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# Assessment of the quality of refrigerated and frozen pork by multivariate exploratory techniques

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ABSTRACT

Pork loin and leg were evaluated 24 h after slaughter and during refrigerated (5 °C) and frozen storage for microbial counts, pH, total volatile base nitrogen (TVB-N), thiobarbituric acid reactive substances (TBARS) and bioactive amines. Spermine was the prevalent amine in fresh pork loin and leg, followed by spermidine and agmatine. During refrigerated storage, pH, TVB-N, mesophilic and psychrotrophic counts increased and no changes ($p < 0.05$) were observed on polyamines; however putrescine, cadaverine and histamine were produced and accumulated throughout storage. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) for all parameters indicated a shelf life of 8 days for both cuts, which was also coherent with safety regarding histamine levels. During frozen storage, there was no change on amines and pH, TVB-N decreased, and TBARS increased. None biogenic amine was produced. PCA and HCA were not able to classify frozen pork based on the analyzed parameters; however, a shelf life of 90 days was suggested for the frozen cuts based on lipid oxidation.

## 1. Introduction

Pork is one of the most widely eaten meat in the world with an estimated world human consumption of 12.43 kg per capita (OECD/FAO, 2017). Meat is an important source of protein and micronutrients, such as iron, zinc and vitamins B (Gratacós-Cubarsí et al., 2013). However, due to its composition, water activity and pH values, which favor microbial growth, meat is recognized as one of the most perishable foods. Therefore, the quality and safety of meat are of much concern to consumers, regulatory agencies and food industries (Liu, Gou, & Li, 2006; Ercolini, Russo, Nasi, Ferranti, & Villani, 2009).

Refrigerated and frozen storage are widely used to delay chemical and biochemical changes and to increase shelf life of meat and meat products. The main changes are related to microbial growth, which can result in spoilage. Furthermore, there can be degradation of meat nutrients, such as sugars and free amino acids, and the release of undesired metabolites, such as amines, aldehydes, and sulfur compounds. All of these compounds can affect the quality and safety of meat (Ercolini et al., 2009; Kuley, Balikci, Özoğul, Gökdogan, & Özoğul, 2012). Since microbial analysis is time-consuming, other parameters associated with chemical changes have been suggested as quality indicators of meat, such as pH, total volatile base nitrogen, sulfhydric gas production and biogenic amines contents (Custodio, Theodoro, & Gloria, 2016; Galgano, Favati, Bonadio, Lorusso, & Romano, 2009; Huang, Zhao, Chen, & Zhang, 2014; Jordan et al., 2009; Nguyen & Nguyen, 2015; Salinas et al., 2014).

Biogenic amines are, along with polyamines, constituents of the bioactive amines' family. The polyamines – spermine and spermidine – are naturally present in meats. Biogenic amines, however, can be naturally present in some tissues, but can also be formed by decarboxylation of free amino acids due to microbial and autolytic protease and decarboxylase activities. In this way, biogenic amines, along with

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polyamines have been proposed as useful indicators of spoilage in several foods (Custodio et al., 2016; Galgano et al., 2009; Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997; Mietz & Karmas, 1977; Silva & Gloria, 2002; Vinci & Antonelli, 2002). Histamine has been considered by the EC Commission Regulation 2073/2005 as a criterion for the safety of some fish species. It has also been adopted by regulatory agencies from several countries and communities (Evangelista et al., 2016). A bioactive amines index – BAI (calculated as (cadaverine + histamine + putrescine) / (1 + spermine + spermidine)) was proposed by Mietz and Karmas (1977) as a freshness index for canned fish. Hernández-Jover et al. (1997) proposed a BAI for pork freshness based on the sum of cadaverine, putrescine, tyramine and histamine; whereas Silva and Gloria (2002) suggested an index based on the ratio of spermidine/spermine contents for the evaluation of chicken meat quality. The availability of reliable parameters to follow meat quality during refrigerated and frozen storage would be important to assess quality, safety and shelf life.

Since no comprehensive study on the shelf life of both frozen and refrigerated pork meat is available in the literature, the objective of this study was to investigate the changes that occur in fresh pork cuts (loin and leg) during refrigerated and frozen storage, including microbial counts, physicochemical characteristics, bioactive amines contents and lipid oxidation. Additionally, indexes of quality based on bioactive amines were calculated. Furthermore, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied to all obtained data to describe the main characteristics attributed to refrigerated and frozen pork during storage.

## 2. Material and methods

### 2.1. Chemicals

All of the reagents used were of analytical grade, except HPLC solvents, which were chromatographic grade. The organic and aqueous solvents were filtered through 0.45 μm membranes, HAWP and HVWP, respectively (Millipore Corp., Milford, MA, USA). Ultrapure water was obtained from Milli-Q Plus System (Millipore Corp., Milford, MA, USA). Spermine (SPM, tetrahydrochloride), spermidine (SPD, trihydrochoride), putrescine (PUT, dihydrochloride), cadaverine (CAD, dihydrochloride), tyramine (TYM, hydrochloride), histamine (HIM, dihydrochloride), agmatine (AGM, sulphate), serotonin (SRT, hydrochloride), phenylethylamine (PHM, hydrochloride), tryptamine (TRM, free base), and *o*-phthalaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. Experimental design

Pork ($n = 6$) of six months' old commercial crossbred male pigs raised under the same conditions were randomly collected 24 h *post-mortem* from a slaughterhouse located in Belo Horizonte, state of Minas Gerais, Brazil. The slaughterhouse operated under typical industry conditions at the auspices of federal inspection. Two cuts were selected from a half carcass of each pig: loin (Longissimus thoracis et lumborum) and leg (Biceps femoris, Semimembranosus, and Semitendinosus). The samples were weighed, packaged individually and transported to the laboratories under refrigerated conditions.

Each loin and leg was divided into nine parts under aseptic conditions in the Food Microbiology Laboratory of Instituto Octávio Magalhães (IOM/FUNED). Each part was portioned in longitudinal cuts (50 mm depth, ~100 g each). Leg samples included randomly the three muscles and were as similar as possible. Cuts were representative of the product commercially available for the consumer. They were wrapped individually in 0.15 μm polyvinyl chloride (PVC) film to simulate household storage. One sample of each cut was analyzed immediately (24 h after slaughter) and the other samples were stored in a refrigerator at 5 ± 1 °C for up to 16 days or in a freezer at −18 ± 1 °C for up to 180 days. The refrigerated and frozen samples were taken at 4 and 45-days intervals, respectively. Refrigerated samples were analyzed for microbial counts, physicochemical characteristics and bioactive amines; whereas frozen samples were analyzed for physicochemical characteristics, bioactive amines, and lipid oxidation.

Two factorial designs were used (refrigerated and frozen storage) with two factors (meat - loin and leg, and storage time) and one block (six different animals). Data were submitted to analysis of variance for normality (Shapiro Wilks) and for homogeneity of variances (F test). Data with normality deviations or heterogeneity of variances were transformed into log10(x + 1). Then, the data were submitted to analysis of variance (one-way ANOVA) and the means were compared by the Tukey test at 95% significance ($p < 0.05$). Correlations among the levels of amines and the microbial and physicochemical characteristics of the pork were determined by Pearson's correlation ($p < 0.01$).

### 2.3. Methods of analysis

#### 2.3.1. Determination of microbial counts

Fresh and refrigerated pork samples were analyzed for coliforms at 35 °C and at 45 °C, mesophilic bacteria and psychrotrophic bacteria according to the American Public Health Association – APHA (Downes & Ito, 2001).

Prior to analysis, the sample packages were disinfected with 70% ethanol and were opened under aseptic conditions. Cuts of about 1 cm depth and 2 cm length were taken at various points up to a total of 25 g. The samples were homogenized in a stomacher (Lab-blender, Seward, London, UK) for 60 s in 225 mL sterile 1% buffered peptone water. Serial decimal dilutions were made and the samples were analyzed for coliforms at 35 °C and at 45 °C, aerobic mesophilic counts and psychrophilic counts at 17 °C.

For the enumeration of coliforms, the most probable number (MPN) procedure was used. Briefly, three replicate tubes of lauryl tryptose (LST) broth per dilution were used. The tubes were incubated at 35 ± 2 °C for 48 h. Gas production was considered presumptive-positive of coliform. Subculture of all positive LST tubes into EC broth was done followed by incubation in a circulating water bath at 45.5 ± 0.2 °C for 48 h.

Total viable count by pour plate on Plate Count Agar (PCA, Oxoid) was used for general enumeration of mesophilic (aerobic incubation at 35 ± 2 °C for 48 h) and psychrotrophic (aerobic incubation at 17 ± 2 °C for 16 h followed by 3 days at 7 ± 2 °C) bacteria. The microbial counts were expressed as logarithmic colony forming units (log CFU) per gram.

#### 2.3.2. Determination of physicochemical characteristics

All samples were analyzed for pH and total volatile base nitrogen (TVB-N). Frozen and fresh pork (24 h after slaughter) were also analyzed for thiobarbituric acid-reactive substances (TBARS) assay. Frozen samples were thawed under refrigeration (5 ± 1 °C) for up to 12 h. Prior to analysis, the samples were ground in a food processor and homogenized thoroughly.

A digital pH meter was used for pH measurements of a suspension of 10 g pork in 100 mL ultrapure water according to AOAC (2000). TVB-N was estimated by titration after steam distillation and alkalization with MgO, and expressed as mg N/100 g (Huang et al., 2014).

TBARS was determined by a spectrophotometric method (Rosmini et al., 1996). Ten grams aliquots of samples were homogenized with 15 mL of 15% trichloroacetic acid (TCA) for 5 min in an orbital shaker (TE Tecnal - 140, Piracicaba, SP, Brazil), centrifuged at 11,180 × *g* at 4 °C for 10 min, and the supernatant was collected. The solid residue was submitted to two additional extractions with 15 mL of 0.25 M HCl. The supernatants were combined, filtered through qualitative filter paper and the volume was brought up to 50 mL. Then, 3 mL of stock solution (0.25 M HCl, 15% TCA and 0.375% 2-thiobarbituric acid) was added to 7 mL filtrate. The mixture was stirred again during 5 min and

heated in a boiling water bath. Pink color formation was measured spectrophotometrically (UV-160A spectrophotometer, Shimadzu, Kyoto, Japan) at 535 nm. Malondialdehyde concentration was calculated using the molar absorption coefficient ($1.56 \times 10^5$/M.cm), and the results were expressed in mg of malondialdehyde/kg of pork sample.

#### 2.3.3. Determination of bioactive amines

The amines were separated by ion-pair reverse phase HPLC and quantified fluorometrically after post-column derivatization with *o*-phthalaldehyde - OPA (Custodio et al., 2016). The bioactive amines were extracted from 5 g pork samples with 7 mL of trichloroacetic acid (5% TCA). The samples were homogenized for 10 min, centrifuged at 11,180 × *g* at 4 °C for 21 min, and the supernatant was collected. The solid residue was extracted two additional times with 7 mL TCA and the supernatants were combined, and filtered through 0.45 μm HAWP pore size membrane (Millipore Corp., Milford, MA, USA) prior to HPLC analysis. A Shimadzu LC-10AD with SIL-10AD VP automatic injector (Shimadzu, Kyoto, Japan) connected to a RF-551 spectrofluorometric detector was used, and the bioactive amines were separated using a Novapak C18 column (3.9 × 300 mm, 4 μm, 60 Å, Waters, MA, USA) and a gradient elution of 0.2 mol/L sodium acetate and 15 mmol/L sodium octanesulfonate with pH adjusted to 4.9 (mobile phase A) and acetonitrile (mobile phase B). The amines were identified by comparison of retention times and co-elution with authentic standards. Detection and quantification were carried out by fluorimetry ($\lambda_{excitation}$ = 340 nm and $\lambda_{emission}$ = 445 nm), after post-column derivatization with OPA, by using analytical curves for each amine ($r^2 > 0.9965$). The results were expressed in mg/kg of pork sample.

### 2.4. Calculation of quality indexes based on bioactive amines

The contents of bioactive amines in the pork samples were used to calculate the following indexes: (i) H-J = [histamine + putrescine + cadaverine + tyramine] (Hernández-Jover et al., 1997); (ii) M&K = [(histamine + putrescine + cadaverine)/(1 + spermidine + spermine)] (Mietz & Karmas, 1977); and (iii) S&G = (spermidine/spermine) (Silva & Gloria, 2002). The adequacy of these indexes to evaluate the quality of pork during refrigerated and frozen storage was investigated.

### 2.5. Principal Component Analysis and Hierarchical Cluster Analysis

Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were used for the characterization of both pork cuts (loin and leg) during the storage days at different conditions (refrigerated and frozen), using Statistica 7.0. In the PCA during refrigerated storage, pH, TVB-N, contents of spermine, spermidine, agmatine, putrescine, cadaverine, histamine, sum of total bioactive amines, sum of total biogenic amines, sum of total polyamines and microbial counts were used as active variables in the derivation of the principal components, and the supplementary variable (quality indexes) was projected onto the factor space. During frozen storage, pH, TVB-N, TBARS, contents of spermine, spermidine, agmatine and sum of total bioactive amines, of total biogenic amines (putrescine + cadaverine + histamine) and of total polyamines (spermidine + spermine) were used as active variables in the derivation of the principal components.

In both cases, PCA analysis was performed using the covariance matrix. For HCA, the hierarchical tree was obtained considering the same active variables applied to PCA and the storage days at different conditions were joined by unweighed pair-group average as the linkage rule, considering the Euclidian distances as the coefficient of similarity.

## 3. Results and discussion

### 3.1. Microbial and physicochemical characteristics and bioactive amines in fresh pork

Mean ± standard error of the mean and the range of mesophilic bacteria counts in fresh pork (24 h after slaughter) were 3.90 ± 0.12 (3.52–4.31) log CFU/g for loin and 4.01 ± 0.17 (3.39–4.80) log CFU/g for leg; whereas mean psychrotrophic bacteria counts were 4.14 ± 0.22 (3.52–4.89) log CFU/g for loin and 4.31 ± 0.12 (3.92–4.68) log CFU/g for leg. The mesophilic counts are similar to values reported for pork (Li, Tian, et al., 2014; Nguyen & Nguyen, 2015). However, the psychrotrophic counts in this study are higher compared to results from Liu, Gou, and Li (2006) and Liu, Yang and Li (2006) in pork shoulders 12 h after slaughter.

Additionally, there were low counts of coliforms at 35 °C and 50% of the samples were positive for coliforms at 45 °C, with counts lower than 23 MPN/g and no confirmation for *Escherichia coli*. Therefore, microbial counts 24 h after slaughter were in accordance with Brazilian legislation (Brasil, 2001).

Concerning the physicochemical characteristics of fresh pork cuts, they were characterized by pH values ranging from 5.5 to 6.0 for loin and from 5.7 to 6.4 for leg. TVB-N contents ranged from 15.4 to 32.9 mg N/100 g for loin and from 9.0 to 29.5 mg N/100 g for leg. TBARS values ranged from 0.08 to 0.30 mg malondialdehyde/kg for loin and from 0.03 to 0.32 mg malonaldehyde/kg for leg. These values are similar to literature reports (Livingston, Brewer, Killifer, Bidner, & McKeith, 2004; Ruiz-Capillas & Jiménez-Colmenero, 2004; Athayde et al., 2012; Li, Tian, et al., 2014; Huang et al., 2014; Nguyen & Nguyen, 2015).

Among the ten bioactive amines investigated, three were detected in pork 24 h after slaughter: spermine, spermidine and agmatine. Total bioactive amines levels ranged from 13.98 to 63.80 mg/kg in loins and from 31.01 to 42.70 mg/kg in legs. Spermine was the prevalent amine (91% of the total amines content for both cuts), followed by spermidine (6 to 7%), and agmatine (2 to 3%). Biogenic amines were not detected in any of the samples 24 h after slaughter. According to the literature, spermine is the predominant polyamine in animal tissues, including pork, followed by spermidine. The levels of spermine ranged from 12.21 to 58.24 mg/kg in loins and from 26.80 to 38.96 mg/kg in legs; whereas the levels of spermidine ranged from 1.21 to 3.67 mg/kg in loins and 1.53 to 3.03 mg/kg in legs. These polyamines are essential factors for cell proliferation and differentiation and for other relevant functions of normal cells (Favaro, Pastore, Saccani, & Cavalli, 2007; Paulsen & Bauer, 2007; Krausová, Kalac, Krizek, & Pelikánová, 2008; Jastrzebska, 2012; Li, Feng, et al., 2014).

The levels of agmatine in the samples varied from not detected to 3.00 mg/kg in loins and from not detected to 1.56 mg/kg in legs. Agmatine is an alternative intermediate precursor in the formation of the polyamines from decarboxylation of arginine (Gloria, 2006; Kalac, 2014). Only few studies investigated agmatine in pork and reported contradictory results. Ruiz-Capillas and Jiménez-Colmenero (2004) reported agmatine in fresh and pickled loin (2.08 mg/kg); however, Favaro et al. (2007) did not find agmatine in pork *Longissimus dorsi* muscle. Interestingly, the coefficients of variation among the levels of agmatine were high (171 and 35% for loin and leg, respectively), suggesting that its content may be affected by individual health status, which deserves further investigation.

### 3.2. Influence of refrigerated storage on the microbial and physicochemical characteristics and bioactive amines contents of pork

The bacterial counts of the meat cuts showed an upward trend during refrigerated storage (Fig. 1). There was no significant difference on the mesophilic, psychrotrophic and coliforms at 35 °C counts between the two cuts. There was a significant effect ($p < 0.05$) of storage

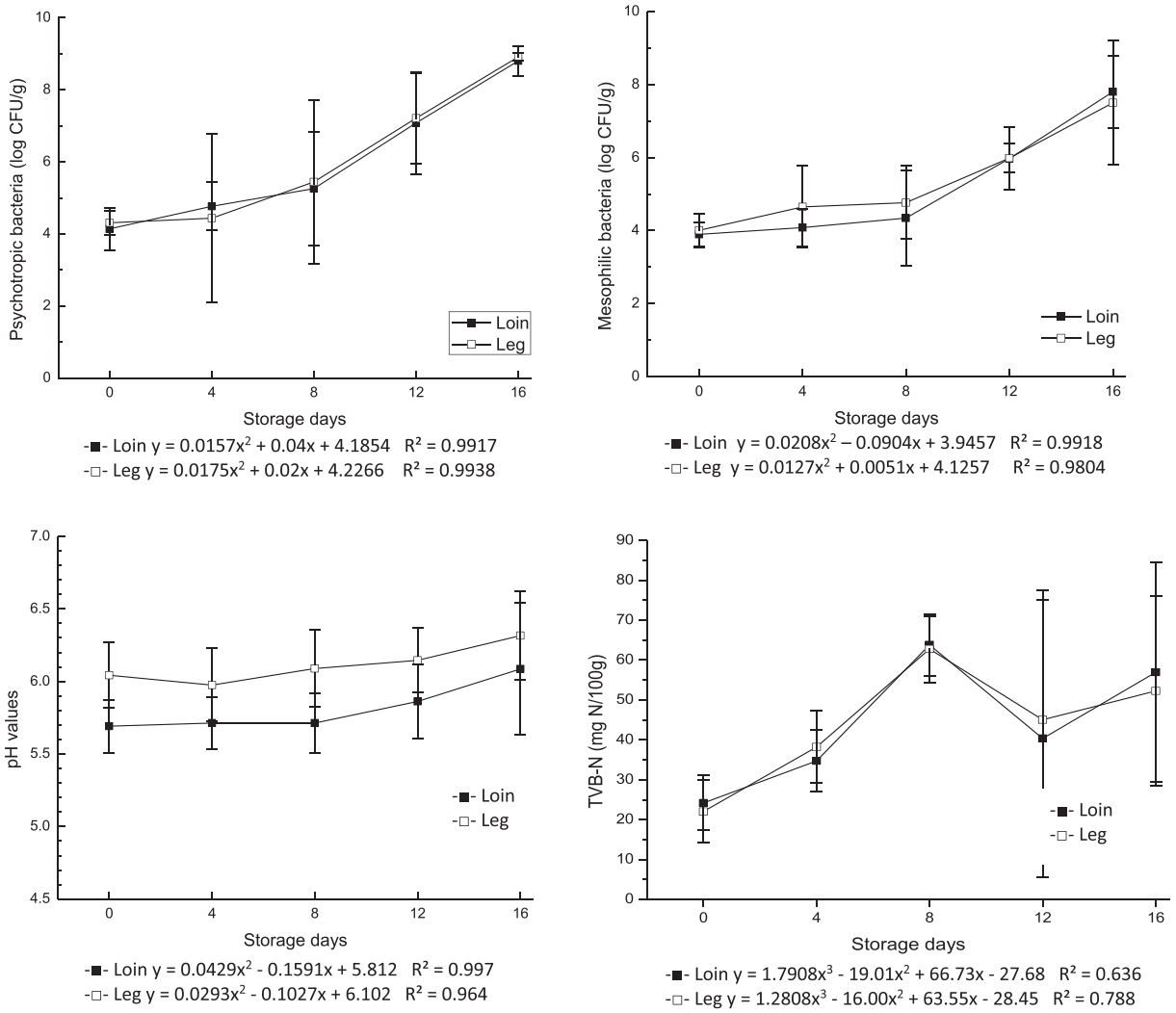


**Fig. 1.** Changes on microbial counts and physico-chemical characteristics – pH and TVB-N – of pork meat (loin -■- and leg -□-) during refrigerated storage (5 ± 1 °C) for 16 days.

time and carcasses (block) for psychrotrophic microflora, but no effect of the pork cut. In general, psychrotrophic counts were significantly higher (Tukey test, $p < 0.05$) than mesophilic during refrigerated storage, which is expected since temperature is an important factor for the selection and development of microorganisms on meat (Liu, Yang, & Li, 2006; Ercolini et al., 2009).

The counts of coliforms at 35 °C during refrigerated storage were low during the whole period and no significant difference was observed throughout storage. Coliforms at 45 °C were detected sporadically at low counts (< 240 MPN/g) in both cuts throughout storage Analysis of variance ($p < 0.05$) indicated that the counts of mesophilic and psychrotrophic bacteria were significantly higher on the 12th and 16th days of storage compared to initial counts.

Regarding physicochemical characteristics, pH values increased significantly in the meat cuts during refrigerated storage, following a second degree polynomial – quadratic model ($R^2 \geq 0.9642$) (Fig. 1). Similar pH increase has been reported in the literature (Bover-Cid, Hernández-Jover, Miguélez-Arrizado, & Vidal-Carou, 2003; Li, Tian, et al., 2014), and can be due to volatile bases production (ammonia and amines) by microorganisms, mainly psychrotrophic bacteria (Liu, Yang, & Li, 2006; Huang et al., 2014). In fact, psychrotrophic and mesophilic counts also followed a second degree polynomial (Fig. 1). Furthermore, an association between pH values and psychrotrophic and mesophilic counts was observed for both pork loin ($r = 0.9563$ and $r = 0.9071$, respectively) and leg (positive correlation, $r = 0.9392$ and $r = 0.8750$, respectively). TVB-N levels increased significantly, for both pork cuts, up to the 8th storage day.

Total levels of bioactive amines (Table 1) remained unchanged until the 12th day for both cuts ($p > 0.05$); however, on the 16th storage day, total levels reached values higher than 70 mg/kg in loin and 120 mg/kg in leg. Similar results were observed for total biogenic amines (putrescine + cadaverine + histamine), reaching values higher than 30 and 70 mg/kg in loin and leg, respectively. No significant change on spermine levels was observed throughout storage for both pork cuts. Similar results were reported in the literature (Paulsen & Bauer, 2007; Krausová et al., 2008; Jastrzebska, 2012; Li, Feng, et al., 2014). No significant change was also observed for total polyamines (spermidine + spermine). The contents of biogenic amines were negligible in fresh pork cuts; however, on the 4th day of storage, cadaverine was detected with levels increasing significantly up to the 16th storage day. Putrescine and histamine were the next biogenic amines produced; they were detected at low levels from the 12th storage day on with a slight increase in pork loins and a more intense increase in legs up to the 16th day of refrigerated storage (Table 1). No detectable levels of the biogenic amines tyramine, phenylethylamine, tryptamine and serotonin were found in any sample.

The physicochemical characteristics and the microbial counts could support the accumulation of biogenic amine. pH values showed positive correlation with psychrotrophic bacteria counts ($r = 0.9563$ and 0.9392 for loin and leg, respectively) and biogenic amines ($r = 0.9784$

**Table 1**
Bioactive amines in pork loin and leg during refrigerated storage (5 ± 1 °C) for 16 days.

| Storage (days) | Mean levels of bioactive amines ± standard error (mg/kg)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Spermine | Spermidine | Agmatine | Putrescine | Cadaverine | Histamine | PA** | BA** | Total |
| Loin | | | | | | | | | |
| 0 | 30.28 ± 5.80$^{b,x}$ | 2.20 ± 0.34$^{a,x}$ | 0.73 ± 0.39$^{a,x}$ | 0.00$^{b,x}$ | 0.00$^{b,x}$ | 0.00$^{a,x}$ | 32.48 ± 6.04$^{a,b,x}$ | 0.00$^{b,x}$ | 33.21 ± 6.37$^{b,x}$ |
| 4 | 16.72 ± 2.33$^{b,y}$ | 4.07 ± 1.81$^{a,x}$ | 0.00$^{b,x}$ | 0.00$^{b,x}$ | 1.26 ± 0.30$^{b,y}$ | 0.00$^{a,x}$ | 20.79 ± 3.61$^{a,b,y}$ | 1.26 ± 0.30$^{b,y}$ | 22.05 ± 3.80$^{b,y}$ |
| 8 | 19.44 ± 3.59$^{a,x}$ | 1.03 ± 0.17$^{a,x}$ | 0.00$^{b,x}$ | 0.00$^{b,x}$ | 2.41 ± 0.20$^{b,x}$ | 0.00$^{a,x}$ | 20.47 ± 3.60$^{b,x}$ | 2.41 ± 0.20$^{b,x}$ | 22.88 ± 3.65$^{b,x}$ |
| 12 | 26.52 ± 6.77$^{a,y}$ | 8.93 ± 7.10$^{a,x}$ | 0.00$^{b,x}$ | 1.40 ± 1.31$^{b,x}$ | 3.29 ± 1.29$^{b,x}$ | 3.65 ± 3.48$^{a,x}$ | 35.45 ± 11.90$^{a,b,x}$ | 8.34 ± 5.98$^{b,x}$ | 43.79 ± 17.44$^{a,b,x}$ |
| 16 | 33.63 ± 5.63$^{a,x}$ | 6.26 ± 3.20$^{a,x}$ | 0.00$^{b,y}$ | 4.65 ± 1.49$^{a,y}$ | 20.18 ± 5.22$^{a,x}$ | 6.69 ± 4.41$^{a,y}$ | 39.89 ± 5.87$^{a,x}$ | 31.52 ± 9.47$^{a,y}$ | 71.41 ± 11.43$^{a,y}$ |
| Leg | | | | | | | | | |
| 0 | 33.71 ± 1.60$^{a,b,x}$ | 2.24 ± 0.20$^{a,x}$ | 1.07 ± 0.15$^{a,x}$ | 0.00$^{b,x}$ | 0.00$^{b,x}$ | 0.00$^{b,x}$ | 35.95 ± 1.57$^{a,b,x}$ | 0.00$^{b,x}$ | 37.01 ± 1.62$^{b,x}$ |
| 4 | 27.06 ± 2.61$^{b,x}$ | 1.64 ± 0.25$^{a,b,x}$ | 0.00$^{b,x}$ | 0.00$^{b,x}$ | 2.13 ± 0.38$^{b,x}$ | 0.00$^{b,x}$ | 28.70 ± 2.65$^{b,x}$ | 2.13 ± 0.38$^{b,x}$ | 30.82 ± 2.87$^{b,x}$ |
| 8 | 24.02 ± 3.00$^{b,x}$ | 1.04 ± 0.09$^{b,c,x}$ | 0.00$^{b,x}$ | 0.00$^{b,x}$ | 1.85 ± 0.41$^{b,x}$ | 0.00$^{b,x}$ | 25.06 ± 2.96$^{b,x}$ | 1.85 ± 0.41$^{b,x}$ | 26.95 ± 2.73$^{b,x}$ |
| 12 | 45.52 ± 6.72$^{a,x}$ | 2.11 ± 0.28$^{a,x}$ | 0.00$^{b,x}$ | 3.73 ± 2.31$^{b,x}$ | 6.98 ± 2.65$^{b,x}$ | 8.55 ± 5.21$^{b,x}$ | 47.62 ± 6.95$^{a,x}$ | 19.27 ± 9.45$^{b,x}$ | 66.89 ± 14.40$^{b,x}$ |
| 16 | 48.43 ± 11.83$^{a,x}$ | 0.77 ± 0.29$^{c,y}$ | 1.52 ± 0.72$^{a,x}$ | 14.60 ± 4.81$^{a,x}$ | 27.17 ± 8.96$^{a,x}$ | 30.89 ± 14.51$^{a,x}$ | 49.20 ± 12.01$^{a,x}$ | 72.66 ± 20.46$^{a,x}$ | 123.4 ± 29.70$^{a,x}$ |

PA = polyamines (spermine + spermidine); BA = biogenic amines (putrescine + cadaverine + histamine).
* Mean ± standard error of the mean (calculated considering < LOD = zero) with different letters for each amine at different storage times (a–c) or for different pork cuts (x–y) indicate significant differences (Tukey test, $p < 0.05$). Limit of detection (LOD) = 0.08 mg/kg, except for histamine (0.15 mg/kg). $n = 6$ samples.

for loin). A positive correlation between the levels of biogenic amines and mesophilic counts was also observed for both pork cuts ($r = 0.9150$ and 0.9290 for loin and leg, respectively), but not for psychrotrophic bacteria counts in leg.

Among amines capable of causing adverse effects to human health, the presence of histamine was detected in both cuts on the 12th day and levels reached 98 mg/kg on the 16th storage day in one leg sample. According to EFSA (2011), the presence of histamine in food, even at low levels, is enough to cause adverse effects to individuals with histamine intolerance; whereas the no-observed-adverse-effect level for healthy individuals is 25 to 50 mg per person per meal. Such high level of histamine detected in pork leg on the 16th storage day could, therefore, cause harmful effects to human health with symptoms, such as abdominal cramps, diarrhea, vomiting, itching and urticaria (EFSA, 2011; Gloria, 2006). Furthermore, the potentiating effect of some bioactive amines (putrescine and cadaverine) on histamine toxicity should be considered (Taylor & Eitenmiller, 1986).

### 3.3. Influence of frozen storage on physicochemical characteristics and bioactive amines levels of pork

There was no significant change on pH values (Table 2) and TVB-N values decreased in pork loins and legs throughout frozen storage (−18 ± 1 °C) for 180 days. TBARS values increased significantly on the 135th storage day in both cuts. In fact, lipid oxidation is an important issue during frozen storage. Since consumers are likely to detect rancidity and off-flavors in meat products at TBARS values above a threshold of ca. 0.5 mg malondialdehyde/kg (Campo et al., 2006; Díaz, Nieto, Garrido, & Bañón, 2008) and both pork cuts reached these limits on the 135th storage day, meat acceptability could be affected from this day on.

The changes on the levels of amines during frozen storage are indicated in Table 2. The levels of polyamines varied during storage. Spermidine levels decreased up to 90 storage days for loin and 45 days for leg, but recovered original levels at 135 and 90 days (loin and leg, respectively). Spermine levels, however, changed without following a pattern in both cuts. Changes in polyamines could be associated with residual enzyme activity from S-adenosylmethionine and also, possibly, from hydrolysis of conjugated polyamines (Custodio et al., 2016; Gloria, 2006; Kalac, 2014). The biogenic amines putrescine, cadaverine and histamine, which were found during refrigerated storage, were not produced and did not accumulate throughout frozen storage.

### 3.4. Quality index based on bioactive amines for quality assessment of pork during refrigerated and frozen storage

The calculated bioactive amines indexes for refrigerated pork cuts are indicated on Table 3. The index proposed by Silva and Gloria (2002) was not adequate for both pork cuts as no significant difference was observed throughout storage; whereas indexes by Mietz and Karmas (1977) and Hernández-Jover et al. (1997) only showed significant differences after the 12th storage day for both pork cuts, when meat quality was already compromised regarding microbial counts (Fig. 1). Furthermore, it was not possible to establish limits to define gradual quality changes due to the large variation among samples. Based on these results, none of the reported indexes of quality based on bioactive amines could be successfully applied in the assessment of pork quality during refrigerated storage.

Since no biogenic amine was produced during frozen storage and the levels of polyamines did not follow a consistent trend, the calculation of a quality index based on amines was not possible.

**Table 2**
Physicochemical characteristics and bioactive amines contents of pork loin and leg during frozen storage (− 18 ± 1 °C) for 180 days.

| Storage time (days) | Physicochemical parameters | | | Bioactive amines (mg/kg) | | | |
|---|---|---|---|---|---|---|---|
| | pH | TVB-N (mg N/100 g) | TBARS (mg/kg) | Spermine | Spermidine | Agmatine | Total |
| Loin | | | | | | | |
| 0 | 5.69 ± 0.07$^{a,y}$ | 24.17 ± 2.82$^{a,x}$ | 0.16 ± 0.02$^{b,x}$ | 30.28 ± 5.80$^{a,b,x}$ | 2.20 ± 0.34$^{a,x}$ | 0.73 ± 0.39$^{a,x}$ | 33.21 ± 6.37$^{b,x}$ |
| 45 | 5.71 ± 0.09$^{a,y}$ | 9.65 ± 3.58$^{b,c,x}$ | 0.20 ± 0.04$^{a,b,x}$ | 10.83 ± 1.85$^{b,x}$ | 0.18 ± 0.18$^{c,x}$ | 0.00$^{b,x}$ | 11.01 ± 1.90$^{c,x}$ |
| 90 | 5.69 ± 0.09$^{a,y}$ | 10.50 ± 2.09$^{b,x}$ | 0.03 ± 0.02$^{b,y}$ | 46.28 ± 9.63$^{a,x}$ | 1.17 ± 0.39$^{b,x}$ | 0.00$^{b,x}$ | 47.55 ± 9.50$^{a,x}$ |
| 135 | 5.66 ± 0.08$^{a,x}$ | 2.27 ± 1.15$^{d,x}$ | 0.87 ± 0.42$^{a,x}$ | 43.34 ± 5.01$^{a,x}$ | 2.12 ± 0.23$^{a}$ | 0.00$^{b,x}$ | 45.46 ± 5.18$^{a,x}$ |
| 180 | 5.73 ± 0.07$^{a,y}$ | 3.58 ± 1.35$^{c,d,x}$ | 0.52 ± 0.31$^{a,b,x}$ | 22.15 ± 7.14$^{b,x}$ | 1.62 ± 0.14$^{a,b}$ | 0.00$^{b,x}$ | 23.91 ± 7.28$^{b,c,x}$ |
| Leg | | | | | | | |
| 0 | 6.04 ± 0.09$^{a,x}$ | 22.04 ± 3.18$^{a,x}$ | 0.17 ± 0.04$^{c,x}$ | 33.71 ± 1.60$^{b,y}$ | 2.24 ± 0.20$^{a,x}$ | 1.07 ± 0.15$^{a,x}$ | 37.01 ± 0.16$^{b,y}$ |
| 45 | 6.09 ± 0.10$^{a,x}$ | 8.73 ± 3.59$^{b,x}$ | 0.31 ± 0.08$^{b,c,x}$ | 23.86 ± 4.85$^{c,y}$ | 0.94 ± 0.16$^{b,x}$ | 0.00$^{b,x}$ | 24.80 ± 5.03$^{c,y}$ |
| 90 | 5.97 ± 0.07$^{a,b,x}$ | 7.13 ± 3.05$^{b,x}$ | 0.10 ± 0.03$^{c,x}$ | 48.31 ± 5.44$^{a,y}$ | 2.52 ± 0.30$^{a,x}$ | 0.00$^{b,x}$ | 50.83 ± 5.67$^{a,y}$ |
| 135 | 5.76 ± 0.06$^{b,x}$ | 3.30 ± 1.56$^{b,x}$ | 0.98 ± 0.25$^{a,x}$ | 44.56 ± 2.62$^{a,b,y}$ | 2.28 ± 0.15$^{a,x}$ | 0.00$^{b,x}$ | 46.85 ± 2.70$^{a,b,y}$ |
| 180 | 6.03 ± 0.10$^{a,x}$ | 11.13 ± 6.85$^{a,b,x}$ | 0.47 ± 0.09$^{b,x}$ | 23.17 ± 5.52$^{c,y}$ | 2.27 ± 0.98$^{a,x}$ | 0.00 $^{b,x}$ | 26.76 ± 5.25$^{c,y}$ |

Mean ± standard error of the mean (calculated considering < LOD = zero) for each parameter with different letters for each amine at different storage time (a–c) or in different pork cuts (x–y) indicate significant differences (Tukey test, $p < 0.05$). Limit of quantification (LOQ) = 0.08 mg/kg, except for histamine (0.15 mg/kg). $n = 6$ samples.

**Table 3**
Application of different indexes of quality based on bioactive amines for pork loin and leg during refrigerated storage (5 ± 1 °C) for 16 days.

| Storage time (days) | Quality indexes | | |
|---|---|---|---|
| | Mietz & Karmas | Hernández-Jover | Silva & Glória |
| Loin | | | |
| 0 | 0.00$^{b}$ | 0.00$^{b}$ | 0.08 ± 0.01$^{a}$ |
| 4 | 0.06 ± 0.01$^{b}$ | 1.46 ± 0.29$^{b}$ | 0.21 ± 0.08$^{a}$ |
| 8 | 0.16 ± 0.06$^{b}$ | 2.42 ± 0.21$^{b}$ | 0.11 ± 0.07$^{a}$ |
| 12 | 0.15 ± 0.07$^{b}$ | 5.62 ± 4.80$^{b}$ | 0.30 ± 0.17$^{a}$ |
| 16 | 0.88 ± 0.25$^{a}$ | 34.26 ± 9.19$^{a}$ | 0.75 ± 0.76$^{a}$ |
| Leg | | | |
| 0 | 0.00$^{b}$ | 0.00$^{b}$ | 0.07 ± 0.008$^{a}$ |
| 4 | 0.08 ± 0.004$^{b}$ | 2.34 ± 0.27$^{b}$ | 0.07 ± 0.01$^{a}$ |
| 8 | 0.09 ± 0.02$^{b}$ | 1.97 ± 0.44$^{b}$ | 0.05 ± 0.008$^{a}$ |
| 12 | 0.34 ± 0.16$^{b}$ | 16.58 ± 9.08$^{b}$ | 0.05 ± 0.004$^{a}$ |
| 16 | 1.48 ± 0.35$^{a}$ | 72.66 ± 20.46$^{a}$ | 0.01 ± 0.008$^{a}$ |

Mean value ± standard error of the mean with different superscripts in each column for each pork cut are significantly different (Tukey test, $p < 0.05$). $n = 6$ samples.

### 3.5. Classification of changes on pork cuts by multivariate statistical analysis

#### 3.5.1. During refrigerated storage

During refrigerated storage, when microbial counts (mesophilic and psychrotrophic bacteria counts and coliforms), physicochemical characteristics (pH, TVB-N), bioactive amines contents (individually, and total sum of polyamines and of biogenic amines) and indexes of quality based on bioactive amines of pork loin and leg are considered to perform PCA analysis, it can be observed that the first two components accounted for 90% (loin) and 88% (leg) of the explained variance (Fig. 2a, b, d, e).

The tree diagram from HCA (Fig. 2c, f) evidenced two groups for the refrigerated storage days, for both cuts, considering their similarities. During storage of pork loin under refrigeration, the first group was characterized by samples from days 0, 4 and 8 (Fig. 2b, c), which were the samples with the lowest values of the physicochemical parameters (pH and TVB-N), microbial counts (mesophilic and psychrotrophic bacteria) and biogenic amines. The second group was formed by pork loin from days 12 and 16 with the highest contents of biogenic amines and the highest counts of mesophilic and psychrotrophic bacteria. Based on these results, changes which occurred during refrigerated storage, affected pork loin quality on the 12th and 16th storage days, and, thereby, a shelf life of 8 days can be assumed for refrigerated pork loin.

Regarding storage of pork leg under refrigeration, the first group was composed by samples from days 0, 4, 8 and 12 (Fig. 2e, f), which were pork samples with the highest contents of spermidine, but the lowest contents of biogenic amines. The second group, with samples from day 16, was characterized by the highest contents of biogenic amines, total bioactive amines and the highest mesophilic and psychrotrophic bacteria counts. Based on this result, a shelf life of 12 days may be considered; however, since detectable levels of histamine were present on the 12th day, a shelf life of 8 days would be safer for human health for pork leg. In addition, putrescine and cadaverine accumulate after 8 days of refrigerated storage for both pork cuts by the breakdown of amino acids in dead organisms. These biogenic amines are largely responsible for the foul odor of putrefying flesh and both are reported to potentiate the toxic effects of histamine (EFSA, 2011; Gloria, 2006).

For both pork cuts under refrigeration storage, pH values showed high positive correlation with the contents of cadaverine ($r = 0.9437$ for loin and $r = 0.9263$ for leg), putrescine ($r = 0.9887$ for loin and $r = 0.9398$ for leg) and histamine ($r = 0.9906$ for loin and $r = 0.9386$ for leg) (Fig. 2a, d), suggesting the formation of biogenic amines concomitant with pH increase. In this same sense, the counts of mesophilic and psychrotrophic bacteria also presented high positive correlation with cadaverine ($r = 0.8884$–$0.9207$ for loin and $r = 0.9130$–$0.9506$ for leg), putrescine ($r = 0.9416$–$0.9743$ for loin and $r = 0.9204$–$0.9482$ for leg) and histamine ($r = 0.9778$–$0.9947$ for loin and $r = 0.9737$–$0.9768$ for leg), suggesting the formation of these biogenic amines as microbial counts increase.

#### 3.5.2. During frozen storage

The variables used in PCA and HCA analyses to characterize pork quality (pH, TVB-N, TBARS and the contents of spermine, spermidine, agmatine and the sum of total amines, biogenic amines and polyamines) during frozen storage were unable to classify and distinguish groups. Thus, additional parameters are needed to characterize pork meat quality during frozen storage by PCA.

However, as lipid oxidation becomes an important issue during frozen storage, and it is well known that consumers are likely to detect rancidity at malonaldehyde levels or higher than 0.5 mg/kg, shelf life of both loin and leg to 90 days at − 18 °C.

## 4. Conclusions

Fresh pork loin and leg were characterized by pH values of 5.5–6.4, TVB-N of 9–32.9 mg N/100 g, and TBARS values of up to 0.32 mg malondialdehyde/kg. The counts of mesophilic and psychrotrophic bacteria and coliforms were low and *Salmonella* and *E. coli* was not detected. Spermine was the prevalent amine followed by spermidine

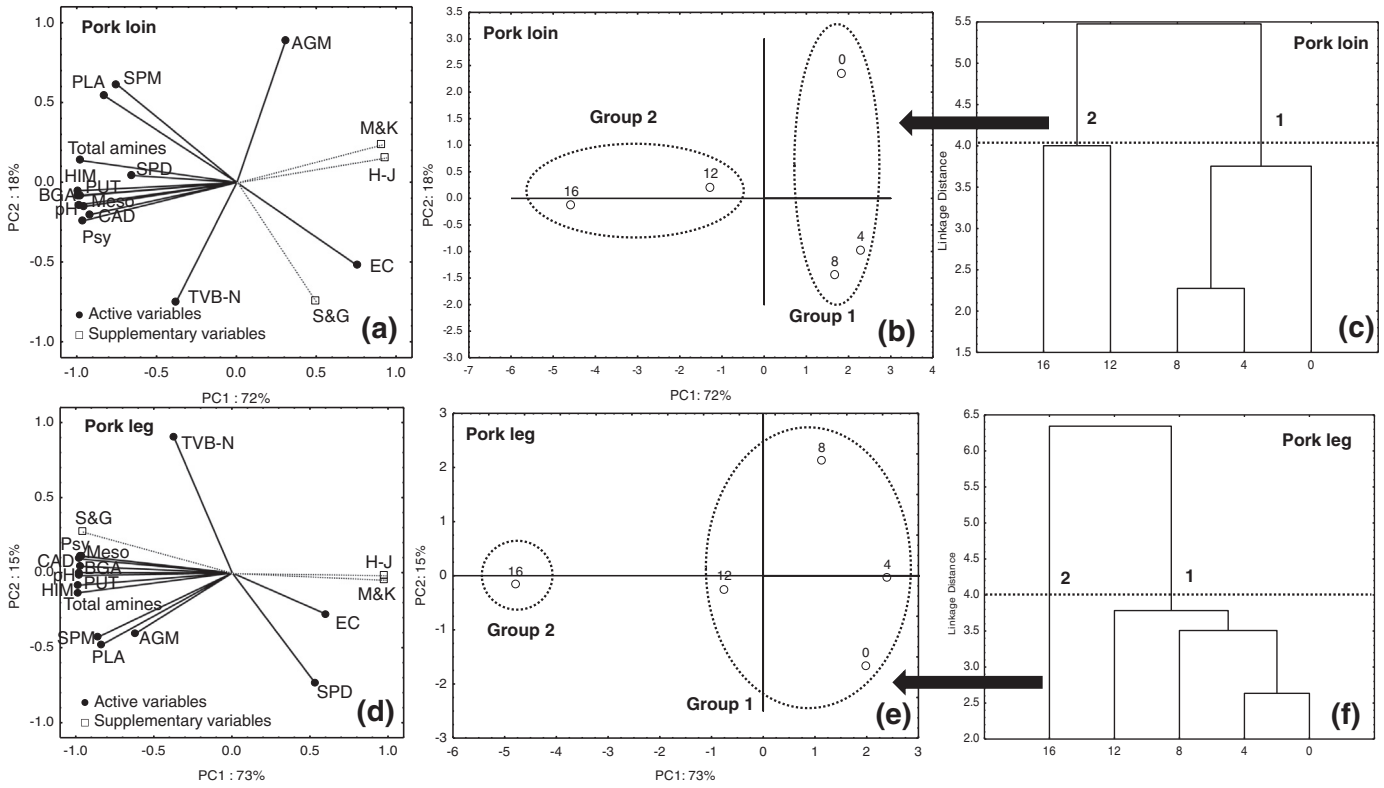


**Fig. 2.** Classification of pork loin (a–c) and leg (d–f) during refrigeration storage based on physicochemical characteristics, microbial counts and bioactive amines contents. (a and d) Variable projection by Principal Component Analysis (PCA), (b and e) Scatterplot for the pork cuts during each storage day by PCA with suggested grouping, in accordance with Hierarchical Cluster Analysis (HCA), and (c and f) dendrogram by HCA analysis. Abbreviations: pH = pH values, TVB-N = total volatile base nitrogen contents, Meso = mesophilic bacteria count, Psy = psychrotrophic bacteria count, EC = coliform count, SPM = spermine, SPD = spermidine, AGM = agmatine, CAD = cadaverine, PUT = putrescine, HIM = histamine, PLA = total sum of polyamines, BGA = total sum of biogenic amines, M&K = Mietz & Karmas index, H-J = Hernández-Jover index and S&G = Silva & Glória index.

and agmatine. No biogenic amine was detected in fresh pork.

During refrigerated storage (5 ± 1 °C) of pork loin and leg, there was a significant increase on pH, TVB-N and mesophilic and psychrotrophic bacteria counts. No significant change was observed on polyamines contents; however, putrescine, cadaverine and histamine were produced and accumulated during storage. Histamine accumulated on the 12th storage day and reached levels capable of causing adverse effects to healthy individuals in pork leg on the 16th day of storage. Multivariate analysis by means of PCA and HCA based on microbial and physicochemical parameters and bioactive amines were effective in following changes in both pork loin and leg during refrigerated storage indicating no changes in these parameters up to 8 days. Based on PCA an HCA and safety regarding histamine contents, a shelf life of 8 days at 5 ± 1 °C is suggested for both pork loins and legs.

During frozen storage (− 18 ± 1 °C) of pork loin and leg for 180 days, there were no significant changes on pH, TVB-N levels decreased, and TBARS increased. No biogenic amine was produced. PCA and HCA were unable to classify pork meat based on the parameters used. However, considering consumers' ability to detect rancidity (≥ 0.5 mg malonaldehyde/kg), a shelf life of 90 days under frozen storage is recommended for both loins and legs.

## Conflict of interest

The authors have no conflict of interest.

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