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DETECTION OF *LISTERIA MONOCYTOGENES* IN RAW MILK AND READY TO EAT DAIRY PRODUCTS WITH EVALUATING THE ANTIMICROBIAL EFFECT OF COLD-PRESSED NIGELLA SATIVA OIL

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Received: 24 January 2016; **Accepted:** 17 February 2016

ABSTRACT

The objectives of this study were to evaluate the public health significance of *Listeria monocytogenes* in milk and some dairy products and to estimate the antimicrobial effect of Nigella sativa oil on artificially contaminated soft Damietta cheese. The highest prevalence rate of *L. monocytogenes* was detected in goat milk (26.7%) followed by buffalo milk & yoghurt (13.3% each) then kareish cheese (10%) and finally sheep milk samples (6.7%). CHROMagar ListeriaTM[TM] medium performed better than Oxford agar medium in detection of *L. monocytogenes*. Out of the 90 samples tested, 21/90 (23.3%) and 13/90 (14.4%) were presumptively positive for Listeria spp. on Oxford agar and CHROMagar Listeria[TM] media, respectively. Further biochemical identification of the suspect colonies on Oxford agar and the chromogenic medium revealed that 5/21 (23.8%) and 12/13 (92.3%) of the isolated organisms, respectively were confirmed as *L. monocytogenes*. by applying PCR technique for detection of listeriolysin (*hly* A) gene, 3/90 (3.3%) of the total examined samples and 3/12 (25%) of biochemically confirmed isolates were positive (gave specific 750 bp band). A trial was made to control *L. monocytogenes* contamination in Damietta cheese by using cold-pressed Nigella sativa oil. The results revealed a progressive highly significant reduction in the logarithmic count of *L. monocytogenes* ($P \leq 0.01$) in the sample treated with this oil.

Key words: *Listeria monocytogenes*, Raw Milk, Dairy Products, N. Sativa oil

INTRODUCTION

Food-borne diseases can cause serious and sometimes lethal infection in humans. Listeriosis is one of the most important food-borne diseases. It is mainly caused by *Listeria monocytogenes*. It is Gram-positive bacterium capable of multiplying at a wide range of pH, osmolarity and temperature (Sleator *et al.*, 2003). Several food types have been implicated in transmission of this organism including milk and dairy products. Post-pasteurization contamination is the major source of *Listeria spp.* in dairy products (Conly and Johston, 2008).

Pregnant women, immune-compromised, children and elderly persons are considered to be at high risk of contracting the disease. The disease is manifested by septicemia, meningitis, spontaneous abortion or stillbirths. However, it has also been associated with gastroenteritis and fever with a high fatality rate 25-30% (CDC, 2013). On farm, the organism can grow in muddy and dusty soil. Moreover, it can be excreted in feces and milk of both symptomatic and asymptomatic animals (ADASC, 2000). An important character that makes *L. monocytogenes* of public health significance is its ability to form biofilms and survive on materials commonly used in food processing equipments (Silva *et al.*, 2014).

According to (EC 2073/2005) (European Commission, 2005), the allowable limit of *L. monocytogenes* in food must not exceed 100 cfu/g throughout the shelf life of the product. Cheeses are considered to be possible vehicles of contamination for *L. monocytogenes* (CDC, 2015). They are exposed to several conditions that are favorable to the development of microorganisms. These conditions include unhygienic manipulation during processing, storage and distribution of the cheese and the mixture of different ingredients included in the cheese. *L. monocytogenes* is capable to grow at refrigerating

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temperatures, so the adoptions of natural antimicrobial strategies to overcome the tolerance of *L. monocytogenes* at low temperatures are essential (Mahgoub *et al.,* 2013).

Therefore, the aim of this study was based to determine the prevalence rate of *L. monocytogenes* in raw buffalo milk, sheep milk, goat milk, yogurt and kareish cheese in Ismailia city and to control this pathogen by using natural antibacterial preservative.

MATERIALS AND METHODS

Sample collection and preparation:

- A total of 90 samples including raw buffalo milk, sheep milk, goat milk, plain yogurt (15 each) and kareish cheese (30) were collected from retail outlets, street vendors and free reared sheep and goat flocks in Ismailia City, Egypt. All samples were transferred in an ice-box at 4°C to the lab to investigate the presence of *Listeria monocytogenes*.

- Preparation of samples for microbiological examination was done according to APHA, 2004.

Isolation and identification of *Listeria monocytogenes* (Roberts *et al.,* 1995):

Twenty five grams (or ml) of each sample were weighed into sterile stomacher bags and diluted with 225 ml of *Listeria* pre-enrichment broth (Oxoid CM 895) then homogenized in a stomacher (Lab blender 400, Seward Medical, London, UK). All samples were first enriched using cold enrichment method (Jami *et al.,* 2010). The inoculated broth was incubated at 4°C for 7-10 days. Two loopful of the enrichment broth were sequentially plated on surface of Listeria oxford agar (Lab122, Lab M Limited, UK) supplemented with X123 and CHROMagar Listeria ™ (LM 851 (B), Paris, France) supplemented with LM851(S). All plates were incubated at 37°C for 48 h. Five presumptive colonies from forementioned media were purified on tryptone soy agar (Oxoid, CM 131) with 0.6% yeast extract (Oxoid, Basingstoke, UK), incubated at 37°C for 24-48 h for further morphological and biochemical identification (CFSAN, 2001). Moreover, two other selective media were used for further confirmation of the suspect colonies; Agosti & Ottavianni Listeria agar [ALOA] (Oxoid CM1080), and PALCAM agar (Oxoid CM 877) supplemented with PALCAM selective supplement (SR150).

Confirmation of the isolates by PCR

DNA extraction and PCR amplification:

DNA extraction was performed according to the manufacturer's guidelines using Bacterial DNA Extraction Kit (Spin-column) (BioTeke Corporation). Amplification reactions were carried out with 5 µl of the eluted solution, 5 µl of 5X Taq Master/ high yield (Jena Bioscience, GMBH, Germany) containing Thermostable DNA Taq polymerase buffer, dNTPs, $(NH4)_2SO4$, $MgCl_2$, and two pairs of primers 50 pmol. Double-distilled water was added to bring the final volume to 25 µl. The PCR assays were performed using a Thermal Cycler (Eppendorf). PCR assays were performed on bacterial DNA with Forward LF: 5´-CAA ACG TTA ACA ACG CAG TA-´3, Reverse LR: 5´-TCC AGA GTG ATC GAT GTT AA-´3 primers according to Bansal (Bansal, 1996). The predicted size based on the published nucleotide sequence of the listeriolysin (*hly* A) gene oligonucleotide primer combinations was 750 bp. The primers were ordered from Operon Company, (Operon, Japan) as nucleotide sequence. The amplification procedure consisted of an initial denaturation step at 95^{o}C for 1 min, followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 51 ^{o}C for 20 sec and extension at 72 ^{o}C for 30 sec. A final extension step was carried out at 72 ^{o}C for 5 min. Aliquots from amplification reactions were analyzed by 1.5 % agarose gel electrophoresis, viewed and photographed under UV light using gel documentation system (Biospectrum UVP, UK). Products of the appropriate sizes were identified by comparisons with a 100-bp DNA ladder (Gibco). In each PCR run, a non-template control was included to detect possible external DNA contamination.

Experimental test for controlling *L. monocytogenes* in soft cheese:

Manufacture of Damietta cheese according to (Abou-Donia, 1986): Cheeses were manufactured in Dairy Technology Unit, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt. Bacterial inoculum was prepared by serial dilution in Ringer's solution to reach a final level of 6 log cfu/mL.

Milk used in the experiment was divided into two parts one part treated with cold pressed Nigella Sativa (N. sativa) oil in 0.2% concentration and the other served as control without treatment. All milk parts were pasteurized at 80°C for 30 min., cooled, adjusted to 45°C, then calcium chloride and sodium chloride were added at levels of 0.02 and 5%, respectively, and then inoculated with 2% of starter culture. Before renneting, N. sativa oil was added to one batch at concentrations of 0.2% and aliquot of 0.1 mL of the pathogen was inoculated into the two batches of pasteurized milk before manufacturing soft cheese, so that the final count of each becomes 5 log cfu/g of cheese samples (Mahgoub *et al.,* 2013).

Cheese samples were packed in sterile plastic containers within its whey and stored in the refrigerator at 5°C±1 for five weeks. Control and treated cheese samples were analyzed for *L.monocytogenes*, count during storage period at times 0, 7, 14, 21, 30 and 35 days.

Statistical analysis:

Data were analyzed using independent t-test for the significance between two groups according to IBM SPSS software version 20.

RESULTS

Table 1: Evaluation of Oxford agar and CHROMagar Listeria for isolation of *L. monocytogenes*

Type of sample	Oxford agar		CHROMagar Listeria	
	No.	%	No.	%
Buffalo milk	1/15	6.7	2/15	13.3
Sheep milk	1/15	6.7	1/15	6.7
Goat milk	1/15	6.7	4/15	26.7
Kareish cheese	2/30	6.7	3/30	10
Yoghurt	0/15	0	2/15	13.3
Total	5/90	5.6	12/90	13.3

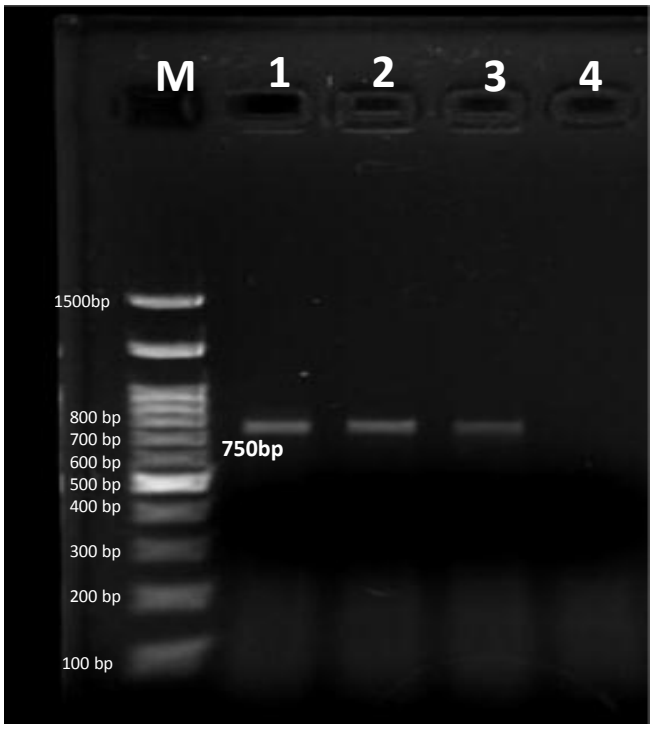

Fig. 1: The figure shows the UV picture after the agarose gel electrophoresis of PCR product. Lane M: the100bp DNA ladder. Lanes from 1 to 3: *L. monocytogenese* positive samples (750 bp), Lane 4: control negative.

Table 2: Effect of N. Sativa oil on total Logarithmic count of *L. monocytogenes* in Damietta cheese

Time / groups	Without treatment	With treatment	P-value
Zero time	5.30103±0.00	5.30103±0.00	---
1 week	6.69897^{a}±0.18875	5.47712^{b}±0.08731	0.005^{**}
2 week	6.84509^{a}±0.132901	5.69897^{b}±0.09281	0.005^{**}
3 week	7.95424^{a}±0.02798	4.90309^{b}±0.03152	0.001^{**}
4 week	7.47712^{a}±0.20621	4.77815^{b}±0.08732	0.001^{**}
5 week	8.90309^{a}±0.03152	3.84510^{b}±0.07394	0.001^{**}
Total means	7.19659^{a}±0.27169	5.00057^{b}±0.14684	
P-value		0.001^{**}	

Log counts with different superscripts showed highly significant differences at ($P \leq 0.01$).
The original counts can be obtained by the antilogarithm (Base 10) of these results.

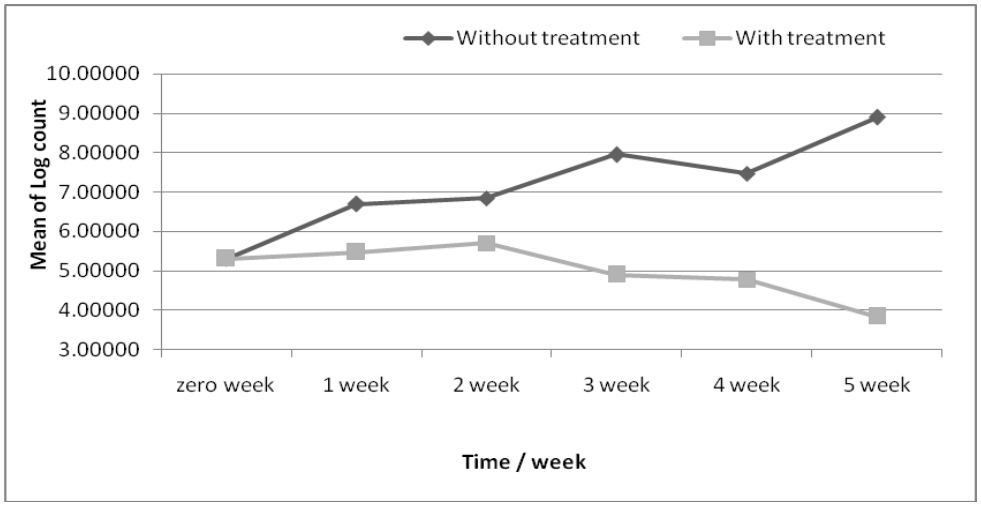

Fig. 2: Reduction in *L. monocytogenes* logarithmic count in cheese treated with N. Sativa oil compared with the non treated one

DISCUSSION

Milk and dairy products are highly perishable foods that may be incriminated in *L. monocytogens* infections. Listeriosis is the third leading cause of death among major pathogens transmitted commonly by food in USA (CDC, 2013). Foods most often associated with human listeriosis are ready to eat (RTE) products that support growth of *L. monocytogenes* through a long refrigerated shelf life and are consumed without further treatments (Jamali *et al.*, 2013). There is a link between animals and their role as a source of infection for humans primarily from consumption of contaminated animal products (OIE, 2008).

Out of the 90 samples tested, 21/90 (23.3%) were presumptively positive for *listeria spp.* on Oxford agar while 13/90 (14.4%) were presumptively positive for *listeria spp.* on CHROMagar Listeria™. Further biochemical identification of the suspect colonies on Oxford agar and the chromogenic medium revealed that 5/21 (23.8%) and 12/13 (92.3%) of the isolated organisms, respectively were confirmed as *L. monocytogenes* (Data not shown in tables).

By applying PCR technique, 3/90 (3.3%) of the total examined samples and 3/12 (25%) of biochemically confirmed isolates gave the specific band (750 bp) (Fig. 1). These results agreed with Jami *et al.* (2010) and Mérdia *et al.* (2010) who recorded low prevalence rates of *L. monocytogenes* (4% and 6.6%) by PCR in raw milk and cheese, respectively.

Data in Table (1) revealed that the chromogenic medium performed better than Oxford agar medium in detection of *L. monocytogenes*. On CHROMagar Listeria™, *L. monocytogenes* produces blue colonies with white halos due to phospholipase activity. These colonies are distinctively different in phenotype from other Listeria species making selection of L. *monocytogenes* much easier, and so improving the accuracy of confirmatory tests (Reissbrodt, 2004; Gouws and Liedemann, 2005 and Jamali *et al.*, 2013). Some *L. monocytogenes* positive samples can go undetected on Oxford agar. Overgrowth by non pathogenic *listeria spp.* and/or phenotypically similar natural background flora can interfere with the isolation of this pathogen (Gouws and Liedemann, 2005). However, if one aims to detect *Listeria spp.*, but not just *L. monocytogenes*, the combination use of traditional selective and chromogenic media are required to achieve higher recovery rate of *Listeria spp*.

The highest prevalence rate of *L. monocytogenes* was detected in goat milk (26.7%) followed by buffalo milk & yoghurt (13.3% each) then kareish cheese (10%) and finally sheep milk samples (6.7%). Several authors could detect *L. monocytogenes* in the examined milk and dairy products. It was detected in 2.2- 2.4%; 1.8% and 2 % in the examined goat milk samples as recorded by Gaya *et al.* (1996); Abou-Eleinin *et al.* (2000) and Nagah and Thabet (2003), respectively. El-Prince and Sayed (2004) recorded *L. monocytogenes* in 2% of examined milk and dairy products. Moreover, El-Malt and Abdel-Hameed (2009) reported the presence of *L. monocytogenes* in 6% of raw cow's milk.

Soft cheeses can be contaminated with *Listeria spp.* because they have a high water content and relatively neutral pH, which facilitate bacterial growth. On the other hand, *L. monocytogenes* will not grow in cottage cheese but can survive. It has also been isolated on rare occasions from yoghurt as post

processing contamination at pH > 4.6 (ADASC, 2000).

Cold-pressed black N. sativa oil contains a high level of hydrophilic phytochemicals including natural antioxidants. The consumption of this product may improve human health and may prevent certain diseases. Moreover, its antimicrobial activity is closely related to their phenolics content (Ahn *et al.*, 2004). The broad spectrum antimicrobial activity of *N. sativa* oil could be attributed to the active constituents mainly Thymoquinon (TQ) and melanin (Bakathir and Abbas 2011 and Monika *et al.*, 2013).

Results illustrated in Table (2) and Fig. (2) revealed a high significant reduction in the logarithmic count of *L. monocytogenes* ($P \leq 0.01$) in the sample treated with cold pressed N. sativa oil. These results agreed with Mahgoub *et al.* (2013) who recorded a reduction of *L. monocytogenes* count by adding N. sativa oil in Damietta cheese during storage period. The inhibitory effect of this oil against *L. monocytogenes* was also recorded by Chaieb *et al.* (2011) and Forouzanfar *et al.* (2014).

It's clearly evident that, *L. monocytogenes* could contaminate milk and ready to eat (RTE) dairy products which imposes a threat to health of consumers. This necessitates the application of strict hygienic measures on farm and during manufacturing of dairy products to prevent contamination of these products. The addition of N. sativa oil; a safe natural antimicrobial and immunostimulant additive, had a lethal effect on *L. monocytogenes* at refrigeration temperature, hence improving the keeping quality of cheese.

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الكشف عن ميكروب الليستيريا مونوسيتوجيناس في الحليب الخام ومنتجات الألبان الجاهزة مع تقييم تأثير زيت حبة البركة كمضاد للميكروبات

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تهدف هذه الدراسة الى تقييم تواجد ميكروب الليستيريا مونوسيتوجيناس فى الحليب الخام وبعض منتجات الألبان مع تقييم تأثير زيت حبة البركة عليها في الجبن الدمياطي وقد أظهرت النتائج أن أعلى معدل تواجد لهذه البكتريا كان في لبن الماعز (٢٦.٧%) يليه اللبن الجاموسي والزبادي (١٣.٣%) ثم الجبن القريش (١٠%) وأقلهم فى لبن الأغنام (٦.٧%). كما أثبتت النتائج ان استخدام الكروم أجاركان أفضل من استخدام الأكسفورد أجار في الكشف عن الليستيريا مونوسيتوجيناس. وقد أظهرت النتائج أنه من بين ٩٠ عينة تم اختبارها، ٩٠/٢١ (٢٣.٣%) و ٩٠/١٣ (١٤.٤%) كانت تحتوي على الليستيريا باستخدام الأكسفورد أجار والكروم أجار على التوالي وبالتحليل البيوكيميائي للمستعمرات المعزولة، أكدت تواجد الليستيريا مونوسيتوجيناس في ٢١/٥ (٢٣.٨%) و ١٣/١٢ (٩٢.٣%) في الأوساط السابق ذكرها على التوالي. تم الكشف عن الجين ليستريوليسين (hyl A) وجد أن ٩٠/٣ (٣.٣%) من كل العينات المختبرة و ١٢/٣ (٢٥%) من العزلات المؤكدة كميائيا كانت ايجابية لهذا الجين. وفي محاولة للسيطرة على ميكروب الليستيريا مونوسيتوجيناس في الجبن الدمياطي باستخدام زيت حبة البركة، أظهرت النتائج انخفاض معنوي تدريجي في العدد اللوغاريتمي الكلي للبكتريا خلال فترة التخزين (٣٥ يوما) .