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Survival assessment of *Salmonella enterica* in inoculated pork salami

Avaliação da sobrevivência de *Salmonella enterica* inoculada em salame de carne suína

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Highlights:
Salmonella enterica is able to multiply during the salami fermentation stage.
Salmonella enterica survives the drying step of artificially contaminated salami.
TAL agar is more efficient than XLD in recovering injured *Salmonella* cells.

Abstract

Pork salami is an embedded, cured and ripened product commonly consumed in Brazil, and the presence of *Salmonella enterica* has already been reported in this product. During its preparation, the microbiological safety depends on the meat quality, addition of ingredients with antimicrobial activity, hygiene during processing, pH and water activity (Aw) reduction during maturation. In Brazil, the maturation protocol has not been determined in food regulation; therefore, the objectives of this study were (a) to identify the fermentation and drying phases during salami maturation; (b) to test the survival of *S. enterica* during salami processing; and (c) to compare xylose lysine deoxycholate (XLD) and thin agar layer (TAL) agar for recovering *Salmonella*. The salami samples were prepared with a cocktail of *S. enterica* strains, fermented at 30°C and dried at 20°C with controlled relative humidity (RH). Periodic sampling for *S. enterica* quantification and Aw and pH analyses were performed during maturation, and curves were constructed. Fermentation occurred during the first 66 hours, and the pH decreased while the population of *S. enterica* increased over the first 21 hours. The drying step was able to reduce the bacterial population by approximately 5 log CFU after 875 hours, reaching an Aw of less than 0.78. However, elimination of *S. enterica* was not achieved. For *Salmonella* recovery, TAL agar was more efficient than XLD agar.
Key words: Fermentation. Maturation. Microorganism. Ripening. Sausage.

Resumo

O salame de carne suína é um produto embutido, curado e maturado comumente consumido no Brasil no qual a presença de *Salmonella enterica* tem sido relatada. Durante a sua elaboração, a segurança microbiológica depende da qualidade da carne, adição de ingredientes com atividade antimicrobiana,

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higiene durante a produção, redução de pH e atividade de água (Aw) durante a sua maturação. O protocolo de maturação ainda não está determinado na legislação brasileira; portanto o estudo objetivou: (a) identificar as fases de fermentação e dessecação durante a maturação de salame; (b) testar a sobrevivência de *S. enterica* durante o processamento de salame e (c) comparar os meios de cultura xilose lisina dextrose (XLD) e *thin agar layer* (TAL) para recuperação de células do referido micro-organismo. Os salames foram elaborados com um coquetel de *S. enterica* e submetidos à fermentação em 30ºC e secagem a 20ºC com umidade relativa (UR) controlada. Amostragens periódicas para quantificação de *S. enterica*, análises de Aw e pH foram realizadas durante a maturação e as curvas foram construídas. A fermentação ocorreu nas primeiras 66 horas, quando houve queda do pH do salame; entretanto *S. enterica* aumentou sua população nas primeiras 21 horas. A etapa de dessecação foi capaz de reduzir aproximadamente 5 log UFC da população bacteriana em 875 horas, alcançando Aw menor que 0,78, mas não foi capaz de eliminar o micro-organismo do alimento. Para enumeração do micro-organismo, o meio sólido TAL foi mais eficiente na recuperação das células submetidas à maturação quando comparado ao ágar XLD comumente utilizado.
Palavras-chave: Dessecação. Embutido. Fermentação. Maturação. Micro-organismo.

Introduction

Salami is a type of sausage consisting of cured and matured meat, mainly pork, and is usually consumed raw by a large part of the Brazilian population. Curing, fermentation and drying are mandatory stages of the manufacturing process; however, Brazilian food regulation does not provide for a specific protocol. Thus, optional ingredients and the duration of fermentation and drying steps are at the discretion of the manufacturer, provided that by the end of the process, the product achieves the maximum permitted values of humidity (40%) and water activity (Aw 0.90) determined by Brazilian regulation (Instrução Normativa nº 22, 2000).

Between 2009 and 2018, *Salmonella enterica* was the second leading cause of foodborne disease outbreaks in Brazil. This pathogen was reported in 11.3% of the confirmed outbreaks, a frequency that is very likely underestimated (Ministério da Saúde [MS], 2019). There are reports on the presence of *S. enterica* in salami at the retail level (Werlang, Haubert, Peter, & Cardoso, 2019), although the Brazilian standard for meat products is the absence of *S. enterica* in a 25 gram sample (Resolução RDC nº 12, 2001). One possible reason for cases of *Salmonella* contamination in salami may be the occurrence of failures in the antimicrobial hurdles (low Aw and pH) imposed during manufacturing. In addition, bacteria may differ in behavior in distinct foodstuffs, or they may develop cellular mechanisms of adaptation to environmental stresses (Álvarez-Ordóñez, Fernández, Bernardo, & López, 2010; Nightingale, Thippareddi, Phebus, Marsden, & Nutsch, 2006).

Different mathematical models allow predicting the inactivation of bacteria and their probability of growth in foodstuffs under adverse conditions such as unfavorable pH and Aw values (Coroller, Kan-King-Yu, Leguerinel, Mafart, & Membré, 2012). In this sense, the adoption of methodologies that are able to recover possible injured bacterial cells after the manufacturing processes is key in order to generate real bacterial behavior data. Selective culture media used for enumeration of *S. enterica* can inhibit the growth of injured cells. Therefore, methodologies that combine layers of nonselective agar with layers of selective solid media, such as Spray, Overlay, and TAL, have shown better results in the recovery of *S. enterica* (Back, Kim, Park, Chung, & Kang, 2012; Wu, Fung, & Kang, 2001). Among these methodologies, the use of xylose lysine deoxycholate (XLD) agar containing a thin overlay of tryptic soy agar (TSA), called thin agar layer (TAL), was shown to be efficient in recovering cells injured by freezing, acid, and heat. Moreover, TAL was reported to be easy to prepare and presented good performance in *S. enterica* isolation (Chang, Mills, & Cutter, 2003; Wu et al.,

2001; Kang & Fung, 2000). Since the decrease in pH and Aw values during the maturation process of salami can cause injuries to *S. enterica* cells, TAL is expected to improve their recovery, generating better estimates of contamination in this foodstuff.

In Brazil, there are no data on the survival of *S. enterica* throughout the manufacturing stages of salami. Thus, the aims of this study were (a) to identify the fermentation and drying phases during the salami maturation process; (b) to assess the survival of *S. enterica* during the maturation process; and (c) to compare XLD and TAL agar for recovering *S. enterica* from artificially contaminated salami.

Material and Methods

Preparation of the Salmonella cocktail

Four strains of *S. enterica* from the culture collection of the Preventive Veterinary Medicine Laboratory of the Federal University of Rio Grande do Sul were selected: two strains of serovar Typhimurium (PL10 and PL1048); S. Infantis (PL7); and S. Derby (PL764). The reference strain S. Typhimurium ATCC 14028 was also added to the cocktail.

The selected strains were individually adapted in BHI broth supplemented with 1% glucose. Afterwards, they were subjected to two consecutive growth rounds in 10 and 20 mL of BHI broth (Oxoid, Basingstoke, UK) incubated at 37°C ± 2 for 18 hours. Next, the cultures were centrifuged at 3,800×g for 40 minutes. The supernatant was discarded, and the cell pellet was resuspended in 2 mL of 0.85% NaCl. The inoculum was adjusted to a concentration of approximately 10 log CFU mL^{-1} on the McFarland scale. The cocktail of the five strains was prepared by transferring a 2 mL aliquot from each strain suspension (approximately 10 log CFU mL^{-1}) to a sterile tube.

Salami manufacturing

The ingredients for the minced meat mass were based on typical recipes of industrialized salami in Brazil: 85% pork (shoulder); 11% bacon; 2% sodium chloride; 0.96% spices; 0.90% glucose; 0.03% curing salts (nitrate and sodium nitrite); 0.11% erythorbate and sodium glutamate; and 0.02% Lyocarni starter culture (SBM-11, Sacco, Campinas, Brasil). After the ingredients were weighed, mixed and homogenized, the *Salmonella* cocktail was added to the dough at a final concentration of ~10^7 CFU g^{-1} for the preparation of contaminated salami. Fifty salami pieces were prepared by inserting the mixture into natural casings. Each salami piece measured approximately 15 cm and weighed approximately 200 g. After encasing, individual salami pieces were identified and hung on stainless steel rods inside a maturation chamber (100 cm x 85 cm x 60 cm). The internal space of the maturation chamber was divided into two areas (A and B) to allow the simultaneous sampling of salami units (n=23) belonging to the two replications of the experiment. At each sampling time, determined after conducting a pilot test (data not shown), one salami piece from area A and one from area B were collected. The samplings for microbiological and pH analysis were first set at short intervals of 0 to 12 hours (11 sampling events performed from the 1st to the 4th day of maturation). Afterwards, the intervals were set at 48 to 72 hours (8 samplings performed from the 5th to the 24th day of maturation), followed by long intervals of 72 to 120 hours (four samplings performed from the 25th to the 39th day of maturation). For the Aw analysis, one salami unit was taken at zero, 192, 528 and 810 hours.

Identification of salami maturation stages

Salami pieces underwent maturation for 941 hours under a controlled temperature and relative humidity (RH): 30°C during fermentation, with 99% to 95% RH, and 20°C during the drying stage,

with the RH gradually reduced from 95% to 70%. The fermentation stage occurred from zero hours until the salami presented a pH equal to or less than 5.4. Thereafter, the drying stage began and lasted until the end of maturation.

Microbiological and physical-chemical analysis

Enumeration of Salmonella enterica and pH analysis

For each salami piece, two internal portions of the salami were taken after removal of the casing: 20 g was added to 200 mL of distilled water for pH analysis, and 25 g was added to 225 mL of 1% buffered peptone water (BPW) for quantification of *S. enterica*. Both portions were homogenized individually for one minute in a stomacher-type homogenizer (LS 1901, Logen Scientific, Brazil).

For enumeration of *S. enterica*, serial dilutions in 9 mL of the initial suspension were performed in 0.1% peptone water (0.1% PW). From each dilution, 0.1 mL was inoculated in duplicate on the surface of XLD agar plates (Oxoid). In parallel, aliquots of equal volume were transferred onto TAL agar (XLD agar + TSA agar, Oxoid), according to Kang and Fung (2000). After incubation for 48 hours at 35°C, typical colonies (red with black center) were counted. Three typical colonies from the XLD and TAL agar were transferred to TSA agar and confirmed phenotypically for *Salmonella* spp. with the following biochemical tests: triple sugar iron agar and lysine iron agar; urea broth and IMViC (indole, methyl red, Voges-Proskauer and citrate), according to Quinn et al. (2011). The confirmation of the genus was carried out by the agglutination test with polyvalent somatic anti-Salmonella spp. (Probac, São Paulo, Brazil).

The pH analysis was performed directly in the homogenate using a pH meter DM-22 (Digimed, São Paulo, Brazil) previously calibrated and used only for contaminated samples. For each sample, three measurements were made, and the average of the readings was calculated. The electrode decontamination was performed with a 20% hydrochloric acid solution (Merck, Darmstadt, Germany) after each measurement round.

Water activity analysis

Aw analysis was performed in four salami pieces at zero, 192, 528, and 810 hours. Three grams of each sample was macerated and placed in disposable ePW capsules (Novasina, Lachen, Switzerland); the Aw was measured in a Lab Touch - Aw device (Novasina). The analysis was performed at 25°C in duplicate for each sample unit of salami, and then, the average of the two readings was calculated.

Statistical analysis

Performance comparison between XLD and TAL

The microbial population density log (CFU g^{-1}) was calculated by multiplying the number of colonies by the inverse of the dilution. The *S. enterica* mean concentrations in XLD and TAL were compared by a generalized linear mixed model using the media (XLD or TAL) as a fixed effect and the paired samples in time as considered a cluster, and it was included as a random effect. The model was assessed with a 95% confidence level.

Water activity (Aw) estimation from the samples

It was assumed that the Aw reduction follows a density-dependent rate described by the following differential equation:

$$\frac{dy}{dt} = -\beta y\,, \qquad (1)$$

where is a constant (measure unit 1/time) fitted by the minimization of the mean squared error.

Solving eq 1 using the initial values observed in the experiment, the following was obtained:

$$y_t = y_0.e^{-t\beta}\,, \qquad (2)$$

where *t* is the time in hours, y_0 is the Aw at time zero and $\beta = 0.000244\ \text{hours}^{-1}$.

The average daily Aw loss from time zero until the end of maturation was obtained by dividing the total Aw variation by the total maturation time in hours.

Results and Discussion

In salami manufacturing, fermentation and drying are necessary steps to achieve the typical characteristics of the product. Moreover, these stages are used for the control of pathogens in products such as salami, which does not undergo heat treatment (Lindqvist & Lindblad, 2009). However, because of the lack of standardization in these steps, salami available in the Brazilian retail market may present physical-chemical parameters outside of the permitted limits (Caccioppoli, Custódio, Vieira, Coelho, & Glória, 2006; Werlang et al., 2019).

In our study, the fermentation step occurred in the period corresponding to the first 66 hours, in which the pH dropped from 6.69 to 5.4. Afterward, the drying step began (875 hours), and the pH decreased until reaching 5.05, followed by an increase to 5.84 at the end of the maturation process. The end of maturation was determined by the loss of the product characteristics due to the very low moisture, which occurred after 941 hours. The decline in pH during the initial hours of fermentation is key to ensuring color development and stabilization as well as the formation of desirable compounds for salami flavor. Moreover, the decrease in pH until reaching a value close to 5.3, which is equivalent to the meat's isoelectric point, signified the beginning of the drying stage. This stage, in turn, ensured the continuity of the technological process since water migration begins from the inner part towards the encased surface (Chasco, Lizaso, & Beriain, 1996). The duration of the fermentation stage, as well as the increase in pH presented throughout the process, were in line with studies conducted in Brazilian salami (Barbosa, Todorov, Jurkiewicz, & Franco, 2015). Moreover, the increase in pH has been related to the development of lactate-oxidizing fungi on the food surface during the process (Mauriello, Casaburi, Blaiotta, & Villani, 2004).

During the drying stage, the Aw gradually decreased, and salami pieces presented Aw values of 0.9570 at zero hours, 0.913 at 192 hours, 0.836 at 528 hours and 0.783 at 810 hours. The analysis by differential equation of the values assessed in the four sampling points allowed determining the Aw at every hour of the maturation process. Thus, at the beginning of the drying stage, after 67 hours of maturation, the salami presented an Aw of 0.9417 and reached an Aw of 0.90 at 252 hours. The average daily decrease in the Aw value was calculated to be 0.0050. Most microorganisms displayed maximum growth between Aw values of 0.990 and 0.995; thus, the reduced Aw value increases the lag phase of the growth curve, leading to a decrease in bacterial multiplication (Beales, 2004).

The quantity of *S. enterica* at zero hours was 7.04 log CFU g^{-1} and 7.13 log CFU g^{-1} on XLD and TAL agar, respectively. The number of *S. enterica* increased until 21 hours of fermentation, reaching 8.27 log CFU g^{-1} on XLD agar and 8.37 log CFU g^{-1} on TAL agar. Afterwards, a decline until 2.59 log CFU g^{-1} (XLD) and 3.03 log CFU g^{-1} (TAL) was observed after 941 hours of maturation. Therefore, the reduction in the *S. enterica* population after fermentation was equivalent to 5.67 log CFU g^{-1} and 5.33 log CFU g^{-1} on XLD and TAL, respectively. The *S. enterica* reduction observed in our study was greater than that in other reported fermentation and drying protocols (Mataragas et al., 2015). It is worth noting that the maturation time in our study was 39 days, which is longer than the period of 14 to 28 days usually adopted in the manufacturing of Brazilian salami (Fieira, Marchi, Marafão, & Alfaro, 2018; Santa et al., 2014)

Studies suggest that the maturation of the salami is completed when the product reaches Aw values

below 0.87 (Campagnol et al., 2011; Cirolini et al., 2010); however, the Brazilian regulation allows Italian salami to present an Aw value of 0.90 (Instrução Normativa nº 22, 2000). In our study, when the salami reached an Aw of 0.90 (at 252 hours of maturation), a *S. enterica* population above 6 log CFU g^{-1} was still present. It should be noted that the initial *S. enterica* concentration was increased in our study to simulate a worse scenario of contamination. Although the initial contamination levels of raw pork are unlikely to be close to 7 log CFU g^{-1}, the occurrence of temperature abuse and cross-contamination during processing can increase the initial bacterial load to values higher than those usually reported (Nightingale et al., 2006).

Pathogens subjected to environmental stress, such as changes in pH and Aw during the maturation of sausages, present different patterns of cell damage and death. As a result, the recovery of injured cells may be difficult, which may cause inaccuracies in determining the behavior of pathogens (Hwang et al., 2009; Riordan et al., 1998). Injured cells are usually repaired and become functionally normal after exposure to favorable environmental conditions, such as cultivation in a nonselective medium; however, they may not recover if transferred directly to selective media (Kang & Fung, 2000). In the present study, to minimize this failure, TAL agar was used. By including an overlay of TSA agar, both types of cells (injured and noninjured) were expected to grow and form typical colonies. In fact, the use of TAL agar enabled a higher mean recovery of *S. enterica* cells, at approximately 0.14 log CFU g^{-1}, than that with XLD agar ($p < 0.05$). In contrast, Ferreira, Horvath and Tondo (2013) found no significant difference in the recovery of injured cells from *S.* Enteritidis, *S.* Typhimurium and *S.* Bredeney when XLD and TAL agar were compared. Other studies, however, reported results that corroborate the greater recovery of injured *S.* Typhimurium in associated culture media such as TAL (Chang et al., 2003; Kang & Siragusa, 1999; Kang & Fung, 2000; Wu et al., 2001). According to Nightingale et al. (2006), the improvement achieved in *Salmonella* quantification when using selective culture media associated with nonselective media suggests the presence of injured but viable cells. These findings highlight the importance of using protocols that are capable of accurately evaluating the survival of *S. enterica* during manufacturing processes of ready-to-eat (RTE) products.

In addition to the fact that the formulation and maturation time of salami manufacturing are not determined in Brazilian food regulation (Instrução Normativa nº 22, 2000), other factors may influence the processing, including the competitive microbiota present in raw meat and differences in the Aw and initial pH between salami units belonging to the same batch or to different batches (Roccato et al., 2017). Thus, it is difficult to determine a processing protocol for salami that excludes the risk of *S. enterica* survival. For this purpose, mathematical models have been proposed to predict the behavior of microorganisms in complex foods. In this sense, mathematical modeling should be applied to the data obtained in this study to predict pathogen behavior and analyze the risks.

Conclusion

In the manufacturing of salami in this study, the fermentation step lasted 66 hours and the drying step lasted 875 hours. After 66 hours of fermentation, the pH decreased from 6.69 to 5.4, while the *S. enterica* count increased in the first 21 hours. At the end of the drying stage (after 39 days of maturation), the Aw was lower than 0.78, and a reduction of approximately 5 log CFU of the initial *S. enterica* population in the mass of the salami was observed. However, total elimination of the bacteria was not achieved at the end of the process. TAL agar was more efficient in recovering *Salmonella* cells after the maturation stage than XLD agar.

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