Vol. 24, No. 1(2025) Alim24358
Revista Mexicana de Ingeniería Química

# Effect of ultrasound on the aging process, physicochemical properties, and lysosomal enzyme activity of *semitendinosus* and *semimembranosus* bovine muscles

# Efecto del ultrasonido sobre el proceso de maduración, propiedades fisicoquímicas y actividad enzimática de enzimas lisosomales del músculo *semitendinosus* y *semimembranosus* de bovino

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Received: June 18, 2024; Accepted: October 16, 2024

## Abstract

Ultrasound has been used to improve meat tenderness and reduce maturation time. In Mexico, information about ultrasound treatment for enhancing meat tenderness of *semimembranosus* (SM) and *semitendinosus* (ST) muscles is scarce. Therefore, this study evaluated the effect of ultrasound on the aging process, physicochemical properties, cooked meat's tenderness, and lysosomal enzymes' activity in SM and ST bovine muscles. Eighteen SM and ST muscles were removed at 24 h *postmortem* from Charolais breed cattle and were submitted to ultrasound treatment for 30 and 60 min at 750 watts and 20 kHz. The pH, weight loss, color, aging index, tenderness, $\beta$-glucuronidase, and cathepsin B+L activity were evaluated at 7 and 14 days *postmortem* at 4°C. The results suggest that ultrasonication did not cause modifications in the pH, color, and weight loss in raw meat ($p > 0.05$). The aging index, cooked meat tenderness, and lysosomal enzymes' activities were enhanced with ultrasound treatment with respect to the control. Finally, the results suggest that the ultrasound could be a useful tool in improving the cooked meat tenderness of ST muscle.

*Keywords*: beef, ultrasound, aging process, physicochemical properties, tenderness, cathepsins.

## Resumen

El ultrasonido ha sido utilizado para mejorar la suavidad de la carne y reducir el tiempo de maduración. En México, información sobre el uso de ultrasonido para mejorar la suavidad de la carne de los músculos *semimembranosus* (SM) y *semitendinosus* (ST) es escasa. Por ello, en este estudio se evalúo el efecto de ultrasonido sobre el proceso de maduración, propiedades fisicoquímicas, suavidad de la carne y la actividad de enzimas lisosomales en músculos SM y ST. Dieciochos músculos SM y ST fueron removidos 24 h *postmortem* de bovinos raza Charolais y sometidos a 750 watts y 20 kHz de ultrasonido durante 30 y 60 min. El pH, la pérdida de peso, color, índice de maduración, suavidad de la carne y la actividad de $\beta$-glucoronidasa y catepsinas B+L fueron evaluadas a 7 y 14 días *postmortem*. El ultrasonido no causó modificaciones sobre el pH, color y pérdida de peso en carne cruda ($p > 0.05$). Con respecto al control, el índice de maduración, la suavidad de la carne cocida y la actividad de las enzimas lisosomales fueron mejoradas con el ultrasonido. Finalmente, los resultados sugieren que el ultrasonido podría ser utilizado como una herramienta útil para mejorar la suavidad de la carne cocida del músculo ST.

*Palabras clave*: bovino, ultrasonido, proceso de maduración, propiedades fisicoquímicas, suavidad y catepsinas.

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https://doi.org/10.24275/rmiq/Alim24358
*ISSN*:1665-2738, *issn-e*: 2395-8472

# 1 Introduction

The tenderness and attractive color are the highest quality traits of beef perceived by the consumer (Got *et al.,* 1999). The major factors that determine meat tenderness are the quantity and quality of the intramuscular collagen of connective tissue and myofibrillar fraction (Archile-Contreras *et al.,* 2010). Collagen is primarily responsible for the underlying toughness of *postmortem* meat and contributes to the variability of meat texture between muscles (Archile-Contreras *et al.,* 2010). During the aging process, for the conversion of muscle to meat, different endogenous proteases like calpains, cathepsins, caspases and proteosomes act on the myofibrillar structure to increase the tenderness (Ouali *et al.,* 2013). This natural process, however, is expensive because it requires a long storage time of approximately 15 days in refrigeration for beef (Marino *et al.,* 2023). To decrease the aging process and improve meat tenderness, several studies have used different treatments, such as intermittent thermal treatments (Herrera-Méndez *et al.,* 2005), papain like protease from plant (Gagaoua *et al.,* 2021), and brining (Jin *et al.,* 2023). Over the last decades, many studies have also used ultrasound (US) as a tenderizing method to improve the biochemical and functional attributes of meat and meat products. Another positive effect of US applications is because of the quaternary, tertiary and secondary structures of muscle proteins are affected, making them more susceptible to degradation by gastrointestinal proteases (Flores-Silva *et al.,* 2022). At low frequency (20-100kHz) and high intensity (>10W/cm$^2$), the acoustic cavitation phenomena of ultrasound waves produce microbubbles that act on myofibrillar and tissue connective structure, cell membranes that release cathepsin and $\beta$-glucuronidase enzymes into cytosol, as well as intracellular calcium ions that activate calpains to accelerate proteolysis and improve meat tenderness (Jayasooriya *et al.,* 2007; Alarcón *et al.,* 2019; Wang *et al.,* 2018, 2022; Abril *et al.,* 2023). There are controversies, however, about the effect of US on meat tenderness. Some studies have found no improvement in tenderness after ultrasound application (Got *et al.,* 1999; Prestes-Fallavena *et al.,* 2020), although others have reported that US improves meat tenderness (Carcel *et al.,* 2007; Jayasooriya *et al.,* 2007; Chang *et al.,* 2015; Peña González *et al.,* 2017; Peña-González *et al.,* 2019; Wang *et al.,* 2018, 2022). These discrepancies may be due to different factors, such as the intensity of ultrasound, the application time (before or after *rigor mortis*), and the muscle type used. In general, the most studied muscle has been *longissimus,* which is a soft muscle because of its low collagen content (4.52 mg/g) (Rhee *et al.,* 2004; Carcel *et al.,* 2007; Jayasooriya *et al.,* 2007; Peña González *et al.,* 2017; Peña-González *et al.,* 2019). Few studies have used muscles with a high collagen content such as *semimembranosus* (7.68 mg/g) (Got *et al.,* 1999; Rhee *et al.,* 2004), *biceps femoris* (8.74mg/g) (Rhee *et al.,* 2004; Prestes-Fallavena *et al.,* 2020), and *semitendinosus* (8.76 mg/g) (Jayasooriya *et al.,* 2007; Chang *et al.,* 2015; Wang *et al.,* 2018, 2022). Most Mexican research has evaluated the effect of ultrasound on beef quality in *longissimus* muscle (Peña González *et al.,* 2017; Peña-González *et al.,* 2019; Carrillo-López *et al.,* 2019; Garcia-Galicia *et al.,* 2020; González-González *et al.,* 2020; Garcia Galicia *et al.,* 2023) and *triceps brachii* (Caraveo-Suarez *et al.,* 2021). As previously stated, studies performed with *semitendinosus* and *semimembranosus* muscles are scarce (Garcia-Galicia *et al.,* 2019). In Mexico, *semitendinosus* and *semimembranosus* are mainly consumed as shredded meat, in stews, and occasionally as steaks, but they can be tough. By considering this, the optimal operating parameters for the meat process must be implemented by local research (Prestes-Fallavena *et al.,* 2020). Thus, performing more studies is necessary regarding the use of ultrasound in muscle with a high collagen content because during the natural aging process collagen degradation is negligible. Therefore, this study aimed to evaluate the effect of low frequency ultrasound on the aging process, physicochemical properties, and lysosomal enzymes' activity in *semitendinosus* and *semimembranosus* bovine muscles.

# 2 Materials and methods

## 2.1 Animals and muscles

Eighteen *semimembranosus* (SM) and 18 *semitendinosus* (ST) muscles were removed from eighteen 3-year-old Charolais male bovines. The muscles were obtained from a commercial slaughterhouse at 24 h *postmortem*. Once transferred to the laboratory, each muscle was cut into nine parallelepiped shapes of 60mm x 30mm x 30 mm, with the longest dimension in the direction of the muscle fiber. The samples were vacuum-packed in polypropylene bags to apply the ultrasound treatment.

## 2.2 Ultrasound treatment

Ultrasound (US) treatments were based on a randomized factorial design of two factors (ultrasound application time and muscle type). There were three levels for ultrasound application time: 0 (used as control), 30, and 60 min, and two levels for muscle type: *semimembranosus* and *semitendinosus* muscle.

Table 1. Experimental conditions of ultrasound treatments.

| Experiment number | Run order | Muscle type | Time of US treatment (min) |
|---|---|---|---|
| 1 | 1 | ST | 0 |
| 2 | 14 | SM | 0 |
| 3 | 11 | ST | 30 |
| 4 | 8 | SM | 30 |
| 5 | 5 | ST | 60 |
| 6 | 3 | SM | 60 |
| 7 | 10 | ST | 0 |
| 8 | 4 | SM | 0 |
| 9 | 6 | ST | 30 |
| 10 | 2 | SM | 30 |
| 11 | 15 | ST | 60 |
| 12 | 9 | SM | 60 |
| 13 | 13 | ST | 0 |
| 14 | 18 | SM | 0 |
| 15 | 17 | ST | 30 |
| 16 | 7 | SM | 30 |
| 17 | 16 | ST | 60 |
| 18 | 12 | SM | 60 |

ST *semitendinosus*, SM *semimembranosus*, US ultrasound

Two replicates for each experimental condition were used. A total of 18 experiments were conducted (Table 1).

Ultrasound treatments were performed using a 750-watt Vibra-Cell ultrasonic processor with a nominal frequency of 20 kHz (Sonics & Materials, Newton, CT) provided with a 13-mm diameter titanium alloy probe (TI-6AL-4V) at 100% amplitude. The methodology proposed by Carcel *et al.* (2007) was used with slight modifications. Each sample was placed separately in a distilled water bath at 4°C. The distance between the sample and the tip of the probe was established at 1.0 cm, and two sonication times were considered: 30 or 60 min; these times were chosen because collagen-rich muscles could require longer ultrasound time to obtain an effect. To ensure homogeneous and well-distributed sonication, the sample was rotated with a half-turn. Each area exposed to the probe was treated individually at time intervals of 15 min (for a sonication time of 30 min) and 30 min (for a sonication time of 60 min). After treatments, the samples were stored at 4°C for 7 or 14 days.

## 2.3 pH measurement

The pH measurements were taken with a HI-9025 pH meter (Hanna Instruments) before and after ultrasound treatment, as well as at the end of the storage stage. Each determination was carried out in triplicate.

## 2.4 Color change

The color of the meat surface was determined before and after ultrasound treatment using a Konica Minolta on color CM-2500d aperture 8 mm, illuminant $D_{65}$ with CIELab parameters L* (luminosity), a* (redness) and b*(yellowness) (American Meat Science Association, 2012). The instrument was calibrated with a white ceramic plate (L*= 99.99, a*= 0.00, b=1.81). Color measurements were determined at six different points of the meat surface. The color change ($\Delta E$) was calculated according to Equation 1.

$$\Delta E = \sqrt{(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2} \quad (1)$$

where $L_0^*$, $a_0^*$ and $b_0^*$ represent the color parameters of meat before the ultrasound treatment. Furthermore, $L^*$, $a^*$ and $b^*$ are the final color values after the treatments at 7 and 14 days.

## 2.5 Weight loss in raw and cooked meat

The weight loss in raw and cooked meat was performed with a gravimetric method:

$$\text{Weight loss } (\%) = \frac{W_1 - W_2}{W_1} \times 100 \quad (2)$$

where $W_1$ is the initial sample weight (g) and $W_2$ is the sample weight after the post-storage ultrasound treatment.

Concerning the weight loss in cooked meat, $W_1$ and $W_2$ represent the sample weight before and after cooking, respectively. The meat was cooked at 7 and 14 days of storage, and the samples were cooked at 80°C for 30 min using a water bath. They were then cooled in a water bath at 10 °C.

### 2.6 *Aging process of raw meat and tenderness in meat cooked through a compression test*

The kinetics of the aging process were determined at 1, 7, and 14 days of storage in control and treated samples. The aging index was mechanically measured through myofibrillar resistance using the methodology of Lepetit *et al.* (1986). Raw meat samples of 3x1x1cm (with the longest dimension parallel to the myofiber axis) were submitted to a compression test at 20% strain perpendicularly to the main myofiber axis at a speed of 50mm/min. An Instron testing machine (model 3365), with Serie IX/s software equipped with a 1 $cm^2$ compression cell with two lateral walls, thus the sample is deformed laterally only in the direction of the muscle's fibers.

A strain of 80% was used with the same methodology to determine the tenderness of cooked meat. All the samples were tested at room temperature; the average was obtained from 10 determinations.

### 2.7 *Assays of cathepsin activities*

Cathepsins B + L were fluorimetrically assayed using the Etherington and Wardale (1982) method. Cathepsin B+L was assayed using N-CBZ-Lphenylalanyl-L-arginine-7-amido-4 methyl-coumarin (Z-Phe-Arg-NHMec Sigma C9521). The activity was measured at excitation and emission wavelengths of 340 nm and 460 nm respectively, using a flurometer Quantech Thermo Scientific LE1095X30. One unit of cathepsin activity was defined as the amount of enzyme hydrolyzing 1 nmol of substrate/min at 37 °C.

### 2.8 *β-glucuronidase activity in cytosol*

*β*-glucuronidase activity was evaluated according to Got *et al.* (1999) with some modifications. Five g of sample was homogenized in a 0.25M sucrose, 0.02M potassium chloride solution with a T25 ULTRA-TURRAX digital at 200 rpm, and a second homogenization was performed at 1500 rpm. The solution was filtered, and the pH was adjusted to 7.3 with 0.1M KOH. The filtered product was centrifuged to 100,000 g for 2 h. For *β*-glucuronidase activity, 30 *μ*l of supernatant was placed in the assay solution (4-methylumbelliferyl-*β*-D-glucuronide 0.5 mM, sodium acetate 0.1 M, and sucrose 0.25 M at pH 4.2). The reaction was performed at 37°C for 30 min and was stopped by the addition of a 0.1M sodium glycinate solution at pH 10. The fluorescence was determined using a Perkin Elmer LS-50B spectrometer, and the excitation and emission wavelengths were 360 and 460 nm, respectively. The fluorescence of 1mM 4-methylumbelliferone was used as the arbitrary unit of fluorescence.

### 2.9 *Statistical analysis*

The data were analyzed through an analysis of variance (ANOVA) using MODDE 7.0 software (Umetrics). The multiple regression method (MLR) (Eq. 3) was employed to evaluate the main effect of the independent variables on each of the response variables. Confidence level was 95%, except for the *β*-glucuronidase and cathepsin B+L activity of 90%.

$$y = b_0 + b_1 t + b_2 M + b_3 t * M + e \tag{3}$$

where Y is the response variable (pH, color change, aging index, weight loss in raw and cooked meat and tenderness of cooked meat, *β*-glucuronidase and cathepsin B+L activity), t is the ultrasound time (30 and 60 min), M is muscle type and t*M is the interaction of ultrasound time and muscle type, e is the residual error and $b_0$, $b_1$, $b_2$ y $b_3$ are the regression coefficients of the model. The minimum significant difference was performed by a Tukey test.

## 3 Results and discussion

### 3.1 *pH and color changes*

Ultrasound had no significant effect on pH ($p > 0.05$); pH values before and after the ultrasound application at different *postmortem* times are shown in Table 2. At 24 h *postmortem* before treatment, the pH value of the SM muscle was higher (5.93±0.17) than the ST muscle (5.79 ±0.13). A slight decrease in pH value for the SM muscle was observed after treatment, and, for the ST muscle, the pH value increased slightly after treatment. After 7 and 14 days of storage, the pH value in treated and untreated samples increased slightly. Our results agree with those reported by Garcia-Galicia *et al.* (2020), who found no significant difference in pH values when they applied US at 90W/$cm^2$ and 37kHz for 40 min. In addition, Prestes-Fallavena *et al.* (2020) reported the same behavior. The observed increase in pH with aging time and ultrasound treatment was also noted by Got *et al.* (1999) and Jayasooriya *et al.* (2007). This behavior could be caused by a release of ions from the cell structure into the cytosol or by a change in the protein structure that led to a modification in some ionic groups' position, which can appear as muscle buffering and the availability of amino acids and basic amines (Got *et al.,* 1999; Alarcon-Rojo *et al.,* 2019; Marino *et al.,* 2023). Our results were slightly higher than those reported by Prestes-Fallavena *et al.* (2020) and Peña-González *et al.* (2019). The meat color is one of the most important visual properties for consumer acceptance. The color changes at 7 and 14 days of storage are shown in Table 2, and they were not affected ($p > 0.05$) by ultrasound treatments.

Table 2. pH, color change, and weight loss in raw and cooked meat at 7 and 14 days of storage.

| Samples | 7 days | | 14 days | |
|---|---|---|---|---|
| | ST | SM | ST | SM |
| | | pH | | |
| NT | 5.73±0.13 | 5.97±0.17 | 5.74±0.31 | 5.87±0.36 |
| $US_{30}$ | 5.75±0.31 | 5.88±0.18 | 5.74±0.30 | 5.89±0.31 |
| $US_{60}$ | 5.98±0.41 | 5.64±0.17 | 5.75±0.52 | 5.93±0.43 |
| *p(F)* | 0.239 | 0.239 | 0.751 | 0.751 |
| | | Color change ($\Delta E$) | | |
| NT | 2.63±0.61 | 3.01±2.61 | 1.68±0.64 | 3.06±2.25 |
| $US_{30}$ | 2.24±1.54 | 2.92±0.19 | 1.95±0.72 | 2.65±0.45 |
| $US_{60}$ | 2.79±1.00 | 3.15±2.12 | 1.96±0.90 | 2.11±1.38 |
| *p(F)* | 0.919 | 0.919 | 0.405 | 0.405 |
| | | Weight loss raw meat (%) | | |
| NT | 8.28±0.99 | 7.39±1.67 | 9.84±0.52 | 8.53±0.59 |
| $US_{30}$ | 7.29±0.15 | 8.46±0.92 | 11.98±4.47 | 9.97±1.22 |
| $US_{60}$ | 5.88±1.69 | 6.90±3.15 | 7.29±1.79 | 9.0±1.94 |
| *p(F)* | 0.347 | 0.347 | 0.633 | 0.633 |
| | | Weight loss cooked meat (%) | | |
| NT | $37.99\pm2.51^{aA}$ | $44.79\pm1.63^{B}$ | $40.89\pm0.54^{aA}$ | $46.36\pm2.16^{B}$ |
| $US_{30}$ | $40.46\pm1.48^{aA}$ | $44.44\pm1.92^{B}$ | $41.25\pm0.92^{aA}$ | $46.16\pm1.38^{B}$ |
| $US_{60}$ | $31.73\pm7.72^{bA}$ | $44.63\pm3.67^{B}$ | $33.03\pm9.56^{bA}$ | $45.67\pm4.73^{B}$ |
| *p(F)* | 0.005 | 0.005 | 0.008 | 0.008 |

SM= *Semimembranosus,* ST= *Semitendinosus,* NT= untreated, $US_{30}$ = ultrasound treated for 30min, $US_{60}$= ultrasound treated for 60 min, *p*(F) = Fisher probability. AB different letters in the same row indicate significant differences $p < 0.05$ at the same day of storage between muscle type, a-c different letters in the same column indicate significant differences $p < 0.05$ among treatments of the same muscle type. Values without letters indicate that there is no significant difference $p>0.05$.

Generally, the greatest color changes were observed for the SM muscle with and without treatment. The color change remained stable after treatment in both muscles because non significant differences were discovered during storage. These results agree with those reported by Jayasooriya *et al.* (2007), who showed that the color parameters were not affected by high-intensity ultrasound treatment. These same authors stated that when the muscle is vacuum stored between 4 and 5°C the exchange of oxygen was prevented and metmyoglobin accumulation was reduced, promoting color stability. Other authors have stated that the ultrasound had no effect on meat color because the heat generated is insufficient for protein or pigment denaturation (Bhargava *et al.,* 2021). Prestes-Fallavena *et al.,* (2020) reported that when the US was applied at 22, 35 and 46 W/cm$^2$ for 15 min at 10°C no significant color changes were observed with respect to the control. When the intensity of US was increased to 84 W/cm$^2$, however, the color change was affected, as well as the parameters a* and b* that decreased the color intensity. This same behavior of high luminosity and less redness was observed by Peña-Gonzalez, *et al.* (2019), who used high-intensity US (40kHz and 11W/cm$^2$) for 60 min. When high-intensity US was employed, the color was more unstable and could limit oxymyoglin formation, which delays the metmyoglobin formation that promotes the color changes (Prestes-Fallavena *et al.,* 2020).

## *3.2 Weight loss in raw and cooked meat*

The average weight loss for raw meat treated with ultrasound at 30 and 60 min was similar ($p>0.05$) to control meat after being stored for 7 and 14 days (Table 2). This suggests that the structural muscle damage caused by ultrasound did not affect the water migration mechanisms in the meat: there was no significant increase in the inter- and intrafibrillar spaces, which would allow greater water movement. The values obtained in this study align with those reported by Jayasooriya *et al.* (2007), as well as those of Prestes-Fallavena *et al.* (2020) and Peña-Gonzalez *et al.* (2019), who found that the water retention capacity was not modified with ultrasound treatment. In contrast, in the case of cooking loss only the ST muscle was affected by ultrasound time ($p<0.05$) (Table 2). In the samples treated for 60 min, cooking loss decreased with respect to the control, as well as in the samples treated for 30 min. These results indicate that the duration of treatment had a positive effect on cooking loss, which was not found

in other studies with cooking losses increasing with ultrasound time (10, 20, 30,40, 50, or 60 min) at 40 kHz and 1500 W (Chang *et al.,* 2015). These same authors reported that the US treatment had no significant effect on cooking loss. The cooking loss was higher ($p < 0.05$) in the SM muscle than in the ST muscle: SM muscles' weight loss was 44 to 46% at 7 and 14 days, respectively. These percentages were similar to the SM control sample ($p > 0.05$). Regarding ST muscle, the percentage of cooking loss was 31 to 41% at 7 and 14 days, respectively, and the lowest cooking loss was observed for ultrasound treatments at 60 min for both storage times. The differences in cooking loss observed between both muscles may be because the SM muscle has more red fibers (type IA and IIA), which cause a more severe contraction during cooking and thus greater weight loss. This contraction is associated with a decrease in sarcomere length where the intermyofibrillar space is reduced, and the water is then released (Lepetit *et al.,* 2000). Additionally, the weight loss observed in both muscles was not only caused by ultrasound application: post-cooking weight loss has also been related to cytoskeletal proteins' degradation, which increases cellular muscle contractions during cooking. Our results corroborate that the weight loss in raw and cooked meat depended on US conditions (Alarcon-Rojo *et al.,* 2019).

### *3.3 Aging process and tenderness in cooked meat*

As previously mentioned, tenderness is one of the most important quality attributes required by consumers. When the tenderness or toughness of raw meat is determined through compression tests at 20% deformation, the force obtained corresponds to the myofibrillar structure because, at this rate of deformation, the contribution of collagen is negligible (Lepetit 1989). Thus, the value attained represents the myofibrillar resistance, which is the main structure that showed changes the most during the aging process for converting muscle into meat. Figure 1 shows the results of myofibrillar resistance at 7 and 14 days of storage. The ST muscle had a higher myofibrillar resistance ($30.59 \pm 3.13$ N/cm$^2$) ($p < 0.05$) than the SM muscle ($22.82 \pm 5.04$ N/cm$^2$) at 24 h *postmortem*. This difference can be associated with the metabolic characteristics of each muscle. At 7 days, there is a significant reduction of myofibrillar resistance observed in both muscles, including the control. For SM muscle, no significant differences ($p > 0.05$) were observed between the muscle treated with 30 min of ultrasound and the control. Moreover, the myofibrillar resistance observed was of 10 N/cm$^2$, and a significant increase ($p < 0.05$) of myofibrillar resistance was observed in SM treated with 60 min

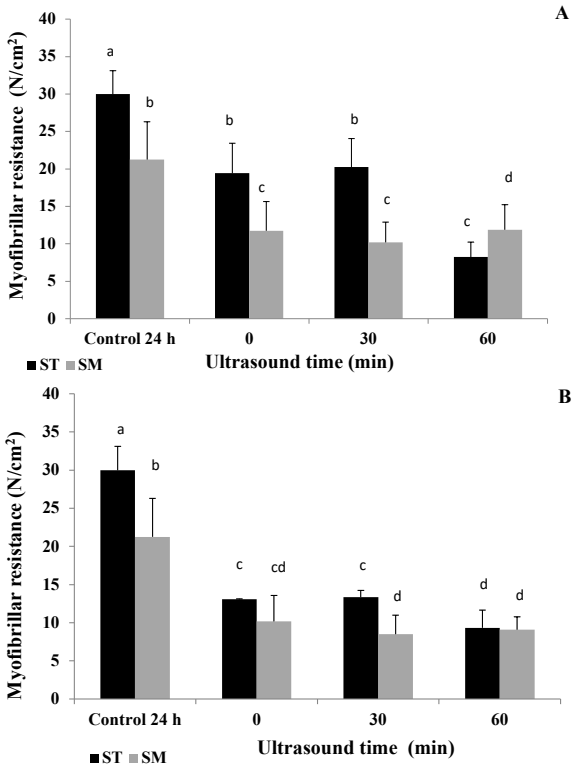


Figure 1. Maturation index in raw meat A) 7 days *postmortem* B) 14 days *postmortem*. SM (*semimembranosus*), ST (*semitendinosus*), 0= untreated, 30= treated for 30 min, 60= treated for 60 min. Different letters indicate significant differences between ultrasound time and muscle type at $p < 0.05$.

of ultrasound. In this same *postmortem,* period, the myofibrillar resistance of the ST muscle treated with ultrasound for 30 and 60 min was significantly lower ($p < 0.05$) than the control, and the lowest values were observed in the muscles treated with ultrasound for 60 min ($8.23 \pm 0.63$ N/cm$^2$). In the literature, it has been reported that when myofibrillar resistance is less than 10 N/cm$^2$ the muscle was converted into meat (Zamora *et al.,* 2005). According to this value, the ultrasound application only affected ST muscle, and 60 min of US was required to accelerate this process at 7 days of storage. In the case of the SM muscle, the control and treated muscles had values of 10 N/cm$^2$. These results corroborate the assumption that the ultrasound effect is dependent on muscle type.

At 14 days *postmortem,* the myofibrillar resistance value of treated and untreated SM muscle was equal to or less than 10 N/cm$^2$ ($p > 0.05$) and was like the samples at 7 days except for the sample treated for 60 min. Furthermore, the myofibrillar resistance value for treated and untreated ST muscle at 14 days was significantly lower than at 7 days *postmortem,* but only the sample treated for 60 min had a value less than 10 N/cm$^2$. These results suggest that for muscles with a high connective tissue content a prolonged ultrasound application promotes the aging process and tenderness

because ST (8.76 mg/g) muscle contains more total collagen than SM (7.68 mg/g) (Rhee *et al.,* 2004). These findings are consistent with those of Jayasooriya *et al.* (2007), who observed a significant reduction in meat toughness after 8.5 days of storage in samples treated with ultrasound, and Kang *et al.* (2017), who reported an increase in tenderness when 300 W of ultrasound was applied for 120 minutes during the curing process. According to the findings reported in the bibliography, the SM and ST muscles of different animal species, including cattle, have always been characterized as requiring long maturation periods: from 10 days for the SM muscle and up to 16 days for the ST muscle (Lepetit *et al.,* 2000; Herrera-Méndez *et al.,* 2005; Marino *et al.,* 2023). The improvement of tenderness and the aging process of ST muscle by the US have been correlated to the rupture of myofibrillar proteins structures and the disintegration of connective tissue structure, which leads to proteolysis activation through the release of calcium ions that activate calpains, as well as to the release of cathepsin and *β*-glucuronidase (Chang *et al.,* 2015; Wang *et al.,* 2018; Alarcón-Rojo *et al.,* 2019). Recently, Marino *et al.* (2023) found that the US promotes the index of myofibrillar fragmentation, and it is associated with an improvement of tenderness. The proteomic approach demonstrated that ultrasound could degrade troponin complex. In addition, these authors suggest that the combination of US and subsequent papain injection could contribute to a greater tenderization until the meat's core without an excessive structure collapse because papain can degrade actin and myosin proteins.

Figure 2 shows results regarding cooked meat tenderness. During 7 days of storage (Figure 2A), the untreated samples presented the highest hardness values of approximately 280 N/cm$^2$ for both muscles. These values agree with those reported by Lepetit *et al.* (2000) for meat samples aged without treatment and cooked under the same conditions.

The ultrasound treatment improved the tenderness of cooked meat; both muscles were more tender than the controls. Furthermore, the average value was less than 250 N/cm$^2$ for both muscles, and the ST muscle treated for 60 min had the lowest value: 185 ± 23.62 N/cm$^2$, however, no significant differences were observed (p>0.05). The tenderness reached after 14 days of storage (Figure 2B) was higher for the muscle ST. These results suggest that in the case of ST muscle the ultrasound application accelerated the aging process and improved the tenderness of cooked meat when the ultrasound was applied for 60 min. Our results concur with those reported by Chang *et al.* (2015), who found ultrasound treatments enhanced tenderness in ST muscle. This improvement in tenderness in cooked meat is not only due to the denaturation of myofibrillar proteins and the rupture

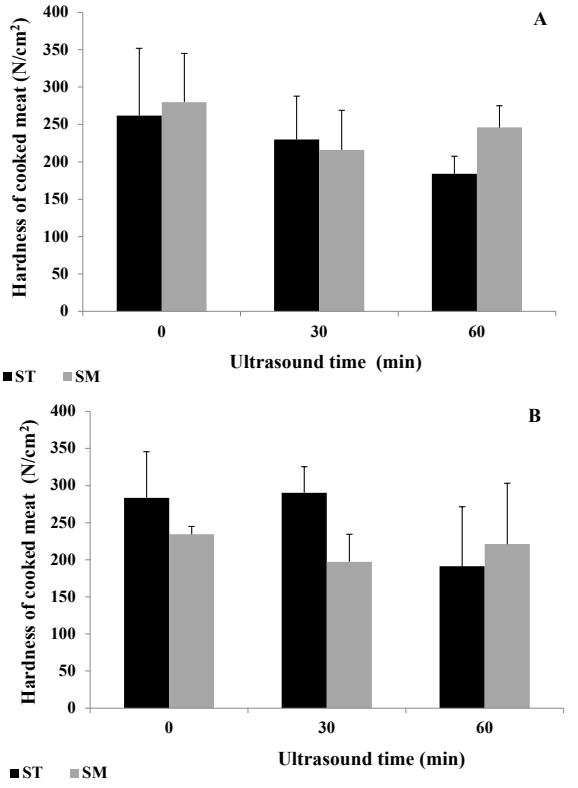


Figure 2. Hardness of cooked meat treated with ultrasound A) at 7 days *postmortem* B) at 14 days *postmortem*. ST= *semitendinosus*, SM= *semimembranosus*, 0= untreated, 30= treated for 30 min, 60= treated for 60 min. Without letters indicate that there is no significant difference p>0.05.

of myofibrils (Barekat and Soltanizadeh, 2018) but also to the structural changes in the connective tissue mainly in collagen. Collagen is responsible for the underlying toughness of meat and plays an important role in the variation of cooked meat tenderness (Purslow, 2018). During cooking, collagen undergoes denaturation, contraction, and finally solubilization. If collagen has a greater capacity for contracting freely, the solubilization increases, and meat hardness decreases (Lepetit *et al.,* 2000; Grajales-Lagunes *et al.,* 2007). It was reported by Wang *et al.* (2022) and Marino *et al*. (2023) that the total collagen content was not affected by ultrasound treatment. but the network of collagen fibers became disorganized and visibly loose, however, and a higher soluble collagen content was obtained, which is consistent with the lowest hardness values (Wang *et al.,* 2022; Marino *et al.,* 2023). Furthermore, conformational changes in the perimysium and endomysium have been observed after ultrasound application at 300 and 600 W. Ultrastructural changes were also observed, which were consistent with collagen solubility (Wang *et al.,* 2022).

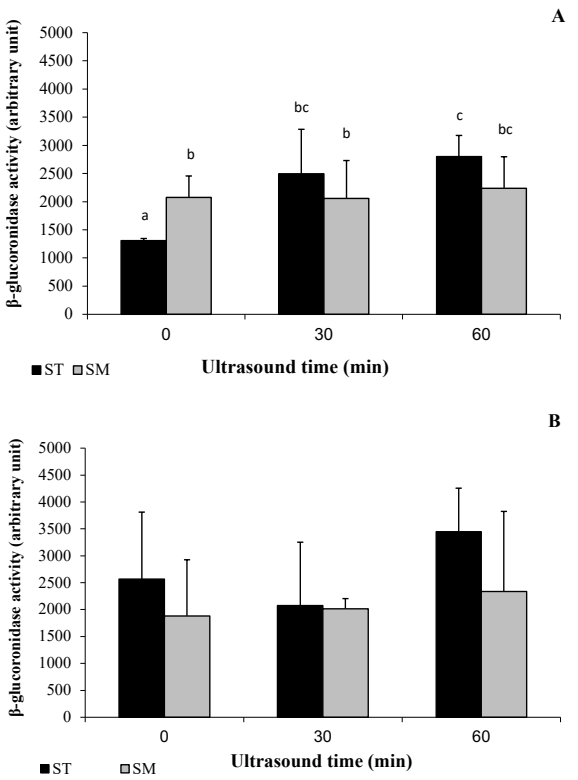


Figure 3. *β*-glucuronidase activity after application of ultrasound A) at 7 days *postmortem*, B) at 14 days *postmortem*. *arbitrary unit: fluorescence of 1 mM methylumbelliferone/*μ*L/min. ST= *semimembranosus*, SM= *semimembranosus*, 0= Untreated, 30= treated for 30 min, 60= treated min. Different letters indicate significant differences between ultrasound time and muscle type at $p < 0.1$.

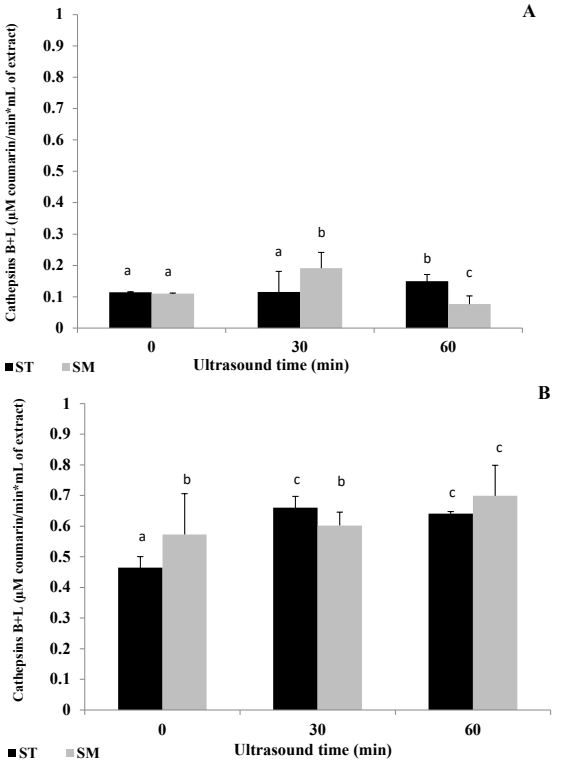


Figure 4. Cathepsin B+L activity after ultrasound treatment A) at 7 days *postmortem*, B) at 14 days *postmortem*. ST= *semitendinosus*, SM= *semimembranosus*, 0= untrated, 30= treated for 30 min, 60= treated for 60 min. Different letters indicate significant differences between ultrasound time and muscle type at $p < 0.1$.

## *3.4 β-glucuronidase and cathepsins activity*

The *β*-glucuronidase and cathepsin are lysosomal enzymes that are released into the cytosol to act on their substrates and improve meat tenderness (Got *et al.,* 1999; Ertbjerg *et al.,* 1999). The changes in *β*-glucuronidase activity on different days *postmortem* appear in Figure 3. Significant differences ($p<0.1$) in *β*-glucuronidase activity were only observed at 7 days *postmortem*, with the factors affecting *β*-glucuronidase activity being treatment time, the interaction time of treatment, and muscle type. At 7 days *postmortem,* significant differences were found between treated and untreated ST muscle, and *β*-glucuronidase activity improved after ultrasound treatment. These results imply that ultrasound application fragments the lysosomal membrane and releases this enzyme into the cytosol. For SM muscle, no significant differences ($p>0.1$) were observed between untreated and treated muscle, and the

*β*-glucuronidase activity remained constant at 7 and 14 days *postmortem.* Higher activity was observed for ST muscle treated with 60 min of ultrasound at 14 days, but there was no significant difference ($p>0.1$). Our results regarding SM muscle agree with those reported by Got *et al.* (1999), who also observed constant enzymatic activity at days 0 and 1. Furthermore, these authors indicated that the *β*-glucuronidase activity can decrease after 2 days of storage. Conversely, on our results show that the activity of this enzyme remained constant during the aging process in treated and untreated SM muscle, in ST muscle this activity increased. Therefore, ultrasound had no significant effect on the release of this enzyme in the cytosol in SM muscle. The values of this study were lower than those reported by Got *et al.* (1999), which could be related to the ultrasound condition used.

With respect to ST muscle, Chang *et al.* (2009) reported only significant differences in *β*-glucuronidase activity between the control and the sample treated for 10 min with ultrasound; minimal activity was found at this time. Additionally, these same authors reported that the enzymatic activity of ST treated for longer times was similar to the control and did not significantly affect the activity. Determinations at different *postmortem* times were not presented, however.

Cathepsin B+L activities are shown in Figure 4 for the ST and SM muscles at 7 and 14 days' *postmortem*. For the ST muscle, non significant differences ($p>0.1$) were observed between the treated for 30 min with US and the control at 7 days *postmortem*; an increase was observed in the sample treated for 60 min ($p<0.01$). Regarding SM muscle, an increase in enzyme activity was observed with 30 min of US ($p<0.01$); the US treatment for 60 min reduced cathepsin B+L activity compared to the control and the US for 30 min. At 14 days *postmortem*, cathepsin B+L activities increased in both the control and the treated samples; the treated ST muscle activity was similar but higher than the control ($p<0.1$). In the case of SM muscles, higher activity was observed in the sample treated for 60 min; significant differences ($p<0.1$) were observed with the control and the sample treated with 30 min. The increase of cathepsin B+L activities in samples treated with ultrasound coincided with previous studies (Wang *et al.,* 2018; 2022) that suggested that US cavitation breaks down lysosomes to release cathepsin. This activity promotes the interaction between the enzyme and substrate, such as actin and myosin, inducing myofibrillar proteolysis, which can explain the results of the aging process and cooked meat tenderness, especially for ST muscle. Additionally, it was reported that this enzyme possibly acts on the non-helical terminal part of native collagen, inducing the depolymerization of cross-linked fibers, which can affect meat tenderness (Wang *et al.,* 2022).

## Conclusion

The present study shows that the low-frequency ultrasound application at 750 W, 20 kHz for 60 min could reduce the aging process time and improve the tenderness and cooking loss of *semitendinosus* muscle after cooking without negatively affecting other meat quality parameters. However, these ultrasound conditions did not affect the aging process time and the toughness of cooked meat in the *semimembranosus* muscle. The ultrasound-induced the release of $\beta$-glucuronidase and cathepsin B+L, reflected in high activity in both muscles during the aging process. In summary, ultrasound can improve muscle quality with high collagen content, like *semitendinosus* muscle. On the other hand, sensory evaluation studies are necessary to determine the consumer's perception of meat tenderness after an ultrasound maturation process.

### *Acknowledgements*

This research was financially supported by the Research Support Fund of the Autonomous University of San Luis Potosí.

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