Assessing Listeria Monocytogenes Survival and Growth in Skim, Partial Skim, and Full-Fat Milk under Refrigeration

Marwa Mahmood [1*], Fouad K. Alsammarraie [1]

Abstract

Background: Milk is a global fundamental dietary staple cherished for its nutritional richness and versatility. However, despite pasteurization efforts, its susceptibility to bacterial contamination, particularly by pathogens like Listeria monocytogenes (L. monocytogenes), poses a significant concern. This study explored how components within cow milk influence L. monocytogenes, particularly in safeguarding bacterial cells against damage or destruction during refrigeration. Method: Forty samples were employed, comprising 30 milk varieties (skim, partial skim, and full-fat) and 10 phosphate buffer solution (PBS) samples serving as controls. Each sample was inoculated with L. monocytogenes and stored in a refrigerator at 4-5°C for one week. Results: The highest survival rate on tryptose agar (TA) was observed in the full-fat milk group at 99.28% after 7 days, showing no significant difference ($p \geq 0.05$) from baseline. Conversely, the control and skim milk groups exhibited the lowest survival rates, at 27.23% and 58.95%, respectively, after the same storage period. Furthermore, an elevated percentage of injured bacterial cells on tryptose salt agar (TSA) was found in the partial skim and skim milk groups (46.02% and 57.14%, respectively), compared to the full-fat milk group (25.85%), with significant differences ($p \leq 0.05$) across all time points (0, 1, 3, 5, and 7 days). Conclusion: The study underscores L. monocytogenes' resilience and capacity to proliferate even under adverse conditions such as refrigeration. Moreover, it highlights the pathogen's ability to withstand high-salt environments, as evidenced by its performance on TSA media containing 5.5% NaCl.

Keywords: Milk, Listeria Monocytogenes, Refrigeration, Bacterial Survival, Bacterial Count.

Significance | This study revealed that preserving dairy products in refrigeration does not protect them from contamination with Listeria.

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Editor Md Asaduzzaman Shishir, and accepted by the Editorial Board Apr 12, 2024 (received for review Jan 28, 2024)

Introduction

Milk is a vital component of diets globally, serving as a rich source of essential nutrients including macro and micro elements. It is a significant provider of lipids, proteins, carbohydrates, minerals, and vitamins such as calcium, vitamin B12, and riboflavin (Duguma & Janssens, 2015). The production of high-quality milk necessitates meticulous preparation of the cow, adherence to proper milking procedures, and rigorous post-milking practices to maintain sanitary standards (Harding, 1995; AL-Shamary, 2009).

However, the handling and consumption of milk also present significant health concerns, particularly due to the risk of zoonotic and food-borne illnesses (Alsammarraie et al., 2018). In many markets, inadequate handling practices, lack of quality control standards, and the consumer preference for raw milk exacerbate these hazards (Kivaria, Noordhuizen, & Kapaga, 2006; Gany Kszaal Atshan & AlGraibawi, 2018; Fathi, 2019). The unregulated sale and distribution of milk increase the potential for contamination with pathogenic bacteria.

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Please cite this article.
Marwa Mahmood and, Fouad K. Alsammarraie (2024). Assessing Listeria Monocytogenes Survival and Growth in Skim, Partial Skim, and Full-Fat Milk under Refrigeration, Journal of Angiotherapy, 8(4), 1-5, 9640

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Instances of listeriosis outbreaks linked to both pasteurized and unpasteurized dairy products highlight the persistent risk of contamination. Although effective pasteurization should eliminate Listeria monocytogenes, failures in the process can lead to serious public health issues (Pricope-Ciolacu et al., 2013, Hoque et al. 2018). Pathogenic bacteria such as Listeria monocytogenes, Yersinia enterocolitica, Campylobacter jejuni, and enteropathogenic Escherichia coli are known to be transmitted through milk and milk products, posing a substantial risk to consumers (Vasavada, 1988).

L. monocytogenes is particularly concerning due to its ability to cause severe illness, especially in vulnerable populations such as the elderly, pregnant women, newborns, and immunocompromised individuals (Abdalla, ALKhatib, & Alwan, 2004; Ibrahim, 2017; Ryser, 2021). Studies have indicated that milk components can exhibit cryoprotective properties, which may help bacterial cells survive the freezing process. For instance, lactose, milk salts, and casein have been shown to provide resistance to Salmonella anatum during storage (El-Kest & Marth, 1992). Furthermore, the presence of milk in products like ice cream and yogurt may facilitate the survival of L. monocytogenes during freezing and refrigeration (Goff et al., 2013).

The present study aims to explore the characteristics of L. monocytogenes in pasteurized cow milk. Specifically, it seeks to determine whether the components of different types of milk (full fat, partial skim, and skim milk) provide a protective environment for L. monocytogenes during refrigeration. This research is critical in understanding how milk's intrinsic properties might influence the survival of harmful pathogens, thereby informing safer dairy processing and handling practices.

Materials and methods

Bacterial isolation

Listeria monocytogenes was isolated from ice cream samples that were collected from local markets of Baghdad city (the data was not included).

Preparation of the Bacterium

The preparation of bacterial broth was done according to (El-Kest and Marth, 1991) with some modifications. *L. monocytogenes* isolate was moved from a tryptose agar (TA) culture to a tube that contained tryptose broth (TB) and then incubated at 37°C for 24 hours(Salman *et al.*, 2021) . One loopful of the culture was moved to another tube containing TB medium and cultured at 37°C for 24 hours. Further sub-cultures were similarly conducted twice. The culture from the 4th sub-culturing was centrifuged at 1100 x g for 7 minutes. The supernatant was removed, and the cell pellet was rinsed twice with phosphate buffer (PBS). A 9 ml solution of PBS was used to resuspend the washed cell pellet before inoculating the required material.

Bacterial plating and serial dilutions of refrigerated milk samples

The experiments were accomplished in triplicate, and the bacterial count was submitted as log CFU/mL. *L. monocytogenes* was aerobically incubated overnight in Brain Heart Infusion broth (BHI) at 37 °C. Then, 200 µL of the bacterial broth was injected into three different types of pasteurized milk, which were skim, partial skim, and full-fat milk, in addition to using PBS as a control group in this experiment. Following the injection of the control and treatment groups with *L. monocytogenes*, the samples were kept at refrigeration temperature (4-5 °C). Then 0, 1, 3, 5, and 7 days of refrigeration, the control and treatment samples were diluted using peptone water in a series of concentrations ranging from 10-1 – 1010 CFU/mL of control and treatments groups. Afterwards, 1 mL from each diluted concentration was poured on TA and TSA agars to calculate the live and injured *L. monocytogen.* After 24-48 hours of incubation, the bacterial counts were done (Al-maqasisi and Al-Samarai, 2023; Alsammarraie, Lin and Mustapha, 2023). The samples were plated on two different types of agar; TA, which supports the development of both unharmed and harmed bacteria, and TSA, which exclusively supports the growth of intact bacterial cells that would not be affected by the temperature of the refrigeration. The TSA media was prepared by adding 5.5% of sodium chloride (NaCl) to TA agar.

Calculations

The proportion of deaths was determined by measuring growth on TA medium and calculating the average from two experiments.

$$Death\ (\%) = 100 - \frac{to - ti}{to} \times 100$$

To = colony-forming units at zero time (before refrigeration). Ti = colony-forming units after time of refrigeration.

The discrepancy in survival percentages between TA and TSA of each time point was utilized to determine the proportion of damaged cells at that specific time. The percentage of harmed bacteria is the ratio of cells on TA that were unable to form colonies on TSA within a certain time frame. Calculations are derived from the mean of two experiments.

$$Injury\ (a) = \frac{survivors\ on\ TA\ (\%) - survivors\ on\ TSA\ (\%)}{survivors\ on\ TA\ (\%)} \times 100$$

survivors on TA (%) = The proportion of surviving is computed using the number of colonies on TA medium (average of two experiments). survivors on TSA (%) = The proportion of surviving is computed using the number of colonies on TSA medium (average of two experiments).

Statistical Analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1). Two-way, three-way ANOVA and Least significant differences (LSD) post hoc test were performed to assess significant differences among means. Chi- square test was applied to identify the significant differences among proportions. SPSS program was used for conducting figures, also Excel was used

for determination of prediction equations P< 0.05 that is considered statistically significant.

Results and Discussion

The results in Table 1 illustrate the effect of cold storage on Listeria monocytogenes growth and recovery in two different media: tryptic soy agar (TSA) and tryptose agar (TA). Overall, bacterial growth was significantly lower on TSA compared to TA at all time points, including the initial measurement. This difference is likely attributable to the presence of NaCl in TSA, which inhibits the growth of injured Listeria cells.

At the initial time point (zero time), the bacterial counts for groups G1, G2, and G3 on TSA were 7.07, 7.11, and 7.07 log CFU/mL, respectively, whereas on TA, groups G2 and G1 had higher counts of 9.36 and 9.37 log CFU/mL, respectively.

During the first and third days of refrigeration, significant variations ($P < 0.05$) were observed among the storage durations for all groups. Notably, G1 on TSA exhibited the most significant reduction in bacterial count, with values dropping to 3.16 and 2.04 log CFU/mL, respectively. In contrast, the smallest reduction in bacterial count was seen in G2 under TA conditions, with counts of 9.09 and 8.86 log CFU/mL, respectively.

On the fifth and seventh days, groups G2, G3, and G4 on TA, along with G2 on TSA, showed the most significant bacterial proliferation. Remarkably, no significant difference ($P > 0.05$) in log counts of L. monocytogenes was observed in G2 on TA, indicating no decline in bacterial growth for this group. Conversely, bacterial counts decreased by 7, 2, and 4 log CFU/mL in G1, G3, and G4, respectively, on TA, and by 7, 2, 4, and 5 log CFU/mL in G1, G2, G3, and G4, respectively, on TSA.

By the end of the seven-day refrigeration period, the highest bacterial log counts were recorded in the full-fat milk group on both TA and TSA media, followed by the partial skim and skim milk groups. In contrast, the control groups exhibited the lowest bacterial counts on both media.

These findings suggest that the components of milk may offer a protective effect to L. monocytogenes during cold storage, with the type of milk and storage medium influencing the extent of bacterial survival and proliferation.

The results depicted in Table 2 and Figure 1 demonstrate the survival percentages of Listeria monocytogenes over time in different storage media. At the initial time point, the survival percentages were identical across all groups, showing no significant differences. However, by the first day, the G2 TA and G2 TSA groups exhibited the highest bacterial survival rates at 97.04% and 95.52%, respectively, while the G1 TSA group showed the lowest survival rate at 44.71%. On the third day, a statistically significant difference ($P<0.05$) emerged between all groups, with the G2 TA group maintaining the highest survival rate at 94.65%, contrasting sharply with the G1 TSA group's low rate of 29.48%. Generally, TSA groups displayed lower survival rates than TA groups during the first three days. By the fifth day, the G2 TA group continued to exhibit the greatest bacterial survival rate at 94.86%, followed by the G3 TA and G4 TA groups at 73.49% and 60.64%, respectively. Notably, the G3 TA group showed a significant increase in survival rate compared to its third-day measurement. On the seventh day, the highest survival rates were observed in the G2 TA (99.28%), G3 TA (82.10%), and G2 TSA (73.61%) groups, with statistically significant differences ($P<0.05$) compared to the fifth day. Ultimately, the G2 TA group exhibited the most substantial survival rate throughout the study, followed by the G3 TA and G2 TSA groups. These findings suggest that the survival rate of Listeria monocytogenes is influenced by both the lipid content and sodium chloride concentration in the storage media.

The findings presented in Table 3 and Figure 2 illustrate the proportion of Listeria monocytogenes bacteria that were injured and their capacity to recuperate under suitable circumstances. On the first day, the G1 TSA group exhibited the highest injury rate at 37.25%, while the G2 TSA group had the lowest at 1.56%. By the third day, a noticeable increase in the percentage of bacterial injuries was observed across all groups except the G4 TSA group, with statistically significant differences ($P<0.05$) compared to the first day. On the fifth day, the G1 TSA group reached the maximum injury rate at 100%, whereas the G2 TSA group saw a reduction in damage, recording 31.73% compared to the third day. By the end of the experiment, all TSA groups showed a significant rise in the proportion of damaged bacteria, except for the G2 TSA group, which exhibited a 25.85% decrease in injury rate. Throughout the study, the G2 TSA group consistently demonstrated the highest recovery rate of L. monocytogenes, while the G1 TSA group experienced complete destruction from the fifth day onwards.

Additionally, the data depicted in Figure 3 highlight the fluctuations in the growth and survival of L. monocytogenes on TA and TSA media. There was a statistically significant disparity ($P<0.05$) between the same groups (G1, G2, G3, and G4) cultivated in TA and TSA, underscoring the impact of a 5.5% sodium chloride concentration on bacterial viability and reproductive capacity. Overall, bacterial survival percentages were consistently lower on TSA media compared to TA media for all treatment groups, attributed to the inhibitory effect of NaCl. Sodium chloride impedes bacterial growth as most bacterial strains struggle to thrive in high NaCl concentrations (Wiktorczyk-Kapischke et al., 2023).

The results of this study highlight the ability of Listeria monocytogenes to recover and thrive in optimal conditions, such as environments with appropriate nutrition and temperature. Several investigations have confirmed the presence of L. monocytogenes in butter derived from both raw and pasteurized milk. Notably, outbreaks of listeriosis in the United States have been linked to the

Table 1. The effect of the bacterial culture medium and periods of cold storage on log count of *Listeria monocytogenes*.

Media Types	Time Group	zero time	1 day	3 days	5 days	7 days
TA	G1	A9.37±0.03a	B6.68±0.04e	C4.68±0.01d	D2.85±0.04f	E2.55±0.04f
	G2	A9.36±0.05a	B9.09±0.05a	C8.86±0.05a	C8.88±0.01a	A9.30±0.01a
	G3	A9.33±0.04ab	B8.58±0.04b	D6.78±0.05b	D6.86±0.04b	C7.66±0.03b
	G4	A9.23±0.02b	B7.45±0.04c	C6.60±0.01c	D5.60±0.01c	E5.44±0.01c
TSA	G1	A7.07±0.06d	B3.16±0.05h	C2.08±0.04h	D0.00±0.00h	D0.00±0.00
	G2	A7.11±0.04d	B6.80±0.01d	D4.56±0.02e	D4.61±0.02d	C5.24±0.02d
	G3	A7.06±0.06d	B5.83±0.08f	C3.39±0.03g	D3.26±0.03e	E3.12±0.05e
	G4	A7.93±0.04c	B4.06±0.02g	C3.57±0.01f	D2.32±0.02g	E2.00±0.01g
LSD		0.10				
Means with a different small letter in the same column are significantly different						

(P<0.05) Means with a different capital letter in the same row are significantly different (P<0.05) Mean ±Standard Error. TA =Tryptose Agar, TSA =Tryptose Salt Agar, G1 = control, G2 = full-fat milk, G3 = partial skim, and G4 = skim milk.

Table 2. The percentage of surviving *Listeria monocytogenes* on TA and TSA agars in three different types of milk and control groups during the various periods of refrigeration.

Media Types	Time Group	zero time	1 day	3 days	5 days	7 days
TA	G1	A100.00±0.00a	B71.26±0.04f	C49.97±0.13e	D30.50±0.32f	E27.23±0.28f
	G2	A100.00±0.00a	B97.04±0.02a	C94.65±0.03a	C94.86±0.60a	A99.28±0.45a
	G3	A100.00±0.00a	B91.95±0.05c	E72.65±0.15b	D73.49±0.07b	C82.10±0.00b
	G4	A100.00±0.00a	B80.69±0.68e	C71.47±0.12c	D60.64±0.31d	E58.95±0.12d
TSA	G1	A100.00±0.00a	B44.71±0.31h	C29.48±0.38h	D00.00±0.00h	D00.00±0.00h
	G2	A100.00±0.00a	B95.52±0.41b	D64.14±0.04d	D64.75±0.09c	C73.61±0.71c
	G3	A100.00±0.00a	B82.58±0.41d	C48.01±0.02f	D46.24±0.12e	E44.31±0.35e
	G4	A100.00±0.00a	B51.24±0.59g	C45.04±0.32g	D29.33±0.44g	E25.27±0.21g
Means with a different small letter in the same column are significantly different						

(P<0.05) Means with a different capital letter in the same row are significantly different (P<0.05) Mean ±Standard Error. TA =Tryptose Agar, TSA =Tryptose Salt Agar, G1 = control, G2 = full-fat milk, G3 = partial skim, and G4 = skim milk.

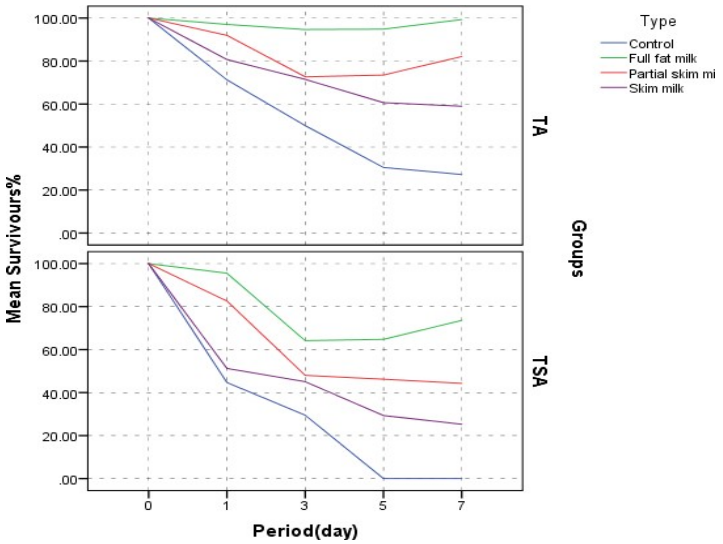

Figure 1. Calculation of the percentage of surviving *Listeria monocytogenes* on different media throughout the cold storage durations.

Table 3. The percentage of injured *Listeria monocytogenes* on TSA agar in three different types of milk and control groups during the various periods of refrigeration.

TSA Groups	Zero-time	1 day	3 days	5 days	7 days
G1	D00.00±0.00a	C37.25±0.30a	B40.98±0.92a	A100.00±0.00a	A100.00±0.00a
G2	D00.00±0.00a	C1.56±0.41d	A32.23±0.07d	A31.73±0.33d	B25.85±0.38d
G3	E00.00±0.00a	D10.18±0.40c	C33.91±0.11c	B37.07±0.11c	A46.02±0.43c
G4	D00.00±0.00a	C36.49±0.19b	C36.97±0.34b	B51.61±0.48b	A57.14±0.26b
LSD	0.97				

Means with a different small letter in the same column are significantly different (P<0.05) Means with a different capital letter in the same row are significantly different (P<0.05) Mean ±Standard Error. TA =Tryptose Agar, TSA =Tryptose Salt Agar, G1 = control, G2 = full-fat milk, G3 = partial skim, and G4 = skim milk.

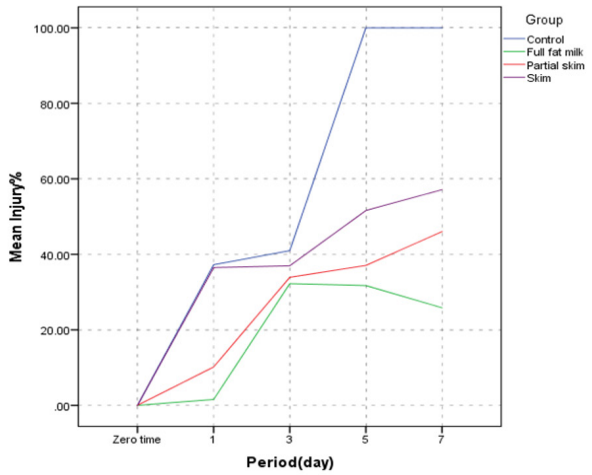

Figure 2. The percentage of injured Listeria monocytogenes on TSA agar in three different types of milk and control groups throughout different cold storage periods.

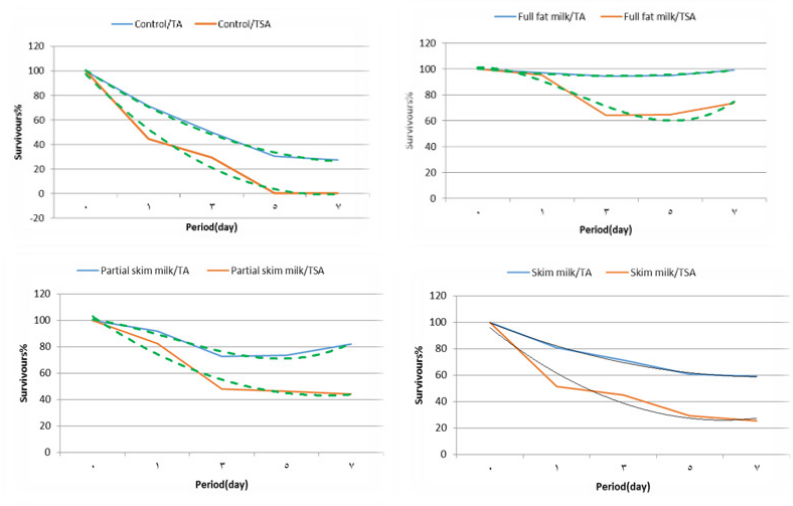

Figure 3. The impact of NaCl concentration on the ability to survive and proliferation of Listeria monocytogenes in different types of milk samples and control groups throughout the time of cold storage.

consumption of contaminated butter (Ryser and Marth, 2007). Additionally, Rosenow and Marth (1987) demonstrated that L. monocytogenes could survive and even proliferate at 4°C, a common refrigeration temperature. Ribeiro et al. (2023) also described L. monocytogenes' capacity to endure and flourish in extreme settings, including refrigeration temperatures.

Research by AL-Shamary (2010) and Jia et al. (2021) emphasized the necessity of avoiding temperature abuse during refrigerated storage, as simulations indicate that L. monocytogenes populations in contaminated milk could reach dangerous levels, posing a significant risk to consumers. Lima et al. (2021) found that pasteurized milk stored at 8°C provided optimal conditions for bacterial proliferation. Similarly, Yang et al. (2022) demonstrated that L. monocytogenes could thrive under the low water activity conditions found in milk powders, indicating that cold storage offers limited defense against this pathogen.

Previous research by Wilkins, Bourgeois, and Murray (1972) showed that the lag phase of L. monocytogenes lengthens as the temperature decreases, suggesting that thorough pasteurization effectively eliminates live Listeria cells present in milk (Farber et al., 1988). However, Doyle et al. (1987) and other researchers have shown that L. monocytogenes can survive short-term high-temperature, short-duration pasteurization in the presence of leukocytes.

Michelon et al. (2016) found that the concentration of Listeria remained stable throughout the shelf life of milk fat percentages (MFPs) in both commercial and homemade products, except for commercial low-fat dairy products where Listeria levels eventually declined. Our results align with prior studies by Kim and Kang (2015), who assessed various physicochemical treatments on milk contaminated with L. monocytogenes and noted an increase in Listeria levels in high-fat milk. They attributed this to the protective effect of milk fat against the adverse impact of refrigeration on the bacterium.

Taylor and Zhu (2021) observed that L. monocytogenes is less stable in foods high in antimicrobials, such as cocoa powder, but more stable in high-fat foods like almond flour. Posada-Izquierdo et al. (2021) found that reducing salt content, regardless of the initial concentration, resulted in higher growth rates of L. monocytogenes at 4°C. Siderakou et al. (2021) indicated that various food processing techniques could affect L. monocytogenes' survival or injury, with the dynamics of injured cells being significant due to their potential to develop new resistance traits.

Finally, our findings correspond with those of Yu et al. (2023), who revealed that low-temperature stress increased the resistance of L. monocytogenes. Collectively, these results underscore the resilience and adaptability of L. monocytogenes in various dairy products and storage conditions, emphasizing the need for stringent control measures to ensure food safety.

Conclusion

Our study conclusively demonstrated that refrigeration alone does not safeguard dairy products from contamination by Listeria monocytogenes. The high-nutrient content of certain dairy products, particularly those with high fat content, provides a protective environment for L. monocytogenes during cold storage. Notably, L. monocytogenes exhibited a high survival rate in full-fat milk samples during refrigeration, while its survival rate was significantly lower in skim milk samples. Additionally, the data indicated that the percentage of injured bacteria was lower in refrigerated full-fat milk compared to skim and partial skim milk samples, where 100% of the bacteria were injured after seven days. These findings suggest that the nutritive elements in dairy products can shield L. monocytogenes from the adverse effects of refrigeration, preventing both killing and injury of the bacteria. Future research should explore the impact of the nutritional composition of various media on the survival and preservation of L. monocytogenes during freezing, to better understand and mitigate this pathogen's resilience in dairy products.

Author contributions

M.M., F.K.A. conducted the practical work and edited the manuscript, performed the statistical analysis, wrote the manuscript.

Acknowledgment

The authors were grateful to Department of Veterinary Public Health, College of Veterinary Medicine/ University of Baghdad, Iraq for their assistance.

Competing financial interests

The authors have no conflict of interest.

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