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Histological and microbiological alterations in sardines subjected to repeated freezethaw cycles: Implications for muscle integrity and food safety

Alteraciones histológicas y microbiológicas en sardinas sometidas a ciclos repetidos de congelación y descongelación: implicaciones para la integridad muscular y la seguridad alimentaria

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ABSTRACT

A total of thirty freshly caught sardines were subjected to one to five freeze–thaw cycles, while unfrozen samples served as controls. Muscle structure was assessed by histological and histomorphometric analyses, including measurements of vacuole area, vacuolated fiber area, vacuolated-to-fiber area ratio, and ice crystal size. Microbiological analyses included aerobic mesophilic bacteria, total coliforms, coagulase-positive *Staphylococcus aureus*, and Salmonella spp. Histological observations revealed a progressive deterioration of muscle architecture with increasing numbers of freeze–thaw cycles, characterized by fiber disorganization, extensive vacuolation, and widening of inter-fiber spaces. Histomorphometric analysis showed significant increases ($P < 0.05$) in ice crystal area, from 1050 ± 300 µm² at one freeze–thaw cycles to 2300 ± 150 µm² at five freeze–thaw cycles, as well as in the vacuolated area ratio, which increased from 25 ± 7 % to 65 ± 6 %. Microbial counts showed a slight but consistent decrease during the first three freeze–thaw cycles and remained well below the spoilage threshold of 7 log CFU/g. A significant negative correlation ($r = −0.78$; $P < 0.05$) was observed between vacuole area and total aerobic mesophilic counts, indicating that structural muscle damage did not promote microbial proliferation under controlled cold storage conditions. In conclusion, repeated freeze–thaw cycles notably impair sardine muscle microstructure and may slightly reduce microbial counts, without favoring bacterial growth when the cold chain is properly maintained. These findings highlight the importance of strict temperature control to preserve both the quality and safety of sardine products.

Key words: *Sardina pilchardus*;histology; food safety; ice crystals; fish quality

RESUMEN

Un total de treinta sardinas capturadas recientemente fueron sometidas a uno a cinco ciclos de congelación–descongelación, mientras que las muestras no congeladas se utilizaron como control. La estructura muscular se evaluó mediante análisis histológicos e histomorfométricos, incluyendo la medición del área de las vacuolas, el área de las fibras vacuoladas, la relación área vacuolada/área de la fibra y el tamaño de los cristales de hielo. Los análisis microbiológicos incluyeron bacterias mesófilas aeróbicas, coliformes totales, *Staphylococcus aureus* coagulasa positivo y Salmonella spp. Las observaciones histológicas revelaron un deterioro progresivo de la arquitectura muscular con el aumento del número de ciclos de congelación–descongelación, caracterizado por desorganización de las fibras, vacuolización extensa y ensanchamiento de los espacios interfibrilares. El análisis histomorfométrico mostró incrementos significativos ($P < 0,05$) en el área de los cristales de hielo, desde 1050 ± 300 µm² en un ciclo de congelación–descongelación hasta 2300 ± 150 µm² en cinco ciclos, así como en la proporción del área vacuolada, que aumentó de 25 ± 7 % a 65 ± 6 %. Los recuentos microbianos mostraron una disminución leve pero constante durante los tres primeros ciclos de congelación–descongelación y se mantuvieron muy por debajo del umbral de deterioro de 7 log UFC/g. Se observó una correlación negativa significativa ($r = −0,78$; $P < 0,05$) entre el área de las vacuolas y los recuentos totales de bacterias mesófilas aeróbicas, lo que indica que el daño estructural del músculo no promovió la proliferación microbiana bajo condiciones controladas de almacenamiento en frío. En conclusión, los ciclos repetidos de congelación–descongelación deterioran notablemente la microestructura muscular de la sardina y pueden reducir ligeramente los recuentos microbianos, sin favorecer el crecimiento bacteriano cuando la cadena de frío se mantiene adecuadamente. Estos hallazgos resaltan la importancia de un estricto control de la temperatura para preservar tanto la calidad como la seguridad de los productos de sardina.

Palabras clave: *Sardina pilchardus*; histología; seguridad alimentaria; cristales de hielo; calidad del pescado

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INTRODUCTION

Seafood is a crucial aspect of a healthy diet, serving as a source of quality protein, essential amino acids, and long-chain polyunsaturated fatty acids. The Sardine (*Sardina pilchardus*) is recognized for their nutritional qualities and play an important economic role in fisheries in Algeria. However, their flesh is highly perishable, and handling after harvest has a significant effect on product quality and the microbiological safety of seafood [1].

Freezing is a very effective storage method because it decreases the rate of the enzymatic activity and microbial growth [2]. However, in the distribution and retail stages, the fluctuations in storage temperature frequently cause commercial fishes to go through multiple freeze–thaw (F–T) cycles that can drastically change the microstructure and functional properties of muscle (fish) tissue [3, 4]. Recrystallization of ice causes mechanical rupture of cell membranes, widening of the inter-fiber spaces, and loss of intracellular water leading to texture deterioration and changes in water-holding capacity [5, 6].

In addition to changes in structural integrity, freezing and thawing cycles also impact microbial die-off and survival rates. Although the formation of ice crystals and osmotic stress can render bacterial cells inactive or destroyed [7], thawing can allow for some potential recovery or growth depending on handling conditions [8, 9].

Notably, most of the past research has explored the documented physicochemical properties - such as pH or color - of small pelagic fish, with many studies not looking at histological and microbiological parameters simultaneously after multiple F–T cycles. Recent studies have examined *Sardinella aurita* [10] and *Oncorhynchus mykiss* [11], revealing the need to establish integrative perspectives that connect tissue integrity with microbiological behaviour within fluctuating thermal conditions.

Histological examination gives direct evidence of muscle tissue integrity and its degradation from ice crystals, while microbiological examination determines and counts the hygienic safety level of the packaged end-product. Collectively both perspectives give a rounded viewpoint of freeze stress and its effect on fish quality and safety (one health perspective) with food quality and consideration for public health and sustainability to the environment [12, 13, 14].

The present study aimed to evaluate the histological and food safety changes in *Sardina pilchardus* muscle subjected to repeated freeze–thaw cycles. The study hypothesized that repeated F–T cycles would degrade muscle structure without promoting bacterial growth under controlled cold-chain conditions. By relating tissue damage to bacterial load, the study provides new insights into structural degradation and microbial stability in a commonly consumed small pelagic fish.

MATERIALS AND METHODS

Sampling

Fresh sardines were sourced from a coastal market in Jijel, Algeria, and transported to the Laboratory of Histology and Microbiology, University of Batna 1, under refrigerated conditions (CRF–NT64GF40, Condor, Algeria) (0–4 °C) within 6 h of capture. Thirty fish of comparable size (mean weight = 28.2 g, measured with an analytical balance KERN, Germany; length = 14.3 cm) were randomly allocated into 6 groups (n = 5 per treatment).

The control group (T0) consisted of fresh, unfrozen samples, while groups T1 to T5 underwent one to five F–T cycles. Each cycle consisted of four days (d) at −20 °C, followed by thawing over 12 h at 4 °C (CRF–NT64GF40, Condor, Algeria). The protocol was adapted from previous protocols [4, 5].

Microbiological analysis

Composite muscle samples were created for each treatment (T0–T3) by pooling the muscle of 2–3 sardines of similar size and weight to obtain approximately 25 g per sample, which is necessary for microbiological analysis to ensure sufficient material. Each composite sample of 25 g was aseptically homogenized in 225 mL of Buffered Peptone Water (HiMedia, India) using a BagMixer® (Interscience, France), and the homogenates were incubated (Memmert INE 400, Germany) at 37 °C for 18 h for enrichment [8].

This approach minimizes individual variability and provides a representative estimate of the microbial load for each treatment. However, it does not allow evaluation of variability between individual fish, which is a limitation of this method. Microbiological analyses were restricted to T0–T3 because bacterial counts dropped to very low levels beyond the third freeze–thaw cycle, making accurate quantification unreliable.

Microbial enumeration

Microbiological assays were performed according to ISO standards:

•Aerobic mesophilic bacteria: ISO 4833-1:2013 (30 °C, 72 h) [15].

•Total coliforms: ISO 4832:2006 (37 °C, 24 h) [16].

•Staphylococcus aureus (coagulase-positive): ISO 6888-2:2021 (37 °C, 48 h) [17].

•Salmonella spp.: ISO 6579-1:2017 (enrichment and selective plating) [18].

Results were expressed as $\log_{10}$ CFU/g. The limit of 7 $\log_{10}$ CFU/g was used as the spoilage threshold for fresh and frozen fish [19].

Microbial enumeration was limited to T0–T3 because bacterial counts became extremely low beyond the third cycle, making reliable quantification impossible, as previously recommended [8].

Histological and histomorphometric analysis

Dorsal muscle samples from each treatment (T0–T5) were fixed in 10 % neutral buffered formalin for 48 h, dehydrated, cleared, and embedded in paraffin. Sections of 5 µm thickness were cut using a rotary microtome (Leica, Jung-histocut, 820). and stained with hematoxylin–eosin following established protocols [8].

Microscopic observations were made using a light microscope (Zeiss Axioskop 20, Zeiss, Germany) at 100× magnification.

Five non-overlapping fields per slide were photographed using a digital camera integrated into the microscope (MShot MD50, China) and the SLS-Mvision image acquisition software. For each sample, 50 fibers and their associated vacuoles were measured in these fields, providing a representative estimate of structural alterations. Thus, a total of 250 fibers per composite sample were analyzed. Histomorphometric parameters were assessed using ImageJ software (v1.52a) in the following way:

- •average vacuole area (μm²),
- •mean area of vacuolated fibers, and
- •ratio of total vacuolated area to total fiber area.

These indices were used to quantify ice-crystal-related damage and loss of fiber cohesion [6, 4].

Statistical analysis

All measurements were performed in triplicate, with each replicate representing a biological replicate (different pooled samples from 2–3 sardines). Data are presented as mean ± standard deviation. Statistical differences between treatments were analyzed using one-way ANOVA, followed by Tukey's post hoc test, with significance set at $P < 0.05$.

RESULTS AND DISCUSSION

Histological observations

Microscopic examination of dorsal muscle from Sardina pilchardus demonstrated a progressive deterioration of muscle microstructure with increasing numbers of F–T cycles (FIG. 1). In fresh samples (T0), muscle fibers were tightly packed, polygonal in shape, and separated by minimal inter-fiber spaces. The sarcoplasm appeared homogeneous, and both endomysium and perimysium were intact, indicating preserved tissue integrity.

After one and two F–T cycles (T1–T2), early structural alterations became evident. Small vacuoles appeared between adjacent muscle fibers, accompanied by slight disruption of the sarcolemma and mild loosening of perimysial connective tissue. These changes resulted in a moderate widening of inter-fiber spaces compared with fresh samples. Such initial alterations are consistent with early ice crystal formation and limited recrystallization during freezing and thawing processes.

More pronounced damage was observed after three and four cycles (T3–T4). Muscle fibers lost their compact arrangement, inter-fiber gaps increased markedly, and individual vacuoles coalesced into larger cavities. In some areas, sarcoplasmic disorganization and partial detachment of muscle fibers from the surrounding endomysium were noted, reflecting progressive mechanical stress induced by repeated ice crystal growth.

At five F–T cycles, muscle architecture was severely compromised. Extensive vacuolation, random orientation of fibers, ruptured cell membranes, and collapse of connective tissue layers were evident. Large irregular voids, attributed to ice crystal recrystallization, replaced normal muscle fiber structure, confirming advanced tissue degradation. These histological findings clearly demonstrate that repeated F–T cycles induce cumulative microstructural damage in sardine muscle (FIG. 1) [3, 4, 5, 6]

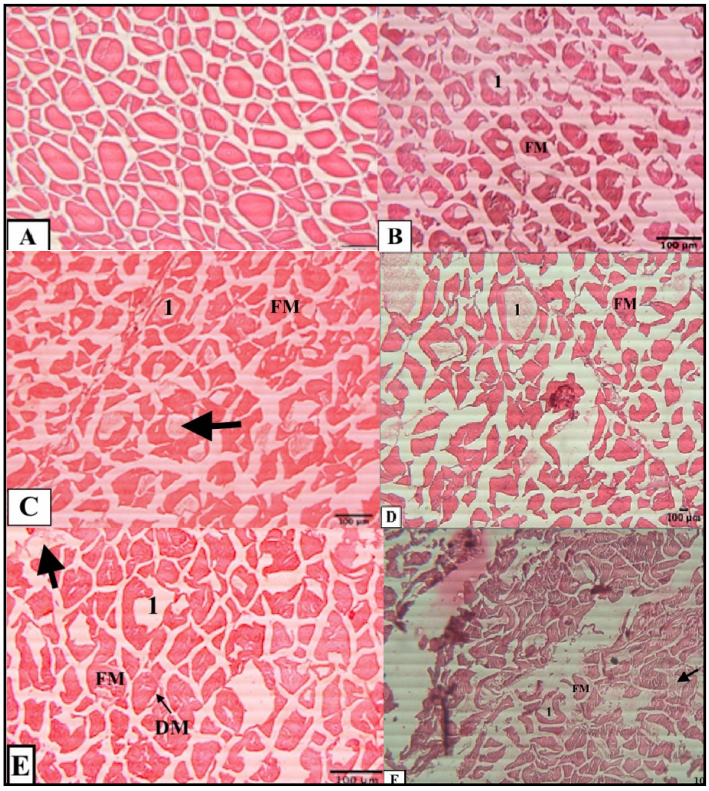

FIGURE 1. Muscle histology of Sardina pilchardus subjected to different freeze–thaw cycles (H&E, ×100). A: T0 (fresh sample); B: T1 (one cycle); C: T2 (two cycles); D: T3 (three cycles); E: T4 (four cycles); F: T5 (five cycles). FM: muscle fiber; DM = muscle fiber disruption; 1: ice crystal; arrow: interstitial protein material.

Histomorphometric quantification

Quantitative histomorphometric measurements confirmed the descriptive histological observations (TABLE I). The mean ice crystal area increased significantly ($P < 0.05$) with each additional F–T cycle, rising from 1050 ± 300 μm² at T1 to 2300 ± 150 μm² at T5. Similarly, the ratio of ice crystal area to total fiber area increased progressively from 25 ± 7 % to 65 ± 6 %, indicating substantial expansion of intra- and inter-fiber spaces.

These increases reflect intensified recrystallization phenomena during repeated freezing and thawing, which promotes mechanical rupture of muscle fibers and loss of cellular cohesion. Comparable trends have been reported in other small pelagic and freshwater fish species, including *Sardinella aurita* and *Oncorhynchus mykiss*, confirming that fish muscle is highly sensitive to fluctuating thermal histories [10, 11].

TABLE I
Effect of repeated freeze–thaw cycles on ice crystal size and proportion of fiber area area in Sardina pilchardus muscle.

Freeze–thaw cycle	T1	T2	T3	T4	T5
Ice crystal area (μm², mean ± SD)	1050 ± 300^{a}	1450 ± 250^{b}	1900 ± 250^{c}	2100 ± 350^{d}	2300 ± 150^{e}
Ice crystal area / fiber area (% mean ± SD)	25 ± 7^{a}	35 ± 8^{b}	45 ± 6^{c}	60 ± 5^{d}	65 ± 6^{e}

Notes: SD = standard deviation; Values with different superscript letters (a–e) within the same row are significantly different ($P < 0.05$)

Microbiological results

Microbiological analyses revealed a slight but consistent reduction in bacterial counts with increasing numbers of F–T cycles (TABLE II). Total aerobic mesophilic bacteria decreased from 4.52 ± 0.23 log10 CFU/g in T0 to 3.96 ± 0.18 log10 CFU/g after T3. A similar declining trend was observed for total coliforms, which decreased from 3.84 ± 0.27 to 3.10 ± 0.19 log10 CFU/g over the same period.

Coagulase-positive *Staphylococcus aureus* counts remained below the detection limit (< 2 $\log_{10}$ CFU/g) in all treatments, and no *Salmonella spp*. weredetected in any sample. Importantly, all microbial counts remained well below the commonly accepted spoilage threshold of 7 $\log_{10}$ CFU/g, indicating satisfactory hygienic quality throughout the experimental period.

The observed reduction in microbial loads can be attributed to the bactericidal and bacteriostatic effects of freezing, including membrane damage caused by ice crystal formation and osmotic stress during thawing [7, 9]. Beyond the third cycle, microbial levels became very low, limiting reliable quantificatin; therefore, microbiological analysis was restricted to T0–T3.

TABLE II
Microbial counts of Sardina pilchardus muscle during freeze–thaw cycles, expressed as $\log_{10}$ CFU/g (mean ± SD)

Microbial group	T0 (fresh)	T1	T2	T3
Aerobic mesophilic bacteria	1050 ± 300^{a}	4.21 ± 0.19^{ab}	4.05 ± 0.17^{ab}	3.96 ± 0.18^{b}
Total coliforms	3.84 ± 0.27^{a}	3.62 ± 0.21^{ab}	3.32 ± 0.22^{ab}	3.10 ± 0.19^{b}
Coagulase-positive *S. aureus*	< 2	< 2	< 2	< 2
Salmonella spp.	Absent	Absent	Absent	Absent

Note: T0: fresh samples; T1–T3: one to three freeze–thaw cycles. Different superscript letters within a row indicate significant differences (P < 0.05).

Correlation between histological damage and microbial trends

Correlation analysis demonstrated a significant negative association between vacuole area and total aerobic mesophilic counts (r = −0.78; P < 0.05) [8, 9]. However, this result should be interpreted with caution due to the small sample size and the observational nature of the study. No causal relationship can be inferred from this correlation. This finding indicates that progressive structural damage to muscle tissue did not favor microbial proliferation under controlled cold-chain conditions. On the contrary, increased tissue disruption coincided with a slight decline in microbial viability.

These results suggest that although repeated F–T cycles severely impair muscle microstructure and, which may indirectly affect textural quality, they do not compromise microbiological safety when appropriate storage temperatures are maintained. This integrative evaluation of histological and microbiological parameters supports previous observations in small pelagic species and highlights the importance of uninterrupted cold-chain management from a One Health perspective, linking food quality, consumer safety, and sustainable use of fishery resources [12, 13, 14, 20, 21].

CONCLUSIONS

The results suggest that multiple freeze-thaw cycles exert a limited bactericidal effect and may cause moderate changes in color and texture, while overall maintaining product safety. Examining histology alongside microbiology provides significant insight into the overall impact of thermal cycles on both structural and hygienic aspects of pelagic fish. Microbial levels were below the detection limit from the third cycle onwards; therefore, microbiological analyses were not performed beyond this point This confirms is consistent with previously established data on cumulative bactericidal effects. Overall, this study emphasizes the importance of maintaining an intact cold chain to preserve both the texture and hygienic quality of sardine products. The work aligns with the One Health philosophy by connecting food quality, safety, and sustainable management of fishery resources. It should be noted that the observed microbial stability applies only under controlled cold-chain conditions (4 °C). In real commercial scenarios, such as ambient temperature storage, microbial proliferation could be significantly higher due to the loss of muscle structural integrity described in this study.

The results suggest that multiple freeze-thaw cycles exert a limited bactericidal effect and may indirectly cause moderate changes in color and texture, while overall maintaining product safety. Examining histology alongside microbiology provides significant insight into the overall impact of thermal cycles on both structural and hygienic aspects of pelagic fish. Microbial levels were below the detection limit (< 2 $\log_{10}$ CFU/g) from the T3 onwards; therefore, microbiological analyses were not performed beyond this point. These findings are consistent with previously established data on the cumulative bactericidal effects of freezing.

Importantly, the observed microbial stability applies only under controlled cold-chain conditions (4 °C). In real commercial scenarios, such as ambient temperature storage, microbial proliferation could be significantly higher due to the loss of muscle structural integrity described in this study. Overall, this study emphasizes the importance of maintaining an intact cold chain to preserve both the texture and hygienic quality of sardine products. The work aligns with the One Health approach by linking food quality, safety, and sustainable management of fishery resources.

Conflict of interests

Authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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