolates in mealworm gut and body surface were mostly Gram-negative and Gram-positive, respectively. Bacterial isolates were identified by DNA *sequencing* of the 16S rRNA gene and were assigned in four families Staphylococcaceae, Bacillaceae, Pseudomonadaceae and Enterobacteriaceae. The two formers, were most prevalent bacteria agents. *Bacillus thuringiensis* and *Pseudomonas aeruginosa* had been counted the maximum colonies at body surface and *Staphylococcus Succinus* in gut of mealworm.

Microbial counts of fresh edible mealworm larvae are presented in Tables 1 and 2. Total bacterial counts for all bacterial strains in body surface and gut were ranged 2.34×10^5-2.14×10^5 CFU/ml. Some isolation of *Bacillus*, *Staphylococcus* and *Pseudomonas* were able to survive even after sterilization on the body surface. Bacilli and Staphylococcal species were found the most common in both gut and body surfaces.

Pathogenicity tests

The pathogenicity test was performed on healthy geranium plant. Symptoms appeared as necrotic lesions in all samples and in bacteria *Pseudomonas aeruginosa, Enterobacter asburiae*, *Bacillus firmus* and *Serratia marcescens*. The other bacterial isolates did not induce such reactions.

جدول ۱ -خصوصیات مورفولوژیکی و بیوشیمیایی ایزوله های باکتریایی در سطح بدن میل ورم

Table 1 Morphological and biochemical characteristics of bacterial isolates on mealworm body surface

Test (آزمون)	**(شناسایی گونه ها) Species identity**				
	Staphylococcus warneri	***Staphylococcus gallinarum***	***Bacillus thurigiensis***	***Bacillus firmus***	***Pseudomonas aeruginosa***
(شمارش)count(cfu/ml) Colony (کلنی)	**1.84×10^5**	**0.9×10^5**	**3×10^5**	**2.08×10^5**	**4×10^5**
(آزمون Gram reaction گرم)	+	+	+	+	-
Motility(حرکت)	-	-	+	+	+
Cell shape(شکل سلول)	**cocci**	**cocci**	**rod**	**rod**	**rod**
(رنگ Colony color کلنی)	**white**	**yellow**	**white**	**cream**	**cream**
(اسپورزایی) Spore formation	-	-	+	+	-
Heat test(حرارت)	-	-	+	+	-
(تستFluorescent on KB فلورسنت)	-	-	-	-	+
Levan(لوان)	-	-	-	-	-
6.5 % NaCl(تحمل شوری)	+	+	+	+	-
10 ٪NaCL	+*	+*	-	-	-
Oxidase(اکسیداز)	-	-	-	-	+
Catalas(کاتالاز)	+	+	+	+	+
Voges Proskaeur	+	-	+	-	-
Methyl red(متیل رد)	-	-	-	-	-
(احیای نیترات)Nitrate reduction	-	_	+	+	+
Acid from arabinose (اسید از قند آرابینوز)	+	+	-	+	+
(اکسیداسیون یا تخمیر گلوکز)O/F test	**F**	**F**	**F**	**F**	**O**
(مصرف سیترات) Citrate utilization	-	-	+	-	+
(هضم Starch hydrolysis نشاسته)	-	-	+	+	-
Cellulose hydrolysis (هضم سلولز)	-	-	-	-	-
Lignin hydrolysis (هضم لیگنین)	-	-	+	+	-
HR reaction (واکنش حساسیت)	-	-	-	++	++

+, Positive, -, Negative

+، مثبت، -، منفی

*Tolerate 16% NaCl

NaCl * تحمل شوری ۱۶٪

HR: Hypersensitivity Reaction on geranium; +: Weak; ++: Moderate; +++: Severe.

HR: واکنش حساسیت روی شمعدانی، +، ضعیف، ++، متوسط، +++، شدید

جدول ۲- خصوصیات مورفولوژیکی و بیوشیمیایی ایزوله های باکتریایی در روده میل ورم

Table 2 Morphological and biochemical of bacterial isolates on mealworm gut

Test (آزمون)	**Species identity**(شناسایی گونه ها)							
	Bacillus cereus	*Serratia marcescens*	*Staphylococcus Succinus*	*Bacillus thurigiensis*	*Pseudomonas mosselii*	*Enterobacter cloacae*	*Enterobacter asburiae*	*Cronobacter* sp.
Colony count(cfu/ml)(شمارش کلنی)	1.8×10^5	2.6×10^5	3.6×10^5	3.2×10^5	2.08×10^5	2×10^5	1.4×10^5	0.5×10^5
Gram reaction (آزمون گرم)	+	-	+	+	-	-	-	-
Motility(حرکت)	+	+	-	+	+	+	+	+
Cell shape(شکل سلول)	Rod	Rod	cocci	Rod	Rod	Rod	Rod	Rod
Colony color (رنگ کلنی)	White	White	White / yellow	White	Yellow	White	White /yellow	Yellow
Spore formation (اسپورزایی)	+	-	-	+	-	-	-	-
Heat test(حرارت)	+	-	-	+	-	-	-	-
Fluorescent on KB (تست فلورسنت)	-	-	-	-	+	-	-	-
Levan(لوان)	-	-	-	-	-	-	-	-
6.5 % NaCl (تحمل شوری)	+	+	+	+	+	+	+	+
10 ٪NaCL	-	-	+*	-	-	-	-	-
Oxidase(اکسیداز)	-	-	-	-	+	-	-	-
Catalas(کاتالاز)	+	+	+	+	+	+	+	+
Voges Proskaeur	+	+	-	+	-	-	-	+
Methyl red(متیل رد)	-	-	-	-	+	-	+	-
Nitrate reduction (احیای نیترات)	+	+	+	+	-	+	+	+
Acid from arabinose (اسید از قند آرابینوز)	-	-	-	-	-	+	+	+
O/F test (اکسیداسیون یا تخمیر گلوکز)	F	F	O	F	O	F	F	F
Citrate utilization (مصرف سیترات)	+	+	-	+	-	+	+	-
Starch hydrolysis (هضم نشاسته)	-	-	-	+	-	-	-	+
Cellulose hydrolysis (هضم سلولز)	-	+	-	-	-	-	-	-
Lignin hydrolysis (هضم لیگنین)	+	+	V	+	+	-	-	-
HR reaction (واکنش حساسیت)	+	++	+	-	-	-	++	-

+، مثبت، -، منفی

+, Positive, -, Negative

NaCl * تحمل شوری ٪۱۶

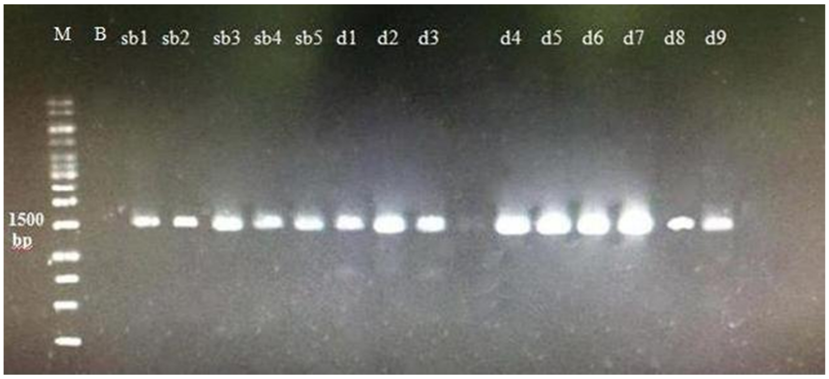
*Tolerate 16% NaCl

HR: Hypersensitivity Reaction on geranium; +: Weak; ++: Moderate; +++: Severe.

HR: واکنش حساسیت روی شمعدانی، +، ضعیف، ++، متوسط، +++، شدید

PCR amplification of 16S rRNA gene

All isolates were subjected to molecular identification using PCR amplification of almost the complete 16S rRNA gene. The size of the generated fragments was in 1.5 kb (Fig. 1).



عکس ۱- تصویر PCR ژن 16S rRNA باکتری های جدا شده از یک قطعه ژن واحد را نشان می دهد طول تقریباً ۱۵۰۰ جفت باز است .

خطوط sb1 تا sb5 (نمونه های سطحی بدن) و d1 تا d9 (نمونه های روده). M: خط کش DNA (100bp)، خط B: شاهد بعنوان کنترل منفی

Fig. 1 PCR amplification of the 16S rRNA gene from the bacterial isolates showing a single gene fragment approximately 1500 bp in length. Lanes sb1 to sb5 (Body surface samples) and d1 to d9 (Gut samples). Lane M: DNA size marker (Gene Ruler 100bp DNA ladder plus, Fermentas), Lane B, Blank as a negative control

DNA Sequencing and Constructing Phylogenetic Tree

All sequences were assembled by DNA Baser Assembler v5.15.0 and compared with the accessions deposited at the 'National Center for Biotechnology Information' (NCBI) database (www.ncbi.nlm.nih.gov)(Table 3).

جدول ۳- کد دسترسی توالی های حاصل از گونه های باکتریایی و ثبت شده درNCBI

Table 3 Accession numbers of the sequences obtained from bacterial species and deposited in NCBI

Samples (نمونه ها)	**Identity of isolates** (ایزوله شناسایی شده)	**Accession No** (کد دسترسی)	**Strain Code** (کد استرین)	**Origin** (ناحیه)
S1-1	*Pseudomonas aeruginosa*	MK956162	Sb1	B
S1-2	*Bacillus firmus*	MK956163	Sb2	B
S1-3	*Bacillus thurigiensis*	MK956164	Sb3	B
S1-4	*Staphylococcus gallinarum*	MK956165	Sb4	B
S1-5	*Staphylococcus warneri*	MK956166	Sb5	B
D3-1	*Staphylococcus succinus*	MK956134	D1	G
D3-2	*Serratia marcescens*	MK956135	D2	G
D3-3	*Bacillus cereus*	MK956136	D3	G
D3-4	*Cronobacter* sp.	MK956137	D4	G
D3-5	*Enterobacter asburiae*	MK956138	D5	G
D3-6	*Enterobacter cloacae*	MK956139	D6	G
D3-7	*Pseudomonas mosselii*	MK956140	D7	G
D3-8	*Bacillus thurigiensis*	MK956141	D8	G
D3-9	*Staphylococcus succinus*	MK956142	D9	G

G, Gut; B, body surface.

G، روده، B، سطح بدن

The phylogenetic tree was constructed using the 16S rRNA gene sequences of the bacterial isolates of gut and surface body. The phylogenetic tree obviously shows that the five bacterial isolates of surface and nine isolates of insect gut could be divided into two and three clades, respectively. Based on 16S rRNA sequencing of 13 surface body and gut mealworm larvae bacteria, they showed the closest relationship with member of families Enterobacteriaceae,Staphylococcaceae, Psedumonadaceae and Bacillaceae in the two phylum Firmicutes and Proteobacteria. A short branch length indicates a limited of nucleotide changes. *Actinetobacter pitti* BB4 was used as an out group (Figures 2 and 3).

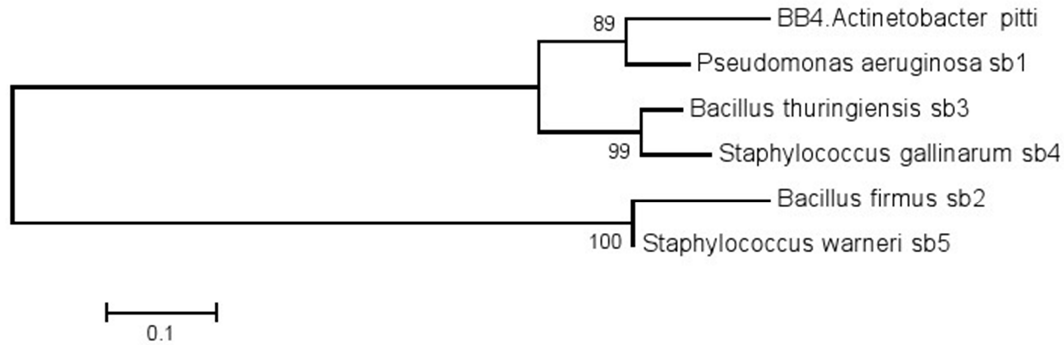


Fig. 2 Phylogenetic tree of bacterial species isolated from the body surface of *T. molitor*. The phylogenetic tree was constructed from rDNA sequences registered in the GenBank, using MEGA 6. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The scale represents a relative evolutionary distance, and *bootstrap values* obtained after *1000* replications

شکل ۲- درخت فیلوژنتیک گونه های باکتریایی جدا شده از سطح بدن *T. molitor* درخت فیلوژنتیک از توالی rDNA ثبت شده در GenBank و با استفاده از نرم افزار MEGA 6 ساخته شد. تاریخچه تکامل با استفاده از روش Maximum Likelihood بر اساس مدل Tamura-Neiاستنباط شد. مقیاس نشان دهنده فاصله تکاملی نسبی و مقادیر بوت استرپ پس از ۱۰۰۰ تکرار بدست آمده است.

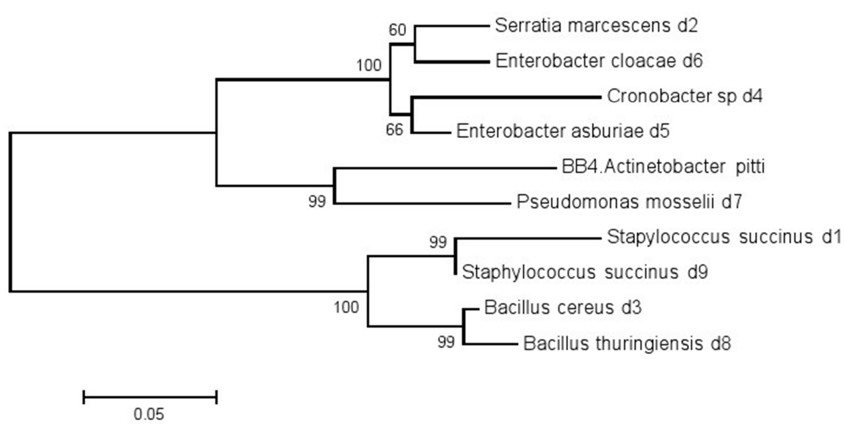


Fig. 3 Phylogenetic tree of bacterial species in the gut of *T. molitor*. The phylogenetic tree was constructed from rDNA sequences registered in the GenBank, using MEGA6. The evolutionary history was inferred by using Maximum Likelihood method based on Tamura-Nei model. The scale represents a relative evolutionary distance, and the whole numbers are bootstrap values for 1000 analyses

شکل ۳- درخت فیلوژنتیک گونه های باکتریایی در روده *T. molitor* درخت فیلوژنتیک از توالی rDNA ثبت شده در GenBank و با استفاده از برنامه MEGA6 ساخته شده است .تاریخ تکاملی با استفاده از روش Maximum Likelihood بر اساس مدل Tamura-Nei استنباط شد. مقیاس فاصله تکاملی نسبی و کل اعداد مقادیر بوت استرپ برای ۱۰۰۰ آنالیز را نشان میدهد.

Antibiogram test

Bacterial isolates were subjected to an antibiotic testing by using amoxicillin, tetracycline, azitromycin, penicillin and chloramphenicol. Results show that the two strains of *Serratia marcescens* and *Pseudomonas mosselii* in mealworm gut were resistant to all tested antibiotics. The isolates of *Staphylococcus succinus*, *Bacillus cereus* and *Cronobacter* sp. from insect guts and *Bacillus firmus*, *Staphylococcus gallinarum* and *Staphylococcus warneri* from body surfaces were showed sensitive to all antibiotics. Most resistances and sensitivity were observed to amoxicillin (53.8%) and chloramphenicol (84.6%), respectively (Table 4)

.

جدول ٤- تست آنتی بیوگرام ایزوله های باکتریایی موجود در روده و سطح بدن لارو میل ورم

Table 4 Antibiogram test of bacterial isolates on mealworm gut and body surface

Identity of isolates (ایزوله های شناسایی شده)	Origin (ناحیه)	Antibiotics (آنتی بیوتیک)				
		AX	**C**	**P**	**AZM**	**TE**
Pseudomonas aeruginosa	B	R	S	R	S	S
Bacillus firmus	B	S	S	S	S	S
Bacillus thurigiensis	B	R	S	R	S	S
Staphylococcus gallinarum	B	S	S	S	S	S
Staphylococcus warneri	B	S	S	S	S	S
Staphylococcus succinus	G	S	S	S	S	S
Serratia marcescens	G	R	R	R	R	R
Bacillus cereus	G	S	S	S	S	S
Cronobacter sp.	G	S	S	S	S	S
Enterobacter asburiae	G	R	S	S	R	R
Enterobacter cloacae	*G*	R	S	R	S	S
Pseudomonas mosselii	G	R	R	R	R	R
Bacillus thurigiensis	G	R	S	R	S	S

R, Resistance; S, Sensitive; C, Chloramphenicol (30 µg); P, Penicillins (10 µg); Ax, Amoxicillin (25 µg); Te, Tetracycline (30 µg); AZM, Azithromycin (15 µg). G, Gut; B, body surface.

R، مقاوم، S، حساس، C) کلرامفنیکل (۳۰ µg، P) پنی سیلین (۱۰ µg، Ax) آموکسی سیلین (۲۵ µg، Te) تتراسایکلین (۳۰ µg، AZM، آزیترمایسین (۱۵ µg). G، روده، B، سطح بدن.

Discussion

The yellow mealworms seem to be an alternative protein source for human and animal's consumption. Insects are widely consumed in many parts of the world as food and feed or supplement. Nevertheless, little attention has been given to the food safety and microbiological content. The analysis of the microbiological content of edible insects as fresh larvae was evaluated. The identification all isolates was performed based on morphological, biochemical characteristics as well as amplification of 16S rRNA gene by PCR.

The results obtained from the microbiological analysis of the fresh *T. molitor* indicate the presence of at least twelve species of bacteria in external body surface and gut. *Staphylococcus succinus*, *S. warneri*, *S. gallinarum*, *Bacillus firmus*, *B. cereus*, *B. thuringiensis*, *Pseudomonas aeruginosa*, *P. mosselii*, *Serratia marcescens*, *Cronobacter* sp., *Enterobacter cloacae* and *E. asburiae*. The total bacterial count of 2.34×10^5 and 2.14×10^5 CFU/ml in body surface and gut suggest the high bacterial contamination which might pose health risk following consumption. Most of these bacteria belonging to the phyla Proteobacteria (45 %) and Firmicutes (55%) which were already reported for mealworm larvae (Colman *et al*., 2012).

At the same time, the dominant bacteria with the largest number of species belonged to *Bacillus* spp. This means that edible mealworm may be contaminated with spores of these bacteria. Undoubtedly, pathogens are an important concern of insect producers who at times experience that whole colonies are eliminated (Szelei *et al.*, 2011). Although some bacterial endospores will survive the low heating treatment.

The fresh mealworm contained a variety of bacteria some of them are common pathogen of human and animal in turn, can be a potential cause of spoilage of larvae as food. While a number of other microbes form the natural population of the intestine, they are either beneficial or not harmful to the hosts. In few cases they might be considered a primary pathogens.

According to numerous report *Staphylococcus*, *Pseudomonas* and *Bacillus* are ubiquitous and main contaminants in food, and they may cause food borne illnesses, which can contain pathogenic species (Prescott *et al.*, 2002). *P.aeruginosa* is an important food contaminant which plays a *key role* in *food contamination* and development of spoilage in food products such as meat, fish, eggs, vegetables and other food stocks. Their presence shows that the samples are susceptible to spoilage through rich in protein and other essential nutrients required for the growth of bacteria. (Harrigan & McCance, 1990; Nester *et al.*, 1998; Prescott *et al.*, 2002, Masson *et al.*, 2002; Stoops *et al.*, 2012).

Gram-positive bacilli on the other hand, cause various food-borne infections, bacterial contamination, food poisoning, and intoxication. *B. cereus* is one of these bacteria responsible for foodborne illnesses, causing nausea, vomiting, and diarrhea (*Kotiranta et al., 2000). Bacillus* foodborne illnesses happen due to survival of endospores when infected food is not, uncooked (*Turnbull PCB, 1996*). On other hand, Staphylococcal food intoxication is *one* of the most common foodborne disease estimated to cause food-borne illness annually (Mead *et al.*, 1999). *S. succinus* has been isolated from human clinical material, but its role in pathogenesis has not been yet known (Prescott *et al.,* 2002; Nováková *et al.*, 2006). *S. gallinarum* was originally *isolated from the skin* of poultry and is widespread in nature (Devriese *et al.*, 1983; Nováková *et al.*, 2006). *S. warneri*, is another species in this genus causes spontaneous abortion in cattle and humans (Barigye *et al.*, 2007). *Cronobacter*, *Serratia* and *Eenterobacter* were also documented for many illnesses in consumers. Some strain of *E. cloacae* is a part of the normal gut of many humans and is not usually a primary pathogen (Keller *et al.*, 1998)

In many ways, including the bacterial analysis of mealworm larvae our research is comparable to those found by Stoops *et al* (2016), who studied the microflora in fresh edible mealworm larvae (*Tenebrio molitor*) and grasshoppers (*Locusta migratoria migratorioide*). The bacterial species they reported from both insects included *Propionibacterium*, *Haemophilus*, *Staphylococcus, Pseudomonas* and *Clostridium.* Grasshoppers were mainly dominated by *Weissella*, *Lactococcus, Yersinia/Rahnella, Pseudomonas, Enterococcus and Klebsiella/Enterobacter.* Total aerobic viable counts of mealworm larvae were 8.3 ± 0.1 CFU/g. *As mentioned earlier, these bacteria in edible insects cause serious problems with consumer's health.* Also, the results of several similar studies have shown that the fresh edible insects have a variety of bacteria that during storage, a part of the microbial species present will become dominant and can cause insects spoilage as food (Stoops *et al.*, 2015).

Some of bacterial strains we found are similar to those identified by Banjo *et al* (2006). These were included human pathogens *S. aureus* Rosenbach, *P. aeruginosa* (Schroeter) Migula and *Bacillus cereus* Frankland & Frankl in African Palm Weevil (*Rhynchophorus phoenicis*) and the non-pathogenic bacteria was *B. firmus* in West Africa, which ultimately creates a potential risk for the consumers. The total viable count expressed 7.5 $\times 10^5$ and 6.8 $\times 10^5$ (CFU/g) in the fresh samples of *O. monoceros* gut and body, respectively.

Finally, *in vitro antibiotic* resistant *tested* to different antibiotics showed that some of these bacteria are resistant to antibiotics. Although this test was performed to alleviate the pathogenic bacteria problem, antibiotics can undoubtedly have potential long-term consequences. Most resistance was observed to amoxicillin and two species of *P. mosselii* and *S. marcescens* were showed resistance to all treated antibiotics. High rates of antibiotics resistance of bacteria may result from inappropriate use of antibiotics in the farming processes (Sunde, 2005; Oladipo **&** Fajemilo**,** 2012**)**. Hence, sanitary conditions during the rearing process are important (Rumpold & Schlüter, 2013; Torcoli *et al*., 2014).

Conclusion

According to our results, we found the most potential food pathogens and spoilage bacteria such as *Staphylococcus*, *Pseudomonas*, *Serratia* and *Bacillus* in reared fresh mealworm larvae within acceptable ranges. One thing is clear: the consumption of insects as food, if it is infected with harmful microbes, could endanger human health. Poor sanitation may be an important reason in the contamination of edible larvae consumed. For this reason it is recommended treating the products in appropriate temperatures in order to reduce microbial population and eliminate the pathogens. Of course, more research works should be carried out to overcome these challenges in the field of healthy food production from insects.

ACKNOWLEDGMENTS

We would like to thank Dr. Ostadi, the laboratory expert of Razi laboratory complexes for his technical support in this study.

Reference

Acharya, T. 2014. Salt tolerance test for *Enterococcus* species: Principle, Procedure and results, Biochemical Tests in Microbiology. Int. J. Food Microbiol, 94: 223–253.

André, S., Zuber, F. and Remize, F. 2013. Thermophilic spore-forming bacteria isolated from spoiled canned food and their heat resistance. Results of a French ten-year survey, Inter. J. Food Microbiol, 165: 134-143.

Baker, F.J. 1967. Handbook of Bacteriological Techniques. 2nd Ed. Butterworths, London, pp: 482.

Banjo, A.D., Lawal, O.A. and Songonuga, E.A. 2006. The nutritional value of fourteen species of edible insects in southwestern Nigeria. African Journal of Biotechnology, 5: 298-301.

Barigye, R., Schaan, L., Gibbs, P.S., Schamber, E. and Dyer, N.W. 2007. Diagnostic evidence of *Staphylococcus warneri* as a possible cause of bovine abortion. J. Vet. Diagn. Invest, 19(6): 694-696.

Belluco, S., Losasso, C., Maggioletti, M., Alonzi, M. C., Paoletti, M. G. and Ricci, A. 2013. Edible insects in a food Safety and nutritional perspective: a critical review, comprehensive reviews. Comp Rev Food Sci Food Saf, 12: 296-313.

Cheesborough, M. 2000. District Laboratory Practice in Tropical Countries, Part 2. Cambridge Univ. Press: UK, 35 – 38, 62–69.

Clinical and Laboratory Standards Institute. 2015. Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. Wayne: Clinical and Laboratory Standards Institute.

Colman, D.R., Toolson, E.C. and Takacs-Vesbach, C.D. 2012. Do diet and taxonomy influence insect gut bacterial communities? Molecular Ecology, 21: 5124-5137.

Devriese, L. A., Poutrel, B., Kilpper-Ba¨ lz, R. and Schleifer, K. H. 1983. *Staphylococcus gallinarum* and *Staphylococcus caprae*, two new species from animals. Int J Syst Bacteriol, 33: 480-486.

Dickey, D. A. and Fuller, W. A. 1979. Distribution of the estimators for autoregressive time series with a unit root. Journal of the American Statistical Association, 74(336): 427-431.

Dobermann, D., Swift,A.J. and Field, L.M. 2017.Opportunities and hurdles of edible insects for food and feed. Nutr Bull, 42(4): 293-308.

Elboutahiri, N., Thami-Alami, I., Zaïd, E. and Udupa, S. M. 2009. Genotypic characterization of indigenous *Sinorhizobium meliloti* and *Rhizobium sullae* by rep- PCR, RAPD and ARDRA analyses. African Journal of Biotechnology, 8 (6): 979-985.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach J. Mol. Evol, 17: 368-376.

Grabowski, N.T., Jansen, W. and Klein, G. 2014. Microbiological status of edible insects sold as pet feed in Germany. Insects to feed the world. Wageningen: Wageningen University and Research Centre.

Harrigan, W.F. and McCance, M.E.1990. Laboratory Methods in Food and Dairy Microbiology (8th edn). Academic Press Inc.: London, 7(23): 286 – 303.

Humphries, J. 1974. Bacteriology. John Murray Albermack Street, London, pp: 452.

Ijong, F, G. 2003. Uji IMVIC. Uraian Teoritis Proses Biokimianya. Laboratorium Mikrobiologi Hasi Perikanan. FPIK Unsrat. Manado.

Keller, R., Pedroso, M.Z., Ritchmann, R. and Silva, R.M. 1998*. Occurrence of virulence-associated properties in Enterobacter cloacae. Infection and Immunity, 66 (2): 645–9.*

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. The Journal of laboratory and clinical medicine, 44(2): 301-307.

Klunder, H.C., Wolkers-Rooijackers, J., Korpela, J.M. and Nout, M.J.R. 2012. Microbiological aspects of processing and storage of edible insects. Food Control, 26:628-631.

Kotiranta, A., Lounatmaa, K. and Haapasalo, M. 2000*. Epidemiology and pathogenesis of Bacillus cereus infections". Microbes Infect, 2 (2): 189–98.*

MacEvilly, C. 2000. Bugs in the system. Nutrition Bulletin 25: 267-268.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. andTauxe, R.V. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis, 5: 607-625.

Masson, Y., Ainsworth, P., Fuller, D., Bozkurt, H. and Ibanoglu, S. 2002. Growth of *Pseudomonas fluorescens* and Candida sake in homogenized mushrooms under modified atmosphere. Journal of Food Engineering, 54: 125–131.

Mlcek, J., Rop, O., Borkovcová, M. and Bednácová, M. 2014. A comprehensive look at the possibilities of edible insects as food in Europe – a review. Polish Journal of Food and Nutrition Sciences, 64(3):1477-157.

Murinda, S. E., Nguyen, L. T., Ivey, S. J., Almeida, R. A. and Oliver, S. P. 2002. Novel single-Tube agar-based test system for motility enhancement and immunocapture of *Escherichia coli* O157:H7 by H7 flagellar antigen-specific antibodies. Journal of Clinical Microbiology, 40(12): 4685–4690.

Nester, E.W., Robert, C.E., Pearsall, N.N., Anderson, D.G. and Nester, M.T. 1998. Microbiology: A Human Perspective, (2nd ed), WBC/McGraw-Hill: New York, U.S.A, 415: 434-435.

Nováková, D., Sedlácek, I., Pantůcek, R., Stetina, V., Svec, P. and Petrás, P. 2006. *Staphylococcus equorum* and *Staphylococcus succinus* isolated from human clinical specimens. J. Med. Microbiol, 55: 523-528.

Ogbalu, O.K. and Williams, J.O. 2015. The edibility, methods of preparation of the raphia palm beetle, *Rhyncophorus Phoenicis* [Coleoptera: Curculionidae] in the Niger Delta and associated microorganisms. Journal of Pharmacy and Biological Sciences, 10: 125-129

Oladipo, I.C. and Fajemilo, Y.O. 2012. Physiological Studies and Antibiotic Resistance Profile of Bacterial Pathogen Isolated from Some Nigerian Fast Food. American Journal of Food Technology, 7 (12): 746-753.

Olutiola, P.O., Famurewa, O. and Sontag, H.G. 1991. An Introduction to General Microbiology. A practical Approach. Cab. Herdelberg Verlay Sanstait and Druckeres, Gmbh. Heidelberg, Germany.

Prescott, M.I,, Harle, J.D. and Klein, D.A. 2002. Microbiology of Food (5th ed). McGraw Hill Limited: New York, U.S.A, pp: 964-976.

Ramin, M., Alimon, A.R., Abdullah, N., Panandam J.M. and Sijam, K. 2008. Isolation and identification of three species of bacteria from the termite *Coptotermes curvignathus* (Holmgren) present in the Vicinity of University Putra Malaysia. Res. J. Microbiol, 3: 288-292.

Rumpold, B.A. and Schlüter, O.K. 2013. Potential and challenges of insects as an innovative source for food and feed production. Innovative Food Science and Emerging Technologies, 17:1-11.

Rumpold, B.A., Fröhling, A., Reineke, K., Knorr, D., Boguslawski, S., Ehlbeck, J. and Schlüter, O. 2014. Comparison of volumetric and surface decontamination techniques for innovative processing of mealworm larvae (*Tenebrio molitor*). Innovative Food Science and Emerging Technologies, 26: 232-241.

Saidi, A., Imani, S., Hassanzadeh, N. and Ahadiyat, A. 2016. Lethal effects of tungsten and boric acid, and three garlic,essential oils on Amitermes vilis (Isoptera: termitidae) and it's endosymbiont's cellulotic activity. J. Bio. & Env. Sci, 9(1): 1-10.

Sangiliyandi, G., Kannan, T. R., Chandra Raj, K. and Gunasekaran, P. 1999. Separation of levan-formation and sucrose-hydrolysis catalyzed by levansucrase of *Zymomonas mobilis* using *in vitro* mutagenesis. Braz. Arch. Biol. Technol, 42(4).

Siemianowska, E., Kosewska, A., Aljewicz, M., Skibniewska, K.A., Polak-Juszczak, L., Jarocki,A. and Jędras, M. 2013. Larvae of mealworm (*Tenebrio molitor* L.) as European novel food. Agricultural Sciences, 4(6): 287-291.

Stoops, J., Claes, J., Maes, P. and Van Campenhout, L. 2012. Growth of *Pseudomonas fluorescens* in modified atmosphere packaged tofu. Letters of Applied Microbiology, 54: 195-202.

Stoops, J., Ruyters, S., Busschaert, P., Spaepen, R., Spaepen, R., Verreth, C., Claes, J., Lievens, B. and Van Campenhout, L. 2015. Bacterial community dynamics during cold storage of minced meat packaged under modified atmosphere and supplemented with different preservatives. Food Microbiology, 48,192-199.

Stoops, J., Crauwels, S., Waud, M., Claes, J., Lievens, B. and Van Campenhout, L. 2016. Microbial community assessment of mealworm larvae (Tenebrio molitor) and grasshoppers (Locusta migratoria migratorioides) sold for human consumption. Food Microbiolog, 53:122-127.

Stock, S.P., Glazer, I., Boemare, N. and Vandenberg, J. 2009. Insect Pathogens: Molecular Approaches and Techniques. CABI 440 pages.

Sunde, M. 2005. Prevalence and characterization of class 1 and class 2 integrons in *Escherichia coli* isolated from meat and meat products of Norwegian origin. J. Antimicrob. Chemother, 56: 1019-1024.

Szelei, J., Woodring, J., Goettel, M.S., Duke, G., Jousset, F.X., Liu, K.Y., Zadori, Z., Li, Y., Styer, E., Boucias, D.G., Kleespies, R.G., Bergoin, M. and Tijssen, P. 2011. Susceptibility of North-American and European crickets to *Acheta domesticus* densovirus (AdDNV) and associated epizootics. Journal of Invertebrate Pathology, 106: 394-399.

Torcoli, E. Amoruso, I., Moretto, E., Caravello, G.U. and Giaccone, V. 2014. Small scale experimental insect farming: compliance of raw insects and processed flour with food hygienic requirements. Insects to feed the world. Wageningen: Wageningen University and Research Centre, P: 169.

Turnbull, P.C.B. 1996*. Baron, S., et al. (eds.). Bacillus. In: Baron's Medical Microbiology (4th ed.). Univ of Texas Medical Branch.*

Van der Spiegel, M., Noordam, M.Y. and VanderFels-Klerx, H.J. 2013. Safety of novel protein sources (insects, microalgae, seaweed, duckweed, and rapeseed) and legislative aspects for their application in food and feed production. Comprehensive Reviews in Food Science and Food Safety 12: 662-678.

Van Huis, A. 2013. Potential of insects as food and feed in assuring food security. The Annual Review of Entomology, 58: 563-583.

Van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G. and Vantomme, P. 2013. Edible insects: future prospects for food and feed security. FAO Forestry, Paper 171.

Van Huis, A., Dicke, M. andVan Loon, J.J.A. 2015. Insects to feed the world**.** Journal of Insects as Food and Feed 1(1): *3*-5.

Vega, F. and Kaya, H. 2012. Insect Pathology. London, Academic Press.

Wenzel, M., Schonig, I., Berchtold, M., Kampfer, P. and Konig, H. 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. J. Applied Microbiol 92: 32-40.

Yen, A.L. 2009. Edible insects: traditional knowledge or western phobia? Entomological Research 39:289-298.

دانشگاه آزاد اسلامی، واحد اراک
شاپا ۴۶۶۸-۲۰۰۸
http://jer.iau-arak.ac.ir

فصلنامه تخصصی تحقیقات حشره‌شناسی
(علمی- پژوهشی)
جلد ۱۲، شماره۳، سال ۱۳۹۹، (۲۸-۱۳)

شناسایی میکروفلور باکتریائی لاروهای خوراکی تازه سوسک زرد آرد (*Tenebrio molitor* L.)

ستاره محمدسلیم[1]، سهراب ایمانی[2*]، محمد عبدی گودرزی[3]، نادر حسن زاده[2]، وحید زرین نیا[2]

۱- دانشجوی دکتری حشره شناسی،گروه گیاهپزشکی، واحد علوم و تحقیقات، دانشگاه آزاد اسلامی، تهران، ایران.
۲- به‌ترتیب استادیار و دانشیار، گروه گیاهپزشکی، واحد علوم و تحقیقات، دانشگاه آزاد اسلامی، تهران، ایران.
۳- دانشیار، گروه پارازیتولوژی، واکسن و سرم سازی موسسه تحقیقات رازی،سازمان تحقیقت،آموزش و ترویج کشاورزی (AREEO)، البرز، ایران.

چکیده

در اکثر کشورهای آسیایی حشرات خوراکی به عنوان یک منبع جایگزین پروتئین حیوانی مصرف می‌گردند. از آنجا که شواهد محدودی در مورد ایمنی آنها به ویژه از جنبه های میکروبیولوژیکی وجود دارد، تلاش شد تا با ارزیابی سوسک زرد آرد (*Tenebrio molitor* L.) در شرایط آزمایشگاهی، این مورد ارزیابی گردد. کلنی اولیه سوسک میل ورم از بازار محلی ساری- ایران خریداری گردیده شد. نمونه های میکروبی از هر دو سطح بدن و روده ها تهیه شد. نمونه ها بصورت جداگانه در محیط کشت نوترینت آگار (NA) کشت داده و در دمای ۲۷ درجه سانتیگراد بمدت ۲۴-۷۲ ساعت انکوبه شدند. کلنی ها متمایز انتخاب و خالص سازی شدند. براساس ویژگی های فنوتیپی، پاسخ حساس (HR) روی برگهای شمعدانی و همچنین آنالیز توالی ژن 16s rRNA، جدایه ها به دو گروه طبقه بندی شدند. جدایه‌های بیماری زا به عنوان *Bacillus cereus*، *Staphylococcus* sp.، *Pseudomonas aeruginosa*، *Enterobacter asburiae*، *Bacillus firmus* و *Serratia marcescens* شناسایی شدند. در حالی‌که، باکتریهای *Enterobacter cloacae* و *Bacillus thuringiensis* بعنوان باکتری های غیر بیماریزا تعیین شدند. بدون شک وجود میکروب های بیماری زا در میکرو فلور لارو میل ورم با مصرف مستقیم و غیرمستقیم حشرات ممکن است تهدیدی برای سلامت انسان و حیوان ایجاد کنند.این یافته ها بیانگر اجرای برخی از روش های فراوری بمنظور کاهش یا ریشه کن کردن خطرات آلودگی میکروبی در رژیم های غذایی با استفاده از حشرات طبیعی را نشان میدهد.

واژه‌های کلیدی: حشرات خوراکی، تغذیه، *Tenebrio molitor*، تنوع باکتریایی، 16S rRNA ژن

* نویسنده رابط، پست الکترونیکی: *Drsohrabimani@gmail.com*

تاریخ دریافت مقاله:۹۹/۴/۱۸ - تاریخ پذیرش مقاله:۹۹/۷/۲