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Effect of different dry aging temperatures on *Listeria innocua* as surrogate for *Listeria monocytogenes*

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ABSTRACT

The objective of this study was to evaluate the effect of different dry aging temperatures on the behavior of *Listeria innocua* used as a surrogate of *L. monocytogenes*. The process was performed in boneless loin pieces for up to 42 days at 2 and 8 °C. The behavior of *L. innocua* was influenced by the temperature, aging time, and the water activity of the beef surface ($P < .05$). After 42 days, reductions of 2.38 and 3.37 log cfu/g were obtained on the beef surface aged at 2 and 8 °C, respectively. According to data predicted by the Weibull model, the samples aged at 2 °C would achieve a 4-log reduction with twice the time required for the process at 8 °C. After trimming, 66.7% of the samples aged at 2 °C were positive for *L. innocua*, whereas at 8 °C the rate was 33.3%. Therefore, the results showed that the increase of process time and temperature as well by decrease of the a_w reduces *L. innocua* counts.

1. Introduction

Meat aging is a process that brings an increase in tenderness, succulence and taste development through the proteolysis of myofibrillar proteins (Koohmaraie & Geesink, 2006; Matarneh, England, Scheffler, & Gerrard, 2017). It can be performed by wet or dry methods. In the wet process, the meat is vacuum packed and stored under refrigeration, while in the dry process, the unpacked meat is placed in refrigeration chambers, with control of air velocity, humidity, and temperature (Dashdorj, Tripathi, Cho, Kim, & Hwang, 2016; Stenström, Li, Hunt, & Lundström, 2014). This process usually lasts between 14 and 40 days (Lepper-Blilie, Berg, Buchanan, & Berg, 2012; Savell, 2008).

Although producers and consumers have been attracted to meat dry aging, few studies assess its impact on beef microbiological safety (Hulánková, Kameník, Saláková, Závodský, & Borilova, 2018; Knudsen, Sommer, Sørensen, Olsen, & Aabo, 2011; Tittor et al., 2011). *Listeria monocytogenes* is one of the main microorganisms that cause foodborne diseases and is able to grow at low temperatures (1 to 45 °C) (Lianou, Panagou, & Nychas, 2017; Ryser & Donnelly, 2015). The latest outbreaks involving *Listeria monocytogenes* suggest that consumption of low doses ($< 10^2$ CFU/g) can cause infection (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017). In 2016, the European Food Safety Authority described > 2500 cases of listeriosis, with 247 deaths (EFSA, 2017). Meat products accounted for 18% of the listeriosis outbreaks registered by the CDC (2018). In Italy *Listeria monocytogenes* was detected in 24.4% of beef samples (Pesavento, Ducci, Nieri, Comodo, & Nostro, 2010). In Turkey the percentage of contamination was 6.2% in fresh meat (Yucel, Cıtak, & Onder, 2005). In bovine carcasses, the pathogen detection ranged from 6.5% to 26% (Iglesias, Kroning, Decol, de Melo Franco, & da Silva, 2017; Loiko et al., 2016). Despite there being no reports on outbreaks involving dry aged beef the high lethality (20 to 30%) of listeriosis (Milillo et al., 2012) associated to the capacity of the pathogen to multiply under refrigeration highlights the need of studies that evaluate the behavior of this bacteria genus when submitted to stress conditions, such as the meat dry aging process. Therefore, the objective of this study was to evaluate the effect of the beef dry aging temperature on the survival of *Listeria innocua* used as a surrogate for L. *monocytogenes*.

2. Material and methods

2.1. Samples

At 3 d postmortem, two boneless loins (*M. longissimus* lumborum; Institutional Meat Purchase Specifications #180; (USMEF, 2014) were obtained from two beef carcasses (Nellore intact bulls) from a commercial beef packing plant. Each loin was divided into three portions yielding a total of six loin pieces (1.5 kg; 20 × 13 × 8 cm) that were

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randomly distributed in two treatments. The experiment was repeated three times, totalizing six boneless loins and 18 pieces.

2.2. Listeria strain

Listeria innocua ATCC 33090 was used as a surrogate of *L. monocytogenes*. The strain was stored at −80 °C in trypticase soy broth (TSB, Difco, MD, USA), supplemented with 15% glycerol (Synth, Brazil).

2.3. Preparation of the inoculum

For the inoculum preparation, the strain was grown in TSB broth (Difco) supplemented with 0.6% yeast extract (YE, Oxoid, UK) at 35 °C for 24 h. After that, a culture loop was streaked on TSA (Difco) plates, supplemented with 0.6% yeast extract (Oxoid) and incubated at 35 °C for 24 h. Isolated colonies were transferred to tubes containing 5 mL of 0.1% peptone water (Difco) to reach 0.5 turbidity on the MacFarland scale (10^8 cells/mL). Serial decimal dilutions were carried out in 0.1% peptone water (Difco) to reach 6 log CFU/g. Cell numbers were determined on TSA-YE (Difco).

2.4. Inoculation and aging process

All samples were previously analyzed for *Listeria* sp. (USDA, 2017). For each treatment two loin sections were inoculated with *L. innocua* and one was used as negative control. A total of 3.9 mL of the inoculum solution (6 log CFU/g) was spread on the surface of each sample (1.5 kg) with a drigalski spatula. The initial concentration of *L. innocua* on the samples' surface was *ca.* 4 log CFU/g.

The inoculum was distributed on the surface of the samples with a drigalski spatula (2.6 mL/kg). After inoculation, the pieces were kept for 10 min in a biosafety cabinet (Vecco, Brazil), with the purpose of ensuring maximum adherence of the inoculum. Then, the samples were transferred to refrigeration chambers (Metalfrio Solutions, model VN50R, Brazil) at 2 ± 1 and 8 ± 1 °C with 75 ± 2% relative humidity (RH) and air velocity of 2 ± 0.5 m/s for up to 42 days (U.S. Meat Export Federation, 2014). Every two days, the samples were randomly relocated inside the chamber to avoid the effect of fixed location. The water activity (a_w) and *Listeria* counts were determined after 0, 7, 14, 21, 28, 35 and 42 days of aging.

To monitor the temperature of the chambers, a digital thermometer (Incoterm, Brazil) with ± 0.1 °C resolution was used. Air velocity was measured using a digital anemometer (Airflow Developments, model TA3, USA). RH was monitored by the aging chamber measuring instrument (Metalfrio Solutions).

2.5. Listeria enumeration

At each analysis point, 10 g of beef fillets measuring ± 2 mm thickness were collected aseptically with a scalpel from the external surface of each piece of striploin. After that, to evaluate the possibility of cross-contamination through the utensils used in the trimming, a layer of approximately 5 mm thick was removed from the meat surface with sterile knives and a 10-g sample was collected. Each sample was homogenized with 90 mL of 0.1% peptone water (Difco) in a stomacher (Stomacher 400 circulator, Seward, UK) for 2 min at 230 rpm. When necessary, decimal dilutions were performed in 0.1% peptone water (Difco).

The *Listeria* population was determined in *Listeria* Oxford agar (Acumedia, MI, USA), plus Modified Oxford Listeria Supplement (Difco) with incubation at 35 °C for 24 h (USDA, 2017). Typical colonies were confirmed by biochemical tests (motility, catalase, Gram stain, Carbohydrate fermentation series and β-hemolysis (Hitchins, Jinneman, & Chen, 2017). The limit of detection was 1 log CFU/g.

2.6. Determination of water activity and pH

The water activity was determined after 0, 7, 14, 21, 28, 35 and 42 days of aging in duplicate at 25 °C with a hygrometer (Aqualab PRE CAP, Decagon Device, Pullman, WA, USA).

The pH was measured before and after aging in duplicate by inserting a calibrate pH probe (MP125 portable pH meter, Mettler Toledo, Brazil) directly into the beef.

2.7. Weibull model

The *Listeria* survival curve on the meat surface was estimated using the Weibull model (Mafart, Couvert, Gaillard, & Leguérinel, 2002), available from GInaFiT (Geeraerd, Valdramidis, & Van Impe, 2005), represented by Eq. (1):

$$\log(N/N_0) = -(t/\delta)\beta \ \log(N/N_0) = -(t/\delta)^{\beta}\log(N/N_0) = -(t/\delta)^{\beta} \quad (1)$$

where N is the final count, N_0 is the initial count, both in log CFU/g; t is the time of the process (days); δ is the time required to obtain the first decimal reduction, and β is the parameter that defines the curve shape. The Root Mean Squared Error (RMSE) and the regression coefficient (R^2) were used to evaluate the model. T_{3d} and T_{4d} are the time in days to achieve three and four decimal reductions of *Listeria*, respectively.

2.8. Statistical analysis

The data were analyzed using the MIXED procedure for ANOVA using a model with temperature (2 and 8 °C), time (0, 7, 14, 21, 28, 35 and 42 days) and their interaction as fixed effect with batch and loin section as random effect. In addition, to verify the effect of the water activity on *Listeria* counts, correlation matrices analysis was performed at 95% significance. The analyses were carried out using Statistica software (version 10.0, StatSoft, CA, USA).

3. Results

3.1. Behavior of Listeria throughout the aging process

Listeria sp. was not detected (< 1 log CFU/g) in the negative control samples nether before nor after trimming throughout the aging at 2 and 8 °C.

On the surface of the meat samples, the highest rate of reduction in the *Listeria innocua* population occurred in the first seven days of aging; the initial count (4.37 log CFU/g) was reduced to 3.13 and 2.85 log CFU/g at 2 and 8 °C, respectively (Fig. 1). From the 28th day on, a significant difference ($P < .05$) in *L. innocua* counts between the aging

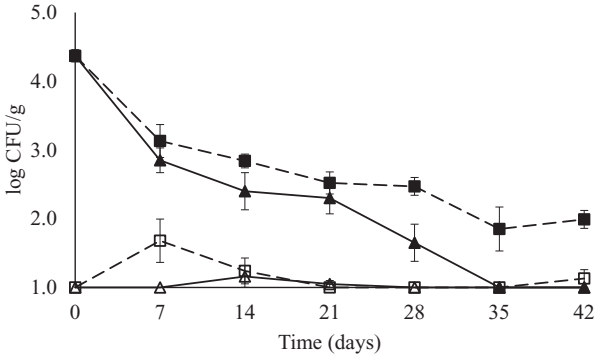

Fig. 1. Behavior of *Listeria innocua* (log CFU/g) during meat dry aging for 42 days. At 2 °C before trimming (—■—); 8 °C before trimming (—▲—); 2 °C after trimming (—□—) and 8 °C after trimming (—Δ—). Values obtained from three independent experiments with standard error. Limit of detection: < 1 log CFU/g.

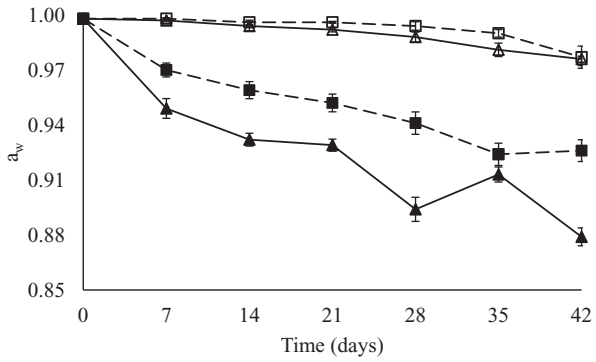

Fig. 2. Water activity evolution in dry aged meat for 42 days. At 2 °C before trimming (—■—); 8 °C before trimming (—▲—); 2 °C after trimming (—□—) and 8 °C before trimming (—Δ—).Values obtained from three independent experiments with standard error.

temperatures was observed. The microorganism population was reduced below the limit of detection (1 log CFU/g) after 35 days at 8 °C, whereas at 2 °C a count of 1.99 log CFU/g was detected even after 42 days.

Similar to the microbial count, the highest slope of the water activity (a_w) was noted during the first week of the process (Fig. 2). At 2 °C, the a_w was 0.970 while at 8 °C it declined to 0.949. From the 7th day, except on day 35, a significant difference ($P < .05$) was observed between the a_w of the samples aged at 2 and 8 °C. After 42 days, the a_w was 0.926 at 2 °C and 0.879 at 8 °C. The statistical analysis showed a correlation of 0.86 (R^2) between the a_w of the meat surface and the *L. innocua* count.

The pH of the samples was not affected by the aging time and the temperature ($P > .05$). After 42 days, the samples aged at 2 and 8 °C had the same pH value, 5.5 (data not shown).

In the samples collected after trimming, the temperature had no significant influence ($P > .05$) on the *Listeria innocua* counts (Fig. 1). However, the a_w was significantly influenced by temperature and time ($P < .05$; Fig. 2). At 2 °C, the a_w remained practically stable for 35 days (0.990) and reduced to 0.977 after 42 days. At 8 °C, the a_w stayed above 0.990 up to 21 days. A gradual decline in the a_w was verified from 28th day, reaching 0.976 on day 42. In addition, *Listeria innocua* was detected in three, two and one samples after 7, 14 and 42 days of aging at 2 °C, respectively, with counts up to 2.58 log CFU/g. Further, at 8 °C, the microorganism was recovered from two samples, one after 14 days and another after 21 days of process.

3.2. Weibull model

Due to the low level of contamination observed in the samples after trimming, the Weibull model was used to predict the *L. innocua* death only on the meat surface (Table 1). The values estimated by the model were very close to those observed in the experiments. For both temperatures evaluated, the R^2 value calculated was 0.976.

Concave curves were obtained for both aged temperatures, with β values ≤0.5. The death rate declined steadily throughout the process. Also a faster loss of viability of *L. innocua* was noted at 8 °C. The time needed to reach the first decimal reduction (δ) was 4.7 and 4.0 days at 2 and 8 °C, respectively (Table 1). According to the Weibull model, the time required for 3-log reductions (T_{3d}) would be 69 and 33 days at 2 and 8 °C, respectively. In addition, it would take 140 days at 2 °C and 58 days at 8 °C to achieve 4-log (T_{4d}) reductions.

4. Discussion

Surrogate is defined as a microorganism used to study the fate of a pathogenic bacterium in a specific condition or environment (Sinclair, Rose, Hashsham, Gerba, & Haas, 2012).The ideal surrogate would be a nonvirulent bacterium that has physiological characteristics nearly identical to the target pathogen (Sinclair et al., 2012). Due to the risk of exposing individuals to *L. monocytogenes* and the risk of the pathogen persistence in the environment, the use of a surrogate is required to understand and model of the behavior of *L. monocytogenes* in foods. This is the first work that has evaluated the behavior of *Listeria innocua* ATCC 33090 as a surrogate of *Listeria monocytogenes* during the dry aging process. *L. innocua* ATCC 33090 has already been proposed as a surrogate in meat and other food matrices (Char, Guerrero, & Alzamora, 2010; Crowe, Skonberg, Bushway, & Baxter, 2012; Friedly et al., 2008; Hnosko, San-Martin Gonzalez, & Clark, 2012; Lorentzen, Ytterstad, Olsen, & Skjerdal, 2010; Milillo et al., 2012; Montazeri, Himelbloom, Oliveira, Leigh, & Crapo, 2013; Skåra, Valdramidis, Rosnes, Noriega, & Van Impe, 2014).

The meat industry is concerned about the presence of *L. monocytogenes* in the process environment and in the final product due to its ability to grow at refrigerated temperature (Buchanan et al., 2017). Monitoring and controlling storage temperature are key control measures for meat products. Based on that, our study evaluated two dry aging temperatures on the behavior of *Listeria innocua*, 2 °C that is within the temperature range recommended by international organizations (U.S. Meat Export Federation, 2014; AMPC & MLA, 2010) and 8 °C a mild abusive temperature. Decrease in *L. innocua* count was noted throughout the dry aging process. After 21 days, reductions of 1.85 and 2.07 log CFU/g at 2 and 8 °C were observed on the surface of the meat pieces. At the end of the process, the *L. innocua* population decreased by 2.38 and 3.37 log CFU/g when aged at 2 and 8 °C, respectively (Fig. 1). The decline in the *Listeria* count during the refrigerated storage of meat products has already been reported (Menendez, Rendueles, Sanz, Capita, & Garcia-Fernandez, 2015; Prendergast, Rowe, & Sheridan, 2007; Saraiva et al., 2016). In addition, the aging time significantly influenced ($P < .05$) the survival of *L. innocua*. Many studies report the duration of the dry aging process between 14 and 40 days (Savell, 2008), with 21 days the most common (Lepper-Blilie et al., 2012).

Predictive microbiology models are used to infer about the

Table 1
Parameters of the Weibull model on the behavior of *Listeria innocua* on the surface of samples submitted to aging processes at 2 ($n = 6$) and 8 °C ($n = 6$).

Temperature (°C)	δ (day)[a]	SE δ[b]	β[c]	SE β[d]	T_{3d}[e] (day)	T_{4d}[f] (day)	RMSE[g]	R^2[h]
2	4.66	2.18	0.41	0.08	69.08	139.93	0.158938	0.97648
8	4.01	1.88	0.52	0.10	33.14	57.63	0.225493	0.97568

[a] Time required for the first decimal reduction (days).
[b] Standard error for the δ values.
[c] Parameter that defines the shape of the curve.
[d] Standard error for the β values.
[e] Time required for three decimal reductions (days).
[f] Time required for four decimal reductions (days).
[g] Root mean square error.
[h] Regression coefficient.

evolution of microbial population considering the initial contamination and food environment, as the responses of microorganism populations in a specific environment are reproducible (Saraiva et al., 2018). Based on the Weibull model, the time required to reach 3 and 4-log reductions (T_{3d} and T_{4d}) of *L. innocua* at 2 °C was 2.2 and 2.4 times greater than at 8 °C. Other authors also reported a higher rate of inactivation of *L. monocytogenes* with the increase of aging temperature of beef (Mataragas, Rantsiou, Alessandria, & Cocolin, 2015). On the other hand, Saraiva et al. (2016) did not note a significant difference on the inactivation of *L. monocytogenes* in air-packaged beef meat stored at 4 and 9 °C. These authors also mentioned that the concavity of the death curves indicates adaptation of the microorganism to the stressful environment.

The microbial growth and/or survival in food matrices are influenced by a complex interaction between extrinsic and intrinsic parameters. According to Mataragas et al. (2015), temperature as an isolated parameter may not result in complete inactivation of *L. monocytogenes*, requiring interaction with other factors such as pH or a_w. Conditions that do not support *Listeria monocytogenes* growth include pH < 4.4, aw < 0.92, NaCl > 16% or the association of pH < 5.0 and aw < 0.94 (Codex Alimentarius, 2009).

In our study as the temperature rose, the water loss increased and consequently the a_w was reduced. At 8 °C the water activity on the surface of the samples decreased to below 0.92 after 28 days, and the count was reduced below the detection limit (< 1 log CFU/g) after 35 days. However, at 2 °C, the a_w remained above the limit throughout the study, and the lowest count was 1.85 log CFU/g. Ferreira (2018) when evaluating the meat dry aging for 42 days obtained greater water loss at 7 °C than 2 °C. Tapia de Daza, Villegas, and Martinez (1991) reported that the minimal water activity for growth of *L. monocytogenes* was affected by the temperature incubation, increasing with the reduction of the temperature. At 4 °C the minimal aw required for growth was 0.94 using NaCl as a solute and 0.92 using glycerol. In another study performed at 21 °C the inactivation of *L. monocytogenes* and *L. innocua* was observed in a_w of 0.92 using NaCl and 0.91 using glycerol (Nolan, Chamblin, & Troller, 1992). Prendergast et al. (2007) observed a reduction of 2 log CFU/g of *L. innocua* in refrigerated carcasses at 4 °C for 72 h with a synergy effect between a_w and temperature. Further, the pH did not seem to have exerted a significant influence on the behavior, since it remained practically stable at around 5.5, *i.e.*, within the growth range of the microorganism.

The background microbiota was not determined in the current study. However, in a previous experiment we observed counts of mesophilic and psychrotrophic microorganisms of 3.0 and 3.8 log cfu/g after 42 days at 2 °C and of 3.3 and 5.4 log cuf/g after the same period at 8 °C (Silva, 2019). Therefore, it is not possible to rule out the influence of microbial interaction, especially psychrotrophics, on the behavior of listeria. Saraiva et al. (2016) suggested that competitive microbiota could not favor the growth of the pathogen.

Microbiological cross-contamination is a major issue with respect to *L. monocytogenes*Codex Alimentarius, 2009). In our study we evaluated the cross-contamination caused by the trimming step. After the trimming, *L. innocua* was recovered from 50, 33 and 17% of the samples aged for 7, 14 and 42 days at 2 °C. Whereas, at 8 °C, two beef pieces - one aged for 14 days and another for 21 days - were positive for the target bacteria (Fig. 1). This cross-contamination may have been due to the utensils used, the handling or by the internalization of the inoculum along the process. During the dry aging, with the water evaporation and consequent reduction of the a_w, muscle fiber shortening can occur, and formation of grooves allows the internalization of the superficial contamination into the samples (Thomas, O'Rourke, & McMeekin, 1987). Although dry aged meat is usually consumed after undergoing a heat process, it can be insufficiently cooked or eaten raw (steak tartare, carpaccio, raw kibbeh). In addition, the contaminated meat may be a source of human contamination during home or retail handling. Therefore, our data points out a concern in the public health area and dry aging producers.

5. Conclusion

The results showed that in dry-aged meat *Listeria innocua* ATCC 33090 count declines with the increase of process time and temperature and decrease of the a_w. However, further studies involving different strains of *Listeria* are required to determine the susceptibility of the microorganism in the dry-aging process.

Declaration of Competing Interests

The authors have no conflict of interest to declare.

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