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Influence of ultimate pH on biochemistry and quality of *Longissimus lumborum* steaks from Nellore bulls during ageing

Clara Lucía Contreras Barón,[1†] Priscila Robertina dos Santos-Donado,[1†] Patricia Maloso Ramos,[2] Carlos M. Donado-Pestana,[3] Eduardo Francisquine Delgado[2] & Carmen J. Contreras-Castillo[1]*

1 Department of Agro-industry, Food and Nutrition, "Luiz de Queiroz" College of Agriculture – University of São Paulo, P.O. Box 13418-900, Av. Padua Dias 11, Piracicaba, SP, Brazil

2 Department of Animal Science, "Luiz de Queiroz" College of Agriculture – University of São Paulo, P.O. Box 13418-900, Av. Padua Dias 11, Piracicaba, SP, Brazil

3 Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, P.O. Box 05508-900, Av. Prof. Lineu Prestes 580, São Paulo, SP, Brazil

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Summary Ultimate pH (pH_u) is an indicator that influences *post-mortem* meat quality. We studied physiological and biochemical changes of steaks obtained from Nellore bulls (*Bos indicus*) during *post-mortem* ageing. To this, *Longissimus lumborum* (LL) muscles were classified into three groups: Normal-pH_u (≤5.79), Intermediate-pH_u (5.80–6.29) and High-pH_u (≥6.30) groups, portioned into steaks, vacuum packaged and matured at 2 °C for 0, 7, 14, 21 and 28 days. High-pH_u steaks exhibited impaired colour stability and were darker compared to the other groups. High- and Normal-pH_u steaks showed improved tenderness and myofibrillar fragmentation linked to proteolysis. Intermediate-pH_u steaks were associated with a lower meat tenderness and decreased collagen solubility. High-pH_u steaks retained a high pH during ageing and increased water-holding capacity. These findings provide evidence that highlight pH_u as a strategy for the classification of pH_u-dependent beef quality from Nellore bulls that can be adopted by the Brazilian meat industry.

Keywords Beef, non-castrated, proteolysis, tenderisation.

Introduction

In 2018, Brazil was the world's largest exporter of beef, providing 20% of worldwide exports (Zia *et al.*, 2019). Brazilian beef production is largely based on *Bos taurus indicus* animals raised primarily on pasture, resulting in meat with lower fat content and higher levels of antioxidants (Daley *et al.*, 2010; Lobato *et al.*, 2014). The Nellore breed (*B. taurus indicus*) is predominant in the industry because of its high productivity, heat tolerance and disease resistance (Carvalho *et al.*, 2014). Bulls represent an important number of animals in the national herd (Instituto Brasileiro De Geografia E Estatistica - IBGE, 2020) due to the producers preference, which is influenced by the superiority in daily weight gain and higher efficiencies in feed conversion compared to steers, heifers and cows. Despite these advantages, it is known that bulls have a flighty, excitable temperament, being behaviourally aggressive and seeking to re-establish social dominance hierarchies, leading to animal stress, mainly if they are mixed prior to slaughter (Holdstock *et al.*, 2014).

The susceptibility to physical and psychological stress has been related to a multitude of factors including transport, age of animal, nutrition level, animal health status, handling, feed restriction, and mixing of unfamiliar animals, among others, thereby influencing the rate and extent of *post-mortem* pH decline (Ponnampalam *et al.*, 2017). After slaughter, skeletal muscle suffers biochemical and physical modifications due to the interruption of blood flow and oxygen supply. Despite these limitations, muscle continues metabolising energy to sustain its homeostasis. Under anaerobic conditions, ATP is produced through the glycogenolysis to finally produce lactate. The hydrolysis of ATP results in the production and accumulation of hydrogen ions responsible for decreasing the muscle pH (from 7.0 to 7.2 in living muscle to an ultimate pH (pH_u) of 5.5–5.8). A stress-associated

*Correspondent: Fax: +55 19 34294288; e-mail: ccastill@usp.br
†These authors contributed equally to this work.

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abnormal rate and extent of *post-mortem* glycolysis and pH decline may lead to a detriment in the meat quality such as dark-cutting (the formation of dark, firm and dry meat; Ponnampalam *et al*., 2017; Chauhan & England, 2018). Furthermore, previous studies have reported that *post-mortem* muscle pH_u can be used as a potential meat colour and tenderness indicator, being a relevant factor related to meat quality (Li *et al*., 2014; Ponnampalam *et al*., 2017). Intermediate-pH_u and high-pH_u are frequently observed in bull carcasses, differing from the low- or normal-pH_u observed in cows (Jeleníková *et al*., 2008). Intermediate- and/or high-pH_u can reflect inferior quality attributes including dark colour, greater tenderness inconsistency, reduced shelf-life, and less flavour and consumer acceptability (Chauhan & England, 2018). In fact, in a previous study by Hughes *et al*. (2014) it was observed that carcasses with pH_u values of 5.8, 6.0 and 6.2, had a dark-cutting incidence of 28%, 74%, and 96%, respectively.

Because several factors influence pH_u, discrepancies are regularly reported for the meat quality and biochemistry aspects, representing a continuing problem for the industry (Li *et al*., 2014). Limited information is available concerning the effects of pH_u on steak quality from Nellore cattle in Brazil. Nellore cattle present divergent temperament that may be associated with lower and inconsistent meat tenderness (Coutinho *et al*., 2017; de Moura Souza *et al*., 2019). Given the high diversity and volume of Brazilian beef originating from multiple geographic locations with continental scale, the national meat industry requires to adopt production strategies to reduce the quality heterogeneity, as well as meeting quality parameters demanded by consumers. The possible segregation and classification of meat by the pH_u is a useful tool for providing valuable information and guidance into decision-makings related to the product quality and processing strategies, minimising operational costs and/or economic penalties. Although the pH_u-dependent effects on meat quality have been reported previously, the specific relationship between pH_u and biochemistry underlying inconsistent beef tenderness from Nellore are still poorly studied. For this reason, we hypothesised that the meat segregation by pH_u can become a predictive control strategy for classifying pH_u-dependent quality characteristics during *post-mortem* ageing that can be adopted by the *Bos indicus*-based Brazilian industry aiming to develop products for specific markets. Thus, in the current study we investigated, for the first time, the influence of the inherent pH_u classified into three groups: Normal-pH_u (≤5.79), Intermediate-pH_u (5.80–6.29) and High-pH_u (≥6.30), on the tenderness and other quality attributes of loin steaks obtained from Nellore bulls during ageing.

Materials and methods

Animals and sampling

Nellore bulls between 30 and 36 months of age were selected from a commercial slaughterhouse (São Paulo, Brazil). All animal procedures were performed strictly in accordance with the Regulation Guideline for the Industrial and Sanitary Inspection of Products of Animal Origin (RIISPOA). Animals were stunned by captive bolt pistol, hung by the Achilles tendon and bled using standard procedures. Carcasses were stored at 4 °C in a cold room. At 24 h *post-mortem*, carcass pH was measured using a potentiometer (HI99163, Hanna Instruments, São Paulo, Brazil, calibrated at cold temperature and pH buffers 4.0 and 7.0 used as standards) inserted between the 12/13th ribs. The machine was regularly re-calibrated to ensure the accuracy of the results. A total of 100 carcasses were measured and finally 12 were selected (taking into account the lower occurrence of High-pH_u in a preliminary analysis at slaughterhouse. See Table S1) and segregated into three groups ($n = 4$ each) as follows: Normal-pH_u (≤5.79), Intermediate-pH_u (5.80–6.29) and High-pH_u (≥6.30). The carcass segregation by the different pH_u ranges used in this study was based on previous studies from our group and others (Lomiwes *et al*., 2014; Contreras-Castillo *et al*., 2016) having as a determining factor the pH_u-dependent tenderness variation. Next, LL muscles were excised from the left half-carcasses, vacuum packaged (VSA 211; Cryovac, Sealed Air, São Paulo, Brazil) and transported at 4 °C to the Meat Science Laboratory at University of São Paulo (Piracicaba, São Paulo-Brazil). Muscles were unpackaged and pH was re-measured at 48 h *post-mortem*. Steaks with an approximate thickness of 2.5 cm (400 g) were cut in an air-conditioned environment at 12 °C and vacuum packaged. Thereafter, loin steaks were held in dark storage at 2 ± 1 °C for 0, 7, 14, 21 and 28 days.

Instrumental colour

The instrumental colour of the surface loin steaks was measured using a HunterLab MiniScan XE Plus spectrophotometer (HunterLab Associates, Reston, VA, USA). The instrument was calibrated with a standard black and white-plate, using an optical geometry of 45/0, a 2.54 cm diameter aperture, illuminant D65, and an observer angle of 10°. After blooming for 1 h at 2 °C, the colour values were recorded for the parameters lightness (L^* = from black to white), redness (a^* = from green to red) and yellowness (b^* = from blue to yellow). The Chroma (C^*, saturation index) and hue angle (h^*) parameters were calculated with the following equations: $C^* = [(a^*2 + b^*2)$

1/2] and $h^* = [\text{ATAN} - 1(b^*/a^*)]$ (AMSA, 2012). The measurements were performed in three replicates, at three different locations on each steak (free of visible connective and adipose tissue) and an average value was used for statistical analyses.

Warner-Bratzler shear force

Shear force measuring was performed as previously described (Shackelford *et al.*, 1991). Steaks were cooked on an electric grill (SSE50, EDANCA, São Paulo, Brazil) until they reached an internal temperature of 71 ± 1 °C at the geometric centre. Subsequently, steaks were stored for 24 h at 4 °C. Prior to analysis, steaks were kept at room temperature for 2 h. Six to ten cores (1.27 cm diameter) were excised, parallel to the longitudinal orientation of the muscle fibres. Each core was sheared perpendicular to the fibre orientation using a Warner-Bratzler shear blade (Warner-Bratzler meat shear, Macmesin BFG 500N; G-R Manufacturing Co. Collins LN, Manhattan, KS, USA) with a 50 kg load cell and a 5.0 mm s^{-1} crosshead speed. The results were expressed in Newton (N).

Myofibril fragmentation index

The myofibril fragmentation index (MFI) was determined using the method described by Culler *et al.* (1978) with slight modifications. Frozen meat samples (1 g) were thawed in 10 mL of ice-cold buffer (100 mM KCl; 20 mM potassium phosphate, pH 7.0; 1.0 mM EDTA; 1.0 mM $MgCl_2$; and 1.0 mM NaN_3, pH 7.0) at room temperature followed by homogenisation using an Ultra-Turrax (T25, IKA) at 18 000 rpm for 30 s (repeated three times with cooling period of 10 s in between). After homogenisation, samples were centrifuged (1000 ***g*** at 4 °C for 15 min). The myofibrils were resuspended with a glass rod and after the third wash with the extraction buffer, the extracted volume was reduced by half and samples were filtered through a 400 μm^2 nylon mesh to remove connective tissue and debris. Protein content was determined through the Biuret method using bovine serum albumin (BSA) as a standard. The myofibrils were diluted to a protein concentration of 0.5 mg mL^{-1} and the absorbance reading at 540 nm was multiplied by a factor of 200 to generate an MFI value.

Total and soluble collagen

The total collagen analysis was performed as previously described (Archile-Contreras *et al.*, 2010) with some modifications. Frozen-dried samples (350 mg) were hydrolysed in 12 mL of 6 N hydrochloric acid for 16 h at 110 °C. The hydrolysate was cooled to room temperature, filtered through a Whatman No. 1 filter paper and neutralised with 12 mL of 6 N sodium hydroxide. The volume was then filled up to 250 mL with distilled water. The hydroxyproline concentration of the diluted samples was determined using a 4 mL aliquot oxidised with 2 mL of chloramine T hydrate and allowed to react for 30 min. After this period, 2 mL of 4-dimethylamino-benzaldehyde reagent was added, and the tubes were placed in a covered water bath at 60 °C for 45 min. The absorbance was measured at 570 nm at room temperature. The results were calculated from the standard curve of hydroxyproline, multiplied by a factor of 7.25, and expressed as g of total collagen per 100 g of dry matter.

Soluble collagen was extracted according to Palka (1999), with some modifications. Samples of 500 mg were homogenised with 10

mL of distilled water. Homogenates were heated for 75 min at 80 °C, and then centrifuged (4000 ***g*** for 15 min). For hydrolysis, the supernatant was mixed with 10 mL 6 N HCL to hydrolysis for 16 h at 110 °C. The hydrolysate was cooled to room temperature and neutralised with 10 mL of 6 N NaOH. The hydroxyproline content was determined as described above. Soluble collagen was calculated by multiplying the hydroxyproline content by 7.25 and expressed as g of soluble collagen per 100 g of dry matter.

Drip loss

Drip loss was determined according to Honikel *et al.* (1986) at 0 days (48 h *post-mortem*). Samples (100 g), without adipose and connective tissues, were weighed and placed in reticulated plastic covered with a polyethylene plastic bag covering at 2 ± 1 °C for 72 h. The samples were carefully removed from the bags, gently dried with towel paper, and weighed. All drip loss measurements were expressed as a percentage of the initial and final weight.

SDS-PAGE

Samples were finely chopped and at 0 (48 h *post-mortem*) and 28 days of ageing, 1 g of meat was homogenised in 10 mL of cold extraction buffer (50 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS, and 2% 2-mercaptoethanol) using an Ultra-Turrax at 20 000 rpm for 30 s (repeated three times). The homogenate was centrifuged at 10 000 ***g*** for 5 min at 4 °C. The supernatant was collected, and the protein concentration determined through the Bradford method with BSA as a standard. Next, 100 μg of protein was denatured through boiling in Laemmli buffer, loaded and separated on SDS-PAGE gel (5%, 35.1:1 acrylamide:bisacrylamide, w/w). HiMark™ Pre-Stained Protein Standards (LC 5699, Invitrogen, São Paulo, Brazil) and Pre-Stained Kaleidoscope Molecular Weight Standards (Cat. 161-0375, Bio-

Rad, Hercules, CA, USA) standards were used as references. SDS-PAGE gels were run on a Mini Protean Tetra Cell system (Bio-Rad Laboratories, Hercules, CA, USA), conducted at 5 mA for 14 h in running buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS; pH 8.3). Gels were stained in Colloid Coomassie Blue G250 for 48 h, and subsequently destained with 5% methanol, 7.5% acetic acid, and scanned using the ChemiDoc MP Imaging System (Bio-Rad Laboratories).

Immunoblotting

Homogenates from samples were obtained in a similar way to SDS-PAGE, as previously described. After determining the protein concentration, samples were adjusted to a concentration of 4 mg mL^{-1} with a Pironin-Y extraction buffer (30 mM Tris-HCl; 30 mM EDTA; 30% glycerol; 3% SDS; 0.003% Pironin-Y, pH 8.0) and 30 μg of protein were loaded onto 12% resolving gel (35.1:1 acrylamide:bisacrylamide, w/w) and resolved in a Mini Protean Tetra Cell system (Bio-Rad Laboratories) at 120 mA for 2 h at room temperature. Proteins were transferred onto Immobilon-P PVDF membranes (Amersham™ Hybond™ ECL; GE Healthcare, Uppsala, Sweden) and blocked by incubating them with 5% non-fat dry milk powder diluted in PBS-Tween for 1 h. Membranes were then washed three times with PBS-Tween, and incubated overnight at 4 °C with primary antibodies against desmin (D1033, Sigma Aldrich, St Louis, MO, USA) and troponin-T monoclonal (T6277, Sigma Aldrich) diluted 1:5000 in 5% non-fat milk. After washing, membranes were subsequently incubated with goat anti-mouse IgG (H + L) HRP conjugate (#172-1011, Bio-Rad Laboratories) diluted to 1:5000 in PBS-Tween at room temperature for 1 h. The membranes were then washed, and the bound antibody was detected using a Pierce ECL Western blotting substrate kit (# 32209, Thermo Scientific, Waltham, MA, USA). Western blot images were captured with a ChemiDoc MP Imaging System (Bio-Rad Laboratories).

pH

After each storage period, the measurement of internal pH for each steak was determined directly using a potentiometer (HI99163; Hanna Instruments) with automatic temperature compensation. The pH probe was calibrated in buffers at pH 4.0 and 7.0 before use. Measurements were taken at least three times for each sample and averaged.

Statistical analysis

The statistical analysis was performed using the SAS 9.4 package (SAS Institute, Inc., Cary, NC, USA). The results were analysed using a completely randomised design with pHu group, storage time, and its interaction fitted as fixed effects, and animal/carcass ($n = 4$, with dependent variables measured in triplicates for each analysis, for a total of 12 replicates) as a random effect. To investigate the differences, a two-way analysis of variance (ANOVA) was conducted. The significant differences found between the mean values were analysed using a Tukey's test for a value of $P < 0.05$.

Results and discussion

Instrumental colour

Our results show that pH_u had no influence on the lightness (L^*) of Nellore bull steaks (Fig. 1a); however, following a prolonged storage time (28 days), a gradual increase of L^* values was observed for steaks in all pH_u groups. As expected, a gradual increase ($P < 0.05$) in the redness (a^*) and Chroma (C^*) values were observed for all groups until the end of the experiment (Fig. 1b,c). Normal- and Intermediate-pH_u groups did not differ in a^* and C^* values during ageing. Steaks in the High-pH_u group showed lower a^* values ($P < 0.05$) when compared to the other groups during the 28 days of storage; C^* values were significantly lower at 0, 21 and 28 days of ageing and a significant increase in the b^* values ($P < 0.05$) was observed for all pH_u groups during the experiment. Normal-pH_u steaks showed higher b^* values ($P < 0.05$) than High-pH_u steaks at 0 and 21 days (Fig. 1d). The hue angle (h^*) values in the steaks revealed that there were no significant differences among the pH_u groups during ageing (Fig. 1e).

Confirming and expanding on previous findings, High-pH_u was associated with dark-cutting beef in Nellore bulls as evidenced by the combination of decreased redness (a^*), yellowness (b^*) and Chroma (C^*) observed in our results. High-pH_u steaks possess a lower reflectance, derived from a high water-holding capacity (WHC) in myofibrillar proteins, resulting in a closed structure with fewer extracellular spaces; consequently, the increase in WHC inhibits oxygen diffusion, allowing higher light absorption and decreased reflectance over the steaks surface (Hulot & Ouhayoun, 1999; Abril *et al.*, 2001). In addition, a High-pH_u environment in the meat also favours a decrease in the oxymyoglobin formation due to the competition for oxygen consumption by mitochondria and a higher activity of oxygen-scavenging enzymes. Thus, a shiny red surface colour is reduced and a purple red colour takes its place, derived from an increase in the deoxymyoglobin formation in the steaks (England *et al.*, 2017).

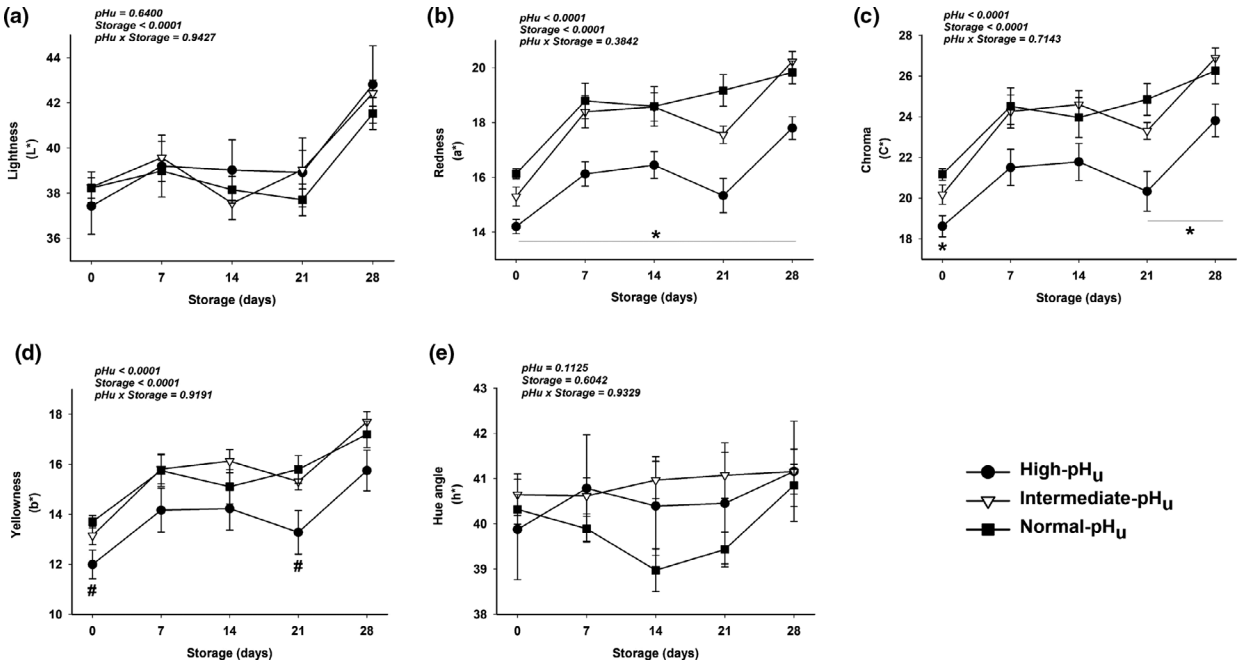

Figure 1 Instrumental colour of *Longissimus lumborum* Nellore bull steaks stored at 2 °C for 28 days. Ultimate pH groups: Normal-pH_u (≤5.79); Intermediate-pH_u (5.80–6.29); High-pH_u (≥6.30). Values are expressed as mean ± SEM (n = 12). *($P < 0.05$) High-pH_u vs. Intermediate- and Normal-pH_u; $^{\#}$($P < 0.05$) High-pH_u vs. Normal-pH_u.

The instrumental colour results from both Normal- and Intermediate-pH_u groups indicated an appearance of fresh red meat, probably due to the metabolic processes including myoglobin oxygenation and the oxidative status of haem iron during the muscle *post-mortem* period (Ramanathan *et al.*, 2019). Indeed, Normal-pH_u promotes denaturation of the globin moiety of myoglobin, an effect which exposes the haem group and increases its susceptibility to oxidation forming metmyoglobin. In addition, Normal-pH_u also impairs the ability to reverse the reduction of metmyoglobin to deoxymyoglobin in meat (England *et al.*, 2017). The *post-mortem* glycolysis and pH decline in Normal- and Intermediate-pH_u can favour the phosphorylation of the myoglobin structure and modify its susceptibility to oxidation as previously observed in a phosphoproteomic study (Li *et al.*, 2018). Moreover, Normal-pH_u in meat can regulate the protein denaturation, decreasing the solubility of proteins and their capacity to bind water. Therefore, water migrates from inside the muscle fibres to the extracellular environment, thereby increasing reflectance on the steak surface (England *et al.*, 2017).

WBSF and MFI

To evaluate the influence of pH_u on tenderness in Nellore bull steaks, both Warner-Bratzler shear force (WBSF) and MFI were measured. WBSF and MFI are recognised as instrumental and biochemical approaches, respectively, of objective measuring meat tenderness (Culler *et al.*, 1978; AMSA, 2015). For all pH_u groups, a gradual increase in the tenderisation was observed during ageing, as evidenced by decreasing WBSF values (Fig. 2a) and increasing MFI (Fig. 2b). Our results showed that Intermediate-pH_u steaks exhibited the highest WBSF values, with significant differences ($P < 0.05$) detected at 0, 21 and 28 days compared to Normal-pH_u steaks, and at 7 and 14 days in relation to both Normal-pH_u and High-pH_u groups ($P < 0.05$). In turn, Normal-pH_u and High-pH_u steaks were tenderer during ageing, with High-pH_u steaks having the highest tenderness values observed at end of the experimental period. Consistent with observations for WBSF, the MFI values were lower ($P < 0.05$) for Intermediate-pH_u steaks when compared to the High- and Normal-pH_u steaks from day 0 until the end of the experiment. The MFI values of High- and Normal-pH_u groups did not differ between 0 and 21 days, but High-pH_u steaks showed an accentuated fragmentation ($P < 0.05$) at the 28th day when compared to the Normal-pH_u and Intermediate-pH_u steaks. Both pH_u and storage time had a significant effect ($P < 0.05$) on instrumental tenderness and fragmentation parameters, but the effect of the interaction

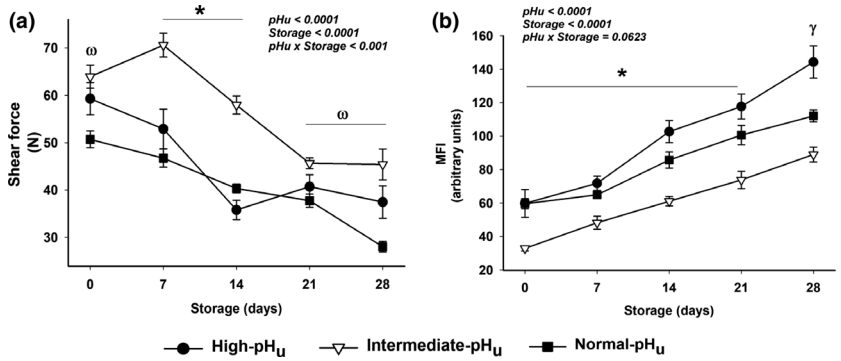

Figure 2 Shear force (N) and myofibrillar fragmentation index of *Longissimus lumborum* Nellore bull steaks stored at 2 °C for 28 days. Ultimate pH groups: Normal-pH_u (≤5.79); Intermediate-pH_u (5.80–6.29); High-pH_u (≥6.30). Values are expressed as mean ± SEM (n = 12). $^{\omega}$($P < 0.05$) Intermediate-pH_u vs. Normal-pH_u; *($P < 0.05$) Intermediate-pH_u vs. High-pH_u and Normal-pH_u; $^{\gamma}$($P < 0.05$) Intermediate-pH_u vs. Normal-pH_u vs. High-pH_u (MFI – 28 days).

between these two factors was significant ($P < 0.05$) only for WBSF, not MFI.

The mechanisms by which tenderisation increased in Nellore bull steaks classified as Normal- and High-pH_u may be related to the functionality and degradation of myofibrillar proteins. Indeed, our results demonstrated that the pH_u regulates tenderisation rate by influencing the degradation of myofibrillar proteins during ageing (Figs 2 and 3). Previously, it was reported that the rapid tenderisation observed in High-pH_u steaks was attributed to the early degradation of filamin, most likely due to the activation of calpain-1 (Lomiwes *et al*., 2014). In turn, tenderisation in Normal-pH_u steaks was associated with the degradation of titin and nebulin most likely by calpain-1 in combination with the extensive degradation of desmin by both calpain-1 and cathepsin activities (Lomiwes *et al*., 2014). Controversially, some studies have disregarded the participation of cathepsin in the meat tenderisation process (Kemp *et al*., 2010), especially because inhibition of cysteine proteinases, like calpain, is associated with a concomitant reduction of myofibrillar protein degradation and tenderisation decreased, without any effect on inhibition of cathepsin (Uytterhaegen *et al*., 1994). Consistent with our findings, High- and Normal-pH_u steaks from Angus bulls tenderised more rapidly when compared to Intermediate-pH_u steaks, which showed a slower tenderisation rate, attaining acceptable tenderness evaluated by shear force at 28 days of *post-mortem* (Contreras-Castillo *et al*., 2016).

Since apoptosis might be involved in the metabolic process related to the conversion of muscle to meat, it was observed that the activation of caspase-3/-7, as an apoptotic signal, was delayed in Intermediate-pH_u ($5.7 < pH_u < 6.3$, values similar to our study) when compared to Normal- and High-pH_u in bull beef (Pulford *et al*., 2009). In our study, the negative impact on tenderness induced by Intermediate-pH_u, evidenced by the combination of increased WBSF and decreased MFI in comparison to the Normal- and High-pH_u in steaks from Nellore bulls may be partially associated with the caspase activation, which is regulated by small heat shock proteins (sHSP). Pulford *et al*. (2009) also reported that in Intermediate-pH_u, sHPS bind onto myofibrillar proteins in response to protein denaturation, protecting them from the enzymatic cleavage. The down-regulation of the apoptotic response mediated by the up-regulation of sHSP in Intermediate-pH_u resulted in a delayed meat tenderisation (Lomiwes *et al*., 2014). Furthermore, down-regulation of HSP-coding genes is associated with improved tenderness (Bernard *et al*., 2007).

Degradation of myofibrillar proteins

As shown in Fig. 3a,b, the degradation pattern of filamin, nebulin, desmin and troponin-T in Nellore bull steaks was influenced by pH_u and ageing period (28 days). It was possible to identify that the High-pH_u steaks exhibited a greater intensity of bands at approximately 250 kDa, most likely associated to intact filamin and its degradation product (F_2) at 48 h *post-mortem* (0 days). This pattern was noticeable in Normal- and Intermediate-pH_u steaks at the 14th and 28th day. The nebulin and its degradation product bands (N_2, above 500 kDa) were clearly detected with greater intensity from 0 to 28 days of ageing in the High- and Normal-pH_u steaks. The pattern of nebulin degradation showed that Intermediate-pH_u was poorly degraded at 14 days and was only degraded at day 28.

Next, we also assessed whether pH_u is associated with degradation of other key proteins involved in the proteolysis process by immunoblotting. Desmin degradation products (50 and 37 kDa) increased with ageing period in all pH_u groups (Fig. 3b). However, Normal-pH_u steaks showed an accentuated degradation when compared to both Intermediate- and High-pH_u steaks as evidenced by the increase in the abundance of bands at 37 kDa from 7th day of storage until the end of the experimental period. In contrast, High-pH_u steaks presented lower or no product band intensity, most likely related to an impaired or advanced desmin degradation during the early stages of ageing. Indeed,

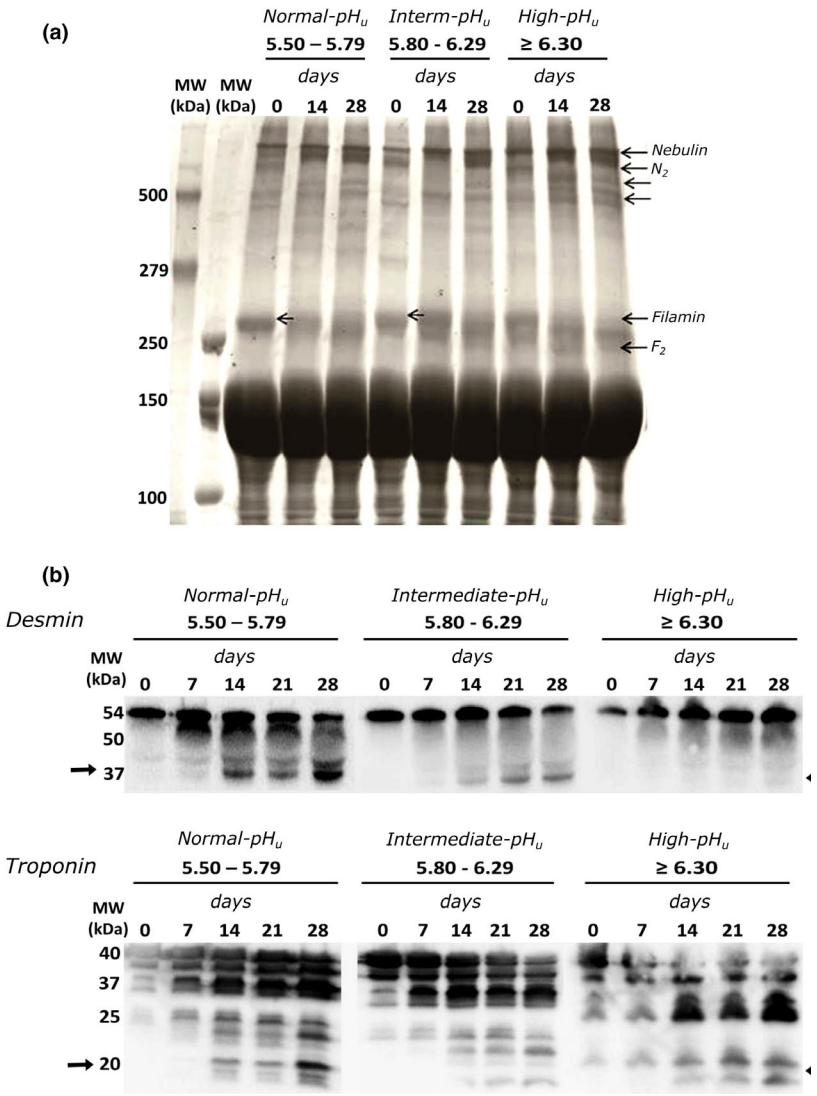

Figure 3 SDS-PAGE and immunoblotting of proteins extracted from sarcoplasmatic fraction of *Longissimus lumborum* Nellore bull steaks stored at 2 °C for 28 days. Ultimate pH groups: Normal-pH_u (≤5.79); Intermediate-pH_u (5.80–6.29); High-pH_u (≥6.30). MW, molecular weight standard (kDa).

Huff Lonergan, *et al*. (2010) reported that desmin is degraded more rapidly in myofibrils from samples with low shear force and higher water-holding capacity, corroborating our results to the High-pH_u group. Intermediate-pH_u samples showed a visible desmin degradation from 14 days of storage but with a low intensity compared to Normal-pH_u steaks.

In turn, the abundance of troponin-T also showed differences among the pH_u groups (Fig. 3b). Normal-pH_u steaks showed the highest intensity of troponin-T degradation product bands (<40 kDa) when compared to High- and Intermediate-pH_u. Troponin-T degradation at ~20 kDa bands was observed with lower intensity in High-pH_u at 0 days (48 h *post-mortem*) while it was not observed for the other groups, corroborating an accelerated proteolysis for this group. Troponin-T was degraded almost completely after 14 days of storage in all pH_u groups.

Our findings suggest that the nature of myofibrillar protein degradation is pH_u-dependent. In agreement with the results of a study by Lomiwes *et al*. (2014), High-pH_u Nellore bull steaks predominately favoured degradation of myofibrillar proteins with larger molecular weights including filamin and nebulin. This degradation probably results in a significant weakening of the thin filament/Z-disk interaction, loss of muscle cell integrity, causing tenderisation of the muscle (Huff Lonergan *et al*., 2010; dos Santos *et al*., 2015). In contrast, Normal-pH_u steaks were tender during ageing due, at least in part, to the faster degradation of myofibrillar proteins with a lower molecular weight including desmin and troponin-T, considered key

substrates for proteolysis in *post-mortem* muscle by calpain-1 (Koohmaraie and Geesink, 2006). The results for Intermediate-pH_u may be associated with an impaired degradation of nebulin but also possibly titin, which occurs around pH 6.0, reducing tenderness. This effect is attributed to a minimal activity of the two major proteolytic enzymes calpain and cathepsin (England *et al*., 2017). As discussed above, for intermediate pH_u, HSPs protect myofibrillar proteins in response to its denaturation, obstructing the enzymatic cleavage by proteases (Pulford *et al*., 2009). In an *in vitro* experimental study, HSP27 restricted the degradation of troponin and desmin in calpain-1- and caspase-3-treated myofibrils extracted from *Longissimus thoracis* muscle from Simmental bulls (Ding *et al*., 2018). These authors reported that HSP27 has the ability to interact directly or indirectly with both proteolytic enzymes, compromising their capacities to facilitate meat tenderisation (Ding *et al*., 2018).

Total and soluble collagen, drip loss and loin steaks pH

The total collagen content in Nellore bull steaks was not influenced by pH_u, (Fig. 4a). When the soluble collagen was evaluated, Intermediate-pH_u steaks showed a reduced content ($P < 0.05$) compared with Normal- and High-pH_u steaks at 0 days; however, after 28 days of storage, pH_u did not have a significant effect on this parameter (Fig. 4b). The differences to collagen solubility seem to be associated, at least in part, with the impaired tenderness observed in the Intermediate-pH_u samples at 0 days. As it is known, intramuscular connective tissue has an important role in the meat quality, and along with intramuscular fat, affects beef tenderness in both Nellore and Angus breeds (Martins *et al*., 2015). Moreover, the collagen concentration, sarcomere length and desmin degradation were the major factors responsible for the variation in *Longissimus* tenderness rating (Wheeler *et al*., 2002). Similar to our results, *Longissimus* muscle from young Nellore bulls showed a negative relation between soluble collagen content and tenderness parameters monitored under different residual feed intake levels (Zorzi *et al*., 2013). The soluble collagen, rather than total collagen, is expected to affect *Longissimus lumborum* tenderness (Warner *et al*., 2010).

One additional consequence associated with pH_u is the ability to influence WHC. We observed that the pH_u did have a significant effect ($P < 0.05$) on drip loss of Nellore bull steaks (Fig. 4c). Specifically, drip loss values from High-pH_u steaks were significantly lower ($P < 0.05$) at 48 h *post-mortem* (0 days). Both Normal- and Intermediate-pH_u steaks showed decreased WHC in Nellore bulls as evidenced by the higher drip loss values. These effects may be related to the isoelectric point (pI) of myofibrillar proteins. During the conversion from muscle to meat, glycogen reserves are used to restore the intracellular ATP levels under anaerobic conditions, accumulating lactate and H^+ and causing the pH decline from 7.2 to 5.5–5.7, similar values to the pI of the main myofibrillar proteins. Since pI is defined as the pH at which a molecule carries almost equal positive and negative charges, protein side groups are attracted to each other, minimising the number of reactive groups available to react with water (Huff-Lonergan & Lonergan, 2005; England *et al*., 2017). In view of that, our findings for Normal-pH_u (<5.7), but also for Intermediate-pH_u (5.8–6.2) Nellore bull steaks, showed higher drip loss. Another plausible mechanism by which pH_u influenced drip loss in Nellore bulls may be linked to the sarcomere length. Indeed, in muscle, drip loss increases with a shortening of sarcomeres (Honikel *et al*., 1986), an event that also induce increased meat toughness (Ertbjerg & Puolanne, 2017). Interestingly, our findings for drip loss and tenderness showed that both High- and Intermediate-pH_u may underlie the sarcomere shortening, probably being a reduced and increased event, respectively. Further studies are required to evaluate the contribution of this hypothesis in Nellore bulls.

Moreover, we examined how loin steak pH would change over the course of ageing (Fig. 4d). The pH_u

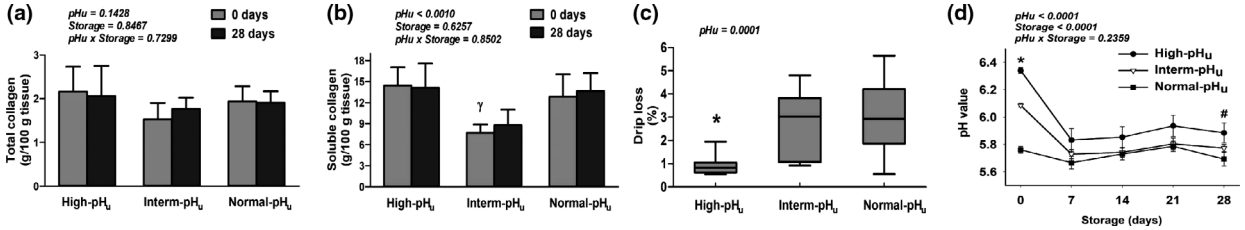

Figure 4 Total and soluble collagen (g/100 g), drip loss (%) and pH of *Longissimus lumborum* Nellore bull steaks stored at 2 °C for 28 days. Ultimate pH groups: Normal-pH_u (≤5.79); Intermediate-pH_u (5.80–6.29); High-pH_u (≥6.30). Values are expressed as mean ± SEM ($n = 12$). *($P < 0.05$) High-pH_u vs. Intermediate- and Normal-pH_u; $^{\gamma}$($P < 0.05$) Intermediate-pH_u vs. Normal- and High-pH_u; $^{\#}$($P < 0.05$) High-pH_u vs. Normal-pH_u.

groups and storage time were shown to have a significant effect ($P < 0.05$) on the stability of steak pH, but no significant difference was found for interaction between both factors. Based on its previous segregation in the different pH_u groups, the pH of the steaks stayed relatively stable according to their assigned group during ageing. The steak pH values in the High-pH_u group were higher ($P < 0.05$) when compared to the Intermediate- and Normal-pH_u groups at 48 h *post-mortem* (day 0). However, a decline in pH was observed after 7 days of storage for all pH_u groups; but only at the end of the experiment was a significant difference ($P < 0.05$) observed between the High- and Normal-pH_u groups. Unexpectedly, High-pH_u steaks reached pH varying between 5.83 and 5.94 after 7 days of storage until the end of the experimental period, but these values remained highest when compared to the Intermediate- and Normal-pH_u groups, which presented a pH varying between 5.73–5.80 and 5.67–5.79, respectively. These findings are most likely caused by muscles having high residual glycogen levels, enough to promote an anaerobic glycolysis, driving further pH decline during the early stages of ageing, as well as by its buffering capacity (Matarneh *et al.*, 2017). After slaughter, the buffering capacity of the muscle is greatly reduced, and the anaerobic carbohydrate metabolism is responsible for the homeostatic response of the resynthesis of ATP. This metabolic perturbation leads to the acidification of the muscle, which depends on the carbohydrate content and structural buffering capacity (Pösö & Puolanne, 2005).

In a comparative breed study, Nellore cattle presented a greater abundance of fast twitch myofilaments in oxidative glycolytic muscle fibres from *L. lumborum* when compared to Angus cattle (Rodrigues *et al.*, 2017). Both glycogen synthesis and glycogenolysis/glycolysis processes are expected to remain metabolically intense in muscle with a greater amount of fast twitch myofilaments (Rodrigues *et al.*, 2017). It has been observed that the 48 h *post-mortem* pH_u was correlated with factors such as glycogen depletion before slaughter (transport farm-slaughter), with consequent lower glycogen concentration at the time of slaughter and lower residual glycogen concentration in the muscle (Immonen *et al.*, 2000). Despite these findings, further studies are required to unravel the regulatory mechanisms that modulate energetic status, focusing on the glycogenolytic/glycolytic pathways that occur in the conversion of muscle to meat, especially during the early stages of ageing in Nellore bulls classified by pH_u.

Conclusions

This study provides novel evidence for the pH_u segregation in three different broad ranges on meat quality of *B. indicus*-based Brazilian production system. These observations revealed that pH_u influences meat quality from Nellore bulls during ageing. Specifically, High-pH_u steaks exhibited impaired colour stability, making them darker compared to other pH_u groups. In turn, both High- and Normal-pH_u steaks showed tenderness and myofibrillar fragmentation improved due to proteolysis. Intermediate-pH_u steaks were associated with a lower and inconsistent meat tenderness and decreased collagen solubility. Furthermore, Normal- and Intermediate-pH_u steaks were associated with decreased WHC as evidenced by drip loss. These findings bring strong support to the notion that pH_u plays an essential role as a critical point of control for beef quality from Nellore bulls during *post-mortem* ageing, which can be adopted by the Brazilian meat industry.

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Conflict of interest

The authors declare that they do not have any conflict of interest before, during and after the development of this study.

Author contributions

Clara Lucía Contreras Barón: Investigation (equal); Methodology (equal). **Priscila Robertina dos Santos-Donado:** Conceptualization (equal); Formal analysis (lead); Investigation (equal); Visualization (lead); Writing-original draft (lead); Writing-review & editing (lead). **Patricia Maloso Ramos:** Investigation (equal); Methodology (equal); Writing-review & editing (supporting). **Carlos M. Donado-Pestana:** Formal analysis (equal); Writing-original draft (equal); Writing-review & editing (equal). **Eduardo Francisquine Delgado:** Conceptualization (equal); Supervision (equal); Writing-review & editing (supporting). **Carmen Josefina Contreras-Castillo:** Conceptualization (equal); Funding acquisition (lead); Project administration (lead); Supervision (lead); Writing-review & editing (equal).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Occurrence of different pH_u (24 h *post-mortem* at *Longissimus lumborum*) classified into three groups: Normal-pH_u (≤5.79), Intermediate-pH_u (5.80–6.29) and High-pH_u (≥6.30) from *Bos taurus indicus* bulls in a commercial slaughterhouse in Brazil.