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Low Temperature-Survivability Behavior of *Salmonella Enterica* Subsp. *Enterica* Serovar Typhimurium *and Salmonella Enterica* Subsp. *Enterica* Serovar Enteritidis in a Minced Beef Meat Model as an Evaluation of the Cold Chain's Preserving-Effectiveness

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ABSTRACT

Salmonella spp. commonly existed in processed meat production and consumption environment. Hence, their transmission to meat products is of great concern. The industry has widely used cold chain low temperatures as a strategy to prevent the bacterial growth, and recently, refrigeration and freezing have been suggested as a preserving method to improve mechanical operations, quality, and safety of meat products. The purpose of this study was to evaluate the effect of the most used low temperatures in the cold chain, on the survival of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) that were artificially inoculated on the raw minced meat products. Samples were refrigerated at (6°C) or completely frozen at (-20°C) for 9weeks. The *Salmonella spp.* were recovered on appropriate selective and non-selective media. Log reductions and their reflection on the extent of bacterial cell injury were calculated and treatments were calculated and compared. No significant differences were observed in the bacterial count log reduction of refrigerated or frozen bacteria on minced beef meat up to the most used minced meat low temperature keeping periods (14days). The average reduction for *Salmonella* Enteritidis was 0.5log CFU/mL, and for *Salmonella* Typhimurium 2logs CFU/mL; therefore, none of the final reductions were greater than (3logs). Bacterial cell injury was not significantly different among any of the treatments in the first 14days, but variant reductions have been recorded further. Data showed no practical significance for the initial bacterial count log reduction of these pathogens from refrigeration and freezing, thus, this technology should not be considered as a strategy for the reduction or elimination of *Salmonella spp*.

Key words: Food, Foodborne Salmonellosis, Cold chain, Minced Beef, Meat.

INTRODUCTION

In Europe and most of the Mediterranean countries including Egypt, minced meat is very popular. Minced beef is a crucial component in many traditional Arabian and Mediterranean dishes, as well as meat-based goods (such as sausages and beef burgers). As a result of the disruption of the meat's cellular structure during the mincing process, which also permits the spread of surface bacteria, the minced meat becomes a very nutrient-rich environment for bacterial development. This creates a very perishable product that must be wrapped or packaged, refrigerated to an internal temperature of no more than 2°C as soon as possible, or frozen to -18°C during storage and transportation (Köppel et al. 2012; Motjaremi et al. 2014; EFSA 2014).

The burden of diseases brought on by foodborne pathogens persists as a significant health and economic issue, despite efforts to manage them under the farm-to-fork concept (Bacon and Sofos 2003; Sofos 2008; Newell et al. 2010; Linscott 2011; Bošković et al. 2013). In both developed and developing nations, some of these diseases, such as *Salmonella spp.*, continue to have a significant negative impact on both public health and the economy (EFSA 2008; Ehuwa et al. 2021). Following *Campylobacter spp*. in terms of frequency of reports, *Salmonella spp*. came as the 2nd most frequent cause of foodborne illness in humans (Carrasco et al. 2012;

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Boskovic et al. 2016). Rather than infected animal-derived *Salmonella*, also, as a result of cross-contamination, meat can become contaminated with *Salmonella* throughout the slaughter, dressing, and deboning procedures as well as during processing, transport, storage, and home consumption (Miya et al. 2014; Sofos 2014; Bashir et al. 2019). The most common serotypes associated with human salmonellosis are *Salmonella* Enteritidis and *Salmonella* Typhimurium (Vugia et al. 2004; CDC 2006; Cheung and Kam 2012; Mukhopadhyay and Ramaswamy 2012; Chironna et al. 2014; Okenyi et al. 2022).

Salmonella is a significant pathogenic bacterium linked to several outbreaks of foodborne illness that can have a substantial impact on public health, the economy, and quality of life. Since processed meat is one of the most important sources of salmonellosis in humans, food quality management is crucial throughout the entire production process. Since *Salmonella* is a mesophilic bacterium, the cold chain is crucial for the preparation of meat, hence investigations of pathogen activity in cool storage that replicate the industrial environment can be very useful to the food industry (Silva et al. 2022).

Extensive and uncontrolled use of antibiotics lead to an observable antibiotic resistance of *Salmonella*. Animal farming employs antibiotics to treat illnesses and boost animal performance by giving regular non-therapeutic and sub-lethal doses. Pathogenic bacteria carry a large number of antibiotic-resistance genes, resulting in the emergence of novel drug-resistant strains, multi-drug resistant (MDR), and extensive drug-resistant (XDR) bacteria. Human and animal health is seriously harmed by resistant microorganisms, as new resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. A growing list of infections – such as pneumonia, tuberculosis, blood poisoning, gonorrhea, and foodborne diseases – are becoming harder, and sometimes impossible, to treat as antibiotics become less effective. The humanity is heading for a post-antibiotic era, in which common infections and minor injuries can once again kill (Pławińska-Czarnak et al. 2022; Rabie et al. 2023). Studies directed toward detecting the antibiotic residues in the food chain (Hemeda et al. 2022) and research work concerned by the foodborne bacteria antibiotic resistant profiles is the need of the hour. Global reports have noted a rise in *Salmonella* that is multidrug-resistant. The centers for disease control and prevention (CDC) estimate that every year, 6200 instances of multidrug-resistant *Salmonella* are reported, accounting for 10% of all *Salmonella* infections in the world (Wu et al. 2021; Aziz et al. 2023).

This highlights the requirement for better management and prevention of *Salmonella spp*. in food. Therefore, the main objective of the current study was to investigate *Salmonella* behavior in meat stored at both refrigeration and freezing temperatures focusing on the effect of those storage temperatures on the antibiotic sensitivity profiles of both involved *Salmonella spp*.

MATERIALS AND METHODS

Ethics Approval: There are no animals involved in the current study, therefore no ethical approvals are required.

Samples source

Minced beef meat was obtained from a local government-inspected meat shop in Cairo, Egypt. The obtained samples were kept in an ice bag during transportation to the laboratory.

Preliminary microbiological inspection of samples

The surface of the obtained meat samples was sterilized by hot spatula, followed by a standardized general laboratory bacteriological investigation protocol as well as a specialized *Salmonella* serovars investigation protocol to ensure that, the incorporated meat sample was *Salmonella*-free.

Molecular Identification and Confirmation of the Involved *Salmonella* Serovars

Two serotypes of pure *Salmonella spp.* cultures were incorporated into the current study. *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) were isolated and preserved. Molecular identification and confirmation of both *Salmonella* serovars were performed as follows;

Primers Design

DNA Extraction: It was performed according to the manufacturer instructions of the QIAamp® DNA extraction mini kit.

PCR Reaction: The formulation of the PCR reaction components was achieved according to Emerald Amp GT PCR Mastermix (TAKARA) Code No. RR310A kit as shown in Table 2, while the cycling condition of the PCR was designed based on both Emerald Amp GT PCR Mastermix (TAKARA) instructions and the melting points of the incorporated forward (F) and reverse (R) primers. The PCR cycling condition program was entered as illustrated in Table 3.

Agarose Gel Electrophoreses with Modification

Electrophoresis grade agarose (1.5g) was prepared in 100mL TBE buffer in a sterile flask, it was heated in the microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5µg/mL ethidium bromide was added and mixed thoroughly. The warm agarose was

Table 1: Illustration of the used *Salmonella* species-specific primers and the target genes as well as the size of the PCR expected outcome amplicon

Target bacteria	Gene	Sequence (5'-3')	Amplified product	Reference
S. Typhimurium	*STM4495*	GGT GGC AAG GGA ATG AA CGC AGC GTA AAG CAA CT	915bp	Tiwari et al. (2022)
S. Enteritidis	*SefA*	GCAGCGGTTACTATTGCAGC TGTGACAGGGACATTTAGCG CTGGATCTTAAATAGTCATC	310bp	Liu et al. (2012)

Table 2: Tabulation of the PCR reaction components

Component	Volume/reaction
PCR Mastermix (2x premix)	12.5µL
PCR grade water (Nuclease free water)	5.5Ml
Forward primer (20pmol)	1Ml
Reverse primer (20pmol)	1µL
Template DNA	5Ml
Total	25Ml

Table 3: Tabulation of the PCR cycle condition of each involved *Salmonella* species

Parameters	S. Typhimurium	S. Entertidis
Gene	*STM4495*	*sefA*
Primary denaturation	94˚C/5min	94˚C/5min
Secondary denaturation	94˚C/30sec	94˚C/30sec
Annealing	50˚C/1min	52˚C/30sec
Extension	72˚C/1min	72˚C/30sec
No. of cycles	35	35
Final extension	72˚C/10min	72˚C/7min

poured directly into the gel casting apparatus with the desired comb in apposition and left at room temperature for polymerization. The comb was then removed, and the electrophoresis tank was filled with TBE buffer. 20µL of each PCR product sample, negative control, and positive control were loaded onto the gel. The power supply was 1-5 volts/cm of tank length. The run was stopped after about 30minutes and the gel was transferred to the UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software (Sambrook et al. 1989).

Preparation of *Salmonella spp.* Inocula

Inoculation of each identified *Salmonella* serovar on 5mL Brain heart infusion (BHI) broth for 24hrs at 37°C, centrifugation of the broth culture at 6000rpm for 10min, discarding the supernatant then re-suspend the pellet in phosphate-buffered saline (PBS) 7.0 pH. A further two washing cycles were performed on the pellet through a consequent PBS resuspension and centrifugation. The final pellet was then re-suspended in PBS and adjusted to McFarland 4 to obtain a viable cell count nearly equal to $12x10^8$ CFU/mL which was confirmed by the plate count on an XLD agar plate (Chaves et al. 2011).

Microbial Survival Analysis Assay Using TVC

A 36 minced meat 0.5g aliquots were prepared and divided into two sets, from which 18 aliquots were inoculated with 0.5mL *Salmonella* Typhimurium suspension, and the rest 18 were inoculated with 0.5mL *Salmonella* enteritidis suspension. Each set was divided equally into two 9 aliquot groups; one group was stored in the refrigerator (6°C) and the other in the freezer (-20°C). Both *Salmonella* Typhimurium and *Salmonella* Enteritidis counts were taken on the day of inoculation and after 1, 2, 3, 4, 5, 6, 7, 8, and 9 weeks of a refrigerator (6°C) and freezer (-20°C) storages for survival assay. On every sampling day, one of the inoculated minced meat samples was placed in 9ml of sterile saline and 10-fold serially diluted till the 7^{th} tube, then total bacterial count was performed by spreading method on both selective XLD and ordinary plate count agar plates using 100µL of each dilution then incubated for 24hrs at 37°C (Al-Nabulsi et al. 2015).

Pre- and Post-Low-Temperature Exposure Antibiotic Sensitivity Profile Evaluation

The susceptibility testing of each isolated bacterial strain was performed according to the standard Kirby-Bauer disc diffusion method (Quinn et al. 1994) and the outcome results were evaluated and interpreted according to the latest clinical and laboratory standard institute (CLSI) manual (2021 version). The selection of the antibiotic panel to be tested through the performed ASTs depended on certain criteria; the updated CLSI guidelines (CLSI 2021), scholarly internationally published articles in the same field of the current study, commercial availability of the used active principle, and the commercial availability of a proper active principle preparation to be used. Beta-lactam with beta-lactamase inhibitors (Amoxicillin/Clavulanic acid and Ampicillin/Sulbactam), Aminoglycosides (Amikacin and Gentamicin), Carbapenem (Imipenem), Beta-lactam (Aztreonam and Ampicillin), Sulfonamides (Sulfamethoxazole/Trimethoprim), 3^{rd} generation cephalosporin (Ceftazidime, Ceftriaxone, and Cefotaxime), 1^{st} generation cephalosporin (Cephalothin), 4^{th} generation cephalosporin (Cefepime), Tetracycline (Doxycycline), Fluoroquinolones (Enrofloxacin and Ciprofloxacin), and Macrolides (Tylosin) were the 10 families and 17 members involved in the AST.

Data Analysis

All data collection, tabulation, presentation, and representation were achieved using an Excel sheet and its associated data analysis tools.

RESULTS AND DISCUSSION

The designed model was initially confirmed by isolation followed by PCR. The incorporated isolates were confirmed to be *Salmonella* Typhimurium and *Salmonella* Enteritidis (Fig. 1).

Through the retrieved studies attempted to study *Salmonella* behavior at low temperatures, storage temperatures between 0°C and 4°C were used more frequently than those between 5°C and 7.5°C (Deblais et al. 2019; Igo and Schaffner 2021; Lobacz and Zulewska 2021; Bashir et al. 2022). Generally, the majority of treatments included beef samples, standard packaging, no longer than 10 days of storage, at a concentration of 6 logs CFU/mL, and *Salmonella* Typhimurium strains were the most common model used in the majority of the previous studies (Silva et al. 2022). The current work tried to modify the commonly used testing model, therefore, two different *Salmonella spp*. used, the testing procedures were applied for 9 weeks with special consideration for the first two weeks as well as higher starting critical *Salmonella* concentrations were used at 8 logs CFU/mL through 6°C refrigeration temperature and -20°C freezing temperature.

In previous literature, the main samples used at refrigerating-storage temperatures were beef, chicken, and pork (Silva et al. 2022). The minced beef model was chosen to be employed in the current work because it is the most common and extensively used type of processed meat throughout the majority of European, Arabian,

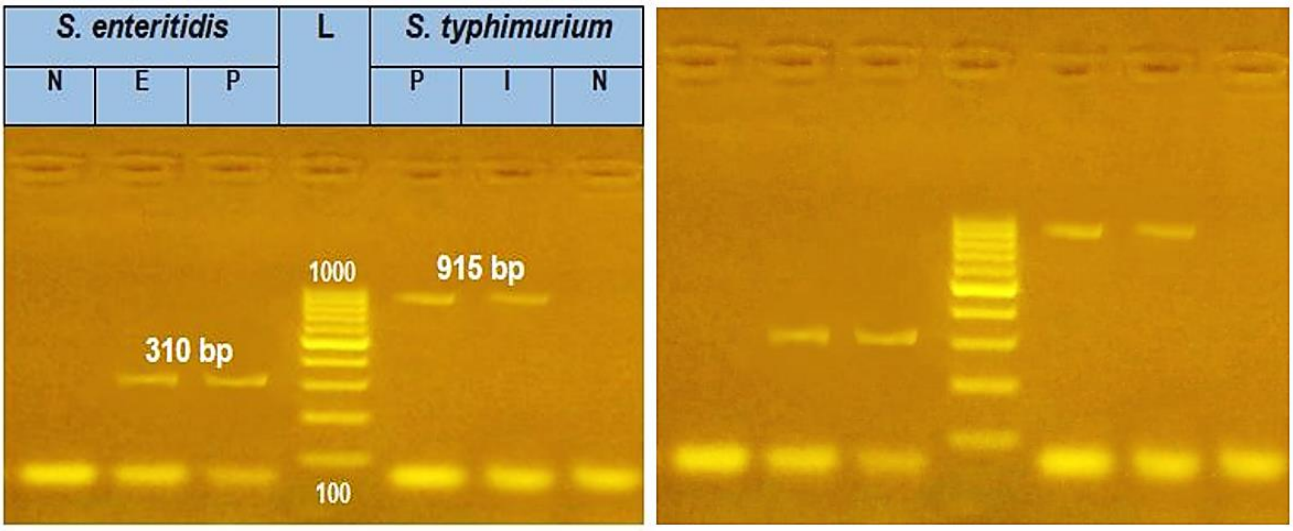

Fig. 1: Gel electrophoresis showing the *Salmonella* Enteritidis and the *Salmonella* Typhimurium positive bands, N: is a negative control, P; is a positive control, E; *Salmonella* Enteritidis, I; *Salmonella* Typhimurium.

African, American, and most South Asian cuisine cultures. Additionally, earlier research using antimicrobial compounds under similar conditions was less successful than research done in a typical setting. The most often inoculated strain in refrigeration trials was *Salmonella* Typhimurium, while in the current study, two *Salmonella spp.* were used.

At both temperatures, a decrease of more than 2 logs CFU was also noted. As well as of the treatments examined showed no growth rates at (6°C) and (-20°C), respectively. Even though the majority of the studies showed drops in *Salmonella* levels, several showed rise in pathogen levels even in cool storage. For instance, one study found that ground beef packed under a vacuum and in a modified environment at 3°C for 12days had lower *Salmonella* counts by 1.4 to 1.9log CFU (Mukhopadhyay and Ramaswamy 2012). In a different study, modified atmosphere packed (MAP) beef held at 7.5°C for 12days showed declines in *Salmonella* concentrations of 0.17 and 0.97log CFU, but the same study's vacuum-packed beef showed a pathogen reduction of 1.69log CFU (Morey and Singh 2012). In the current work, there were no total viable count (TVC) changes that have been recorded up to 14days whatever the Salmonella spp. was involved and whatever temperature there were exposed to. The TVC changes have been recorded after the 14days as follows; the refrigerated *S*. Typhimurium-infected samples showed 1log reduction from the 3rdweek up to the 5thweek followed by 1log reduction from the 6thweek up to the 8th week and a final 1log reduction at the 9thweek with overall 3logs reduction. On the other hand, the frozen *S*. Typhimurium-infected samples showed stability in the TVC till the 5thweek with only 1log reduction from the 6thweek till the end of the study. In the case of the refrigerated *S*. Enteritidis-infected samples showed TVC stability up to the 6th week with only 1log reduction from the 7thweek till the end of the conducted treatment at the 9th week. In contrast to the log reduction results obtained in the previous cases, the frozen *S*. Enteritidis-infected samples showed TVC stability till the end of the study without recording any log reductions.

The incorporated two *Salmonella spp*. growth is influenced by the competitive microbiota as well as the initial number of bacterial cells. The literature describes *Salmonella spp.* as a rather weak competitor, even though they may develop and compete with other microorganisms (Djordjević et al. 2018; Guerrini et al. 2021). For these reasons, it is assumed that low temperatures, the predominant bacteria in minced meat held at refrigerated temperatures (6°C), inhibit this group of bacteria in the current investigation until the 4thweek while the freezing temperature (-20°C) was sufficient to inhibit this group till the end of the study at the 9th week (Table 4).

The shelf-life of meat and the pathogen survivability through the cold chain is a metric that can easily be determined by TVC (Morey and Singh 2012; Blixt and Borch 2002). So, the current work was based on the TVC result to monitor the survivability status of the incorporated bacterial pathogen model. One of the most crucial factors affecting the meat's shelf life is the initial microbial load. Mesophilic and psychrotrophic bacteria make up the majority of the initial microbiota on fresh meat, and the latter group of bacteria is primarily in charge of meat deterioration. These factors make TVC a crucial microbiological quantitative indication of production process cleanliness, a safety assessment tool, and a raw meat rotting indicator. A TVC value of 10^7 CFU in beef is regarded as a crucial number for determining the degree of rotting based on various experiments, so using the cold chain may truly have an efficient impact in harnessing the meat's deteriorating microbiota with more effective control in cases of freezing status with less control can be achieved in refrigerating temperature, but overall the lowest range of the cold chain temperatures can inhibit the naturally associating microbiota for up to one month.

Salmonella Typhimurium and *Salmonella* Enteritidis are inherently resistant to a number of antibacterial and exhibit multi-drug resistance. Reduced outer membrane permeability, modifications to lactamase and penicillin-binding proteins, and activation and synthesis of efflux pumps are the major mechanisms of antibiotic resistance

Table 4: Tabulation and representation of the TVC through the whole flow of the conducted study

	S. Typhimurium			S. Enteritidis		
	Refrigerator (6°C)		Freezer (-20°C)	Refrigerator (6°C)		Freezer (-20°C)
0	$13.0x10^8$			$12.6x10^8$		
1	$9.0x10^8$		$10.0x10^8$	$12.4x10^8$		$11.6x10^8$
2	$3.0x10^8$		$8.5x10^8$	$11.8x10^8$		$11.1x10^8$
3	$24.0x10^7$		$7.3x10^8$	$11.0x10^8$		$10.9x10^8$
4	$9.7x10^7$		$5.8x10^8$	$10.1x10^8$		$9.4x10^8$
5	$4.3x10^7$	Non-mannitol fermenter	$3.2x10^8$	$9.8x10^8$	Non-mannitol fermenter	$7.2x10^8$
6	$20.0x10^6$	orange *Staphylococci*	$25.0x10^7$	$3.9x10^8$	orange *Staphylococci*	$6.8x10^8$
7	$13.0x10^6$	colonies	$22.0x10^7$	$27.0x10^7$	colonies	$5.3x10^8$
8	$5.5x10^6$		$18.0x10^7$	$20.6x10^7$		$4.7x10^8$
9	$30.0x10^5$		$15.0x10^7$	$11.0x10^7$		$4.9x10^8$

Broad-spectrum resistance *Salmonella* Typhimurium and *Salmonella* Enteritidis bacteria are now more common than ever because of the growing number of clinically used antibacterial drugs. In addition to plasmids, the kind of chromosomal gene and the strain gene plays a role in the development of drug resistance. Environmental selection and mutations are intimately intertwined (Fàbrega and Vila 2013; Knudsen et al. 2014; Ryan et al. 2018). In the current work, there were no changes in the antimicrobial resistance profiles of the used *Salmonella* isolates pre- and post-low temperature exposure which reveals no significant effect of low temperatures on the control of the antimicrobial resistance of the *Salmonella spp.* through the food processing chain.

Conclusion

Minced beef meat stored in two different cold environments (6°C representing a refrigeration temperature and -20°C representing a freezing temperature), simulating the cold chain for handling meet and its processed products, showed different log reductions in the *Salmonella* levels based on the species of the *Salmonella* and the temperature to which they were exposed. Both *Salmonella* Typhimurium and *Salmonella* Enteritidis treated samples showed bacterial count log reduction in *Salmonella* concentrations throughout the overall period through the (6°C and -20°C) storage. *Salmonella* growth wasn't effectively controlled by cool storage, with declines in the meat between 1 and 3logs CFU reported at lower temperatures. This finding highlights the significance of cold chains for both the industry and consumers. *Salmonella* was not completely eradicated from the samples, despite the fact that most previous trials showed prominent reductions in the infection during cool storage. *Salmonellosis* transmission through meat is still a possibility, thus it should be taken into account with sufficient and efficient international public health concerns and the need for publicity awareness.

Availability of Data and Material (ADM)

The data used and/or analyzed related to the animal cases tested during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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Authors' Contributions

All authors contributed to the article and approved the submitted version. All authors contributed equally through different stages of the study.

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