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The effect of cold storage on physicochemical and microbiological properties of beef *Semitendinosus* muscle subjected to ultrasonic treatment in different systems (bath or probe)

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Abstract

This study was to evaluate the effects of two systems for US application on the physical (Warner Bratzler Shear force - WBS, and color) chemical (pH, lipid oxidation,) and microbiological (mesophilic, lactic acid and psychotropic bacteria) properties of *Semitendinosus* beef during storage (16 days at 7±1ºC). Samples obtained 48 h post-mortem were submitted to US using bath (45 kHz) and probe (20 kHz) in different time (0, 60, 120 or 240 s). After application the samples were vacuum packaged and evaluated during at 0, 3, 5, 9 and 16 days. The sonication presented an influence ($P < 0.05$) in pH, wherein sonicated samples presented higher pH than the control up to 5th day of storage. US reduced WBS force after treatment, but this effect was not maintained during storage, even because cold storage improves toughness of meat. Lipid oxidation and color values did not changed and microbial flora were not damaged by the use of US. Discriminant analysis shows that storage time was the most important factor, while US system and time of exposure presented slightly differences regarding to their effect on meat. The results suggested that for improvement of US effect on meat the application should be performed not only before packaging, but also during storage.

Introduction

Biochemical processes have an influence on meat tenderization (Lawrie, 2005), and it has long been known that tenderness is improved post-mortem during the ageing of meat due to the action of proteolytic enzymes as cathepsins and calpains (Lonergan *et al*., 2010; Kemp and Parr, 2012). Although calpains could be considered the most important proteases responsible for softening, calcium is required for their activation at micromolar and millimolar concentration for calpain I and calpain II, respectively (Koohmaraie and Geesink, 2006). In this way, increased calcium availability could improve post-mortem muscle tenderness (Pohlman *et al.*, 1997; Kemp *et al.*, 2010). Some methods have been used for this purpose as electrical stimulation (Hwang *et al*., 2003; Barbut, 2014) and injection of calcium chloride (Gerelt *et al.*, 2002; Bunmee *et al.,* 2014). However, these methods have been shown to affect the appearance, color stability and water-holding capacity of meat (van Laak and Smulders, 1990; Varnam and Sutherland, 1994).

Ultrasound (US) has been used as an alternative technique to promote meat tenderness (Pohlman *et al*., 1997; Got *et al.*, 1999; Sikes *et al*., 2014). US refers to sound waves inaudible to the human ear frequency (>20 kHz) (Cárcel *et al*., 2012; Chandrapala *et al*., 2012) and its main effect for meat tenderization is related to the cavitation phenomenon observed generally at lower frequencies (20 to 100 kHz). Under US application, a disruption of sub-cellular components (e.g., sarcoplasmic reticulum and mitochondria) could occur, with increased concentration of calcium ions and a subsequent improvement of calpain activity (Alliger, 1975; Pohlman *et al*., 1997; Got *et al*., 1999). Therefore, several papers have been published that investigate the effects of ultrasound on meat tenderization (Pohlman *et al*., 1997 a,b; Got *et al.*, 1999; Jayasooriya *et al*., 2004, 2007; Stadnik *et al.*, 2008, 2011; Chang *et al.*, 2012), but the results obtained were not conclusive. The differences observed in these works could be explained due to the high variation of experimental conditions such as frequency, time, and intensity of US application (Alves *et al*., 2013). The use of different US systems could lead to different effects on meat tenderization, but there is a lack of information about the comparison of bath and probe systems for meat tenderization in literature.

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Cavitation also accelerates chemical reactions (Suslick, 1989) and could affect the quality of meat because of oxidative reactions (Chang and Wong, 2012; Cichoski *et al*., 2015). Microbiological characteristics also subject to ultrasound influence and both inhibition (Sams and Feria, 1991; Piyasena *et al*., 2003) and stimulation (Nguyen *et al*., 2009; Ewe *et al*., 2012; Yeo and Liong, 2013) of growth effects have been reported.

The aim of this work is to investigate the effect of two systems of US application (bath at 45 kHz an probe at 20 kHz) on *Semitendinosus* at different exposure times (0, 60, 120 or 240 s). For this purpose, an evaluation of US effect on the cold storage time of *Semitendinosus* beef (7± 1°C, up to 16 days) was performed. In this way, physical (Warner Bratzler Shear (WBS) force and color), chemical (pH and lipid oxidation), and microbiological properties of meat were evaluated.

Materials and Methods

Samples and reagents

Semitendinosus beef muscle samples were collected from zebu × charolais crossbred bull carcasses (2-3 years old, 370-400 kg) slaughtered in a commercial meat processing industry following guidelines recommended in Brazil (Brasil 2000). The samples were excised after 48 h post-mortem into blocks (40×60×20 mm of length, width and height, respectively) with around 50 g. The individual samples were vacuum-packaged in polyethylene bags and identified according to ultrasound system treatment (bath (B) or probe (P)) and times of US application (60, 120 or 240 s) to each system. In addition, a control sample without ultrasound application (C) was used. Experiments were replicated twice. All reagents and chemicals were of analytical-reagent grade.

Ultrasound treatment

For ultrasound application, a bath (Elma® TI-H 5, Singen, Germany, 45 kHz, 500 W, 1.8 W cm^{-2}) and a probe (VC 750, Sonics and Materials®, Newtown, EUA, 20 kHz, 750 W, 26.5 W cm^{-2}) systems were used. Ultrasound was applied using these systems for 60, 120 or 240 s perpendicularly to muscle fibers. Samples were analyzed immediately after ultrasound exposure (day 0) or remained stored at 7± 1°C for 3, 5, 9 and 16 days in order to evaluate the effect of storage time.

pH determination

The pH was determined using a potentiometer (Digimed – DM-22, São Paulo, SP, Brazil) with 5 g of homogenized in 50 mL of distilled water (AOAC, 2006). Measurements were carried out in triplicate.

Texture determination

Texture was determined in cooked samples in order to simulate the consumption conditions. One portion of sample in each run was placed in a water bath at 70ºC for 45 min within a vacuum-packed polyethylene bag, then cooled with tap water (approximately 25ºC) for 30 min and held at 5ºC. After, the samples were removed from their plastic bags and dried with paper towel to remove moisture excess before cutting for texture determination (Jayasooriya *et al*., 2007). Each meat sample was cut in a parallel way to the muscle fibers into six parts (10 x 15 mm).

Texture analysis was carried out on TA-XT2 Plus equipment (Stable Microsystems Ltd., Surrey, England) using software Exponent version 6.1.1.0 (Texture Technologies Corp., New York, USA), according to the guidelines of AMSA (1995). Samples were sheared using a V-shaped WBS blade and the force and the peak force (N) to cut them were registered for each piece.

Lipid oxidation analysis (TBARS)

Lipid oxidation was assessed by an evaluation of thiobarbituric acid reactive substances (TBARS), following the methodology described by Raharjo *et al.* (1992) and with modifications proposed by Wang *et al*. (2002), in triplicate. The results were expressed as mg malondialdehyde kg^{-1} (MDA mg kg^{-1}).

Instrumental color determination

Samples were evaluated for instrumental parameters of color using a Minolta Chroma Meter CR- 300 (Minolta Corp., Ramsey, NJ) with illuminant D65 into six points of surface samples (angle 10º), in triplicate. Before color determination, meat samples were allowed to bloom for at least 30 min at 5 ºC. Color measurement followed the CIE color convention (1975) with outputs of L^* (lightness), a^* (redness), and b^* (yellowness).

Microbiological analysis

The external part of the bag was disinfected to prevent contamination of the sample and then the package was opened. Microbiological evaluation was carried out by counting the number of colonies of mesophilic (MESO), lactic acid (LACTIC) and psychotropic (PSY) bacteria following sampling proposed by Silva *et al*. (2001) and the methodology described in guidelines recommended in Brazil

(Brasil, 2000). Results were expressed as log CFU cm^{-2}.

Experimental design and statistical analysis

Statistical analysis was carried out by means of orthogonal contrasts and canonical discriminant analysis in order to identify the differences among treatments.

A completely randomized design with repeated measure in time was adopted, using the MIXED procedure and employing special parametric structure in the matrices of (co)variance as the following statistical model:

$$Y_{ijk}=\mu+\alpha_i+\gamma_k+(\alpha\gamma)_{ik}+e_{ijk}$$

where Y_{ijk} denotes the measurement in time, k is the j^{-th} repetition assigned to treatment i, $\mu+\alpha_i+\gamma_k+(\alpha\gamma)_{ik}$ is the mean for treatment i at time k (containing the fixed effects for treatment, time, and treatment × time interaction), and e_{ijk} is the random error associated with the measurement at time k in the j^{-th} repetition assigned to treatment i, so that $Var[Y]=I_k \otimes \Sigma$, where I_k is an identity matrix of a dimension equal to the number of replicates, and Σ is the matrix of (co)variance due to residue obtaining several measurements of the same experimental unit j. The structures of (co)variance of the errors e_{ijk} tested were VC, CS, CSH, UN, AR (1), ARH (1), ARMA (1,1), TOEP, TOEPH, ANTE (1), and HF. The (co) variance and the solutions for the fixed effects were estimated by restricted maximum residual likelihood method and the number of degrees of freedom of the denominator for the F test was calculated by Kenward-Rogers's method.

An analysis of orthogonal contrasts was used to assess differences among treatments and for trends between the shelf life from the coefficients of the orthogonal polynomials for interpolating estimated by the Interactive Matrix Language procedure. Means were adjusted by the least square method with LSMEANS command and compared using the Tukey test.

Multivariate analysis of variance was performed with MANOVA command, complemented by canonical discriminant technique with CANDISC procedure (Khattree and Naik 2000), completely randomized disregarding the factorial arrangement, as in the following statistical model:

$$Y_{ijk}=\mu_k+\alpha_{ik}+\varepsilon_{ijk}$$

where Y_{ijk} is the observed value of k^{th} variable under the i^{-th} treatment in the j^{-th} repetition, μ_k is the overall mean of the k^{-th} variable, α_{ik} is the effect of the i^{-th} treatment in the k^{-th} variable, and ε_{ijk} is the random effect associated with the ijk observation supposed in $\varepsilon_{ijk} \overset{iid}{\sim} N(0,\sigma^2)$.

For this, T, H, and E are, respectively, matrices of sums of squares and total treatments and residues were obtained. Then the Wilks test was performed to test the hypothesis that the vectors of means of treatments were zero $(H_0:\bar{\mu}_1=\bar{\mu}_2=\cdots=\bar{\mu}_i)$, as follows:

$$\Lambda=|E|/|H+E|$$

where $|E|$ is the determinant of the residual sum of the square and product matrix E, and $|H+E|$ is the determinant of the matrix $H+E$, H being the matrix of the sum of the squares and associated products to the hypothesis in question.

From the multivariate analysis, the eigenvalues were calculated in order to determine the characteristic roots of the following equation (Harris 1975):

$$\left|E^{-1}H-\lambda_j I\right|=0$$

where $E^{-1}$is the inverse of the common matrix of the sum of the squares and waste products, H is the matrix of the sum of the squares and products related to treatments, λ_j is the j^{-th} eigenvalue of the matrix $E^{-1}H$, and I is the identity matrix of order k.

Afterwards, we estimated the eigenvectors associated with the eigenvalues for the solution of the linear system as a constraint:

$$\bar{\ell}_j' \frac{E}{n_e} \bar{\ell}_j = 1$$

where is the j^{-th} eigenvector (canonical vector) associated with each eigenvalue λ_j, $\bar{\ell}_j'$ is the transpose of the j^{-th} canonical vector, E is the matrix of the sum of the squares and residual products, and n_e is the number of degrees of freedom of residue.

Biplot graphics (scores and loadings) were prepared following the work of Lipkovich and Smith (2002), using scaling JK (RMP). All statistical analyses were performed using SAS® software - Statistical Analysis System version 9.4 (2008) at 5% level of significance.

Results and Discussion

pH

An interaction between treatment and storage time was observed for pH (Table 1), which presented values that differed ($P<0.05$) between control versus (vs) sonicated samples throughout shelf life.

Table 1. Probability values for orthogonal contrasts.

	Effect of interaction / Variable	pH	WBS	TBARS	L*	a*	b*	Meso	Lactic	Psy
P value	Treatment	<0.0001	0.0086	0.0010	<0.0001	<0.0001	0.0011	0.0022	<0.0001	<0.0001
	Storage time	<0.0001	<0.0001	<0.0001	<0.0001	0.0063	0.0003	<0.0001	<0.0001	<0.0001
	Treatment*storage time	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0671	<0.0001	<0.0001	<0.0001
Orthogonal contrasts	Control × Sonicated	<0.0001	0.0570	0.0703	0.0027	0.0117	0.0882	0.0045	0.0040	0.0954
	Bath × Probe	0.9484	0.1637	0.0027	0.1250	<0.0001	0.0002	0.0006	<0.0001	<0.0001
	Bath: 60-120 s × 240 s	<0.0001	0.0026	0.8871	0.0979	0.0002	0.9173	0.3100	0.0762	0.0062
	Bath: 60 s × 120 s	0.0002	0.7098	0.1874	0.4300	0.0002	0.0531	0.2065	0.2707	0.0791
	Probe: 60-120 s × 240 s	<0.0001	0.1522	0.0027	<0.0001	0.0119	0.2364	0.8615	0.0993	0.1747
	Probe: 60 s × 120 s	0.1594	0.0768	0.0444	<0.0001	<0.0001	0.0305	0.1990	0.0074	0.8860
Orthogonal polynomial contrasts for shelf life	Storage time: Linear	<0.0001	<0.0001	0.0001	0.5868	0.0709	0.0004	<0.0001	<0.0001	<0.0001
	Storage time: Quadratic	<0.0001	0.0016	0.3857	0.0001	0.0449	0.0016	<0.0001	<0.0001	<0.0001
Orthogonal polynomial contrasts	d=0 vs d=16 × control × sonicated	0.0030	0.0001	<0.0001	<0.0001	0.7089	0.3479	0.7340	0.8368	0.9150
	d=0 vs d=16 × bath × probe	0.0014	0.0002	0.2405	0.1793	0.0368	0.1949	0.0287	0.0277	0.3019
	d=0 vs d=16 × B60-120 s × B240	0.2297	<0.0001	0.1318	0.0059	0.7514	0.8742	0.0481	0.5288	0.7986
	d=0 vs d=16 × B60 × B120	0.0045	0.4352	0.0002	0.0561	0.0014	0.3994	0.9848	0.3475	0.4375
	d=0 vs d=16 × P60-120 × P240	0.0008	0.0113	<0.0001	<0.0001	0.0051	0.7679	0.5765	0.1604	0.4060
	d=0 vs d=16 × P60 × P120	<0.0001	0.0065	0.0239	0.0001	0.0118	0.2277	0.2505	0.4426	0.0121

WBS: Warner-Bratzler blade force; TBARS: thiobarbituric acid reactive substances; L*: lightness-darkness; a*: redness; b*: yellowness; Meso: mesophilic bacteria; Lactic: lactic bacteria; Psy: psychotropic bacteria.

Moreover, the sonication system (bath vs probe) also presented an influence ($P < 0.05$) in pH along shelf life.

The initial pH value on day 0 for the control sample (5.4, Table 2) indicated a rigor mortis process of muscle used in the experiment, whereas after US application samples reached post rigor mortis and became susceptible to proteolytic enzyme softening (Lawrie 2005).

Sonicated samples presented higher pH than the control at day 0, probably due to cell structure damage from ion released into the cytosol and changes in the conformation of proteins with concealment of acidic groups (Got *et al*., 1999; Jayasooriya *et al*., 2007). For samples on days 3 and 5 of storage, the pH increase in sonicated samples remained higher than in the control. The pH increase in the post rigor mortis phase is typical of the aging process, resulting in the release of peptides and basic amino acids from proteolysis, sodium and calcium from the sarcoplasmic reticulum (Prändl *et al*., 1994). However, a reduction of pH was observed on day 9, and is related to the growth of lactic bacteria (Figure 1b).

Jayasooriya *et al.* (2007) report similar results of pH behavior in *Semitendinosus* and *Longissimus* bovine muscles treated with US (probe 24 kHz for 60, 120 and 240 s), whereas other studies have found no significant effect of US on pH (Stadnik and Dolatowski, 2011; Chang and Wong, 2012).

WBS force

Significant interaction between treatment and storage time was observed for WBS force ($P < 0.05$, Table 1), with differences between control vs US ($P = 0.0001$) and bath vs probe systems ($P < 0.05$). Except for P60, the US significantly reduced WBS force on day 0 ($P < 0.05$). The destructive nature of cavitation and turbulence in water media could lead the muscular structures to relax, resulting in fragmentation of myofibrils (Got *et al.*, 1999; Ahmad and Hasnain, 2013). In addition to rupturing the membranes of mitochondria and sarcoplasmic reticulum, US could increase the release of calcium, which activates the calpain proteolytic enzymes present in the sarcoplasm (Alliger, 1975; Lyng *et al*., 1998). These enzymes have been recognized as primarily responsible for the maturation of meat for acting in certain myofibrillar proteins (nebulin, titin, troponin and desmin), but without acting on actin and myosin (Kemp and Parr 2012). The calpains have a maximum activity at pH close to neutrality (Koohmaraie and Geesink, 2006; Lonergan *et al*., 2010) and are present in large quantities in *Semitendinosus* beef (Lawrie, 2005). As mentioned previously, the sonicated samples showed higher pH than the control on day 0, which may have favored the enzymatic action. Dransfield (1993) states that when close to pH 6.1, calcium reaches its maximum concentration and initiates activation of calpain.

In addition to action on calpain, cavitation could improved the activity of other enzymes related to the softening of meat, such as cathepsins, which could be released by weakening and rupturing membranes from the lysosomes where they are stored (Got *et al*., 1999). Xiong *et al.* (2012) and Chen *et al.* (2015) also report a positive effect of US systems on caspase proteases, recently associated with post-mortem tenderization (Lonergan *et al.*, 2010). The US may also have an effect on the increase of sarcomere length after its application, which may have contributed to the softness of samples after sonication at day 0 (Got

Table 2. Evaluation of pH, WBS force, and TBARS in Semitendinosus beef after ultrasound exposure during storage (7 ±1ºC).

	Days of storage						Tendency	
	0	3	5	9	16	Mean	L	Q
pH								
C	5.4^{bB}	5.7^{aB}	5.7^{abB}	5.6^{abB}	5.4^{bB}	5.6		**
B60	5.6^{bB}	6.2^{aAB}	6.3^{aAB}	5.3^{cC}	5.6^{bB}	5.8	**	**
B120	6.0^{bA}	6.5^{aA}	6.0^{bcB}	5.6^{cBC}	5.7^{cB}	6.0	*	
B240	6.0^{bA}	6.4^{aA}	6.4^{aAB}	5.7^{cAB}	5.7^{cAB}	6.0	**	**
P60	5.6^{cB}	5.9^{abB}	6.2^{aAB}	5.8^{bA}	5.7^{bcAB}	5.8		**
P120	6.1^{aA}	6.2^{aAB}	6.2^{aAB}	5.4^{bC}	5.6^{bB}	5.9	**	
P240	5.8^{bcAB}	6.3^{abA}	6.5^{aA}	5.7^{cAB}	5.9^{bAB}	6.1	**	**
Mean	5.8	6.2	6.2	5.6	5.7			
WBS force (N)								
C	56.2^{aA}	50.1^{aA}	45.3^{aAB}	55.5^{aA}	46.6^{aAB}	50.7		
B60	44.8^{bB}	51.0^{abA}	49.1^{abAB}	54.4^{aA}	52.1^{abA}	50.3	**	**
B120	40.3^{bB}	56.2^{aA}	54.4^{aA}	53.5^{aAB}	49.6^{aAB}	50.8	**	**
B240	47.8^{abB}	51.4^{abA}	51.4^{aAB}	41.9^{bB}	39.4^{bB}	46.4	**	
P60	56.5^{aA}	57.8^{aA}	46.0^{bAB}	42.4^{bB}	46.7^{bAB}	49.9	**	**
P120	45.6^{aB}	50.3^{aA}	51.8^{aAB}	45.1^{aB}	43.5^{aAB}	47.3	**	**
P240	45.2^{aB}	52.9^{aA}	42.7^{aB}	47.6^{aAB}	45.4^{aAB}	46.8		
Mean	48.1	52.8	48.7	48.6	46.2			
TBARS (malondialdehyde mg kg^{-1})								
C	0.04^{bA}	0.04^{abB}	0.03^{bA}	0.03^{bAB}	0.10^{aA}	0.05	**	**
B60	0.03^{bA}	0.05^{abAB}	0.08^{aA}	0.02^{bB}	0.09^{aAB}	0.05	**	
B120	0.03^{aA}	0.08^{aAB}	0.03^{aA}	0.04^{aAB}	0.05^{aB}	0.05		
B240	0.02^{aA}	0.08^{aAB}	0.06^{aA}	0.04^{aAB}	0.05^{aB}	0.05		**
P60	0.03^{bA}	0.08^{abAB}	0.03^{bA}	0.03^{bB}	0.08^{aAB}	0.05	**	**
P120	0.04^{aA}	0.10^{aAB}	0.06^{aA}	0.04^{aAB}	0.07^{aB}	0.06		
P240	0.04^{bA}	0.14^{aA}	0.08^{abA}	0.05^{bA}	0.04^{bB}	0.07	**	**
Mean	0.03	0.08	0.05	0.03	0.07			

Least square means with standard error of mean (SEM). Values with the same small letter in a row and the same capital letter in a column do not differ significantly at $P < 0.05$. C: control. untreated; B60: bath 60 s; B120: bath 120 s; B240: bath 240 s; P60: probe 60 s; P120: probe 120 s; P240: probe 240 s. Tendency: L: linear; Q: quadratic. **Significant coefficient at $P < 0.05$.

et al., 1999). Moreover, cavitation destroys muscle cell integrity, and could cause selective heating of collagen, thus reducing meat toughness (Zayas and Garbatow, 1978; Stadnik and Dolatowski, 2011; Chang *et al.*, 2012).

Stadnik and Dolatowski (2011) observed a significant reduction in shear force 48h and 72 h post-mortem when using an US bath (45 kHz) for 120 s in bovine *Semimembranosus* muscle. Chang *et al.*, (2012) also report the positive effect of using an US bath (40 kHz) for 10, 20, 30, 40, 50 or 60 min on the hardness of bovine *Semitendinosus* muscle, where collagen fibers were disorganized and weakened proportional to exposure time.

Nevertheless, the response of US on the texture did not persist after the third day of storage, so from this point the results of WBS force were similar to those of the control. Jayasooriya *et al.* (2007) also found that the use of US probe (24 kHz, 12 W cm^{-2} for 60 s, 120 s and 240 s) had no effect on *Semitendinosus* and *Longissimus* bovine muscle on WBS force during storage for 8.5 days. Stadnik and Dolatowski (2011) observed the effect of US on shear force was perceived only 48h and 72 h post-mortem, indicating that the US promotes a positive effect on the texture of meat after its application, but that this effect did not remains during storage. This could have occurred because the cavitation allowed the formation of pores in the mitochondrial membrane, since sarcoplasmic reticulum and lysosomes are opened temporary and reversible, returning to normal permeability minutes or hours after exposure to the US (Nguyen *et al*., 2009; Yeo and Liong, 2013). Furthermore, the effect of proteinases upon US is limited since after a few days they undergo autolysis (Lonergan *et al*., 2010). *Semitendinosus* is a muscle that has naturally increased softness after four days of storage (Lawrie, 2005), which may contribute to an explanation of the tenderness of the control in comparison with the sonicated samples during storage. The type of plastic packaging used and the large amount of collagen muscle may have influenced by hindering the penetration of waves and prolonged action of the US (Pohlman *et al*., 1997).

TBARS

Interaction treatment and storage time ($P < 0.0001$) was observed for TBARS values, with differences between the US and control samples ($P < 0.0001$), independent of US system (bath vs probe, $P > 0.05$). The increase in temperature caused by sonication yields hydroxyl radicals (•OH) and peroxide (H_2O_2) (Kentish and Ashokkumar, 2008) that can oxidize unsaturated fatty acids in meat. Although the use

Table 3. Evaluation of instrumental color parameters in *Semitendinosus* beef after ultrasound exposure during storage (7 ±1ºC).

	Days of storage						Tendency	
	0	3	5	9	16	Mean	L	Q
				L*				
C	47.2^{bC}	56.0^{aAB}	54.5^{abB}	53.0^{abC}	57.1^{aB}	53.5	**	
B60	52.3^{bABC}	57.7^{abAB}	62.3^{aA}	54.4^{bC}	52.1^{bBC}	55.8		**
B120	57.8^{abAB}	64.8^{aA}	54.0^{bB}	53.9^{bC}	52.3^{bBC}	56.6	**	
B240	55.6^{aABC}	58.9^{aA}	56.8^{aAB}	57.6^{aBC}	59.4^{aB}	57.7		
P60	58.6^{aA}	49.9^{bB}	46.0^{bC}	50.0^{bC}	47.8^{bC}	50.5	**	**
P120	48.8^{bBC}	62.2^{aA}	63.0^{aA}	67.0^{aA}	49.2^{bC}	58.0		**
P240	54.1^{bcABC}	59.0^{bcA}	51.4^{cBC}	60.0^{bB}	69.0^{aA}	58.7	**	**
Mean	53.5	58.4	55.4	56.5	55.3			
				a*				
C	26.4^{aAB}	25.1^{aAB}	29.0^{aA}	25.0^{aAB}	25.0^{aA}	26.1		
B60	21.7^{aB}	26.6^{aAB}	28.6^{aA}	26.2^{aA}	26.5^{aA}	25.9		**
B120	30.8^{aA}	30.8^{aA}	30.3^{aA}	26.2^{aA}	27.3^{aA}	29.1	**	
B240	24.0^{aB}	24.5^{aAB}	27.3^{aAB}	22.7^{aAB}	25.3^{aA}	24.8		
P60	22.2^{aB}	23.9^{aAB}	25.4^{aAB}	27.3^{aA}	25.3^{aA}	24.8		**
P120	24.9^{aAB}	21.7^{aB}	20.1^{aB}	18.8^{aB}	21.5^{aAB}	21.4		**
P240	22.2^{abB}	19.6^{abB}	23.6^{abAB}	25.5^{aAB}	15.8^{bB}	21.3	**	**
Mean	24.6	24.6	26.3	24.5	23.8			
				b*				
C	10.4	10.2	13.2	11.5	11.6	11.4^{A}		
B60	6.3	10.0	13.0	11.7	11.0	10.4^{A}	**	**
B120	9.6	14.1	14.0	11.3	12.4	12.3^{A}		
B240	9.2	11.1	14.3	10.0	12.6	11.4^{A}		
P60	7.7	7.4	11.1	11.4	10.8	9.7^{AB}	**	
P120	8.6	5.0	7.3	8.1	9.0	7.6^{B}		
P240	7.1	10.3	10.4	10.8	9.4	9.6^{AB}		**
Mean	8.4^{c}	9.7^{b}	11.9^{a}	10.7^{b}	11.0^{ab}			

Least square means with standard error of mean (SEM). Values with the same small letter in a row and capital letter in a column do not differ significantly at $P < 0.05$. L^*: lightness/darkness; a^*: red/green; and b^*: yellow/blue. C: control. untreated; B60: bath 60 s; B120: bath 120 s; B240: bath 240 s; P60: probe 60 s; P120: probe 120 s; P240: probe 240 s. Tendency: L: linear; Q: quadratic. **Significant coefficient at $P < 0.05$.

of US affected TBARS more than the control ($P < 0.0001$), the values were below the threshold for detection of unpleasant odor in beef. According to Melton (1983), TBARS values over 0.3 mg MDA kg^{-1} are needed for this change, while Connell (1990) indicates that odor is only modified above 2 mg MDA kg^{-1}. Chang and Wong (2012) observed an increase in TBARS content in cobia sashimi (Rachycentron canadum) exposed to an US bath (60 kHz, 0 to 90 min), but likewise the values were below the detection limits for unpleasant odor. Ashokkumar *et al.* (2008) evaluated the effect of frequency (20 kHz, 358 kHz and 1062 kHz) on the formation of hydroxyl radicals and its impact on phenolic substrate radicals, noting that for 20 kHz oxidative reactions were minimal because transient cavitation bubbles are less active for sonolysis in this frequency. As a result, the frequencies tested (bath 45 kHz and probe 20 kHz) could have been underpowered to provide significant change in lipid oxidation. Furthermore, the vacuum packaging contributes to the oxidative stability by lack of oxygen to the reaction.

Color

Interaction effect of treatment and storage time was significant ($P < 0.05$) to color parameters L^* (lightness) and a^* (redness) (Table 1). Application of ultrasound (control vs sonicated) affected only L^* ($P < 0.05$), while system (bath vs probe) was significant only to a^* ($P < 0.05$). Ultrasound application increased L^* value (Table 3) in sonicated samples at day 0, due to the heating caused by sonication that promotes denaturation of myoglobin and hemoglobin pigments with an increase on L^* (Paniwnyk, 2014). Caraveo *et al.* (2014) observed the same tendency when using US in *Semitendinosus* beef (bath 40 kHz, 0, 60 or 90 min).

Denaturation of myoglobin and hemoglobin directly affects the reddish color of meat, thus reducing a^* (Pohlman *et al*., 1997). It was possible to detect the difference between bath vs probe (Table 1) samples because samples treated with US probe are generally less a^* than samples treated in bath samples or the control as a result of the higher energy intensity of probe compared with bath (Mason and Peters, 2002). Parameter b^* showed a difference between bath and probe ($P<0.05$, Table 1) since samples treated with probe generally presented lower b^* values (Table 3).

Pohlman *et al*. (1997) report similar behavior to

L^*, a^* and b^* in *Pectoralis* bovine when using an US bath at 20 kHz with intensity (22 W cm^{-2}) and time of exposure higher (0, 5 or 10 min) than the present study. By reducing the intensity to 1.55 W cm^{-2} and using the same bath for 8, 16 or 24 min, the authors observed no significant effect on the parameters of color (L^*, a^* and b^*) (Pohlman *et al*., 1997); the same was the case for Stadnik and Dolatowski (2011) and Sikes *et al.* (2014) after ultrasound interventions on beef.

Microbiological analysis

Treatment and storage time had significant interaction effects (P<0.05, Table 1) on the three groups regarding the microorganism content of the evaluated samples (mesophilic, lactic bacteria and psychotropic). Application of ultrasound (control vs sonicated) had no significant effect (P> 0.05) on any of the groups of microorganisms. System (bath vs probe) was significant (P<0.05) only for mesophilic and lactic acid bacteria (P <0.05, Table 1).

US is generally associated with its deleterious effect on microorganisms (Piyasena *et al.*, 2003), although some studies have shown that it can be used to stimulate the growth of fermentative microorganisms (Wang and Sakakibara, 1997; Nguyen *et al.*, 2009; Ewe *et al*., 2012). However, the use of US on the microbiology of meat remains underexplored. Caraveo *et al.* (2014) show that the application of US decreased mesophilic and psychrophilic bacteria in *Semitendinosus* beef stored at 4ºC for 10 days; nevertheless, time of US exposure was higher (60 or 90 min). Cichoski *et al.* (2015) show that the application of US the 25 kHz in bath decreased mesophilic and lactic bacteria in sausages stored at 15°C for 60 days with time of US exposure was low (10.53 min at 74°C). Moreover, Pohlmann *et al*. (1997) and Sams and Feria (1991) found no significant influence of application of US in beef and chicken thigh. The lack of effect of sonication here could be caused due to the temporary effect of US on the growth of microorganisms, which decrease soon after exposure (Ewe *et al*., 2012; Yeo and Liong 2013). However, the low sonication time carried out or even the mild conditions of temperature or pressure used in the experiments could also have influence on this behaviour.

In order to verify the microbiological quality of meat, a total count of mesophilic microorganisms was performed, as evidenced by the low number of colonies of control and other samples at day zero, as well as throughout the experiment (Figure 1a). Signs of deterioration of the meat, especially smell, are only detected at concentrations above 6 log CFU

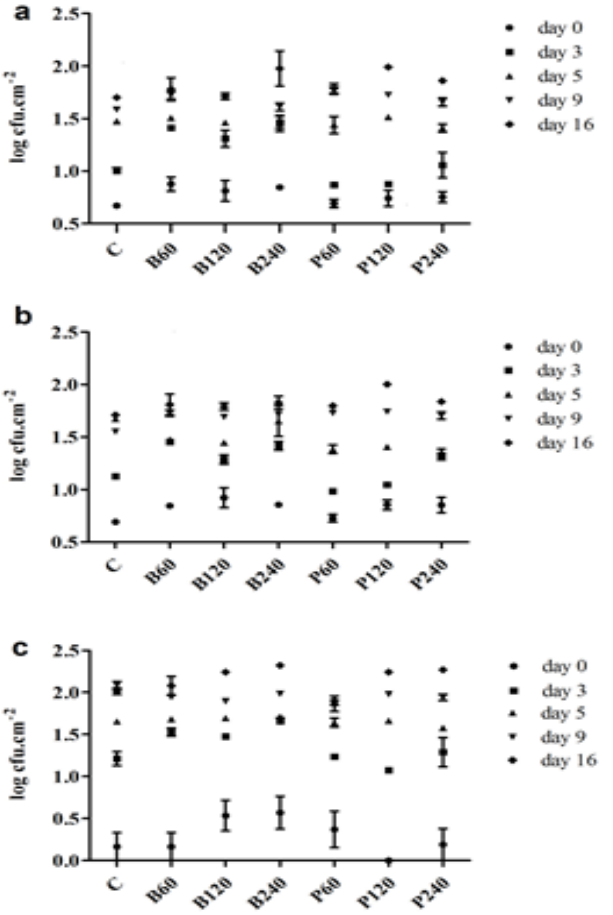

Figure. 1. Effect of ultrasound exposure of *Semitendinosus* beef on microbial growth during storage (7 ±1ºC) over the course of 16 days. (A) Mesophilic microorganisms, (B) Lactic acid bacteria, and (C) Psychotropic microorganisms. C: control. untreated; B60: bath 60 s; B120: bath 120 s; B240: bath 240 s; P60: probe 60 s; P120: probe 120 s; P240: probe 240 s. d0: day 0; d3: day 3; d5: day 5; d9: day 9; d16: day 16. Bars represent the standard error of the mean.

g^{-1} (Dainty and Mackey, 1992), and therefore until the 16th day of storage no evidences of deterioration were observed, probably due to the low count (≤ 2 log CFU cm^{-2}) of microorganisms in the samples. The cold temperature of 7±1ºC during storage and vacuum packaging could extend the validity period of beef for up to 21 days (Blixt and Borch, 2002) and certainly contributed to maintain the microbiological quality of the samples.

However, vacuum packaging promotes anaerobiosis, which generally favors the development of lactic acid bacteria associated with the release of acid flavor (Lawrie, 2005). Li *et al*. (2013) observed a significant increase in lactic acid bacteria on vacuum-packed beef for 14 days compared with permeable packaging. In our study, the number of colonies of lactic acid bacteria increased throughout the storage time, but low count values (≤ 2 log CFU

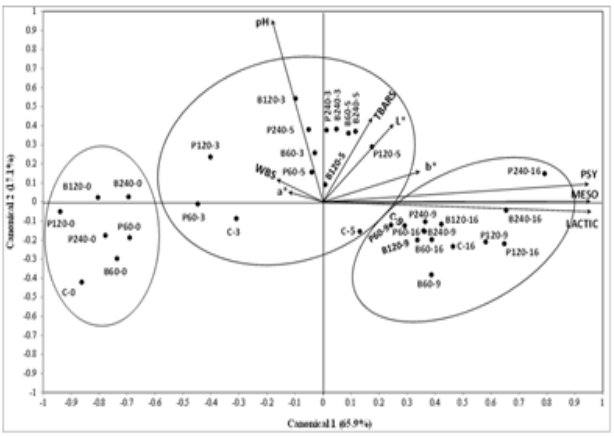

Figure. 2. Discrimination of treatments on the basis of canonical discriminant analysis. C: control. untreated; B60: bath 60 s; B120: bath 120 s; B240: bath 240 s; P60: probe 60 s; P120: probe 120 s; P240: probe 240 s.; after treatment/trace: 0: day 0; 3: day 3; 5: day 5; 9: day 9; 16: day 16.

cm^{-2}) until the 16th day (Figure 1b) were observed. A significant difference between US systems (bath vs probe, Table 1) was observed with values slightly higher for samples treated in baths.

Cold storage encourages proliferation of psychotropic microorganisms, especially *Pseudomonas*. As expected, the number of psychotropic colonies increased during storage for all samples, although remained low (Figure 1c). Fernández-López *et al*. (2008) and Lorenzo and Gómez (2012) report a significant inhibitory effect of vacuum packaging on the number of colonies of psychotropic and *Pseudomonas* when compared with those exposed to air, so packaging used may have contributed to the good quality of refrigerated samples. Psychotropic counts above 6 log CFU g^{-1} are indicative of causing an unpleasant odor and above 7 log CFU g^{-1} result in the appearance of slime on the surface of the meat (Jay, 2005), far from the values observed after 16 days of refrigerated storage in this work. Thus, the use of ultrasound did not affect the growth of microorganisms, as well as did not affect the meat quality characteristics evaluated in this work.

Canonical discriminant analysis

For a deep insight into the differences between treatments, the canonical discriminant technique was used (Figure 2). Analysis of the two functions together explained 83% of variance of the data, with the first function (Canonical 1) responsible for the majority of the variation (65.9%). There was formation of the three groups, separated according to storage time. Samples from day 0 were distinct from the group of days 3 and 5 as well as the group of days 9 and 16. The first function discriminated expressively samples analyzed on day 0 in comparison with samples on days 9 and 16, mainly by microbiological evaluation of mesophilic, lactic acid bacteria, and psychotropic. This result was expected, since the counts increased from day 0 to day 16. Days 3 and 5 are mainly distinguished from the others by TBARS and pH, since these days experienced the highest values for both variables (Table 2). It is observed that control days 0, 3 and 5 (C-0, C-3 and C-5, respectively) are slightly apart from the other samples relating to the day of analysis, suggesting that US may have primarily influenced the beginning of storage for some variables. The system (bath/probe) and time of exposure to US had no influence on grouping, as well as WBS force and parameters of color (L^*, a^* and b^*).

Conclusion

Ultrasound treatment applied (probe 20 kHz and bath 45 kHz during 60, 120 and 240 seconds) significantly reduced the WBS force of meat after application when compared with untreated meat. However, this effect was not maintained during storage, suggesting that ultrasound can only be used to advance the ripening of meat. Other quality parameters such as color, lipid oxidation and microbial flora were not influenced by ultrasound treatments (probe 20 kHz and bath 45 kHz during 60, 120 and 240 second). In general, under the conditions studied, storage time was the most important factor for all parameters evaluated, while a few differences among application system (bath or probe) and time of US application were observed. Therefore, the results suggested that for improvement of US effect on meat the application should be performed not only before packaging, but also during storage.

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