



Prevalence of *Listeria monocytogenes* in ready-to-eat fish and its control by fresh lemon juice

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Abstract

In this work, the prevalence of *L. monocytogenes* in ready-to-eat (RTE) fish from different restaurants in Assiut city was investigated by cultural and PCR methods. Also, the antimicrobial activity of fresh lemon juice against *L. monocytogenes* in fish meat was evaluated. Results showed that *L. monocytogenes* could be isolated from 6 RTE fish samples with incidence of 6%. By PCR, 5 were confirmed to be *L. monocytogenes* out of 6 isolates by cultural method. Treatments of lemon juice for different exposure times caused reduction ranging between 1.4 and 4.7 log CFU/g for *L. monocytogenes*. Conclusion: The results of this study showed that consumption of RTE fish especially grilled fish may constitute a public health hazard, as it may be associated with food poisoning microorganisms such as *L. monocytogenes*. Inactivation effect of lemon juice on *L. monocytogenes* may give a practical and easy way of providing food safety for RTE fish.

Keywords: microbiological safety, RTE fish, *L. monocytogenes*, PCR, fresh lemon juice

1. Introduction

Fish is known to be an excellent source of proteins, very long-unsaturated chains of omega-3 fatty acids, vitamin D, vitamin B₁₂ and many useful trace elements such as selenium and iodine (James *et al.*, 2013) [27]. Ready-to-eat (RTE) fish are food prepared in advance, which can be eaten as when purchased without the need for cooking or other processing (Anonymous, 2013). Fish is prepared for human consumption by different ways as grilling with charcoal or pan frying. Grilling generally refers to cooking food directly under a source of direct dry heat. While pan frying is a cooking method where by fish is submerged in hot oil (Nathan, 2007) [39]. Pan frying requires adequate oil to cover the bottom of the pan. Oil should be heated to 375F (191 °C). Fish is typically cooked until one side is golden brown; the piece is turned once and cooked until the second side is browned (cooking time is 3–5 min) (NACMCF, 2008) [38]. Charcoal grilling mostly employs the direct grilling method. In this method, fish is placed directly over the radiant heat source to cook. The heat conducted through the cooking grid burns grill marks or crosshatches onto the fish thus imparts characteristic 'charcoal flavor'. Fish must be turned when cooked halfway through.

A wide variety of bacterial pathogens are linked to fish and seafood. The main safety concern with RTE fish is that people consume them without prior heating or additional cooking (Kin *et al.*, 2012) [29]. Consequently, consumption of RTE fish may cause diseases due to infection or intoxication; some of these diseases have been specifically associated with pathogens such as *L. monocytogenes* (Adebayo-Tayo *et al.*, 2012) [1].

Listeria monocytogenes has several important virulence markers. Among them, is p60, encoded by *iap* gene which plays a vital role in intestinal invasion. This gene is indispensable for species-specific identification of *L. monocytogenes*. Primers targeting *iap* gene of *L.*

monocytogenes yielding a 131 bp product (Aurora *et al.*, 2007) [5].

With the modern trend toward more natural preservatives, the use of organic acids can achieve a good microbiological safety in food. Recently weak organic acids such as citric acid, acetic acid and lactic acid are frequently used in food technology. Citric acid is preferred for the tenderness of meat (Burke and Monahan, 2003) [8].

Treatment of lemon juice appears to be a potential technique to preserve meat through inactivating pathogenic bacteria without adversely affecting the characteristic freshness of products (Bingol *et al.*, 2011) [6]. Furthermore, Lemon juice has an important role as antimicrobial agents against microorganisms. They are natural, cheap, safe, and due to increase antibiotic resistance among bacteria.

Ready-to-eat (RTE) fish doesn't undergo any treatment to ensure its safety before consumption, and therefore risk of foodborne disease must be considered if these pathogens are present in the food (Cabedo *et al.*, 2008) [9]. Little information about the microbiological safety of RTE fish especially, bagrus, tilapia and mackerel in Assiut city, as well as limited studies about the antibacterial activity of fresh lemon juice on the growth inhibition of food borne pathogens of public health importance, therefore, this study aimed to evaluate the prevalence of *L. monocytogenes* in fried bagrus, fried tilapia, grilled tilapia and grilled mackerel in Assiut city. In addition, the antibacterial properties of fresh lemon juice which is squeezed generally before eating RTE fish against *L. monocytogenes* isolated in this study in food model system were investigated.

2. Materials and methods

2.1 Sampling

A total of 100 random samples of RTE fish were collected from different restaurants at Assiut city. The collected samples

comprised of 25 fried bagrus, 25 fried tilapia, 25 grilled tilapia and 25 grilled mackerel. The samples were obtained from the restaurants as sold to the consumers and transferred directly to the laboratory in pre-sterilized plastic bags with a minimum of delay where they were prepared for bacteriological examination.

2.2 Preparation of samples

At the laboratory, each sample was aseptically and carefully freed from its sterilized plastic bag and cut the RTE fish muscle with the skin into fine particles by sterile scissor, then 10 gm of each prepared sample were added to the appropriate selective enrichment broth (ISO 6887-3, 2003) [24].

2.3 Isolation techniques

2.3.1 Isolation of *Listeria* spp. (Hudson, *et al.* 1994) [21]:

For the isolation of *Listeria* spp., a two-stage enrichment procedure in Fraser broth was used. After sterilization, *Listeria* Oxford Antimicrobial supplements CCCFA (Biolife, 4240038) were added to the broth. Fish meat samples were incubated in flasks with Fraser broth (Biolife, 401600) at 30°C for 24 h. After that, 0.1 mL of broth were transferred into 10 mL tubes of Fraser broth for secondary enrichment and incubated at 37°C for the next 48 h. The content was transferred with inoculation loop to Oxford agar plates (Biolife, 401600). The plates were incubated at 37°C for 48 h. The suspect *Listeria* colonies were further confirmed on the basis of Gram staining, motility, haemolysis, catalase, and CAMP tests, Rhamnose, Xylose and Mannitol fermentation according to Macfaddin (2000) [34].

- Serological identification of *Listeria* spp. (Pagotto *et al.*, (2001) [43]. The Oxoid *Listeria* Test Kit (Oxoid, Basingstoke, Hampshire, England) is a rapid latex agglutination test for the presumptive identification of *Listeria* spp. in selective and/or enrichment cultures. The Oxoid *Listeria* Test Kit confirms the presence of *Listeria* spp. in culture and should be used in conjunction with biochemical identification for full identification of *Listeria* spp.

Technique: Accurately, 1 drop of sterile normal saline was dispensed onto 2 wells of the reaction card of Latex Agglutination kit. Using a loop, a culture of typical tested organism was taken and a heavy suspension in each drop of saline was carried out. Furthermore, 1 drop of the test latex was added to one well to which 1 drop of control latex was also added and mixed well by using a mixing stick. Finally,

the tested card was gently hesitated for up to 2 min and then examined for agglutination. The reaction was considered positive when the latex particles were rapidly agglutinated forming visible clumps.

- Confirmation of *L. monocytogenes* by Polymerase Chain Reaction (PCR)

2.3.2 Materials used for PCR

Reagents used for agarose gel electrophoresis

Agarose powder, Biotechnology grade (Bioshop^R, Canda inc. lot No: OE16323).

It prepared in concentration 2% in 1× TAE buffer.

Tris acetate EDTA (TAE) electrophoresis buffer (50× liquid concentration) (Bioshop^R, Canda inc. lot No: 9E11854).

The solution diluted to 1× by adding 1 ml stock solution to 49 ml double dist. Water to be used in the preparation of the gel or as a running buffer.

Ethidium bromide solution (stock solution) biotechnology grade (Bioshop @ Canda Inc, Lot No: 0A14667)

The stock solution was diluted by 25µl /200ml double distilled water and stored covered at 4°C. It was used for staining of PCR products that electrophoreses on agarose gel to be visualized by UV light.

Gel loading buffer (6× stock solution) (Fermentas, lot No: 00056239)

The components were dissolved in sterile double distilled water and stored covered with aluminum foil at room temperature.

DNA ladder (molecular marker)

100 bp (Fermentas, lot No: 00052518)

5X Taq master (Fermentas)

Containing polymerase enzyme, Magnesium chloride (Mg Cl₂), Deoxy nucleotide triphosphate (dNTP) and PCR grade water.

Primer sequences of *L. monocytogenes* used for PCR

Application of PCR for molecular characterization of invasive associated protein (*iap*) species specific gene of *L. monocytogenes* was carried out using the following primers (Pharmacia Biotech):

Table 1: Primers used

Target gene	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
<i>iap</i> (F)	5' ACAAGCTGCACCTGTTGCAG '3	131	Swetha <i>et al.</i> (2013) [51]
<i>iap</i> (R)	5' TGACAGCGTGTGTAGTAGCA '3		

DNA Extraction using QIA amp kit

The technique recommended by Shah *et al.* (2009) [46] was applied with some suitable modifications. Generally, all detected *L. monocytogenes* strains were grown overnight in Brain Heart Infusion (BHI) broth at 37°C, and the suspension was then heated at 100°C for 20 min. Accurately, 50-200 µl of the culture were placed in Eppendorf tube and kept frozen at -40°C till use. The obtained lysate (5 µl) was used as DNA

template in PCR reaction mixture.

2.4 Amplification reaction of *L. monocytogenes* (Kaur *et al.*, 2007) [28]:

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). A PCR was attempted with comprising associated gene (*iap*). The PCR was set up in 50 µl reaction volume. Based on the results of

various trials, the reaction mixture was optimized as follows: 10 µl PCR buffer (consisting of 100 mmol l), Tris-HCl, pH 8.3; 500 mmol KCl; 15 mmol MgCl₂ and 0.01% gelatin), 1 mmol dNTP mix, 7.5 mmol MgCl₂ and 10 µmol forward and reverse primer, 5 U of Taq DNA polymerase, 5 µl of cell lysate and sterilized milliQ water to make up the reaction volume.

The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 2 min followed by 35 cycles each of 15 sec denaturation at 95°C, 30 sec annealing at 60°C and 1 min extension at 72°C, followed by a final extension of 10 min at 72°C. Amplified DNA fragments were analyzed by 1.5% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with ethidium bromide and captured as well as visualized on UV transilluminator. A 100 bp DNA ladder was used as a marker for PCR products

Effect of fresh lemon juice on *L. monocytogenes* (Bingol *et al.*, 2011) [6].

i) Cultures and preparation of inoculum

Bacterial strain, *L. monocytogenes* isolated from examined fish samples in the present study were used for this investigation. Serotyping of the strains was done at the Food Analysis Center, Faculty of Veterinary Medicine, Benha University by PCR. This strain was maintained in tryptone soy agar slants at 5°C until use. Stock culture of *L. monocytogenes* was grown on tryptic soya broth for 24 h at 37°C to obtain cells in early stationary growth phase.

Concentrations were then adjusted to 1 X 10⁷ CFU/ml using saline peptone water. An aliquot of 1 ml of bacterial suspension (*L. monocytogenes*) at approximately 1 X 10⁷ CFU/ml was added in a sterile jar containing 100 ml of saline peptone water to obtain 1 X 10⁵ CFU/ml.

ii) Preparation of treatment solutions

Undiluted (concentrated) freshly squeezed lemon juice was used as treatment solutions in all experiments. Lemons were purchased from a local supermarket in Assiut city. Lemons were washed with cold water and cut with a sterile knife. A household juicer was used to obtain fresh lemon juice (100 per cent) which was stored in a sterile jar during the experiment. Acidity of test solutions was determined by titration against 0.1 N sodium hydroxide and expressed as citric acid percentage (vol/vol), and also the pH value of lemon juice was measured by a portable pH meter (Gallenkamp pH stick).

iii) Aseptically obtained fish muscle

Fresh tilapia collected from local fish market was utilized in the preparation of fillets. Fish fillets were prepared by removing skin and gut regions. The fish was then filleted aseptically using a sharp knife. The fillets were made into smaller size cubes weighing about 1 g were transferred into individual sterile petridish (Kumar *et al.*, 2015) [31].

iv) Inoculation of fish meat and treatment with lemon juice

Fish meat samples were analyzed before inoculation for the presence of *L. monocytogenes* according to Hudson, *et al.* (1994) [21] procedures and any *L. monocytogenes* were detected. After that, a portion of 10 g fish meat samples were

weighed and transferred into sterile petri plates for inoculation with bacteria.

Each fish meat sample was plunged separately into a glass of inoculums and stayed in for 10 sec., then was placed in sterile petri plates and stored at room temperature for 10 min to allow the attachment of *L. monocytogenes* cells. Two, five, ten and fifteen milliliters of treatment solution were poured onto surfaces of fish meat samples and let them absorb in the petri plates depending on exposure times (30 sec, 1 min, 2 min, 3 min and 5 min) at room temperature. Fish meat samples were then placed in sterile stomacher bags containing 90 ml of sterile saline peptone water and analyzed.

v) Detection and enumeration of *L. monocytogenes*

A pre-detection step was applied to determine if there were any *L. monocytogenes* in the samples before inoculation. For this purpose, 1 gram of fish meat was added into 9 ml of Fraser broth (Biolife, 401600) with selective Listeria Oxford Antimicrobial Supplement CCCFA (Biolife, 4240038) were added and incubated at 37°C for 24 h. for the detection of *L. monocytogenes*. The test cultures Fraser Broth were streaked onto Oxford Agar (Biolife, 401600) and incubated for 48 h at 37°C (Hudson, *et al.*, 1994) [21].

Randomly selected typical colonies from the agar plates were confirmed to be *L. monocytogenes* using the following biochemical haemolysis, catalase, and CAMP tests, Rhamnose, Xylose and Mannitol fermentation according to ISO 11290-1 (1996) [23].

To enumerate the level of *L. monocytogenes* on the surface of inoculated fish meat samples, 1 gm of fish meat was added in 9 ml of saline peptone water and homogenized in a stomacher (Lab. blender 400, Seward Lab., London, UK). Then, an aliquot of 0.1 ml of decimal dilution was transferred to Oxford agar plates and incubated for 48 h at 37°C. The typical colonies were enumerated and the initial *L. monocytogenes* levels were defined as 1 x 10⁵ CFU/g.

Enumeration of *L. monocytogenes* in the treated samples was carried out as mentioned.

vi) Determination of pH

The pH value of each fish meat sample was measured at each exposure time after blended separately with 100 ml of distilled-deionised water by a portable pH meter (Gallenkamp pH stick) (AOAC, 1990) [4].

vii) Sensory evaluation

Of fried fish by deep-frying in sunflower oil at 160°C for 7 min. (frying was carried out immediately during evaluation). Uninfected and treated with lemon juice fish meat samples were evaluated by a five-member panel, post graduate students of Food Hygiene Department, Faculty of Vet. Med., Assiut Univ., who had usually habit to eat fish meat. The panellists were trained in three separate sessions approximately 2 h, in which panellists were served fish meat samples for evaluation of selected attributes. The attributes studied were: flavour intensity, sustained dryness/juiciness, acidic flavour and overall acceptability. All attributes were scored using a five-point descriptive scale (1 ¼ dislike extremely 2 ¼ dislike slightly, 3 ¼ neither like nor dislike, 4

¼ like slightly, and 5 ¼ like extremely) according to Sørheim *et al.* (1996) [48] and all values were recorded. The panel members were seated in individual booths in a temperature-controlled and light-controlled room, receiving a set of four samples served in a complete randomized order. Each sample was labeled, at random, with a two-digit code number. Unsalted crackers and water were served to panellists to freshen their mouth between each sub-samples assessment (ISO 8589, 1988) [25]. Sensory panel was carried out duplicate.

viii) Statistical analysis

To investigate the effectiveness of lemon juice in the elimination of *L. monocytogenes* two replicate experiments were conducted and all counts of bacteria (cfu.g⁻¹) recovered from the fish samples were transformed to logarithms before

computing means and standard deviations. The Decimal Reduction (DR) in the population of the bacteria was calculated by the difference between the counts obtained in the control and each treatment.

In order to determine the effect of lemon juice in fish meat samples, a one way analysis of variance (ANOVA) was performed using SPSS 10.0 statistical package (SPSS, 1999) [50]. Moreover, ANOVA for a four (treatments) x five (exposure times) factorial design was used to explain whether the interactions between treatment and exposure times were significant (p < 0.05). If the effect of lemon juice was found significant, Duncan’s multiple range tests was used to evaluate the significance of the difference in populations of *L. monocytogenes* existed among mean log values.

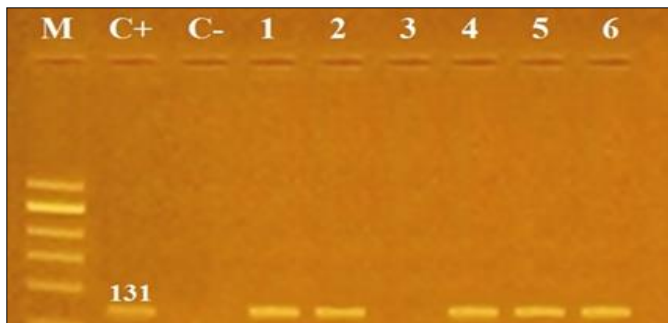
3. Results & Discussion

Table 2: Prevalence of *Listeria* spp, in the examined RTE fish samples

Samples	No.	<i>Listeria</i> spp.	
		No.	%
Fried Bagrus	25	6	24
Fried tilapia	25	2	6
Grilled tilapia	25	3	12
Grilled mackerel	25	8	32
Total	100	19	19

Table 3: Incidence of different types of *Listeria* spp. in the examined RTE fish samples

Samples	No.	<i>L. monocytogenes</i>		<i>L. innocua</i>		<i>L. welshimeri</i>		<i>L. ivanovii</i>		<i>L. grayi</i>	
		+ve	%	+ve	%	+ve	%	+ve	%	+ve	%
Fried Bagrus	25	3	12	2	8	1	4	0	0	0	0
Fried tilapia	25	0	0	1	4	0	0	0	0	1	4
Grilled tilapia	25	1	4	1	4	0	0	0	0	1	4
Grilled mackerel	25	2	8	3	12	1	4	1	4	1	4
Total	100	6	6	7	7	2	2	1	1	3	3



Lane M: 100 bp ladder as molecular size DNA marker.
Lane C+: Control positive *L. monocytogenes* for *iap* gene.
Lane C-: Control negative.
Lanes 1, 2, 4, 5 & 6: Positive *L. monocytogenes* strains.
Lane 3: Negative *L.monocytogenes* strain.

Fig 1: Agarose gel electrophoresis of PCR of *iap* (131 bp) specific gene for characterization of *Listeria monocytogenes*.

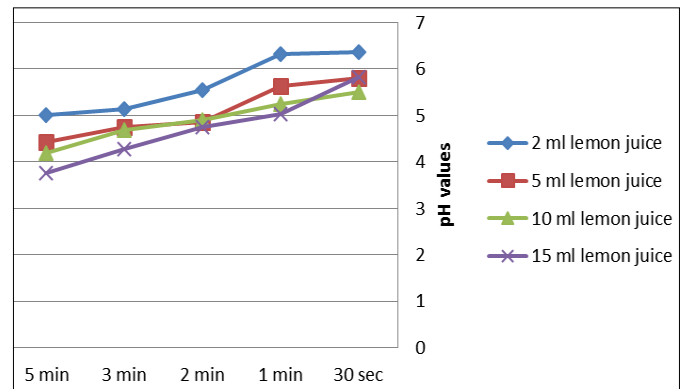


Fig 2: pH values of fish meat samples

Table 4: Sensory evaluation for Flavour intensity, juiciness and overall acceptability of cooked fish meat treated with fresh lemon juice by a 5-point test scale

Treatment	Exposure time	Characteristic		
		Mean score + SD		
		Flavour intensity	Juiciness	Overall acceptability
2 ml lemon juice	30 sec	4.6 ±0.55	4.2 ±0.84	4.2±0.45
5 ml lemon juice	30 sec	4.4 ±0.55	4.4 ±0.55	4.2 ±0.84
10 ml lemon juice	30 sec	2.6±0.89	4.6 ±0.55	2.8±1.10
15 ml lemon juice	30 sec	1.6±0.55	4±1.41	1.6±0.55
2 ml lemon juice	1 min	4.6 ±0.55	3.8 ±0.84	4.2±0.45
5 ml lemon juice	1 min	4.6 ±0.55	4.6 ±0.55	4.8±0.45
10 ml lemon juice	1 min	3±1.2	4.4±0.55	2.8±1.10
15 ml lemon juice	1 min	1.6±0.55	4.2±1.30	1.6±0.55
2 ml lemon juice	2 min	4.8±0.45	4±0.77	4.2±0.45
5 ml lemon juice	2 min	4.8±0.45	4.6 ±0.55	4.6 ±0.55
10 ml lemon juice	2 min	2.8±1.10	4.4±0.55	2.8±1.10
15 ml lemon juice	2 min	1.4±0.55	4.2±1.30	1.4±0.55
2 ml lemon juice	3 min	4.4 ±0.55	4.6 ±0.55	4.4 ±0.55
5 ml lemon juice	3 min	3.8±0.45	4.4±0.55	3.8±0.45
10 ml lemon juice	3 min	2.6±1.14	4.4±0.89	2.6±0.89
15 ml lemon juice	3 min	1.2±0.45	4.4±0.89	1.2±0.45
2 ml lemon juice	5 min	4.4 ±0.55	4±0.77	4±0.77
5 ml lemon juice	5 min	4.2±0.45	4.4±0.55	4±0.00
10 ml lemon juice	5 min	2.4±1.14	4.6 ±0.55	2.4±0.55
15 ml lemon juice	5 min	1.2±0.45	4.8±0.45	1.00±0.00

Sensory scale: 1 ¼ = dislike extremely, 2 ¼ = dislike slightly, 3 ¼ = neither like nor dislike, 4 ¼ = like slightly, and 5 ¼ = like extremely.

Table 5: Effects of lemon juice treatment on *L. monocytogenes* in fish meat samples (log₁₀ CFU/g)

Treatment with lemon juice	30 sec	1 min	2 min	3 min	5 min	p
2 ml lemon juice	4.3	3	2.2	1.8	1.7	*
5 ml lemon juice	4.3	3	2.2	1.8	1.6	*
10 ml lemon juice	4.2	2.9	1.8	1.6	1.3	**
15 ml lemon juice	3.8	2.8	1.3	1	1	**

* p<0.05; ** p<0.01;

Table 6: Reduction rate of lemon juice treatment on *L. monocytogenes* in fish meat samples (log₁₀ CFU/g)

Treatment with lemon juice	30 sec	Log reduction (log cfu.g ⁻¹)	1 min	Log reduction (log cfu.g ⁻¹)	2 min	Log reduction (log cfu.g ⁻¹)	3 min	Log reduction (log cfu.g ⁻¹)	5 min	Log reduction (log cfu.g ⁻¹)
2 ml lemon juice	4.3	1.4	3	2.7	2.2	3.5	1.8	3.9	1.7	4.0
5 ml lemon juice	4.3	1.4	3	2.7	2.2	3.5	1.8	3.9	1.6	4.1
10 ml lemon juice	4.2	1.5	2.9	2.8	1.8	3.9	1.6	4.1	1.3	4.4
15 ml lemon juice	3.8	1.9	2.8	2.9	1.3	4.4	1	4.7	1	4.7

^aData are averages from two independent trials

Incidence of *Listeria* spp. in RTE bagrus, mackerel and tilapia

The association of *L. monocytogenes* with RTE seafood is most likely due to environmental contamination occurring during processing. The raw fish material as well as food handlers may be sources of *L. monocytogenes* contamination (Cruz *et al.*, 2004) [11].

The results of the current study demonstrate that 18% of the retail RTE fish samples tested were contaminated with *Listeria* spp. and 3% were contaminated with *L. monocytogenes*. The incidence of *L. monocytogenes* observed in this study was lower than with published data in Malaysia and Thessaloniki (Northern Greece), which reported the presence of the pathogen in 6.7 and 6.1% of RTE seafood-based products, respectively (Jamali *et al.*, 2013 and Soutos *et al.*, 2014) [26, 49].

The microbiological criteria for *L. monocytogenes* in foodstuffs are specified in Commission Regulation (EC, 2005) No 2073/2005 [17]. For RTE foods which are able to support the growth of the pathogen, absence in 25 g of the product is required at the end of the production.

Concerning fried bagrus bayad, it is an important food fish, with flesh of good eating and of economic importance in Egypt ((Lamai, 1993) [32]. The obtained results of this study showed that 24% of fried bagrus bayad samples tested were contaminated with *Listeria* spp. and 12% were contaminated with *L. monocytogenes*. Other *listeria* spp. isolated included *L. innocua* (8%) and *L. welshimeri* (4%) as recorded in Tables 2 and 3.

Regarding grilled mackerel, from data shown in Table 2 and 3, it could be concluded that out of 25 examined grilled mackerel fish, 8 samples were positive for *Listeria* spp. with a

percentage of 32%. Among these, *L. monocytogenes* was identified and represented 8% of the examined samples. The other species of listeria were classified as *L. innocua* (12%), *L. welshimeri* (4%), *L. ivanovii* (4%) and *L. grayi* (4%). Comparatively, Zarei *et al.* (2012) [54] couldn't isolate *L. monocytogenes* from seafood products. While, lower incidence (2.7% and 6.6%) of *L. monocytogenes* was obtained by Yu and Jiang (2014) [53] and Mohamed-Ghada (2012) [35], respectively. In contrast, higher incidences (10.4%) of *L. monocytogenes* were obtained by EFSA (2013) [14].

Contamination of grilled mackerel by *L. monocytogenes* may be due to grilling process does not always ensure complete heat treatment. Norrung *et al.* (1999) [41] concluded that insufficient heat treatment of food allowed the survival of *L. monocytogenes*. Moreover, RTE fish are especially a concern since these consumed without further cooking and are known to be good growth substrates for pathogenic microorganisms such as *L. monocytogenes*, and might be a health risk to the consumer (Zhu *et al.*, 2005) [55].

Concerning grilled tilapia, the findings outlined in Tables 2 and 3 showed that *Listeria* spp. were isolated from 3 out of 25 examined grilled tilapia fish samples with an incidence of 12%. *L. monocytogenes* was isolated from one of these examined samples with a percentage 4%. Moreover, *L. innocua* (4%) and *L. grayi* (4%) were the other isolated *Listeria* spp. In contrast, higher prevalence rate (16.6%) of *Listeria* spp. was recorded by Mohamed-Ghada (2012) [35] while *L. monocytogenes* was 6.6%. On the other hand, the results obtained in the present study was disagree with Hosein *et al.* (2008) [20] and Ibraheem-Shimaa (2012) [22] who couldn't detect *L. monocytogenes* from the examined RTE fish.

The presence of *L. monocytogenes* in grilled tilapia may be attributed to food handlers who can spread or contaminate the final products by *L. monocytogenes* and facilitate the cross contamination (Saludes *et al.*, 2015) [44]. Also, vegetables such as lettuce, cucumber and tomatoes added to grilled fish after finishing the grilling process may be the source of contamination by *L. monocytogenes* as reported by Jamali *et al.* (2013) [26] who conducted a study shown a high prevalence rate (14.7%) of *L. monocytogenes* in vegetables.

With regard to fried tilapia, out of 25 examined fried tilapia fish in the present study as shown in Tables 1 and 2, two samples with an incidence of 8% were contaminated with *Listeria* spp. One sample was identified as *L. innocua* while the other sample was classified as *L. grayi*. Meanwhile, all the examined samples of fried tilapia fish were free from *L. monocytogenes*. This obtained result was in agreement with El-shenawy *et al.* (2011) [15] and Helmy-Nariman (2015) [19] who couldn't detect *L. monocytogenes* in fried fish. On the contrary, other researchers recorded higher percentage of *L. monocytogenes* in fried fish as Mohamed-Ghada (2012) [35] (3.3%) and Jamali *et al.* (2013) [26] (5.9%).

The absence of *L. monocytogenes* in the examined fried fish samples may be due to the fact that the heat treatment used in preparation of RTE fried fish are in most cases sufficient to kill *L. monocytogenes*. While contamination with other *Listeria* spp. may be occurred at the post processing stage and reflect poor hygienic practices in the preparation of this fish (Bouayad and Hamdi, 2012) [7]. Thus, Frying of fish is more efficient than grilling as frying lead to complete destruction of

the microorganisms in tilapia fish, while grilling not able to destruct these microorganisms.

Our results indicated that *L. monocytogenes* (6%) and *L. innocua* (7%) were the most commonly recovered species in the all examined RTE samples as shown in Table (2), although other species were also detected, but in lower frequency. Concerning the high incidence of *L. innocua* (7%) in the examined samples, this was in agreement with Silva-angulo *et al.* (2015) [47] who reported that *L. innocua* was the most common species of *Listeria* to be detected in the food industry. Generally, RTE fish are in a higher risk category than other foods as the heat step of cooking, which would kill any *L. monocytogenes* present, is missing in these foods (Anonymous, 2013) [3].

The discrepancies in the prevalence of *L. monocytogenes* among different studies and countries may be due to type of seafood sample, differences in food-processing environment, differences in cooking methods, source of the samples (retail level and factories), sampling season, isolation method, human activity etc. (Soultos *et al.*, 2014) [49].

Concerning confirmation of 6 isolated *L. monocytogenes* strains by PCR, 5 were confirmed to be *L. monocytogenes* which possessed invasive associated protein (*iap*) species specific gene (Figure 1).

L. monocytogenes bacteria had *iap* putative virulence gene. Higher incidences (23.9 and 8%) of *L. monocytogenes* by PCR method than the present study (2.5%) were recorded by Van Coillie *et al.*, (2004) [52] and Swetha *et al.*, (2013) [51], respectively whereas Moharem *et al.*, (2006) [36] reported very low incidence i.e. 1.83% (only 3 out of 132 fish samples). All of the *L. monocytogenes* bacteria had *plcA*, *prfA*, *actA*, *hlyA* and *iap* putative virulence genes (Momtaz and Yadollahi, 2013) [37].

Antibacterial activities of fresh lemon juice against *L. monocytogenes* isolated in this study

Properties of lemon juice concentrates used in this study: The pH of lemon juice was 3.20 and the level of citric acid was 0.841% (Titratable acidity).

The initial pH of fish fillet was measured 7.34. When it was treated with lemon juice, the average pH of the mixture became to 5.26 and decreased to 3.77 at the end of the experiments i.e. results showed a reduction in the value of pH (Figure 2).

Sensorial data (Table 4) indicate that the panel members could not detect negative effects in treated fish samples, being the scores observed above 3 until concentration of 5 ml of freshly squeezed lemon juice. Thus, freshly squeezed lemon juice treatment until concentration of 5 ml did not have adverse effects on fish meat quality characteristics. Lemon juice strengthened the flavour of fish meat samples without causing any sensory problems and they were gratefully overall acceptable approximately for 5 min.

Table 5 shows the effects of different doses of lemon juice (2 ml, 5 ml, 10 ml and 15 ml lemon juice) on *L. monocytogenes* inoculated to fish meat at different exposure times. Results of this experiment revealed that all juices concentrations have inhibiting effects against *L. monocytogenes* due to the presence of citric acid and some volatile oils e.g.(limonene) and some materials e.g. linalyl acetate, linalool, turpinol and

cymen (Lee *et al.*, 1987) ^[33].

Although there seemed to be slight reductions in the counts for each inhibitory group, the immediate antimicrobial effect of lemon juice against *L. monocytogenes* at 30 sec, 1 min and 2 min was observed at statistically significant level ($P < 0.05$). There was a significant difference between 5 and 15 min treatments by using lemon juice ($P < 0.01$).

These obtained results were in agreement with Dimic *et al.*, (2012) ^[13] who observed that Lemon extract exhibited greater inhibitory effect against *Listeria* spp., with *L. monocytogenes* being the most susceptible to it than other species of listeria. In vitro studies showed that lemon extracts (both lemon juice and essential oil) have potential antibacterial properties against wide range of both Gram- positive as well as Gram-negative bacterial agents (Naz *et al.*, 2010) ^[40]. Lemon extract contains many biologically active compounds that give rise to their antimicrobial action. The most important active ingredients of lemon are monoterpenes such as limonene, γ -terpinene, β -pinene and the aldehydes geranial and neral (Espina *et al.*, 2011) ^[16].

Giuseppe *et al.*, (2007) ^[18] have reported the presence of limonoids in Citrus species, which can be considered responsible for activity against many clinically, isolated bacterial strains.

Furthermore, lemon juice contains 5 to 6% citric acid (pH from 2 to 3). This property can be exploited to make lemon a good antibacterial agent. Scientific studies proved that it can successfully inhibit vegetative cells and spores of some food spoilage microorganisms (Conte *et al.*, 2007) ^[10].

The results of the experimental study revealed that treatments of lemon juice for different exposure times caused reduction ranging between 1.4 and 4.7 log CFU/g for *L. monocytogenes* (Table 6).

As shown in Table (6) the mean log reductions observed in the present study were in agreement with those reported by other authors when using organic acids. Generally, treatments with organic acids at varying concentrations result in population reductions ranging from 1 to 3 log units on meat surfaces (Dickson and Anderson, 1992) ^[12].

Lemon fruit is an inexpensive, easily available citrus fruit, popular for its culinary and medicinal uses (NPCS, 2012) ^[42]. The lemon fruit juice consists of about 5% citric acid that gives a sour (tarty) taste to the lemon. It is a rich source of vitamin C (Andrew, 2010) ^[2].

The presence of weak acids at different concentrations in some common household products, such as lemon juice led to the hypothesis that these products could be used as sanitizers for in-home use of foods like raw products (Kisla, 2008) ^[30]. These natural products and their mixture can be considered to be potential antimicrobial agents in preventing foodborne outbreaks related to fresh products at household levels (Sengun and Karapinar, 2005) ^[45].

4. Conclusions

The obtained results of the present study showed that consumption of RTE fish especially grilled fish may be constitute a potential hazards to the consumers, as it may be associated with food poisoning microorganisms such as *L. monocytogenes*. The presence of *L. monocytogenes* in RTE fish should be considered as having significance public health

implications, particularly among the persons who are at greater risk. PCR was found to be a suitable test for confirmation of fish samples for *L. monocytogenes* in a rapid way. Also, the achieved results in this work declared that fresh lemon juice was the most potent against *L. monocytogenes*. Inactivation effect of lemon juice on *L. monocytogenes* may give a practical and easy way of providing food safety for RTE fish. Consumers generally prefer to eat RTE fish just after squeezing fresh lemon juice and these could be a way to minimize the amount of existing microorganisms such as *L. monocytogenes*.

5. References

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