

Molecular Identification of *Listeria* Species from Some Processed Meat Products Using Specific *Iap* Gene and 16S rRNA Gene

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ABSTRACT

Three kinds of processed meat (Luncheon, Burger, Minced meat) were examined for the presence of *Listeria* species. Collected samples purchased from local supermarkets were from three different Egyptian companies. All the bacterial isolates were cultured on specific media after pre-enrichment and enrichment cultures. The colonial morphology depends strongly up on the media used and the incubation conditions provided. (Oxford *Listeria* Agar Base) was used as specific medium for *Listeria*. The bacterial distribution of *Listeria* was, 2.22% in Beef burger, 13.6% in luncheon and there was no bacterial growth in minced meat. In this study; gram staining, morphological characterization, molecular identification using 16S rDNA partial sequencing and specific gene *Iap* gene of *Listeria* were carried out.

Keywords: *Listeria* Species- specific PCR.

INTRODUCTION

Listeria considers anaerobic, non-spore, psychrophilic gram positive bacteria. It has the ability for salt tolerant and pathogenic for humans and animals that causing clinical manifestations such as gastroenteritis, encephalitis, meningitis and abortion (Ruppitsch *et al.*, 2015; Lakicevic *et al.*, 2014). Moreover, *listeria* can produce a biofilm at low temperatures to facilitate persistent dissemination of this pathogen during food production (Allerberger *et al.*, 2015).

Listeria entrance through the intestines to the liver where it replicates considers the first step in its pathogenesis and the cell mediated immunity is specific to clear and attack listeria infection. One of the most common clinical diagnosis and effect of *listeria* is the diarrhea that occurred due to direct invasion of the intestinal mucosal epithelium by the organism (Schuppler and Loessner, 2010).

The main means of prevention is through the promotion of safe handling, cooking and consumption of food. This includes washing raw vegetables and cooking

raw food thoroughly, as well as reheating leftover or ready-to-eat foods like hot dogs until steaming hot (CDC, 2011) preventing listeriosis as a food illness.

MATERIALS AND METHODS

Bacterial isolation

A total of (18) samples of three kinds of processed meat from three different Egyptian companies collected from local supermarkets at Alexandria Governorate. The samples included (6) luncheon (6) beef burger, and (6) minced meat. The date production of all samples in July 2016 (Table1).

Table 1. the percentage of bacteria in each sample

Name of company	Kind of procced meat	Percentage of <i>Listeria</i> (%)
P	Beef burger (P)	2.22%
	Luncheon(P)	13.6%
	Minced meat(P)	----
A	Beef burger(A)	0.44%
	Luncheon(A)	7.33%
	Minced meat(A)	----
F	Beef burger(F)	----
	Luncheon(F)	3.78%
	Minced meat(F)	----

For isolation of *Listeria* from the chosen processed meat samples, 2 g from each sample were mixed with 20 ml of Pre-enrichment broth medium(1.5 g of beef extract was added to 2.5 g of peptone then fill up to 500 ml ddH₂O at pH 6.8 (Banwart, 1989), and left over night at 37°C with shaking. 4 ml pre-enrichment media were added to 20 ml of enrichment broth media that prepared with 2.5 g of Protease peptone, 0.5 g of Difco bile salt, 5 g of calcium carbonate and 15 g of sodium thiosulfate were prepared in 500 ml ddH₂O at pH 6.8 (Difco, 1984), and incubated with shaking over night at 37°C. Each sample was diluted after growth into two concentration 10⁻⁵ and 10⁻¹⁰ and transferred to specific

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media Oxford Listeria Agar Base (57.5 g of Oxford Listeria Agar Base powder was dissolved in 1000 ml ddH₂O) (Vanderzant and Splittstoesser, 1992). Samples were incubated at 37°C overnight with agitation at 110 rpm. The bacterial morphology identification was carried out visually and by microscopic examination after Gram staining test.

Morphological identification of *Listeria* species.

Bacterial isolates were grown Oxford Listeria Agar Base according to (MacFaddin, 1985) and (Vanderzant and Splittstoesser, 1992). Morphological identification was assessed visually and microscopically. A clean, grease free slide was taken and the smear of suspension on the clean slide with a loop full of sample was prepared then Air dried and heat fixed Crystal Violet was poured and kept for about 30 seconds to 1 minute and rinsed with water. The gram's iodine was flooded for 1 minute and washed with water. Then washed with 95% alcohol or acetone for about 10-20 seconds and rinsed with water. Safranin was added and left for about 1 minute and washed with water then Air dried, Blot dry and Observed under Microscope (Jacquelyn and Black, 1993).

Molecular analysis

Iap gene-specific PCR amplification, 16S rDNA sequencing and phylogenetic analysis were used to more confirm the identification of the *Listeria* spp.

Extraction of DNA

DNA was extracted from Bacterial samples using modified method instruction reported by (John *et al.*, 1990). The bacterial samples were renewed and cultured for 18 hours on nutrient broth and centrifuged at 1000 rpm for 15 minutes to get the pellet. 467µl TE buffer (pH 8), (30µl 10% SDS, 3µl Protienase K (20 mg/ml) were added on pellet in 1.5 sterilized 1.5ml tube and incubated at 37°C for 1 hour. After incubation, 500µl phenol chloroform isoamyl alcohol (24:25:1), (pH 8) was added and gently inverted for 5 minutes and centrifuged at 1000 rpm for 10 minutes. The DNA was precipitated with 3M sodium acetate (pH 5.2) and 600 µl cold isopropanol and kept overnight at -20°C, then centrifuged at 12000 rpm for 15 minutes at 4°C. The pellet washed twice with 70% Ethanol and then centrifuged at 12000 rpm for 5 minutes at 4°C. The pellet was washed with 70% ethanol, dried and finally dissolved in Tris-EDTA (TE) buffer, pH 7.4. The concentrations and purities of DNA were determined by using nanodrop machine (A260 / A280) and kept at -20°C for furthers molecular examinations.

Specific PCR for detection of *Listeria* spp

The Iap gene was used as specific gene to identify the *Listeria* species as described by (Veriti™ thermal cycler USA). The primer Iap (one of a set of virulence genes specific for *Listeria*) F:(CAAACCTGCTAACACAGCTACT) and Iap R:(TTATACGCGACCGAAGCCAAC) were used for amplification of Iap gene from bacterial DNA. The PCR reaction was carried out in 25µl total volume containing (12.5µl master mix (thermos), 10 p.mol each of forward and reverse primers, and 10 ng of genomic DNA in (BioRad, USA) thermo-cycler. PCR conditions were: 94°C for 3 min: 30 cycles of 95°C for 15 sec, Annealing: 58°C for 30 sec., extension: 72°C for 50 sec, and final extension: 72°C for 10 min. Amplified products were fractionated by electrophoresis in 2% (w/v) agarose/TBE gels, visualized, and documented using a gel documentation and image analysis system (Alfa Imager M 1220, Documentation and Analysis System, Canada) as shown by (Bubert *et al.*, 1999).

Amplification of 16S rRNA gene and Sequencing

16S rRNA gene was amplified for all the examined isolates using universal primer 16SF:(AGAGTTTGATCCTGGCTCAG), and 16S R:(GGTTACCTTGTTACGACTT (James *et al.*, 2010). Amplification was done in 25 µl reaction with 12.5 µl master mix (thermos), 10 ng of genomic DNA and 10 p.mol each of forward and reverse primers. The PCR program was performed in thermo-cycler (BioRad, USA) as follow: Initial denaturation at 95°C for 5 mins then followed by 34 cycles of 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 10 mins. PCR products were separated on agarose gel electrophoresis using 2% agarose (w/v) in 0.5X TBE buffer that stained with 0.5µg/ml ethidiumbromide. The gel was then photographed using gel documentation system. PCR products were purified by EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Canada), following the manufacturer's instruction. The DNA sequences for 16S rDNA were determined by Macro gene Company (South Korea). The sequences were subjected to pairwise and multiple alignment using CLUSTAL W version 2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>, Thompson *et al.*, 1997).

RESULTS AND DISCUSSION

Listeria sp including *L. monocytogenes* have ubiquitous nature and psychotropic growth to be found in food samples. Due to raw food consumption or re-contaminated heat processed food stuffs cause listeriosis that may be led to death in some sensitive groups. Many precautions should be considered to prevent listeriosis

such as no consumption of raw and insufficiently cooked food of animal origin, storage conditions and cross contamination (Ray, 2004). In addition to, many advanced approaches such as understand the characteristic of *listeria*, environmental impact and influence of virulence factors to control and prevent listeriosis (Buchanan *et al.*, 2017). In this study, three kinds of processed meat products (Luncheon, Burger, and Minced meat) were used and checked to examine the presence of *listeria* using morphological and molecular methods including specific medial culture, gram staining specific -specific PCR, 16S rRNA amplification and sequence confirmation.

Morphological characterization of bacteria on specific medium and by Gram staining:

The results of bacterial colonies were shown in Fig (1) in which the bacteria gave black colored or short

rods colonies on specific medium (fig. 2) respectively, after gram staining.

The results indicated that *Listeria* can grow on a variety of processed meat products. The distribution of *Listeria* in beef burger was 2.22%, and 0.44% for the company P and A respectively. While the highest percentage of *Listeria* was 13.6%, 7.33%, 3.78% and found in Luncheon for the three companies. The minced meat samples were totally negative in all the three companies (table 1). These results were agreed with (Mohamed *et al.*, 2016) who found that *L. monocytogenes* were confirmed distributed in beef burgers samples (4%), minced meat (4%) and Luncheon (4%).

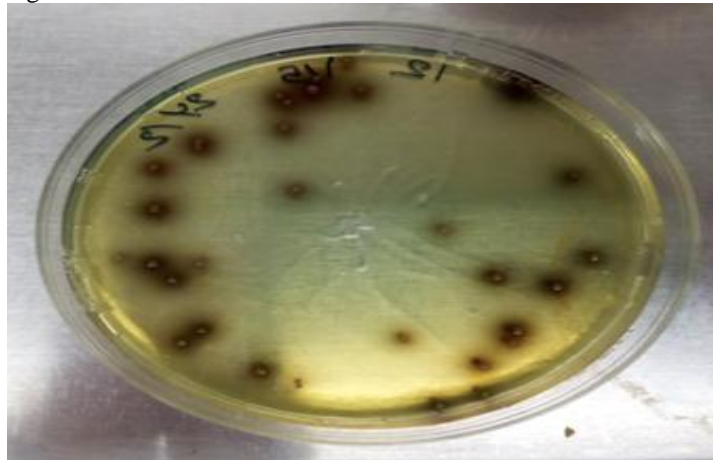


Fig. 1. Colony morphology of *Listeria spp.* after culture on Oxford Listeria Agar Base medium (appeared a black colonies)



Fig. 2. Gram staining of *Listeria spp*

Molecular identification

16S rRNA sequencing analysis:

16S rRNA gene PCR products were appeared as specific fragment (about 1500 bp) in all samples after specific amplification of 16S rRNA gene using universal primer as shown in fig (3). The obtained amplicons were later subjected to further analysis.

The purified 1500 bp band of the 16S rRNA gene from two isolates was subjected to sequencing using 3110 automated DNA sequencer model (SENSQUIST Lab cyclor GmbH Germany) At the city of scientific researches and technological applications. The following partial sequence was obtained for samples.

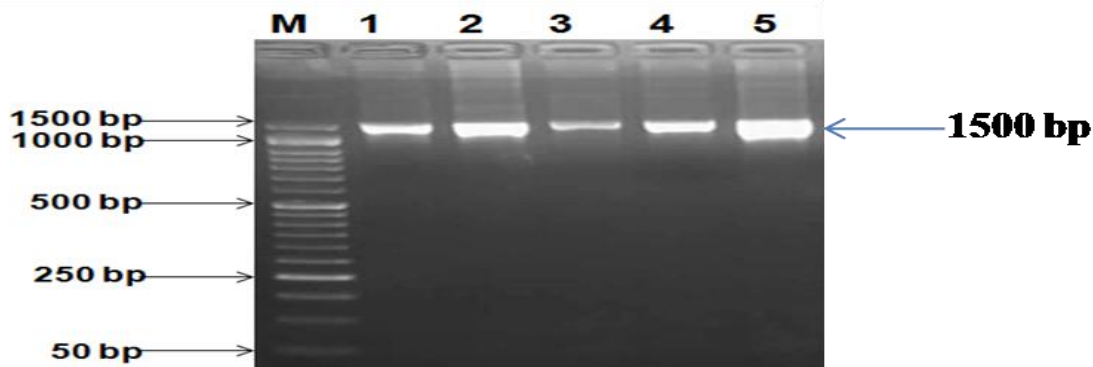


Fig.3. Agarose gel electrophoresis of 16s rRNA gene PCR products. Lane M: 1.5 Kb DNA ladder, lanes 1-5: 16s rRNA PCR products (1500 bp)

>*Listeria spp1*

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ACGTC TTTGTG TTCATGCAGTCGTGCGGAAGACGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTG
AGTAACACGTGGGTAACCAGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATA
GTTCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTTCCGGCTGTCACTTACAGATGGACCCGCGGC
GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCCGGATCGTAAAGCTCTGTTGTT
AGGGAAGAACAAGTGCAAGAGTAACTGCTTGCACCTTGACGGTACCTAACCAGAAAGCCTCGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCT
CGCAGGCCGTTTTCTTAAGTCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATAGGAAACTGGG
AAACTTGAGTGCAGAAGAGGAGAGTGGAATCCCACGCGTTGCGGAGAAATGCGTAGAGATGTGGACT
AACACGGGTGGTGATCGTGTCTGTTTCGTCTGTTACTGATGCTCAGGATCGAATGCCGGCCGGGAGCGA
ACACCATTAGATCCGGTGTTCCTCCACGTCTCTAACGATGTCTGCTCAGC GCGAGGGAGTTCCCTCTCC
TTTGTTGGTGCTCATGACTTCATAAAGTACTCCGACCGGTCCAGTGCTGACACGAGGTGTGTTTTATA
TCAGGATTTAAACAAGCCCCCTGCAAGAGCCGTGGCAGCCCTGATGATTTATTGAGAAGCATCGCCAA
ACATAACGTTATCACGCGCTGTGGACTCGCTCTGAAAACCCGTAGAGATATGGCTTTTACAGTCCCGGT
ACAAGAGTGCACGGATGGTAGCATGGCCAGTCTGTGAGTCCGGTAACAGTGAGAATGTTTGGTAGCTC
ACGCACCTACGTCCATCCTGGACGTTAGCTGCCGAGGAT
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>*Listeria spp2*

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CCCCCTCTGTCATTTATGCAGTCGAGCGATGGATTAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGG
ATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCCGGCTGTCACTTATGGATGGACCCG
GTTCGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACCTCTGTTG
TTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGC
GCGCGCAGGTGGTTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACT
GGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCATGTGTAGCGGTGAAATGCGTAGAGATATGG
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AGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAGTACTGACACTGAGGCGCGAAAGCGTGGGGGA
 GCAAACAGGATTAGATACCTGTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTT
 CCGCCCCTTTTAGTGCTGAAGGTTAACGCATTTAAGCCACTCCGCCCTGGGGGGAGTACGGCCCGCGA
 AGGTCTTGAAACTTCAAAGGTAAATTAGAACGGGGCGGCCCGGCACAAGTCTGGTGGCAGCATGAT
 GGGTTTAAATTACGAAAGTCAACGCCGCAAGAATCCTTTACCCAGGTCTTTGGACGATTTCATTCTGAC
 ACCCGTAGAGATTAGGTGCCTTTCTCCTTCGGGAGGCAGAGCTGACCAGGATGCGTGCATGGCTTGTC
 GTCCAGCTTCGTGTCGTGAGAATGTTTTGCGATTACAGTAACTGCTATCGTAGACGCAA

The increasing signal generation of 16S rRNA analysis due to its a target for genes and species bacteria identification by detection the conserved and variable regions of the gene (Gopo *et al.*, 1988; Maureau *et al.*, 1989). Otherwise, 16S rRNA gene test has been dictated by multiple copies (104) of rRNA in microorganisms. On the other hand, 16S rRNA is the most famous gene used in phylogenetic studies of the genera of archaea and bacteria. Recently, universal primers have been used to amplify the conserved region of 16S rRNA through the PCR technique (Leight *et al.*, 2018).

Species-specific primers targeted against virulence genes of *Listeria* .

The virulence factor occurrence in *L. monocytogenes* which are encoded on a multigene family was effective in the pathogenesis. Some of these virulence genes like *Iap* gene were attractive molecular markers for detection of *Listeria* sp by development of PCR-based assays. *Iap* gene encodes the protein p60, which is common to all *Listeria* species (Bubert, *et al.*, 1999). So, in this study, we used one set of primers based on Iap gene in its PCR assays. Molecular identification of *Listeria* through Iap specific gene (780 bp) was illustrated in Fig. (4).

Specific primers were designed as selective target of virulence genes in *listeria monocytogenes* such as Iap gene to delineate the different species of *listeria* spp as reported by Klein and Juneja (1997). Iap gene was detected in all *Listeria* strains (100%) and these results were similar to those obtained by Gelbicova and

Karpiskova (2012) in the Czech Republic, Jamali *et al.*, (2015) in Iran, Moreno *et al.* (2014) in Brazil, and Wang *et al.*, (2015) in China. Moreover, (Bhunia, 2008; Furrer *et al.*, 1991, and Swaminathan *et al.*, 2007) have been reported that the presence of multiple virulence factors in *L. monocytogenes* strains such as Iap gene may be act as a potential cause of human listeriosis due to improved entry into cells, escape from the vacuole and intracellular spreading. For this purpose, Iap gene was selected in this study because it is a common virulence factor in all members of the genus *Listeria* and it has been indicated that there were conserved regions at 50 and 30 ends, while the internal portions are highly specific (Bubert *et al.*, 1992). In addition to, Iap gene of *L. monocytogenes* encodes the major extracellular protein (P60) (Kuhn and Goebel 1989), which has been shown as a bio-vital role and essential murein hydrolase required for adherence/invasion of the organism to the targeted eukaryotic cell. Recently, it has been shown that the corresponding Iap gene portion is also hypervariable in length in different isolates belonging to the same serotypes, thereby, can be used in identification of different strains of *L. monocytogene*. It can't be neglected that the differences among the results of various studies regarding the prevalence of *Listeria* spp. may be due to several factors such as different isolation methods, type of food samples, season, geographical location and the conditions of packaging, handling, and storage (Bhunia, 2008; Ray, 2004).

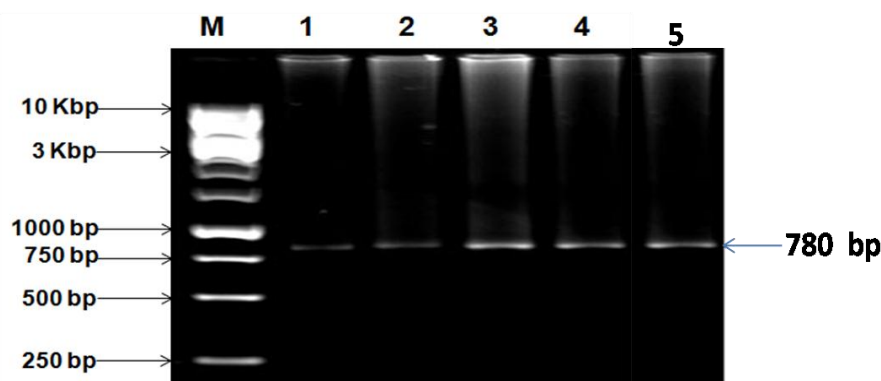


Fig. 4. Agarose gel electrophoresis of *Listeria* Iap gene PCR products. Lane M: 10 Kbp DNA ladder, lanes 1-5: *Listeria* Iap gene PCR products (780 bp)

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الملخص العربي

دراسات وراثية جزيئية على تأثير بعض المستخلصات النباتية المضادة للجراثيم على التعبير الجيني للبكتيريا المعزولة من بعض اللحوم المصنعة

منى مخيمر حسن, سامية عبدالله عبد العزيز, أسماء محمد مجاهد الفرماوي, هاجر أبو العزائم عبد الرسول, ياسر محمد مبروك

عينات البرجر بنسبة ٢,٢٢% و بنسبة ١٣,٦% في عينات الانشون ولم تظهر النتائج أي تواجد لبكتيريا الليزيتيريا في عينات اللحم المفروم. وللتأكد من تواجد وتوزيع بكتيريا الليزيتيريا في عينات اللحوم المختلفة تم إجراء اختبار صبغة جرام والتوصيف الشكلي والجزيئي من خلال إجراء تفاعل البلمرة المتسلسل المتخصص لجين rRNA ١٦s والتأكد الدقيق بإجراء تحليل تتابع الجينات وأيضاً إجراء تفاعل PCR specific لجين Iap الخاص ببكتيريا الليزيتيريا. وأكدت جميع الاختبارات الميكروبيولوجية والوراثية تواجد هذه البكتيريا *Listeria* في عينات اللحوم المصنعة بالنسب المذكورة أعلاه.

في هذه الدراسة تم الكشف عن وجود بكتيريا الليزيتيريا وذلك بفحص ثلاثة أنواع من اللحوم المصنعة وهي الانشون والبرجر واللحم المفروم وذلك من خلال تجميع عينات من الأسواق المحلية من ثلاث شركات مختلفة. وتم تنمية جميع العزلات البكتيرية علي أوساط غذائية متخصصة بعد الزراعة المبدئية للعزلات. وتعتمد شكل المستعمرات البكتيرية علي نوع الوسط الغذائي المستخدم وايضاً ظروف تنمية هذه العزلات. وتم اختيار الوسط الغذائي Oxford listeria agar base medium وذلك لأنه متخصص بشكل دقيق للكشف عن بكتيريا الليزيتيريا. ووضحت نتائج هذه الدراسة أن توزيع بكتيريا الليزيتيريا في