# Behavior of *Listeria monocytogenes* during the Manufacture and After HPP of Dry-cured Sausages: Effect of Fat Content and Curing Salts

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Abstract—To evaluate the behavior of Listeria monocytogenes "chorizo" with high or medium fat level and with or without curing salt were intentionally contaminated and manufactured according to a traditional process. After drying (60 days), a HPP of 500 MPa for 7 min was applied to different batches. At the end of drying process, the sausages presented a<sub>w</sub> values of 0.775-0.808 and pH values of 4.8-4.9, typical values of Spanish "chorizo". A clear effect of fat content and curing salts addition was observed on Listeria monocytogenes counts. This pathogen grew during fermentation process in all batches except in "chorizo" made with medium fat content and curing salts; in this "chorizo" type, Listeria monocytogenes counts decreased. Finally, no inactivation on Listeria monocytogenes due to HPP was observed.

*Index Terms*—Chorizo, *Listeria monocytogenes*, fat level, curing salt and high hydrostatic pressure

# I. INTRODUCTION

"Chorizo" is a typical Spanish dry-fermented sausage manufactured with traditional technologies using meat and fat, together with salt, garlic, Spanish paprika and oregano. Traditional fermented sausages are meat products with a fat content of 35 to 50% [1] although currently growing demand for healthier products is stimulating development of low-fat meat product. On the other hand, in industrial formulation curing agents such as nitrate or nitrite are usually added, aiming at growth inhibition of undesirable bacteria in combination to pH reduction, which occurs during fermentation [2]. However, nowadays it is more common to prepare "chorizo" without curing agents because consumers relate nitrite in meat products with an unhealthy image. Taking into account that when the nitrite is added to meat products, it slows the growth of pathogens such as Listeria monocytogenes since lag-phase duration increases [3], from a safety point of view, not to use nitrate or nitrite would be of concern in relation to the control of L. monocytogenes in the meat industry [4].

High pressure processing (HPP) in the meat industry has primarily been used to improve the microbiological safety and shelf-life of ready-to-eat (RTE) meat products

pre/post-packaging non-thermal as а novel decontamination technology [5]. HPP has been recognized by several organizations and administrations as a useful listericidal post-processing treatment for RTE foods [6], [7]. In general, pathogen lethality during HPP depends on various processing parameters such as the pressure level and holding time. References [8,9] have reported that pressure treatments of up to 300 MPa are insufficient to inactivate L. monocytogenes in different meat products. Reference [10] pointed out a reduction of L. monocytogenes counts when HPP of 400-600 MPa during 5-10 min were applied in dry fermented sausages. Regarding time, nowadays meat industry, within a production line, apply the shortest HPP, from 3 to 6 min maximum [11], [12]. In addition to processing parameters, intrinsic factors of food matrices also have an effect on the inactivation of bacteria during pressure treatment [13], [14]. Several studies have shown that L. monocytogenes baroresistance increases when meat products present a low a<sub>w</sub> [9], [10]. On the other hand, some studies have revealed that the increase in fat content results in an increase in the pressure resistance of microorganisms to HPP [13], [15].

This work aimed to study the behavior of *L. monocytogenes* during the manufacture of a Spanish "chorizo" made with different fat level and different curing salts content and to evaluate the effect of HPP on this kind of dry fermented sausages.

## II. MATERIAL AND METHODS

# A. Experimental Design

Four different types of "chorizo" were intentionally contaminated with L. monocytogenes: (1) HF, sausages manufactured with a high content of fat (30% pork back fat and 70% pork meat); (2) HFN, sausages manufactured with a high content of fat (30% pork backfat and 70% pork meat) and with 300 ppm of curing salts (potassium nitrate/sodium nitrite, 1:1), (3)MF. sausages manufactured with a medium content of fat (20% pork back fat and 80% pork meat) and (4) MFN, sausages manufactured with a medium content of fat (20% pork back fat and 80% pork meat) and with 300 ppm of curing salts (potassium nitrate/sodium nitrite, 1:1).

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# B. Bacterial Strains and Culture Preparation

For inoculation, a four-strain cocktail mixture of L. monocytogenes was used. Three strains of L. monocytogenes were isolated from dry fermented meat products and the other one was obtained from Spanish Type Culture Collection (CECT935, serotype 4b).

To prepare the inoculums, *L. monocytogenes* cultures were individually grow. Initially, 100 µl of the stock cultures (stored in 20% glycerol at -80 °C) were transferred to 10 ml Brain Heart Infusion (BHI, from Scharlau, Barcelona, Spain) broth and incubated for 24 h at 37 °C. One ml from each individual strain was transferred to a second tube of 9 ml BHI and incubated overnight for 18 h at 37 °C, resulting in an early stationary phase culture. The cocktail for inoculation was prepared by mixing equal volumes of all four culture strains in 0.1 % peptone water in a sterile container in order to obtain a level of inoculum of about  $10^{6-7}$  cfu/g of sausage mixture.

## C. "Chorizo" Manufacture and Sampling

All sausages were prepared using the same technology and according to a traditional formulation. Lean pork meat and pork back fat were minced (P-32 FUERPLA, Valencia, Spain) to a particle size of about 15 mm and subsequently mixed in a vacuum mixer (A- 85 FUERPLA, Valencia, Spain) with the following common ingredients per kilogram of meat mixture: 20 g sodium chloride, 20 g paprika, 10 g dextrose, 1.5 g garlic, 1.0 g oregano, 1.0 g black pepper and 1.0 g polyphosphates. To manufacture batches HFN and MFN potassium nitrate (150 ppm) and sodium nitrite (150 ppm) were also added. The sausage mixture for each type of "chorizo" was mixed with the cocktail cultures for 1 min and stuffed into 60 mm ø casings. The sausages were fermented in a drying chamber (Hermekit, Cenfrio, Spain) at 15 °C and 90-100% relative humidity (RH) for 18 h, 22-23 °C and 90% RH for 48 h, 14–15 °C and 80–90% RH for 10 days. Then the RH was slowly reduced to 75% until the end of the ripening process (a total of 60 days of processing).

The proximate composition was evaluated on sausage mixture just before inoculation *L. monocytogenes.* To calculate the weight losses, the weights of five sausages for each types of "chorizo" were recorded just after stuffing (0 day) and periodically during ripening (35 and 60 days). Analysis of pH, water activity and *L. monocytogenes* counts were performed during the "chorizo" production stages (day 0: sausage mixture prior to stuffing, day 3: after fermentation, day 35: half of ripening and day 60: end of ripening).

# D. High Pressure Processing

"Chorizos" were individually packed with plastic bags (polyamide/polyethylene with an oxygen transmission rate of 30–40 cm<sup>3</sup>/m<sup>2</sup>/24 h/bar at 23 °C and 50% RH and a water vapour transmission rate of 2.5 g/m<sup>2</sup>/24 h at 23 °C and 50% RH, supplied by WK Thomas España S.L., Rub fSpain) which were subjected to vacuum and sealed using a packer (mod. EVT-7-TD Tecnotrip, Barcelona, Spain). Once vacuum packaged, the outside of the packages were sanitized with 70% ethanol, and the vacuum packaging process and sanitizing procedure were repeated two more times, such that all samples were triple bagged. Then, the packed sausages were subjected to high pressure of 500 MPa for 7 min. at an industrial hydrostatic pressure unit (Wave 6000/135. NC Hyperbaric, Burgos, Spain) equipped with a 135 l volume high-pressure vessel using additive-free water as the pressure transmitting fluid. In all cases, the initial water temperature was 18  $^{\circ}$ C, the treatment pressure was reached in approximately 4 min and decompression was instantaneous.

After the HPP of the sausages, *L. monocytogenes* counts were carried out. The inactivation of *L. monocytogenes* by HPP was evaluated in terms of logarithmic reductions as the difference between counts after the treatment (N, log cfu/g) and the initial inoculum level (N<sub>0</sub>, log cfu/g) (i.e. log (N/N<sub>0</sub>).

# E. Physico-chemical Analysis

Moisture content of the different types of sausages was determined by drying following the ISO 1442:1997 method. Fat content was evaluated by Sohxlet extraction with diethyl ether according to the ISO method 1443:1973. Protein was measured using the AOAC official method 990.03 (2000), the Dumas nitrogen combustion method, using a Leco TruSpec Nitrogen Determinator (LECO INSTRUMENTOS, S.A. MADRID). Water activity was measured using a Decagon CX-2 AQUALAB hygrometer (Decagon Devices Inc., Pullman, WA, USA) at 20 °C. The pH was measured using a Crison model 507 pH meter with a puncture electrode.

#### F. Microbiological Analysis

For the microbiological determinations, the sausages were sampled by aseptically opening the casings with a sterile lancet and removing 10 g from different places to long of the sausage. Samples were placed in a sterile plastic bag, mixed (1:10) with buffered peptone water (Scharlau, Barcelona Spain) in a PK 400 Masticator (IUL, S.A., Barcelona, Spain) for 2 min and then incubated for 1 h  $\pm$ 5 min at 20 °C  $\pm$ 2 °C. The homogenate was serially diluted in sterile tryptone water (Scharlau, Barcelona Spain) plated onto the selective media ALOA (Biomerieux, Madrid, Spain) and incubated at 37 °C  $\pm$  1 °C for 48 h  $\pm$ 3 hours.

#### G. Statistical Analysis

Data were subjected to variance analysis using an ANOVA to examine the effect of fat content, curing salts contents, manufacturing time and HPP. Differences between particular sample means were analysed according to Fisher's least significant difference (LSD) test. All statistical analyses were performed using the Statgraphics Centurion, XVI computer package.

# III. RESULTS AND DISCUSSION

# A. Chemical Composition and Processing Characteristics

The proximate composition of the "chorizos" at day 0 of processing is shown in Table I. As expected

statistically significant differences were found among types of sausages (P<0.05). "Chorizo" HF and HFN presented the higher fat content and the lower moisture and protein content. The fat contents were  $36.75\pm9.26$  g/100g in sausages with a high fat content (HF and HFN) and  $13.93\pm0.78$  g/100g in "chorizo" with a medium fat content (MF, MFN). Variations in the trimming of meats from visible fat may have contributed to the deviations

observed among these values and the targeted values (30 and 20% fat content). Fat content in sausages with a high fat content (HF and HFN) was similar to dry sausages made with a normal recipe, which have fat contents around 32% in sausage mixture and as a result of drying these values rise to about 40–50% at end of processing [16].

"Chorizo" type <sup>1</sup>	Moisture (g/100g)	Protein (g/100g)	Fat (g/100g)
High fat (HF, HFN)	$48.35\ \pm 8.70^{\rm A}$	$13.05 \pm 3.18^{\rm A}$	$36.75 \pm 9.26^{B}$
Medium fat (MF, MFN)	$64.75 \pm 0.19^{B}$	$17.93 \pm 0.17^{B}$	$13.93 \pm 0.78^{\rm A}$

TABLE I. PROXIMATE COMPOSITION (MEAN ±SD) OF "CHORIZOS" IN SAUSAGE MIXTURE (0 DAY).

<sup>1</sup>HF: high fat content, HFN: high fat content and curing salts, MF: medium fat content, MFN: medium fat content and curing salts. <sup>A-B</sup> Values within the same column with different superscript letters are different (P<0.05).

The weight losses throughout the drying process of the different types of "chorizo" are summarized in Fig. 1



Figure 1. Weight losses (%) throughout the drying process of the different "chorizo" types (HF: high fat content, HFN: high fat content and curing salts, MF: medium fat content, MFN: medium fat content and curing salt). Different letters in each drying time, indicate significant differences between "chorizo" types (LSD test: P<0.05).

No effects (P>0.05) were observed due to the addition of curing salts. On the contrary, weight losses were significantly affected by the fat level (P <0.05). At the

end of drying, weight losses achieved a value of 33% on HF and HFN sausages and a value of 52% on MF and MFN sausages. As is known, weight losses depend as processing factor (temperature, relative humidity and air movement of the ripening room and ripening time), as sausage characteristic (centesimal composition).

# B. Evolution of $a_w$ and pH

Results of the  $a_w$  measurements are shown in Table II. In general,  $a_w$  values decreased (P<0.05) throughout the manufacturing process. At 0 day, HF and HFN sausages had lower  $a_w$  than the other batches (P<0.05). However, these differences between sausages with high fat content and medium fat content may be due to small differences in chemical composition of raw matter. Initially, two batches of raw meat were prepared, one to obtain the high fat batches (HF and HFN) and the other to obtain medium fat batches (HF and HFN). Besides, after 35 days of drying, no differences were observed. As in our work, references [17]-[19] pointed out that fat level had no effect on  $a_w$  decrease throughout the manufactured process of fermented sausages. At the end of drying process, the sausages presented  $a_w$  values of 0.775-0.808.

"Chorizo" type <sup>1</sup>	0 days <sup>2</sup>	3 days <sup>2</sup>	35 days <sup>2</sup>	60 days <sup>2</sup>
HF	<sub>c</sub> 0.967 ±0.001 <sup>A</sup>	$_{c}0.953 \pm 0.008^{A}$	$_{b}0.875 \pm 0.033$	$_{a}0.779 \pm 0.013$
HFN	$_{b}0.965 \pm 0.008^{A}$	$_{b}0.952\ \pm 0.008^{\rm A}$	$_{b}0.924 \pm 0.019$	$_{a}0.779 \pm 0.023$
MF	$_{\rm c}0.983 \pm 0.006^{\rm B}$	$_{c}0.980 \pm 0.007^{B}$	$_{b}0.889 \pm 0.008$	$_a0.808 \pm 0.017$
MFN	$_{\rm c}0.985 \pm 0.003^{\rm B}$	$_{\rm c}0.971 \pm 0.002^{\rm AB}$	<sub>b</sub> 0.882 ±0.012	$_{a}0.795 \pm 0.018$

TABLE II. EVOLUTION OF WATER ACTIVITY (MEAN ±SD) DURING THE MANUFACTURE PROCESS OF DIFFERENT "CHORIZOS".

<sup>1</sup>HF: high fat content, HFN: high fat content and curing salts, MF: medium fat content, MFN: medium fat content and curing salts. <sup>2</sup>Day 0: sausage mixture prior to stuffing, day 3: after fermentation, day 35: half of ripening and day 60: end of ripening.

bay 0: sausage mixture prior to starting, day 5: after rementation, day 55: nan of ripening and day 60: end of  $a_{abc}$  Values within the same row with different subscript letters are different (P<0.05).

 $^{A-B}$  Values within the same column with different subscript letters are different (P<0.05).

The results of pH obtained during the manufacture process of the "chorizos" are shown in Table III. Throughout the process pH values of fermented sausages decreased (P<0.05) until day 35 and then remained constant (P>0.05). In the final product, the pH value was 4.8-4.9. As is known, the lactic acid bacteria derived from the raw materials or the environment are responsible for

both lactic acid production resulting from carbohydrate utilization, and of a low pH value (5.9-4.6).

Regarding batches, differences were only found at day 3. "Chorizos" manufactured with curing salts added presented the highest pH values, whereas MF sausages shown the lowest (P < 0.05).

"Chorizo" type <sup>1</sup>	0 days <sup>2</sup>	3 days <sup>2</sup>	35 days <sup>2</sup>	60 days <sup>2</sup>
HF	<sub>b</sub> 5.50 ±0.20	$_{b}5.37 \pm 0.03^{B}$	$_{a}5.02 \pm 0.13$	$_{a}4.88 \pm 0.01$
HFN	<sub>b</sub> 5.55 ±0.07	$_{b}5.48 \pm 0.03^{C}$	$_{a}4.79 \pm 0.17$	$_{a}4.93 \pm 0.08$
MF	c5.68 ±0.04	$_{b}5.20 \pm 0.06^{A}$	$_{a}4.85 \pm 0.04$	$_{a}4.90 \pm 0.01$
MFN	<sub>c</sub> 5.78 ±0.05	$_{b}5.48 \pm 0.02^{C}$	<sub>a</sub> 4.73 ±0.00	$_{a}4.80 \pm 0.04$

TABLE III. EVOLUTION OF PH (MEAN ±SD) DURING THE MANUFACTURE PROCESS OF THE DIFFERENT "CHORIZOS".

<sup>1</sup>HF: high fat content, HFN: high fat content and curing salts, MF: medium fat content, MFN: medium fat content and curing salts. <sup>2</sup>Day 0: sausage mixture prior to stuffing, day 3: after fermentation, day 35: half of ripening and day 60: end of ripening. Values within the same row with different subscript letters are different (P<0.05).

 $^{A-B}$  Values within the same column with different superscript letters are different (P<0.05).

TABLE IV. EVOLUTION OF L. MONOCYTOGENES COUNTS (MEAN ±SD) DURING THE MANUFACTURE PROCESS OF DIFFERENT "CHORIZOS" (LOG CFU/G)

"Chorizo" type <sup>1</sup>	0 days <sup>2</sup>	3 days <sup>2</sup>	35 days <sup>2</sup>	60 days <sup>2</sup>
HF	$_{a}6.86 \pm 0.04^{A}$	$_{ab}7.45 \pm 0.64^{B}$	$_{\rm b}8.11 \pm 0.08^{\rm C}$	$_{\rm b}8.23 \pm 0.04^{\rm C}$
HFN	$_{ab}6.79 \pm 0.06^{A}$	$_{c}7.42 \pm 0.22^{B}$	$_{bc}7.13 \pm 0.03^{B}$	$_{a}6.31 \pm 0.28^{B}$
MF	$_{a}7.32 \pm 0.00^{B}$	$_{c}8.78 \pm 0.06^{C}$	$_{c}8.82 \pm 0.08^{D}$	$_{\rm b}8.23 \pm 0.11^{\rm C}$
MFN	$_{\rm c}7.32 \pm 0.13^{\rm B}$	$_{b}5.99 \pm 0.49^{A}$	$_{b}5.47 \pm 0.08^{A}$	$_{a}4.59 \pm 0.11^{A}$

<sup>1</sup>HF: high fat content, HFN: high fat content and curing salts, MF: medium fat content, MFN: medium fat content and curing salts. <sup>2</sup>Day 0: sausage mixture prior to stuffing, day 3: after fermentation, day 35: half of ripening and day 60: end of ripening.  $_{\text{a-b-c}}$ Values within the same row with different subscript letters are different (P<0.05).  $_{\text{A-B}}$ Values within the same column with different superscript letters are different (P<0.05).

Reference [20] pointed out that when nitrate was added in sausages manufacture, a weak inhibition of lactic acid bacteria and thereby a lower pH decrease was observed. This fact could explain the highest pH values in HFN and MFN. On the other hand, studies have shown a significant difference in pH drop between high and low fat level salami during the fermentation process [17], [21]. In this sense, reference [17] concluded that the high water activity of low fat sausages could be related to a higher pH decline. In our work, similar results were obtained.

# C. Evaluation of L. monocytogenes Counts

Results of L. monocytogenes counts recorded during the manufacture process of the "chorizos" are included in Table IV. Regarding to effect of manufacturing time, L. monocytogenes grew during fermentation process in all batches except in MFN batch. In this "chorizo" type, L. monocytogenes counts decreased (P<0.05).

Although some authors have reported that curing salts and low pH values acted as the first hurdles or at least delayed the growth of unwanted microorganisms, reference [22] indicated that the fermentation was a critical point, which could promote the growth of L. After fermentation monocytogenes. process, L. monocytogenes counts remained constant in HF and MF sausages whereas a decrease (P<0.05) was observed at 60 days in the "chorizo" with curing salts added. "Chorizos" without curing salts presented the highest values of L. monocytogenes counts, which support the importance of curing salts addition in the control of this pathogen into this kind of meat product.

Regarding batches, at day 0 L. monocytogenes counts were in agreement with the established value of the inoculum. At days 3, 35 and 60 a clear effect (P<0.05) of fat content and curing salts addition was observed. Taking into account fat content of the "chorizo", a faster growth of L. monocytogenes was observed when the fat content decreased. Similar results were found by [23] in liver pat é manufactured with a reduction of the fat level by 30%. These authors pointed out that a significantly (P<0.05) longer lag phase occurred on the reference pate (35 days) than on the low fat pate (13 days) and that this difference observed in the growth of L. monocytogenes may be due to the differences in the a<sub>w</sub>.

On the other hand, as it has been mentioned in introduction section, curing salts addition affects the growth of L. monocytogenes. Thereby, in our study, the growth of L. monocytogenes was inhibited in MFN "chorizos". However, this effect was omitted when the "chorizos" were manufactured with high fat content and curing salts. Reference [24] found an antagonise antilisterial effect when both added nitrite (20 ppm) and a high-fat content (43%) were used in pork meat mixtures incubated at 4 °C for six weeks.

# D. Inactivation of L. monocytogenes by HPP

No differences were found between L. monocytogenes counts recorded before and after treatment with HPP for each type of "chorizo" (Table V). For that, no inactivation on L. monocytogenes due to HPP was observed (P>0.05).

"Chorizo" type <sup>1</sup>	Untreated	Treated	Inactivation <sup>2</sup>
HF	$8.23\ \pm 0.04$	$8.16\ \pm 0.17$	$-0.07 \pm 0.13$
HFN	$6.31 \pm 0.28$	$6.69 \pm 0.81$	$0.38 \pm 0.53$
MF	$8.23 \pm 0.11$	$7.89 \pm 0.22$	$-0.34 \pm 0.11$
MFN	$4.59 \pm 0.11$	$4.35 \pm 0.07$	$-0.24 \pm 0.18$

TABLE V. Evolution of L. monocytogenes Counts (mean  $\pm$ SD) during the Manufacture Process of Different "Chorizos" (log CFU/G).

<sup>1</sup>HF: high fat content, HFN: high fat content and curing salts, MF: medium fat content, MFN: medium fat content and curing salts. <sup>2</sup>Logarithmic reductions as the difference between counts after the treatment (N, log cfu/g) and the initial inoculum level (N0, log cfu/g) (i.e. log (N/N0).

These results may be explained because of low  $a_w$  of the "chorizos". Reference [25] observed that when the  $a_w$  of the "chorizo" was equal to 0.82, it was necessary to apply a pressure of 550 MPa to obtain an increase in the reduction of *L. monocytogenes* counts when the duration of HPP increased.

# IV. CONCLUSION

These results indicate that for the control of *L. monocytogenes* in the elaboration of Spanish "chorizo", it is necessary the incorporation of curing salts and the control of the fat level in the product. On the other hand, a HPP of 500 MPa for 7 min did not involve a reduction of *L. monocytogenes* in this kind of dry fermented sausage.

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