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MICROBIOLOGICAL QUALITY OF FILLETED FISH WITH SPECIAL REFERENCE TO *LISTERIA MONOCYTOGENES*

(With 2 Tables and One Figure)

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الجودة الميكروبيولوجية للسمك الفليه مع الأهتمام بميكروب الليستريا مونوسيتوجينز

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لتحديد جودة السمك الفليه المستورد (السابا) والمحلى (البلطى النيلى وقشر البياض)، أجرى تحليل لمعرفة العد الكلى للبكتريا الهوائية والمعوية وايضا لمعرفة مدى تواجد الليستريا مونو سيتوجينز. وجد أن السمك الفليه المستورد كان أقل فى العد الكلى للبكتريا الهوائية والمعوية وايضا تواجد الليستريا مونو سيتوجينز. كان العد الكلى للبكتريا الهوائية فى الفليه المستورد (السابا) ٣,٣±٧,٧×١٠^٦ مستعمرة، وكان فى البلطى المحلى وقشر البيـــاض المحلى ١,٨±٤,٠ ×١٠^٧ و١,٨ ±٥,٠ ×١٠^٧ مستعمرة أما العد الكلى للبكتريا المعويـــة كان ١,٣±٢,٨ ×١٠^٢ مستعمرة فى الفليـــه المستـــورد (السابا) وكان ٤,٣ ±٨,٤ ×١٠^٢ ، ٥,١±٣,٢ × ١٠^٢ مستعمرة فى كل من البلطى وقشر البياض الفليه المحلى على الترتيب. تم التعرف على الليستريا مونو سيتوجينز بإجراء الأختبارات المعملية وتم التأكد من المعزولات بإجراء أختبار البلمرة.كان تواجد الليستريا مونو سيتوجينز بمعدل ٦,٧% فى السمك الفليه المستورد وبمعدل ٢٣,٣ % فى السمك المحلى (٢٦,٧ % فى البلطى النيلى و٢٠ % فى قشر البياض).

SUMMARY

Imported (Saba) and local (*Tilapia nilotica and Nile perch)* flitted fish (60 samples) were analyzed for aerobic colony count (ACC), total coliform count and for presence of *Listeria monocytogenes* to detect their quality. Imported flitted fish samples were lower in aerobic colony count, coliform count and also in *listeria monocytogenes* incidence. Aerobic colony count (ACC) was $3.3\pm7.7x10^6$cfu/g in imported (Saba) filleted fish, $1.8\pm4.0x10^7$and$1.8\pm5.0x10^7$ cfu/g in local filleted *Tilapia nilotica and Nile perch,* respectively. Coliforms counts were $1.3\pm2.8x10^2$, $4.3\pm8.4x10^2$ and $5.1\pm3.2x10^2$ cfu/g in imported (Saba) filleted, local filleted *Tilapia nilotica and Nile perch*, respectively. The overall incidence of *Listeria*

monocytogenes was 6.7% in imported flitted fish samples, 23.3% in local flitted fish samples (26.7% in *flitted Tilapia nilotica* samples and 20% in *Nile perch* samples), which was identified by conventional PCR reaction.

Key words: *Quality, filleted fish, Listeria monocytogenes.*

INTRODUCTION

Fish form an important part of human diet, and is suspected as one of the important causes of gastroenteritis. The microbiological quality and safety of fish especially flitted fish are largely unknown. According to ICMSF (1986), most aquatic animals at the time of harvest have counts in the region of 10^2– 10^5organisms per gram. While an increase in aerobic plate count (APC) to levels $>10^6$ per gram is an indicative of long storage at chill temperatures or temperature abuse prior to processing. APC is thus an indicative of quality and effectiveness of handling procedures and storage conditions. The microbial upper limit for fresh fish was 7 log cfu/g aerobic mesophilic counts. The shelf life of tilapia fillets were about 10-12 days considered for microbial safety and sensory acceptability. Rapid increase in total volatile base nitrogen (TVB-N) corresponded with high count (> 8 log10 cfu/g) of APC in chilled fillets was recorded by Chytiri *et al.* (2004) and Mhongole (2009).

Fish are recognized as being highly perishable, having a relatively short shelf life, which is defined as the length of time from the day of catch that fresh fish can be in the marketplace unspoiled (Regenstein and Regenstein, 1991). Quality is defined as the aesthetic appearance and freshness or degree of spoilage which the fish has undergone. It may also involve safety aspects such as being free from harmful bacteria, parasites or chemicals (Huss, 1995). Immediately as fish is caught, it loses its natural resistance to be attacked by microorganisms and also starts to undergo both physical and chemical changes that in return bring changes in appearance, taste, smell and texture.

Listeriosis caused by *L. monocytogenes* is a life-threatening disease in fetuses, newborns, immunocompromised people and the elderly (Schuchat *et al.*, 1991). *L. monocytogenes* is ubiquitous in nature; it can be isolated from soil, silage, fresh and marine water, sediments and plants. Therefore aquatic creatures are also potential sources of the bacterium. Part of the seafood products undergoes various processing steps that can inactivate the bacterium if present on the raw product. *L. monocytogenes*, however, can also enter the product both during and after processing due to poor sanitation conditions or inadequate manufacturing practices (Jinneman *et al.*, 1999). *L. monocytogenes* survives freezing well and the

frozen storage causes a limited reduction in the viable population of *L. monocytogenes* (Lou and Yousef 1999).

The contamination with *L. monocytogenes* on whole fish and its processing environment could be the source of post processing contamination on fresh fillets and ready-to-eat products (Hu *et al.,* 2006). A plant-specific *Listeria* control program should also include strategies to minimize both the raw material and the environmental contaminations, procedures to prevent cross-contamination and employee training (Lappi *et al.,* 2004). Even when handled under the best possible conditions, raw seafood or processing environment will probably never be completely free from *L. monocytogenes* (Autio *et al.,* 2004).

Polymerase Chain Reaction (PCR) is a technique used for the rapid, specific and highly sensitive detection of pathogens in food (Somer and Kashi, 2003)

The objective of this study was to evaluate the sanitary quality and safety of imported flitted fish as well as local flitted fish with special emphasis to *Listeria monocytogenes*.

MATERIALS and METHODS

Samples:

Sixty samples of local (*Tilapia nilotica* (15 samples) *and Nile perch* (15 samples)) and imported (Saba) flitted fish (30 samples) were collected from Assiut city markets during the first half of year 2010 and transferred immediately to the laboratory. The samples were analyzed for aerobic colony count (ACC), total coliform count and for the presence of *Listeria monocytogenes* to detect their quality.

Procedures

Aerobic colony count (ACC) and coliforms.

Preparation of the samples:

Samples were kept frozen until analysis. After thawing of samples by overnight chilling, 10 gm sections of fish fillets were cut under sterile conditions and homogenized in Phosphate Buffered Saline (PBS) to 10% (w/v) suspension. Homogenized tissue samples were serially diluted in PBS and plated onto plate count agar (PCA) (DIFCO, Becton Dickinson and Company, Sparks, Md., U.S.A.) for aerobic colony count (ACC) and for enumeration of coliforms by pour plated using violet red bile agar (VRBA) (DIFCO) at 37 °C for 18 to 20 h. Dishes that contain no more than 150 colonies were counted. All colonies on PCA were counted, while on VRBA, coliform colonies are purplish red colonies that have a diameter of

0.5 mm or greater, usually surrounded by a reddish zone were counted (Roberts and Greenwood, 2003).

Isolation of *Listeria monocytogenes*:

Preparation of the samples:

Twenty five grams of fish sample were homogenized with 225 mL of modified tryptone soya broth (modified tryptone soya broth containing yeast extract 6 g/L used as selective primary enrichment medium, made selective by the addition of acriflavine hydrochloride (10 mg/L), nalidixic acid sodium salt (40 mg/L) and cycloheximide (50mg/L)) and incubated at 30°C for 48 ±2h. Enrichment broth was streaked on to Oxford agar and plates were incubated at 37°C for 48 h (Roberts and Greenwood, 2003).

Plates were examined for the presence of typical colonies after 24h and 48 h. Morphologically typical colonies were verified by Gram's staining, catalase reaction, tumbling motility at 20–25 °C, methyl red-Voges Proskauer (MR-VP) reactions, CAMP test with *S. aureus*, and *R. equi*, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and a-methyle-D-mannopyranoside) and haemolysis on 5% sheep blood agar.

Genomic DNA Extraction of *Listeria monocytogenes*:

The strains were further identified by conventional PCR reaction. For each Listeria strain, a 10-ml culture was grown to mid-log phase in Tryptose Soya (TSY) broth, and 1 ml of cells was pelleted by centrifugation (13,000 xg for 5 min). The cell pellets were resuspended in 1 ml of sterile phosphate buffer saline. The resuspended cells were re-centrifuged at 12,500 xg for 15 min. The pelleted cells were then used for DNA extraction. Genomic DNA from suspected Listeria strains was extracted using the Wizard genomic DNA purification kit (Promega, USA) as recommended by the manufactures. DNA samples were stored at -20 °C until use.

PCR identification of *Listeria monocytogenes*:

For *L. monocytogenes* PCR identification, 2 primers were selected based on the prfA (transcriptional activator of the virulence factor) gene for *L. monocytogenes* according to Germini *et al.* (2009). All PCR reactions were performed in a final volume of 25 ul using 2 ul of extracted DNA as template. Each reaction mixture contained 12.5 µl GoTaq® Green Master Mix (Promega, M7122) 1 µl of 500 M forward primer (LISF); 1 ul of 500 M reverse primer (LIS-R) and 8 µl of Ultra-Pure DNase/RNase-Free distilled water (Gibco, Grand Island, NY, USA). The DNA amplification reactions were performed in thermal cycler (Techne Cyclgene, Germany). The cycling conditions for PCR were as follows: preincubation at 95°C for 5 min; 40 cycles consisting of dsDNA denaturation at 95°C for 30 s, primer

annealing at 54°C for 30 s, primer extension at 72°C for 30 s; final elongation at 72°C for 10 min. All amplification products were resolved in 1% agarose gel, stained with ethidium bromide, detected under a short-wavelength UV light source, and photographed with EDVOTEK Gel documentation system. The 1-KB plus DNA Ladder (Invitrogen) was used as molecular size marker. In order to test the specificity of primers, PCR was carried out using purified bacterial DNA from *E. coli*.

RESULTS

Table 1: Aerobic colony count and coliform bacterial count in examined flitted fish samples:

Type of flitted fish	Aerobic colony count			Coliform count		
	Min	Max	Mean± SD	Min	Max	Mean± SD
Imported fish(Saba)	$5x10^3$	$3x10^7$	$3.3±7.7x10^6$	0	$1.2x10^3$	$1.3±2.8x10^2$
Tilapia nilotica	$3x10^3$	$1.6x10^8$	$1.8±4.0x10^7$	0	$3x10^3$	$4.3±8.4x10^2$
Nile perch	$7x10^3$	$1.7x10^8$	$1.8±5.0x10^7$	0	$1.6x10^3$	$5.1±3.2x10^2$
Total	$3x10^3$	$1.7x10^8$	$1.3±0.8x10^7$	0	$3x10^3$	$3.5±2.0x10^2$

SD= Standard deviation.

Table 2: Incidence of *listeria monocytogenes* in examined flitted fish samples:

Type of flitted fish	Incidence of *L.monocytogenes*		
	No.	%	Total
Imported fish(Saba)	2	6.7%	6.7%
Tilapia nilotica	4	26.7%	23.3%
Nile perch	3	20%	
Total	9	15%	15%

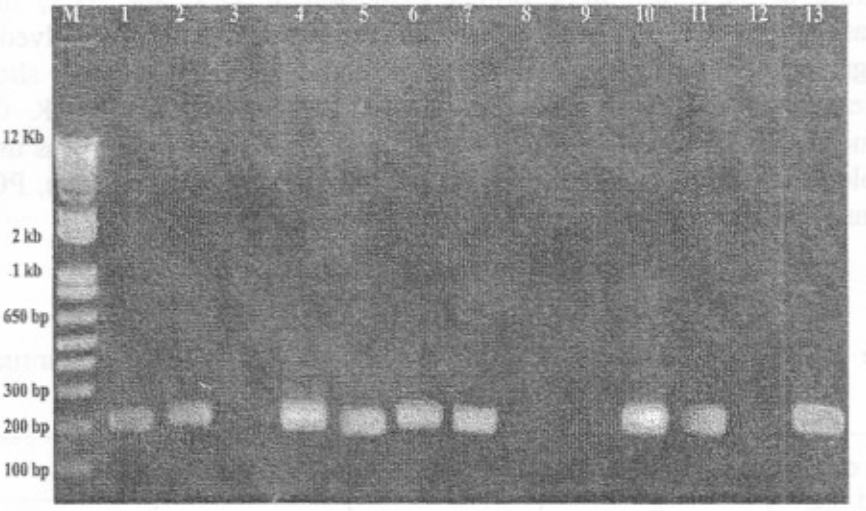

Fig. 1: Showing PCR amplicon for *Listeria monocytogenes* from lane 1, 2, 4, 5, 6, 7, 10, 11 and 13 were positive at band 217bp.

DISCUSSION

Aerobic colony count

The mean aerobic colony count as shown in Table 1 for imported filleted fish was $3.3\pm7.7x10^6$, while for filleted *Tilapia nilotica* and *Nile perch* were $1.8\pm4.0x10^7$ and $1.8\pm5.0x10^7$cfu/g, respectively. The present result agreed with ICMSF (1986) who recorded that the microbial upper limit for fresh fish was 7 log cfu/g of aerobic mesophilic counts as well as with Gram *et al.* (1990) who found the total viable counts of 5×10^7cfu/g at the end of storage time (3 weeks) in chilled Nile perch fillets stored at 0 °C. Lee and Levin (2007) found that the plate count of fish fillet ranged from 4.97 ± 0.02 to 7.25 ± 0.02 log cfu/g.

Manna *et al.* (2008) recorded lower results where the mean aerobic colony count in Tilapia flesh obtained from various fish markets at Kolkata, India, during 2003 and 2004 was $1.2\pm 0.8 \times 10^4$cfu/g. Also the present results were higher than that reported by Goktepe and Moody (1998) who showed that the aerobic plate count in raw catfish fillets was 4.03 logs cfu/g and Silva (2002) who found that the APC was 4.6 ± 1.5 logs cfu/g of fresh non-treated catfish fillet samples. Mhongole (2009) reported that total viable count (TVC) count prior to online fillets washing was5, 49 – 6, 27 log10 cfu/g. This is indicating a likely contamination and or growth of microorganisms during the subsequent processing steps at establishments.

On the other hand the present result was less than that recorded by Chytiri *et al.* (2004) who reported that mesophilic plate counts for filleted rainbow trout exceeded 7 log cfu/ g after 10 days of storage in filleted rainbow trout samples and Hozbor *et al.* (2006) in sea salmon, the bacterial count reached 12 log10 cfu/g by the end of storage period. This indicated the importance of these organisms in the product shelf-life.

Lee and Levin (2007) found that the plate counts increased to 9.35 to 9.76 log cfu/g after 7 days storage at 4 °C of three fillets of tissue. Liu *et al.* (2010) indicated that the shelf life of Tilapia fillets stored at 0 °C was about 10-12 days considered for microbial safety and sensory acceptability.

A guideline on APC level for fresh fish and fish products microbiological quality including fillets is $5x10^5$-10^7colony forming units per gram (cfu/g) of muscle as proposed by International Commission on Microbiological Specifications for Foods (ICMSF, 1986). High aerobic plate counts (2.6 x 10^7cfu/g) for catfish fillets indicated that fillets were heavily contaminated during processing (Ramos and Lyon, 2000).

Coliforms count

The coliforms count for filleted imported fish, *Tilapia nilotica* and *Nile perch* were 1.3± $2.8x10^2$, 4.3± $8.4x10^2$ and 5.1± $3.2x10^2$cfu/g, respectively. Silva (2002) mentioned that the mean coliform count of fresh non-treated sample was 2.8 ± 1.5 logs cfu/g in catfish fillet

The present result was higher than that found by Manna *et al.* (2008) who found that the coliforms count was 4.9 ± 3.3 x 10 cfu/g in tilapia flesh and the author refer this may be due to surface contamination during preparation.

Incidence of *Listeria monocytogenes*

The contamination of *L. monocytogenes* on whole catfish and its processing environment could be the source of post processing contamination on fresh fillets and ready-to-eat products (Hu *et al.*, 2006).

Incidence of *Listeria monocytogenes* in filleted imported fish, *Tilapia nilotica* and *Nile perch* was 6.7, 26.7 and 20%, respectively (Table 2, Figure 1). So the filleted local fish is considered of higher incidence of *Listeria monocytogenes* than the filleted imported fish. This result is higher than that recorded by Gesche and Ferrer (1995) who analyzed 24 samples of fish fillet and found *Listeria monocytogenes* in only two samples. The result in local filleted fish nearly in harmony with Chou *et al.* (2006) who showed that 25% to 47% of fresh channel catfish fillets were contaminated with *Listeria monocytogenes* and Pao *et al.* (2008) who reported that *L. monocytogenes* was present in 23.5% of catfish fillet that was obtained from various retail stores in the United States.

The present result was less than that recorded by Chen *et al.* (2010) who studied the prevelance of *Listeria monocytogenes* in catfish fresh fillets at different production stages. The strains were found with a frequency of 76.7% in chilled fresh catfish fillets and 43.3% in un-chilled fillets. He suggested that *L. monocytogenes* contamination in the processed catfish fillets originates from the processing environment, rather than directly from catfish.

As shown in Table 2, the incidence of *Listeria monocytogenes* in all filleted fish samples was 15%. This result was less than that recorded by Johansson *et al.* (1999) who examined 55 samples from the six fish farms providing filleted fish to this plant, during September 1997–January 1998. The isolates were characterized by serotyping and pulsed-field gel electrophoresis (PFGE). *L .monocytogenes* was isolated in 20% (22/110) of the samples from the retail market. Also less than that recorded by Cao *et al.* (2005) analyzed different fish species fillets from two retail markets in USA for presence of *Listeria monocytogenes* and found that 23% of fish fillets were contaminated with *Listeria monocytogenes*, which seems to be higher than this result.

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