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Freezing effects on survival of *Listeria monocytogenes* in artificially contaminated cold fresh-salmon

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Abstract - The effects of freezing at -20 °C were examined on surviving of *Listeria monocytogenes* contaminating food during freezing. Slices of fresh salmon were inoculated with three strains of *Listeria monocytogenes* (10^8 CFU/g). The inoculated pieces of fish were dried for 1 h at 24 °C and then stored in closed containers at -20 °C for ten months. The identification of atypical cells found after freezing was achieved by PCR. This technique was based on the amplification of two specific genes of *L. monocytogenes hly*A and *iap*. The results showed that after ten months of frozen storage, the concentration of *L. monocytogenes* determined on Trypticase Soya agar-Yeast Extract had declined by 2.39 ± 0.01 log CFU/g and 2.22 ± 0.01 log CFU/g for the strains isolated from meat, S1 and S2 respectively, and by 3.69 ± 0.03 log CFU/g for the reference strain. The effect of frozen storage of *L. monocytogenes* in salmon did not decrease the potential survival of food-borne pathogens' over a period of ten months.

Key words: *Listeria monocytogenes*, freezing, salmon, PCR, artificial contamination, survival.

INTRODUCTION

Listeria monocytogenes is classified as an opportunistic pathogen because it targets specific populations, such as pregnant women and immunocompromised patients (Gellin and Broome, 1989). Several researchers have studied the contamination of meat products by *Listeria monocytogenes* as well as the agents suppressing such contamination, such as the addition of biopreservatives (Nilsson *et al*., 1997; Nufer *et al*., 2007), the use of high hydrostatic pressure (Murano *et al*., 1999), pulsed light systems (Elmnasser *et al*., 2007) and pasteurisation (Muriana *et al*., 2002; Juncher *et al*., 2000). It was concluded that preservation processes which are expected to limit microbial activity do not have a strong effect on meat quality.

The salt concentration, the pH and the water activity values are normally within a range which allows the growth of *Listeria monocytogenes* (Seeliger and Jones, 1984; Lin and Chou, 2004). This bacterium was described as a Gram-positive facultative anaerobic, halotolerant rod with considerable variations in morphology, depending on physiological and environmental conditions (Janda and Abbott, 1999). *Listeria monocytogenes* is increasingly recognised as a highly significant foodborne pathogen, and it causes 10% of fatal foodborne illnesses in the US (Ferreira *et al*., 2003). It was isolated from a variety of foods and ready-to-eat products and generated a serious public health problem (Beuchat *et al*., 1986; Johansson *et al*., 1999; Gao *et al*., 2006). Because of its growth capacity at refrigerating temperatures and its survival in diverse environments, the control of *L. monocytogenes* is becoming a challenge. Generally speaking, the pathogen was reported to have higher heat resistance compared to many other species (Farber and Peterkin, 1991; Gao *et al*., 2006; Hagens and Loessner, 2007). While decreasing the temperature during the production process, the stress by thermal shock of *L. monocytogenes* was described and reproduced experimentally to evaluate the impact of relatively low temperature (+5 °C) on the bacterial growth (Mastronicolis *et al*., 1998; Bergis, 2002). More recently, the survival of *L. monocytogenes* while freezing at least four weeks on fresh and frozen strawberries was reported (Flessa *et al*., 2005). Medrala *et al*. (2003) have shown that both sporadic and epidemic cases of listeriosis are predominantly food related. *L. monocytogenes* is occasionally isolated in seafood, e.g. ready-to-eat cold- or hot-smoked salmon, 'gravad' salmon, shrimp, mussels, fermented fish and fish salads, and the link between seafood consumption and listeriosis cases was recently assessed. A few sporadic cases and minor outbreaks or clusters caused by ingestion of fish and fish-products in comparison with infections caused by the other kinds of foods pathogens have been explained by a lower level of contamination with *L. monocytogenes* (<100 CFU/g) (Rôrvik *et al*., 2000). Although the inhibition of *L. monocytogenes* on cold smoked salmon was confirmed by using nisin and carbon dioxide atmosphere (Nilsson *et al*., 1997), to the author's knowledge, the effect of freezing on the viability of *L. monocytogenes* in salmon has not yet been studied.

The objective of this work is to study the effect of freezing on the viability of *L. monocytogenes* to contaminate freshly sliced salmon stored at -20 °C. The biochemical modifications induced

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by cold stress were determined as well as the identification of non-typical cells assessed by the molecular method, based on the amplification by PCR of the specific virulent genes.

MATERIALS AND METHODS

Bacterial strains isolation and biochemical characterisation. Three strains of *L. monocytogenes*: one reference strain ATCC 19115 and two strains (S1 and S2) isolated from meat according to the standardised French method NF V 08-055 (AFNOR, 1999), for the detection of *L. monocytogenes* in food, were used to artificially contaminate cold salmon. The isolation was done on Palcam and Oxford media (Merk, Germany). The cells were preserved on 20% glycerol at -80 °C and cultivated on Trypticase Soya agar-Yeast Extract (TSA-YE) (BIO-RAD) at 37 °C prior to use. Freshly grown colonies were used to inoculate 10 ml Brain Heart Infusion broth (BHIB) tubes and incubated at 37 °C for 20 h. The identification of strains was performed by API *Listeria* strips and the result was explored using an automated mini-API method (bio-Mérieux, France).

Effect of freezing on survival of *Listeria monocytogenes*. A sample of 500 g of fresh cold Atlantic salmon was provided by a commercial importer (Monastir, Tunisia). The middle flank's fish was sterilised by UV irradiation (no germ was developed on PCA) and then stored at 4 °C. This sample was cut into small cubes of 1 g. At each analysis, a sample of 1 g was analysed.

The three strains of *L. monocytogenes* were separately cultured in BHIB and used to surface inoculate 1 g of sterile salmon slices (10^8 CFU/g) (Flessa *et al*., 2005). Each piece of 1 g of salmon was homogenised in 9 ml of peptone water (Stomacher, Lab-System 400, London, UK) for 2 min in stomacher bags (Bagfilter, France). The homogenate was serially 10-fold diluted in peptone water and speared on TSA-YE agar plates for bacterial enumeration. This analysis was conducted first every two days for ten days, and then every 15 days for three months, followed by once a month for four months and finally after three months to reach ten months of incubation. For each strain, three separate slices were analysed at each sampling time. A total number of 51 samples of salmon were analysed by strain.

Morphological changes and macroscopic examinations of colonies cultivated on TSA-YE plate were recorded. In order to assess the enzymatic activity variations during freezing (-20 °C), strip Api *Listeria* (bio-Mérieux) were used.

Effect of refrigeration and ambient temperature on the survival of *Listeria monocytogenes* in salmon. Stationary phase cultures of each strain of *L. monocytogenes*: ATCC 19115 and two strains isolated from meat, grown in BHIB tubes at 37 °C for 20 h were used to inoculate samples (1 g) of sterile fresh salmon with 10^6 CFU/g. The inoculated salmon were then incubated at +4 °C and at room temperature (25 °C) followed by the enumeration for the *L. monocytogenes* cells at 0, 2, 3, 7, 10 and 15 days of storage. For each treatment, three replicates were performed.

On each day of sampling, 9 ml of peptone water was added to each piece of salmon incubated at the corresponding temperature, and the contents were placed in stomacher bags (Bagfilter, France) and homogenised in a Stomacher (Lab-System 400) for 2 min. Samples were serially diluted (1:10) and 0.1 ml was spread on TSA-YE agar plates in triplicate for bacterial enumeration.

The growth rate for each experimented condition was determined according to the curve slope of the total number of bacteria versus time.

Molecular identification of atypical cells. Atypical strains, exhibiting an enzymatic profile modification, were obtained on a TSA-YE plate after ten months of freezing and identified using PCR. Two specific genes (*hly*A and *iap*) were considered. Chromosomal DNA was isolated using a Wizard Genomic Purification Kit (Promega, Lyon, France), according to the manufacture's recommendations. The concentration of purified DNA was adjusted at 50 ng/µl using spectroscopy (Ultraspec 2100 pro; Amersham Biosciences Europe GmbH, France).

The presence of *iap* coding for invasion protein P60 and the *hly*A gene coding for the listeriolysine O were detected by the polymerase chain reaction. For the *iap* gene, the forward primer used had the following sequence: 5'-GGG CTT TAT CCA TAA AAT A-3'; and the reverse primer sequence was: 5'-TTG GAA GAA CCT TGA TTA-3' giving a 453 pb fragment (Medrala *et al*., 2003). The primer for the *hly* A gene as forward, 5'-GAA TGT AAA CTT CGG CGC AAT CAG-3', and reverse, 5'-GCC GTC GAT GAT TTG AAC TTC ATC-3' giving a 388 bp amplicon size (Garrec *et al*., 2003). The PCR mixture (25 µL) contained 1 µM forward and reverse primer, dNTP mix (100 µM each of dATP, dCTP, dGTP and dTTP), 1 U of Go *Taq* polymerase (Promega), 5 µl green Go *Taq* buffer (5X), and DNA template (50 ng). The PCR for the *iap* gene included an initial step (95 °C for 5 min), followed by 35 denaturation cycles (95 °C for 1.30 min), annealing (46 °C for 1 min) and extension (72 °C for 1.30 min), and concluded at the end of cycling by a final extension (72 °C for 8 min). The PCR conditions of the *hly*A gene included an initial step (94 °C for 5 min), followed by 30 cycles of denaturation (94 °C for 1 min), annealing (65 °C for 1 min) and extension (72 °C for 1 min) and concluded at the end of cycling by a final extension (72 °C for 10 min). The PCR was performed in a PTC Thermocycler (Bio-Rad, USA.). The PCR products (5 µl) were analysed on 1% agarose gel stained with ethidium bromide (0.5 µg/ml), and visualised under ultraviolet transillumination.

Statistical analysis. Triplicate samples were analysed and statistical analysis was performed on STAT VIEW statistics software. The ANOVA test was used to assess inter-group significance. In addition, statistical significance was set at $P < 0.0001$.

RESULTS

Survival of *Listeria monocytogenes* cells during freezing

Fresh salmon slices were inoculated with the three studied strains of *L. monocytogenes* and incubated at -20 °C for 10 months. Bacterial enumeration showed a reduction in the number of bacterial cells during the storage. The initial inoculate (10^8 CFU/g) declined after 10 days of incubation, and a reduction of bacterial concentration up to 10^6 CFU/g was noted for the three tested strains (Fig. 1).

During storage at -20 °C, the reference strain was less resistant to freezing, compared to the strains isolated from meat. This could be explained by the easy adaptation of these bacteria to starvation conditions in the initial food environment ($P < 0.0001$).

Furthermore, after ten months of incubation, the number of cells dropped to 3-5 $\log_{10}$ CFU/g (Fig. 1). These results showed a significant difference in the three concentrations of tested strains

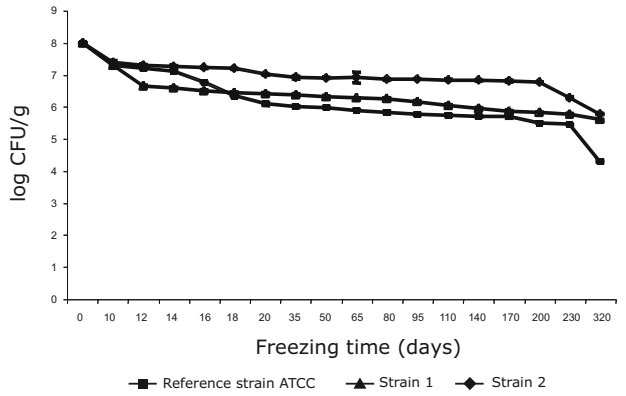

FIG. 1 - Bacterial growth of the three strains of *Listeria monocytogenes* (ATCC 19115, Strain 1 and Strain 2) incubated in fresh salmon and stored at -20 °C.

during freezing ($P < 0.0001$). After 10 months of freezing, a reduction in the bacterial concentration of 2.39 ± 0.01 $\log_{10}$ CFU/g and 2.22 ± 0.01 $\log_{10}$ CFU/g for the strains S1 and S2 respectively was noted. In addition, the cell concentration of the reference strain was reduced by 3.69 ± 0.03 $\log_{10}$ CFU/g.

On the TSA-YE plate, before any incubation in salmon at -20 °C, all the studied strains gave characteristic cell of *L. monocytogenes*: smooth, small size, transparent, not pigmented, and with regular shape. Therefore, after the incubation in salmon at -20 °C, the grown colonies obtained on TSA-YE shrank dramatically. During their incubation in salmon at -20 °C, the evolution of the biochemical characters of *Listeria monocytogenes* evidenced by API *Listeria* strips showed a modification in the enzymatic profile (Table 1). Among the ten biochemical reactions tested on the API strip (arylamidase or DIM, esculin hydrolysis, α-mannosidase, and acid production from D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucoside, D-ribose, glucose-1-phosphate, and D-tagatose), DIM was the first character modified after three months of starvation. It was the only characteristic that unequivocally distinguished *L. monocytogenes* from *Listeria innocua*, with the former being negative for DIM. The basis of the test is the hydrolysis of an unspecified naphtylamide substrate by an aminopeptidase produced by all *Listeria* species except *L. monocytogenes*.

After six months of freezing, other enzymatic tests were modified (Table 1), and the reference strain was unable to acidify D-arabitol. Furthermore, the acidific metabolism of ribose and D-tagatose became positive in strain 1. Similarly to the reference strain, Strain 2 underwent modifications in assimilating the α-mannitol and xylose; however it became unable to produce acids through the metabolism of rhamnose and MDG. After ten months, changes in DIM, D-xylose and L-rhamnose metabolism were noted; these tests became positive for the three tested strains (Table 1).

Survival of *Listeria monocytogenes* cells during refrigeration and ambient temperature

During 15 days of storage in salmon at +4 °C, *L. monocytogenes* populations increased by 2.25 ± 0.24 $\log_{10}$ CFU/g and 1.46 ± 0.20 $\log_{10}$ CFU/g for the strains S1 and S2 respectively and by 1.59 ± 0.10 $\log_{10}$ CFU/g for the reference strain. The final population counts were observed to be 7.46 – 8.25 $\log_{10}$ CFU/g (Fig. 2). The results indicated that all strains of *L. monocytogenes* survived and grew significantly ($P < 0.0001$) at +4 °C.

When the salmon were stored at 25 °C, *L. monocytogenes* populations exhibited higher growth rates. After 2 weeks of storage at 25 °C, the *L. monocytogenes* populations increased by 2.58-2.62 $\log_{10}$ CFU/g. The final population counts were observed to be in the range of 8.58-8.62 $\log_{10}$ CFU/g (Fig. 3) and were significantly different ($P < 0.0001$) than the inoculated populations stored at +4 °C.

The variation of the rate growth (μ_{max}) according to the incubation temperature of *L. monocytogenes* (Table 2) was also noted, in that, the incubated temperature strongly affected the growth rate (μ_{max}).

Amplification of iap and hlyA genes

After ten months of incubation in salmon at -20 °C, the *L. monocytogenes* strains become non-identifiable by API *Listeria* strips. To highlight atypical cells, PCR was used. This technique was

TABLE 1 - Evolution of *Listeria monocytogenes* modification of the enzymatic profile during preservation at -20 °C

Incubation period (months)	*Listeria monocytogenes* strain	Enzymatic reaction (API *Listeria*)									
		DIM	ESC	αMAN	DARL	XYL	RHA	MDG	RIB	G1P	TAG
0	ATCC 19115	-	+	+	+	-	+	+	-	-	-
	S1	-	+	+	+	-	+	+	-	-	-
	S2	-	+	+	+	-	+	+	-	-	-
1	ATCC 19115	-	+	+	+	-	+	+	-	-	-
	S1	-	+	+	+	-	+	+	-	-	-
	S2	-	+	+	+	-	+	+	-	-	-
3	ATCC 19115	(+/-)	+	+	+	-	+	+	-	-	-
	S1	(+/-)	+	+	+	-	+	+	-	-	-
	S2	(+/-)	+	+	+	-	+	+	-	-	-
6	ATCC 19115	(+/-)	+	+	(-)	-	+	+	(+)	-	(+)
	S1	(+/-)	+	(-)	+	(+)	(-)	(-)	-	-	-
	S2	(+/-)	+	(-)	(-)	(+)	+	+	-	-	(+)
10	ATCC 19115	(+)	+	+	(-)	(+)	(-)	+	(+)	-	(+)
	S1	(+)	+	(-)	(-)	(+)	(-)	(-)	(+)	-	(+)
	S2	(+)	+	(-)	(-)	(+)	(-)	+	(+)	-	(+)

DIM: arylamidase; ESC: hydrolysis esculin; αMAN: αMannosidase; DARL: acidification of D-arabitol; XYL: acidification of xylose; RHA: acidification of rhamnose; MDG; acidification of methyl-αD-glucopyranoside; RIB: acidification of ribose; G1P: acidification of glucose-1-phosphate; TAG: acidification of tagatose.
-: negative reaction; +: positive reaction; (+/-): reaction is positive or negative; (+): altered character; (-): non altered character.

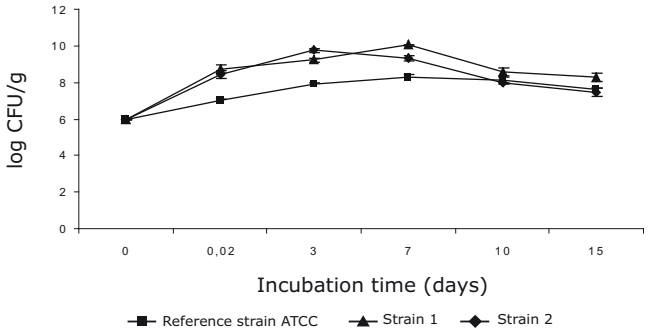

FIG. 2 - Bacterial growth of the three strains of *Listeria monocytogenes* (ATCC 19115, *S*train 1 and *S*train 2) incubated in fresh salmon at +4 °C. Cultivable cells were enumerated by spread Plate Count agar.

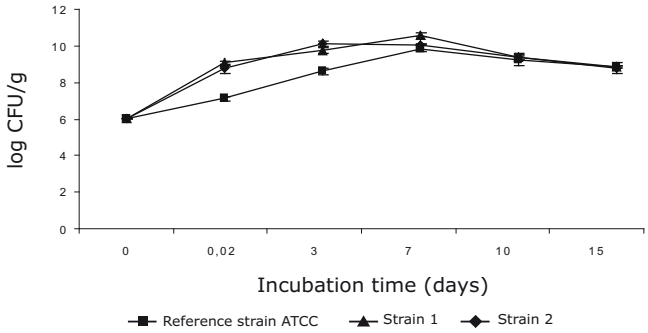

FIG. 3 - Bacterial growth of the three strains of *Listeria monocytogenes* (ATCC 19115, Strain 1 and Strain 2) incubated in fresh salmon at 25 °C. Cultivable cells were enumerated by spread Plate Count agar.

applied to the three studied strains: the reference strain (*Listeria monocytogenes* ATCC 19115) and two strains isolated from food before and after incubation in salmon during ten months of freezing at -20 °C. One reference strain of *Staphylococcus aureus* ATCC 25923 was used as non-related group which was not amplifiable. As shown in figures 4 and 5, all three studied strains were positive for the *iap* gene and for the *hly*A gene, giving respectively 453-bp and 388-bp confirming their identification as *L. monocytogenes.*

DISCUSSION

Listeria monocytogenes is responsible for foodborne disease in susceptible patients, including pregnant women and immunocompromised patients. This species is considered as one of the most difficult pathogens to eradicate, due to its ubiquity in the environment and its relatively low infectivity (Larpent, 1995).

In this study, reduction in the concentration of *L. monocytogenes* was observed during its incubation in salmon at -20 °C. The non-recoverable cells on the culture media after cold starvation could have became viable but non-cultivable cells (VBNC). Therefore, the disappearance of the bacteria is only apparent and corresponds partly to an evolution into non-cultivable forms. Several works showed that stress conditions such as temperature variations could induce the transformation of *L. monocytogenes* into VBNC state (Besnard *et al*., 2000). In the present study, it was noted that *L. monocytogenes* can survive at negative temperature (-20 °C) during a long period. Indeed, according to Fig. 1, the number of cells decreased during the freezing period.

Guyer and Jemmi (1991) suggested that the freezing temperature (-25 °C) does not allow the growth of *L. monocytogenes* SLCC 2755. This was in agreement with Kaya and Schmidt (1989) who experimented with contaminated minced meat, and which was stored them at -18 °C for more than 6 months. A study undertaken by Flessa *et al*. (2005) on the survival of *L. monocytogenes* in the cutters expenses or frozen suggested that *L. monocytogenes* is able to survive but not grow on the surface of fresh strawberries for periods of at least four weeks.

As shown in Fig. 1, the reference strain is more sensitive to freezing. Indeed, the numbers of CFU/g decreased by 5 log after incubation during ten months, whereas the two other strains decreased by 3.5 log; this result is in agreement with that of Guyer and Jemmi (1991). Macroscopic observations of cell morphology after ten months of freezing showed a reduction of colony sizes. This fact is explained by the effect of environmental conditions as mentioned by Janda and Abbott (1999).

In our study, we noted that *L. monocytogenes* has the ability to grow at room temperature as well as at refrigerating temperature (2-4 °C). These results are in agreement with those of Rocourt and Cossart (1997) who showed that *L. monocytogenes* has the ability to grow over a wide range of temperatures (2 to 45 °C). Its survival and growth at refrigerating temperatures are two factors among many others that make the control of this foodborne pathogen difficult.

During the *L. monocytogenes* incubation in salmon at -20 °C, the colonies dramatically reduced in size on TSA-YE. In addition, a modification in the enzymatic profile was noticed. The first character which was modified after three months of starvation was the enzymatic substrate DIM (Table 1). The acidification of D-arabitol remained negative for the reference strain. Furthermore, the RIB test and TAG metabolisms remained positive. In addition, after ten months, changes of D-xylose and L-rhamnose assimilation were observed as shown in Table 1. The biochemical modifications observed in the strain of *L. monocytogenes* after ten months can be explained by the cold starvation effect. In a recent study, it has been reported that the enzymatic modifications are observed after treatment of *L. monocytogenes* by high pressure (Ritz *et al*., 2001). The instability of the enzymatic characters made the classic identification of this pathogenic bacterium very difficult. Nevertheless, the PCR technique, used for the identification of atypical *L. monocytogenes* cells after ten months of freezing is very rapid and more sensitive than the traditional methods. The presence of VBNC cells creates a major public health problem since they cannot be detected by tradition-

TABLE 2 - Variation of maximum growth yield (h^{-1}) of *Listeria monocytogenes* according to the incubation temperature

Listeria monocytogenes strain	Temperatures		
	-20 °C	+4 °C	+25 °C
ATCC 19115	-0.0124	0.3022	0.4707
S1	-0.0042	0.1554	0.2124
S2	-0.0045	0.4388	0.4204

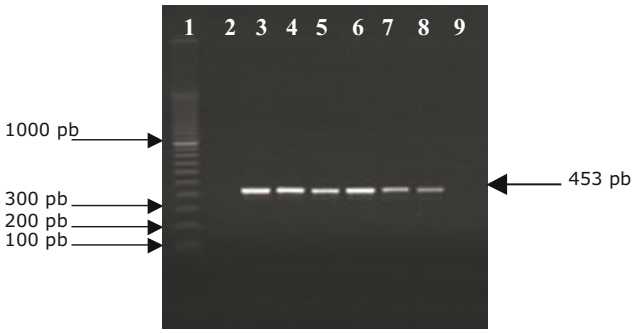

FIG. 4 - Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of *iap* gene. Lane 1: 100 bp DNA molecular size marker; lane 2: negative control; lanes 3 to 5: *Listeria* strains before freezing and after incubation in fresh minced salmon incubated at -20 °C for ten months (lane 3: *L. monocytogenes* ATCC 19115; lane 4: strain 1; lane 5: strain 2); lanes 6 to 8: *Listeria* strains after incubation in fresh minced salmon incubated at -20 °C for ten months (lane 6: *L. monocytogenes* ATCC 19115; lane 7: strain 1; lane 8: strain 2); lane 9: *Staphylococcus aureus* ATCC 25923.

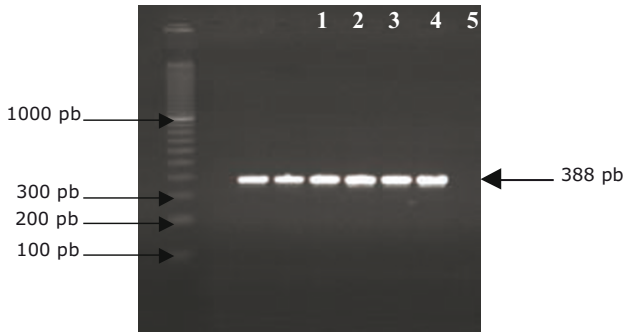

FIG. 5 - Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of *hly*A gene. Lane 1: 100 bp DNA molecular size marker; lane 2: negative control; lanes 3 to 5: *Listeria* strains before freezing and after incubation in fresh minced salmon incubated at -20 °C for ten months (lane 3: *L. monocytogenes* ATCC 19115; lane 4: strain 1; lane 5: strain 2); lanes 6 to 8: *Listeria* strains after incubation in fresh minced salmon incubated at -20 °C for ten months (lane 6: *L. monocytogenes* ATCC 19115; lane 7: strain 1; lane 8: strain 2); lane 9: *Staphylococcus aureus* ATCC 25923.

al culturing methods and the cells remain potentially pathogenic under favourable conditions.

As shown in figures 2 and 3, all the studied strains which were positive for the *iap* gene were also positive for the *hly*A gene, confirming their identification as *L. monocytogenes.* Previous reports suggested that atypical species of *Listeria* with unusual biochemical characters can be identified using the PCR technique (Vaneechoutte *et al*., 1998). Several authors showed that the activities of the stressed cells depend on their preliminary history and in particular on the environmental conditions of preculture (Medama *et al*., 1992).

An important finding of the results is the ability of *L. monocytogenes* cells to survive at low temperature. The number of cells decline and the enzymatic profiles change during starvation. The PCR remains the most sensitive method for identifying atypical cells (Hagens and Loessner, 2007).

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