



First Isolation and Identification of *Listeria monocytogenes* Isolated from Frozen and Shock Frozen Dressed Broiler Chicken in Sudan

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Authors' contributions

This work was carried out in collaboration between all authors. Author GEM designed the study and wrote the protocol. Author ADIA carried out the experiments performed the statistical analysis and wrote the first draft of the manuscript. Authors MAA and AOB managed the analyses of the study. Author AOB managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: To isolate and identify *Listeria monocytogenes* from frozen and shock frozen raw dressed broiler chicken in Khartoum State, Sudan.

Place and Duration of Study: Department of Pathology, Microbiology and Parasitology, Sudan University of Science and Technology, Khartoum-North, Sudan, between July 2011 and June 2012.

Methodology: Eight hundred samples were used in this the study. Five hundred frozen (-18°) raw dressed broiler chickens samples were collected from five station chicken abattoirs in Khartoum State. Three hundred samples were collected as fresh, -18°C and shock frozen (-40°C) raw dressed broiler chickens. Detection and isolation of *L. monocytogenes* was carried out using the conventional International Organization for Standardization method.

Results: Out of the 500 samples, 195(39%) were found to be contaminated with *listeria* spp.; *L. monocytogenes* 64(12.8%), *Listeria ivanovii* 97(19.4%), *Listeria grayi* 20(4%), *Listeria seeligeri* 5(1%), *Listeria welshimeri* 9(1.8%). Out of the 300 samples, 111(37%)

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were found to be contaminated with *Listeria* spp.; *L. monocytogenes* 39(13%), *Listeria ivanovii* 54(18%), *Listeria grayi* 11(3.6%), *Listeria seeligeri* 3(1%) and *Listeria welshimeri* 4(1.3%).

Conclusion: The results presented in this study indicated that *L. monocytogenes* was found in frozen (-18°C) raw dressed broiler chicken and shock frozen (-40°C) raw dressed broiler chickens.

Keywords: *Listeria* spp; broiler; frozen; shock frozen.

1. INTRODUCTION

Listeria spp. is widely distributed in environment and the genus of *Listeria* comprises six species: *L. monocytogenes*, *L. ivanovii*, *L. grayi*, *L. seeligeri*, *L. welshimeri* and *L. innocua*. In 2009, two newly identified species (*L. marthii* and *L. rocourtiae*) were reported [1,2]. *L. monocytogenes* is pathogenic for human, while *L. ivanovii* is rarely pathogenic for humans [3]. *L. monocytogenes* are the causal agent of *Listeriosis*, the disease that can be serious and fatal to human. It is a halo tolerant, Gram-positive, facultative anaerobic, non-spore forming rod bacterium [4] and can grow in a wide pH range from 4.6 to 9.5 and in low water activity environments as 0.90 [5-7]. *Listeria* spp. are considered as an important cause of zoonoses infecting many types of animals such as domestic pets, livestock, avian species, rodents, amphibians, fish, and arthropods. In mammals, *L. monocytogenes* can cause spontaneous abortions and is the cause of circling disease which is a manifestation of basilar meningitis. Fecal-oral transmission is the probable means by which *Listeria* spp. is spread in animals. *Listeria* spp. can be transmitted directly from animals to humans and has been documented in veterinarians, farmers, and abattoir workers. Vertical transmission from mother to neonate occurs trans placentally or through an infected birth canal. The approximate fatality rate is 30% that may increase up to 75% in high risk groups, such as pregnant women, neonates, and immunocompromised adults [8-10]. *Listeriosis* is unique disease that represents a considerable public health concern because of its high mortality rate that reaches 20-40% [11-13]. Most cases of *Listeriosis* appear to be foodborne, including those acquired during pregnancy. Different food items can be contaminated by *L. monocytogenes* including raw vegetables, raw milk, soft cheeses, fish, poultry, processed chickens, and beef. Approximately 15-70% of chicken hotdogs are reported to be contaminated with *Listeria* species [14]. The important characteristics of *L. monocytogenes* contributing to foodborne transmission are the ability to grow as low as -0.4°C, resist heat, salt, nitrite, acidity, withstand osmotic stress and survive mild preservation treatment measures commonly used to control the growth of organisms in food [8]. *L. monocytogenes* may cross-contaminate RTE meat and poultry products during post-processing steps such as slicing, peeling, and packaging [15]. As a facultative anaerobic and psychrotrophic bacterium, *L. monocytogenes* can grow in vacuum-packaged and cold-stored. Ready to eat (RTE) meat foods poultry products are widely consumed in Sudan. To our knowledge no published data has been found concerning isolation and characterization of *L. monocytogenes* recovered from RTE meat and poultry products in Sudan. *L. monocytogenes* easily spreads by direct food contact with contaminated surfaces. The nature of strain persistence is unknown but biofilm formation in food-processing facilities could be one of the important reasons [16]. A USDA-FSIS survey published in 2001 showed that 1-10% of retail RTE meat and poultry products were contaminated with *L. monocytogenes* [17].

In this study the standard method for isolation of *L. monocytogenes* comprises selective enrichments (24 to 48 h) and isolation on selective media (48 h), followed by biochemical species-specific identification were examined. The recommended standard methods for isolation of *L. monocytogenes* take five days to confirm a negative result and up to 10 days to confirm a positive result [18]. The food safety regulations of most countries required zero tolerance of *L. monocytogenes* in RTE food, especially food produced for specific subgroups of the population that are at risk [19]. Because the lack of surveillance about *L. monocytogenes* outbreaks in frozen raw dressed broiler chicken and shock frozen raw dressed broiler chicken in Sudan, the objectives of this research work were to investigate, isolate and identify *L. monocytogenes* using conventional method.

2. MATERIALS AND METHODS

2.1 Samples

First experiments: This study was conducted during July 2011 and June 2012, five hundred frozen (-18°) raw dressed broiler chickens samples were used for detection of *L. monocytogenes*. Samples were collected from five station chicken abattoirs in Khartoum State, one hundred samples from each station. Second experiments: This study was conducted during January 2012 - June 2012, three hundred samples were collected: 100 fresh raw dressed broiler chickens, 100 frozen (-18°C) raw dressed broiler chickens and one hundred shock frozen (-40°C) raw dressed broiler chickens were used for detection of *L. monocytogenes*. Samples were collected from one station chicken abattoir in Khartoum state. All samples were taken from chicken-neck skin after thawing and transported to Sudan University of Science Technology College of Veterinary Medicine Microbiology laboratory under aseptic and refrigerated conditions in portable insulated cold-boxes. Samples were kept at 4°C and analyzed within 24 h.

2.2 Isolation and Identification of *L. monocytogenes*

All samples were tested for the presence of *L. monocytogenes* following the procedure recommended by using the International Organization for Standardization [20,18] procedure. 25 g representative portion from each sample was introduced aseptically into a sterile stomacher bag containing 225 ml of Half Fraser Broth (Oxoid, Ltd., Basingstoke, UK, CM0895) (primary enrichment medium) to obtain a 1:10 sample dilution. The samples were then homogenized for 1 minute at 260 rpm in a stomacher circulator unit 400 (Seward, UK) followed by incubation for 24 h at 30°C. After incubation period, 0.1 ml sub-sample from each Half Fraser Broth culture was added to 10 ml of Fraser Broth (Oxoid, CM0895) (secondary enrichment medium), and incubated for 48 h at 37°C. A loopful of the Fraser Broth enrichment culture was streaked on the surface of Chromogenic Listeria Agar (Oxoid, CM1084) and on Listeria Selective Agar (Oxford Formulation) (Oxoid, CM0856). These selective agars were then incubated for up to 48 h at 37°C. Selective agars were observed for suspected colonies at 24 h and 48 h of incubation. Suspected colonies were those that appeared grayish colonies surrounded by black halos and sunken centers with possible greenish sheen on Oxford agar or green-blue colonies surrounded by an opaque halo zone on Chromogenic Listeria agar. Whenever possible, up to 5 suspected colonies showing typical morphology of Listeriae on these isolation media were streaked onto Tryptone Soya Agar (Oxoid, M290) supplemented by 0.6% of Yeast Extract Powder (Oxoid, LP0021) (TSYEA) and incubated at 37°C for 24 h. The following tests were used for confirmation; Gram's staining, motility test, catalase reaction, and oxidase test.

2.3 Confirmation of *Listeria* spp.

For confirmation, the suspected colonies were streaked onto Tryptone Soya Agar (Oxoid, CM0131) supplemented by 0.6% of Yeast Extract Powder (Oxoid, LP0021) and incubated at 37°C for 24 h, and the following tests were used for confirmation; Gram's staining, motility test, catalase reaction, and oxidase test.

2.4 Confirmation of *L. monocytogenes*

2.4.1 Haemolysis test

An inoculating needle was used to stab the Sheep Blood Agar Base (Oxoid, CM0854), supplemented with 7% sterile sheep blood, with a culture taken from a typical colony on TSYEA and incubated at 37°C for 24 h [20]. After incubation positive test cultures show narrow, clear and light zones (β -haemolysis).

2.4.2 Carbohydrate utilization

The Microbact™ *Listeria* 12L Kit System (Oxoid, MB1128A) for rapid biochemical testing. Microbact™ *Listeria* 12L Kit System (Oxoid, MB1128A) is a standardized micro-substrate system designed to stimulate conventional biochemical substrates. Each identification strip consists of 12 tests, (11 sugar utilization tests (Esculin, Mannitol, Xylose, Arabinol, Ribose, Rhamnose, Trehalose, Tagatose, Glucose-1-Phosphate, Methyl-D-Glucose, and Methyl-D-Mannose) plus a rapid haemolysis test. The reactions occurring during the incubation period is demonstrated through either a color change in the sugar utilization tests or in the lyses of sheep red blood cells in the haemolysis test. The results were analyzed by Microbact Software (Oxoid, MB1244A) to determine the *L. monocytogenes* with percent probability number.

2.5 Statistical Analysis

A paired T-test was used to compare the prevalence of *Listeria* (i.e., all *Listeria* spp.) and *L. monocytogenes* observed. Data were analyzed using the statistical software package SPSS for Windows (SPSS Inc, Chicago, IL).

3. RESULTS

3.1 Isolation of *Listeria* spp. From Frozen Raw Dressed Broiler Chicken using of Conventional Method

According to the growth on selective media, Gram stain reaction, oxidase test and catalase test, a total of 195 (39%) suspected *Listeria* spp. were isolated from 500 samples of frozen raw dressed broiler chicken. The isolation was distributed between five station chicken abattoirs in Khartoum State (100 samples from each station). Station one 51(51%), station two 25(25%), station three 40(40%), station four 42(42%), station five 37(37%) isolates Table 1.

Table 1. Prevalence of *Listeria* spp. in frozen raw dressed broiler chickens in Khartoum, Sudan using conventional methods

Food Items	No. of samples	<i>Listeria</i> spp. No. (%)	<i>L. monocytogenes</i> No. (%)	<i>L. ivanovii</i> No. (%)	<i>L. grayi</i> No.(%)	<i>L. seeligeri</i> No. (%)	<i>L. welshimeri</i> No. (%)
Frozen broiler chicken (1)	100	51 (51)	19 (19)	26 (26)	3(3)	1 (1)	2 (2)
Frozen broiler chicken (2)	100	25 (25)	8 (8)	12 (12)	3(3)	1 (1)	1 (1)
Frozen broiler chicken (3)	100	40 (40)	12 (12)	22 (22)	3(3)	1 (1)	2 (2)
Frozen broiler chicken (4)	100	42 (42)	13 (13)	23 (24)	3(3)	1 (1)	2 (2)
Frozen broiler chicken (5)	100	37 (37)	12 (12)	14 (14)	8(8)	1 (1)	2 (2)
Total	500	195(39)	64 (12.8)	97(19.4)	20(4)	5 (1)	9 (1.8)

(1): Station one, (2): station two, (3): station three, (4): station four, (5): station five.

3.2 Isolation of Suspected *Listeria* Species using of Conventional Method for Effect of Frozen and Shock Frozen Dressed Broiler Chicken

A total of 111(37%) suspected *Listeria* spp. were isolated from fresh dressed broiler chickens, frozen broiler chickens and shocked frozen broiler chickens. the isolation was as fresh dressed broiler chickens 47(47%), frozen broiler chickens 43(43%) and shock frozen broiler chickens 21(21%) isolates, this was done by the growth on selective media, Gram stain reaction, oxidase test and catalase test Table 2.

3.3 Confirmation of *L. monocytogenes* using the Microbact™ *Listeria* 12L Kit System (Oxoid, MB1128A) Frozen Raw Dressed Broiler Chicken

Among 500 samples in five station chicken abattoir in Khartoum State, the isolated *Listeria* spp.195(39%) were distributed as follows:- *L. monocytogenes* 64(12.8%), *Listeria ivanovii* 97(19.4%), *Listeria grayi* 20(4%), *Listeria seeligeri* 5(1%) and *Listeria welshimeri* 9(1.8%) Table 1.

Fig. 1 Shows the percentage of each types of *Listeria* from 195 isolated *Listeria* spp.:- *L. monocytogenes* 64(32.82%), *Listeria ivanovii* 97(49.74%), *Listeria grayi* 20(10.25%), *Listeria seeligeri* 5 (2.56%), *Listeria welshimeri* 9(4.61%).

Table 2. Prevalence of *Listeria* spp. in fresh dressed broiler chickens, frozen dressed broiler chickens and shock frozen dressed broiler chickens in Khartoum Sudan using conventional methods

Food Items	No. of samples	<i>Listeria</i> spp. No. (%)	<i>L. monocytogenes</i> No. (%)	<i>L. ivanovii</i> No. (%)	<i>L. grayi</i> No. (%)	<i>L. seeligeri</i> No. (%)	<i>L. welshimeri</i> No. (%)
Fresh broiler chickens	100	47 (47)	17 (17)	23 (23)	5 (5)	1 (1)	2 (2)
Frozen broiler Chickens	100	43 (43)	15 (15)	21 (21)	4 (4)	1 (1)	2 (2)
Shock frozen broiler chickens	100	21 (21)	7 (7)	10 (10)	2 (2)	1 (1)	1 (1)
Total	300	111 (37)	39 (13)	54 (18)	11(3.6)	3 (1)	4 (1.3)

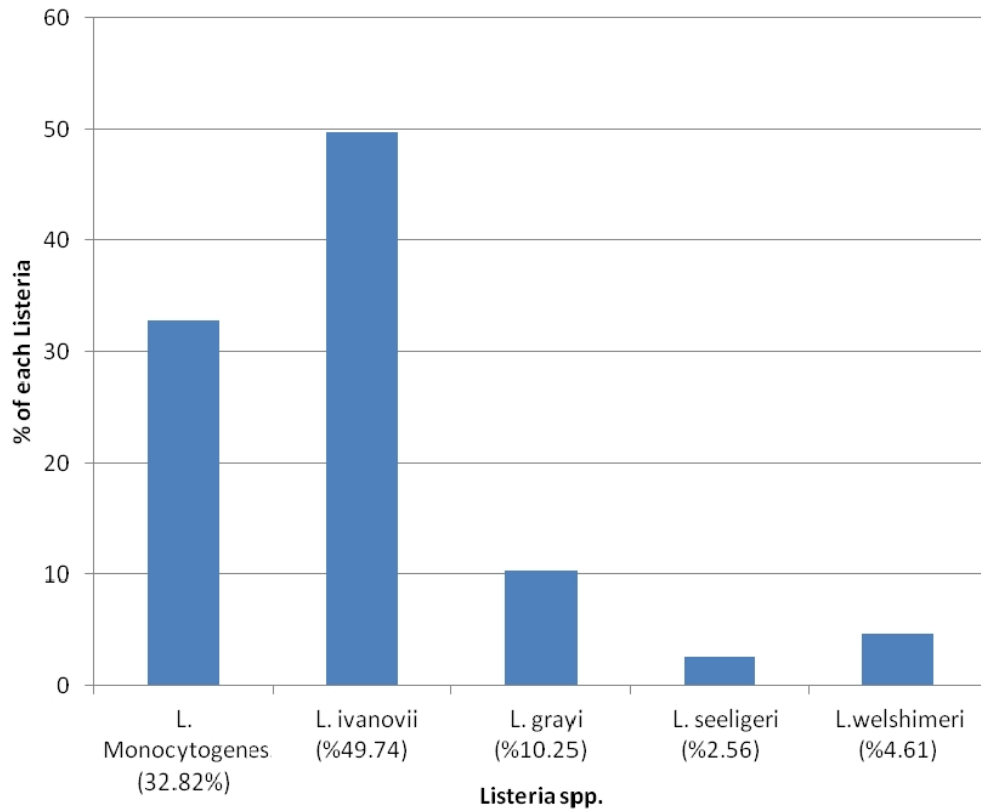


Fig. 1. The isolation percentage of different types of *Listeria* spp. from 195 isolates of *Listeria* spp. from in frozen raw dressed broiler

3.4 Confirmation of *L. monocytogenes* using the Microbact™ Listeria 12L Kit System (Oxoid, MB1128A) Frozen and Shock Frozen Raw Dressed Broiler Chicken

Among 300 samples collected from one station chicken abattoir in Khartoum state as listeria spp. 111(37%) were isolated these distributed as follows *L. monocytogenes* 39(13%), *Listeria ivanovii* 54(18%), *Listeria grayi* 11(3.6%), *Listeria seeligeri* 3(1%), *Listeria welshimeri* 4(1.3%) (Table 2).

Fig. 2 Shows the percentage of each types of *listeria* from 111 isolated *listeria* spp.:- *L. monocytogenes* 39 (35.13%), *L. ivanovii* 54 (48.64%), *L. grayi* 11 (9.90%), *L. seeligeri* 3 (2.70%), *L. welshimeri* 4 (3.60%).

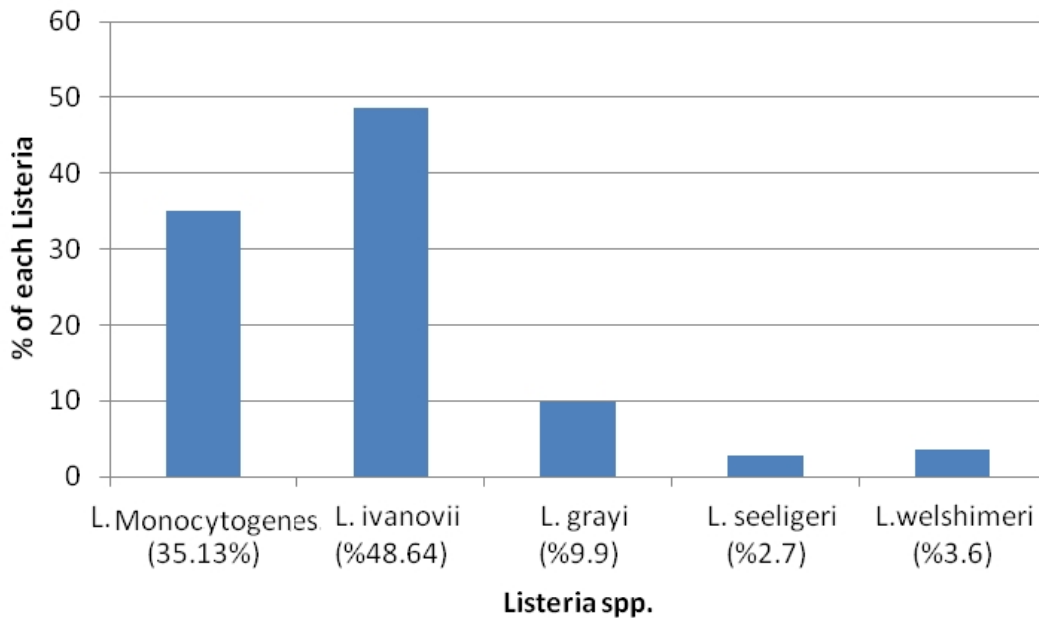


Fig. 2. The isolation percentage of different types of *Listeria* spp. from 111 isolates of *Listeria* spp. from fresh dressed broiler chickens, frozen dressed broiler chickens & shock frozen dressed broiler chickens.

4. DISCUSSION

The real situation of *listeriosis* in Sudan is unknown, and no information is available on the presence of *L. monocytogenes* in frozen raw fresh dressed broiler chickens in Sudan.

The first step to convince regulatory authorities and private industry about importance of *L. monocytogenes* in frozen raw fresh dressed broiler chickens is to provide data on the isolation and distribution of the bacterium in these foods.

The standard method for isolation and detection of *listeria* was used during this study confirms the findings of Vlaemyneck et al. [21] and Beumer and Kusumaningrum [22]. ALOA medium has proved to be a useful and significantly better assay than other media (Oxford

agar, UVM agar, and PALCAM agar) for the isolation and differentiation of *L. monocytogenes* from non-pathogenic *Listeria* species, this is because *L. monocytogenes* colonies on ALOA agar exhibited clear halo zone, this involves cleavage of the substrate, L- α -phosphatidyl-inositol by the virulence factor phosphatidylinositol-phospholipase-C (PI-PLC) and phosphatidylcholin-phospholipase-C (PC-PLC) produced by pathogenic *L. monocytogenes* resulting in the formation of a white precipitation zone (halo) around the colony.

Bailey et al. [23] had examined the factors of colonization of broiler chickens with *L. monocytogenes* (orally inoculated) did not colonize chickens as easily as did *Salmonellae* or *C. jejuni*. Younger birds were more susceptible to colonization than older birds, and there was a dose-related colonization response. It is evident that poultry can become contaminated either environmentally during production or farm healthy carrier chickens in the processing plant Genigeorgis et al. [24], Bailey et al. [23].

L. monocytogenes has ability to grow at 5°C means that any food sample taken for qualitative or quantitative determination of *L. monocytogenes* should be analyzed immediately or frozen to avoid skewing the results. Based on the isolation of *L. monocytogenes* from ice cream Buchanan et al. [25] and *Listeria* spp. from frozen seafood Weagant et al. [26], it is generally recognized that the organism can withstand freezing.

In first experimental study (19%, 8%, 12%, 13% and 12%) *L. monocytogenes* isolates were recovered from frozen raw dressed broiler chickens in station 1, 2, 3, 4 and 5 respectively using the conventional methods for isolation. Prevalence of *L. monocytogenes* in frozen raw dressed broiler chicken was only 12.8%. The demonstrated highest incidence among *Listeria* spp. were *L. ivanovii* (19.4%) and *L. monocytogenes* (12.8%) but, *L. grayi* (4%), *L. seeligeri* (1%) and *L. welshimeri* (1.8%) were low incidence in processed meat products.

While second experimental study (17%, 15% and 7%) *L. monocytogenes* isolates were recovered from fresh raw dressed broiler chickens, frozen raw dressed broiler chickens and shock frozen raw dressed broiler chickens one station using the conventional methods for isolation. Prevalence of *L. monocytogenes* was only 13%. There are no big effect of temperature on *L. monocytogenes* between fresh dressed broiler chickens and frozen dressed broiler chickens 17% and 15% respectively because *L. monocytogenes* has ability to survive freezing and frozen storage at -18°C. Novak and Juneja [27] reported that, the freezing and frozen storage of foods do not induce a marked inactivation of *Listeria monocytogenes* in under-cooked ground beef. Also Flessa et al. [28] reported that, the *L. monocytogenes* can survive on frozen strawberries at -20°C \pm 2°C for periods at least 4 weeks. Palumbo and Williams [29] (1991) studied the ability of *L. monocytogenes* to survive freezing and frozen storage at -18°C in ground beef, ground turkey, frankfurters, canned corn, ice-cream mix, and tomato soup. Their results showed *L. monocytogenes* survived freezing and frozen storage well in five of the examined foods, was not injured, and was quantitatively recovered on *Listeria*-selective media. In contrast, the organism showed a decline in viable count after extended frozen storage in tomato soup, was injured, and could not be quantitatively recovered on *Listeria*-selective media. These results indicate that for most foods, freezing prior to analysis for *L. monocytogenes* should not hamper quantitative determination of the organism. *L. monocytogenes* affected by freezing temperatures.

El-Kest and Marth [30] reported that, the higher (-18°C) freezing temperatures were more lethal than lower (-198°C) temperatures. And freeze-thaw cycles were more lethal with freezing to -18°C than with freezing to -198°C. Also freezing at 198°C and storage at -18°C

was more lethal than freezing and storing at -198°C . Palumbo and Williams [29] reported that, there is variability among strains of *L. monocytogenes* in susceptibility to freezing. In another study using five foods with a pH of 5.8 or above and one with a pH of 4.74, there was little effect of freezing and frozen storage at -18°C on *L. monocytogenes* in the pH 5.8 foods, but declines in viability, and evidence of injury during storage in the pH 4.74 foods.

Listeria monocytogenes cells respond to a decrease in temperature by inducing a set of proteins, called cold shock proteins (csps). These proteins are thought to play a role in the protection of cells against damage caused by freezing. The *Listeria monocytogenes* has investigated the cold shock response [31].

Wemekamp-Kamphuis et al. [32] reported the mechanisms of microbial resistance / inactivation. It has been shown that cold-shocked (4 h at -10°C) cells of *Listeria monocytogenes* synthesized cold-shock proteins and became much more resistant to either freezing or to pressurization at 200MPa.

The demonstrated highest incidence among *Listeria* spp. were *L. ivanovii* (18%) and *L. monocytogenes* (13%) but, *L. grayi* (3.6 %), *L. seeligeri* (1 %) and *L. welshimeri* (1.3%) were low incidence in processed meat products.

In second experimental study (17%, 15% and 7%) *L. monocytogenes* isolates were recovered from fresh raw dressed broiler chickens, frozen raw dressed broiler chickens and shock frozen raw dressed broiler chickens, showed shock frozen raw dressed broiler chickens 7% lower than fresh and frozen this may be decrease temperature suddenly at -40°C lead to injury of *L. monocytogenes*.

Chou [33] studied the inactivation and injury of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in Tryptic soy broth stored at -5°C , -18°C and -28°C the results are population reduction of *E. coli* O157:H7 determined with TSA was ca 1.72 log CFU/ml. On the other hand, a population reduction of only 0.64 log CFU/ml was noted with *L. monocytogenes*. Besides, the surviving population of *E. coli* O157:H7 contained a larger proportion of injured cells than *L. monocytogenes*.

5. CONCLUSION

The results presented in this study indicated that *L. monocytogenes* was found in frozen (-18°C) raw dressed broiler chicken and shock frozen (-40°C) raw dressed broiler chickens.

We recommend that the hygienic conditions described in Sudanese HACCP program should be enforced in order to minimize presence of *L. monocytogenes* in frozen and shock frozen dressed broiler chickens during manufacturing, handling and storage process at plant and retail stores level.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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