



# High pressure processing at the early stages of ripening enhances the safety and quality of dry fermented sausages elaborated with or without starter culture

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## ABSTRACT

To study the quality of *chorizo de León* dry fermented sausages (DFS), high pressure processing (HPP) applied at the early stages of ripening and the use of a functional starter culture were evaluated as additional safety measures. Furthermore, the ability to control the populations of artificially inoculated *Listeria monocytogenes* and *Salmonella* Typhimurium was investigated and the evolution of microbial communities was assessed by amplicon 16S rRNA metataxonomics. The use of HPP and the starter culture, independently or combined, induced a reduction of *Listeria monocytogenes* of 1.5, 4.3 and > 4.8 log CFU/g respectively, as compared to control. *Salmonella* Typhimurium counts were under the detection limit (<1 log) in all treated end-product samples. Both additional measures reduced the activity of undesirable microbiota, such as *Serratia* and *Brochothrix*, during the production of DFS. Moreover, the starter culture highly influenced the taxonomic profile of samples. No adverse sensory effects were observed, and panelists showed preference for HPP treated DFS. In conclusion, this new approach of applying HPP at the early stages of ripening of DFS in combination with the use of a defined starter culture improved the safety and quality of the meat product.

## 1. Introduction

Dry fermented sausages (DFS) are generally considered microbiologically stable products. However, these products may represent a health risk for consumers when raw materials are contaminated with high levels of pathogenic bacteria, and/or the control of antimicrobial factors is deficient. Indeed, in the last two decades DFS have been involved in cases of foodborne disease, and outbreaks occurring worldwide (Ducic, Blagojevic, Markov, Velicanski, & Buncic, 2014; Omer et al., 2018).

*Chorizo de León* is a DFS from the north-west of Spain, being

commonly produced in an artisanal manner without the use of starter cultures, which leads to the proliferation of an autochthonous beneficial microbiota during the ripening process. However, this artisanal way of manufacturing can also lead to the development of undesirable microorganisms which can compromise food safety and quality (Gormley et al., 2010).

High pressure processing (HPP) is nowadays used as an additional post-packaging safety measure in meat products (Rendueles et al., 2011). Its positive effects, such as inactivation of pathogens and better preservation of flavor compounds and nutritional value, as compared to heating, have pushed forward this technology. On the other hand,

**Abbreviations:** A-INP, Sausages without starter, without pathogens, and without HPP; A-IP, sausages without starter, without pathogens and with HPP; A+INP, sausages without starter, with pathogens and without HPP; A+IP, sausages without starter, with pathogens and with HPP; S-INP, sausages with starter, without pathogens and without HPP; S-IP, sausages with starter, without pathogens and with starter; S+INP, sausages with starter, with pathogens and without HPP; S+IP, sausages with starter, with pathogens and with HPP.

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negative effects on color, texture and water holding capacity may occur in certain meat products (Bak, Bolumar, Karlsson, Lindahl, & Orlien, 2017).

HPP is mostly applied to ready-to-eat meat products when the product is already packaged and has developed its final sensory characteristics, requiring high pressure intensity (usually up to 600 MPa), due to the relatively low water activity ( $a_w$ ) of the final product (Bajovic, Bolumar, & Heinz, 2012). On the other hand, HPP can also be applied to raw materials (Omer et al., 2015) or sausages just after stuffing (Marcos, Aymerich & Garriga, 2005), but this approach modifies negatively the quality of the meat products. In that sense, the application of HPP in the early stages of ripening could be more efficient to inactivate undesired microorganisms and preserve quality, when beneficial bacterial populations have already grown to their maximum level, and physical and biochemical transformation processes in the sausage matrix have already started.

Starter cultures are applied to DFS in order to facilitate the fermentation process. Lactic acid bacteria (LAB) with beneficial features (e.g., bacteriocin producers) can be included in the formulation to control pathogenic and spoilage microorganisms (Campus, 2010; Fadda, López, & Vignolo, 2010) and prevent the production of compounds responsible for off-flavors by spoilers. These approaches are alternative, natural preservation methods which do not generally lead to organoleptic defects (Cui & Fan, 2019). The increased demand for safe and minimally processed, additive-free products has promoted the adoption of these alternative, promising methodologies (i.e., HPP and food cultures) (Komora et al., 2021).

The study of the evolution of microbial communities during fermentation by high-throughput sequencing and metagenomic analysis is able to provide a better understanding of microbial ecology. The sequencing of taxonomically relevant genes, such as the 16S rRNA gene, provides information on the taxonomic composition of microbial communities (i.e., the relative abundance of the different genera or gene amplicons (ASVs) present in the product) (Filippis, Parente, & Ercolini, 2017; Quijada et al., 2018).

The objective of this study was to investigate the preservation effect obtained with the combined use of HPP applied in the early stages of ripening (7 days after stuffing) and a starter culture on chorizo de León DFS artificially contaminated with *Salmonella* Typhimurium and *L. monocytogenes*. In addition, the impact on the main background microbiota, physico-chemical and sensorial characteristics of the treated meat products was also assessed. Classic culture-based methods and metataxonomic analyses were combined to shed light on the evolution of beneficial and pathogenic bacterial populations during ripening.

## 2. Materials and methods

### 2.1. Production of DFS

Meat batters were obtained from an industry setting using a traditional recipe of *chorizo de León*. The ingredients used were: pork meat (72 %), pork back fat (21 %), spices (3.5 %; paprika, garlic, oregano), NaCl (1.6 %), and Ligavi 384® (1.8 %; dextrin and dextrose 82.5 %, pork proteins 17.5 %). The meat batter was pre-ripened by keeping it at 5 °C for two days, and then it was divided into eight batches following an experimental design (Fig. 1) to include the use of starter culture, HPP, and the artificial inoculation with pathogenic microorganisms. Three time independent production batches of the DFS were carried out.

### 2.2. Inoculation of strains

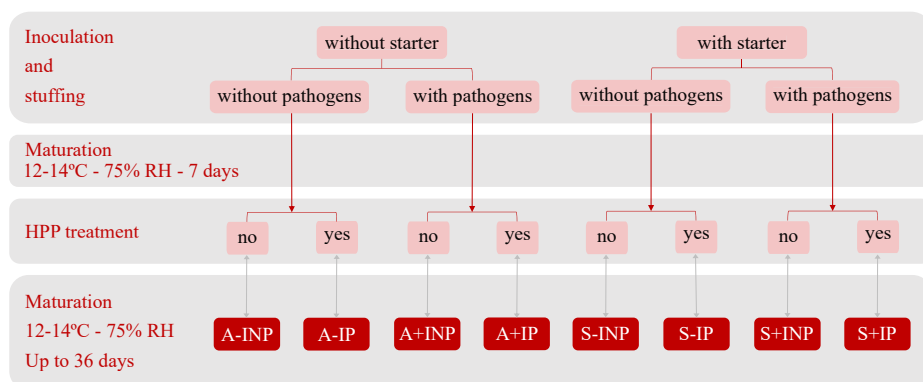
According to the experimental design (Fig. 1), four batches of the meat batter were inoculated with the lyophilized starter culture Safe-Pro® Flora Italia LC (Chr. Hansen), containing *Lactilactobacillus sakei*, *Staphylococcus carnosus* and a pediocin producing strain of *Pediococcus acidilactici* (DSM 28307). The culture was added to the batches following the manufacturer's instructions and thoroughly mixed (Mixer AS-60, Mainca) for 10 min.

Likewise, according to the experimental design, pathogenic microorganisms were added to 4 batches of meat batter. Two strains of *Salmonella* Typhimurium (CECT (Colección Española de Cultivos Tipo) 443 and CECT 722, serotype 4,5,12:i:1,2 for both strains) and two strains of *Listeria monocytogenes* (CECT 911 and CECT 940, serovar 1/2c and 4d, respectively) were used. Stock cultures stored at -20 °C in cryovials (Cryoinstant Mixed, VWR Chemicals) were used to inoculate Brain Heart Infusion broth (BHI, Oxoid), which was incubated at 37 °C for 24 h. For each strain, cell counts in broth cultures were determined by spread plating 100 µl of various decimal dilutions on xylose lysine deoxycholate (XLD, Oxoid) agar for *Salmonella*, and Agar Listeria acc. to Ottaviani & Agosti (ALOA, VWR Chemicals) for *L. monocytogenes*. For both pathogens, cultures were then mixed in approximately equal proportions to obtain an inoculum with a mixture of both strains, and an appropriate volume of each inoculum was added to the sausage batter to obtain a concentration of approximately 6 log CFU/g of each species of pathogen in the batter.

The batches were subsequently stuffed (EB-25 Mainca) into natural pork casings (32–34 mm, CDS). Sausages were labeled and stored in a ripening chamber with temperature and humidity control (12–14 °C, 75 % RH) for 5 weeks.

### 2.3. High pressure processing

HPP treatment was applied to 4 batches seven days after stuffing (Fig. 1). For that purpose, sausages were vacuum packed (20 µm



**Fig. 1.** Experimental design of the study, with 8 DFS batches, including all manufacturing stages: inoculation of starter culture and/or pathogenic microorganisms, sausages stuffing, initial 7-days maturation, HPP treatment after the 7-days maturation, and continuation of the ripening process. Abbreviations: A-INP, sausages without starter, without pathogens, and without HPP; A-IP, sausages without starter, without pathogens and with HPP; A + INP, sausages without starter, with pathogens and without HPP; A + IP, sausages without starter, with pathogens and with HPP; S-INP, sausages with starter, without pathogens and without HPP; S-IP, sausages with starter, without pathogens and with HPP; S + INP, sausages with starter, with pathogens and without HPP; S + IP, sausages with starter, with pathogens and with HPP.

polyamide/100 µm polyethylene bags), transported to the processing facility and processed (300 MPa, 5 min) in a 135 L chamber, industrial hydrostatic pressure unit (Wave 6000/135, Hyperbaric) using additive-free water as the pressure transmitting fluid. The initial water temperature was 12 °C. The time taken to achieve the holding pressure (300 MPa) was 40 s and decompression time was about 2 s. After pressure treatment, plastic bags were removed, and the processed sausages were returned to the ripening chamber for ripening.

#### 2.4. Microbiological analyses

Twenty-five g of meat batter and of cross sections from the center of the sausages were sampled after processing and during ripening (0, 7, 18 and 36 days) and added to 225 ml of Buffered Peptone Water (BPW, Merck) in filter bags (15–23 cm, Nasco, Whirl-pack), then homogenized with a stomacher (IUL Instruments) for 5 min, and decimally diluted in BPW. Counts of *S. Typhimurium* and *L. monocytogenes* were determined by spread plating various decimal dilutions on XLD agar and ALOA agar plates, respectively, which were incubated at 37 °C for 48 h. The detection limit for both pathogens was 1 log CFU/g. Enterobacteria counts (EBC) were determined on Enterobacteriaceae Count Plate Petrifilms (3 M Health Care) incubated at 37 °C for 24 h, LAB on Lactic Acid Bacteria Count Plates (3 M Health Care) incubated at 30 °C for 48 h, and Coagulase negative cocci (CNC) on Mannitol Salt Agar (MSA, Oxoid) incubated at 37 °C for 48 h. Detection limits were 1 log CFU/g for EBC and LAB, and 2 log CFU/g for CNC. Heterofermentative activity of LAB was also evaluated on Lactic Acid Bacteria Count Plates and its manifestation was presented as LAB colonies with bubbles. The microbiological analyses were conducted in three independent experiments with two technical replicates each (n = 6).

#### 2.5. Physicochemical analyses

Water activity and pH of the DFS were measured after processing and during ripening (0, 7, 18, 23 and 36 days). The pH was measured using a pH-meter Crison pH 25 (Crison Instruments). Water activity was measured at 25 °C by using a Decagon CX-2 hygrometer (Decagon Devices Inc.).

#### 2.6. Sensorial evaluation

A sensory analysis was performed by a 16-member panel, whose members were acquainted with the methodology and procedure of sensory analysis. Samples were sliced (approx. 2.5 mm of thickness) and served at room temperature. All samples were given a three-digit number and served randomly.

Three triangular comparisons were carried out: 1) A-INP vs A-IP, to evaluate the effect of pressurization in DFS elaborated without starter culture; 2) S-INP vs S-IP, to evaluate the effect of pressurization in DFS elaborated with starter culture; 3) A-INP vs S-INP, to evaluate the effect of the starter culture.

A preference test was also performed when the panelists were able to differentiate between samples. The degree of difference in regard to appearance, aroma and taste was annotated.

Finally, the attributes evaluated for each pair were odor (intensity), flavor characteristics (flavor intensity and aftertaste) and general aspect (overall appreciation). The panel members evaluated these attributes using a 5-point scale, where the left side of the scale corresponded to “dislike very much” (1) and the right side of the scale corresponded to “like very much” (5). DFS were evaluated after a 5-week ripening.

#### 2.7. Instrumental color and texture measurement

Color determinations were performed on finished vacuum packed DFS after 15 days of storage (4–6 °C) as described by Chen et al. (2019) using a CM-5/CR-5 Konica Minolta. The procedure of Marcos et al.

(2007) was adopted for texture analysis on finished vacuum packed DFS after 15 days of storage (4–6 °C). TA-XT2i Texture Analyzer was employed for texture analysis, using a speed of 5 mm/s instead of 1 mm/s and compression of 60 % instead of 75 %.

#### 2.8. Total DNA extraction

Samples for extraction of total metagenomic DNA were taken from meat batters and sausages at days 7, 18 and 36. 10 g were homogenized with 90 ml Phosphate Buffered Saline (PBS, Sigma-Aldrich) using a stomacher (IUL micro) for 2 min. Then, the homogenate was centrifuged at 5,000 g for 15 min at room temperature, and cell pellets were resuspended with PBS. This step was repeated three times. Total metagenomic DNA was extracted using the DNeasy® PowerSoil® Pro Kit (QIAGEN) following the manufacturer's specifications. In addition, a ZymoBIOMICS Microbial Community Standard (cat. no. D6300, Zymo Research) was used as a positive control. DNA concentrations were quantified using the fluorometric assay Qubit™ dsDNA HS assay kit with a Qubit 3.0 fluorometer (Invitrogen).

#### 2.9. 16S rRNA gene amplicon sequencing

Amplicon sequencing of the 16S rRNA gene was done with the Illumina Miseq platform using 300 bp pair-end sequencing. The primers used were S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (Carrasco et al., 2020), which amplify the V3-V4 hypervariable region, giving an average amplicon length of 430 bp. PCR amplification, preparation of Miseq libraries, and sequencing were performed at the sequencing platform of Centro de Investigación Biomédica de La Rioja (CIBIR), Spain.

#### 2.10. Bioinformatic analysis and data processing

Raw reads were initially processed using DADA2 v1.8.0 (Callahan et al., 2016) and following the authors tutorial. Firstly, primer sequences were removed using the *cutadapt* (Martin, 2011), and ambiguous bases were removed using the *truncLen = c(275,250)*. Chimeras were removed after the construction of the sequence table, and an Amplicon Sequence Variant (ASV) table was obtained by comparing the clean sequences with the reference RDP database version 18 (Ribosomal Database Project) by using the *assignTaxonomy* command in DADA2. Alpha-diversity indexes were calculated using the *specnumber* and diversity commands from *vegan* while beta-diversity analysis was done using Bray Curtis dissimilarity distances with the *cmdscale* command. Significant differences in alpha-diversity indices were determined using the Wilcoxon test by using the *compare means* command from *ggpubr*. Significant differences in the beta-diversity analysis were calculated through an analysis of dissimilarity test (ADONIS), using the *adonis* command from *vegan*. Plots were produced using the *ggplot2*. All analyses and plots were carried out in RStudio version 4.0.2.

#### 2.11. Accession numbers

All the raw reads have been deposited at the National Center for Biotechnology Information and are available under the Bioproject ID PRJNA906057.

#### 2.12. Statistical analyses

Data obtained from the experimental runs in replicate (n = 3) were subjected to the determination of mean values, standard deviations, coefficient of determination and the analysis of variance (ANOVA) followed by Tukey test for the significance of differences between grouped data. Statistical analysis was conducted by using Statistica Version 14 software. (Stat. Soft. Inc.). For sensory analyses, Roessler tables were

used for estimating the significance in a triangle test with 16 member-panels. Also, the table for establishing significant differences in a two-sample preference test was employed according to Anzaldúa-Morales (1994).

### 3. Results and discussion

#### 3.1. Evolution of physicochemical parameters

The evolution of pH and  $a_w$  throughout ripening was measured to evaluate the impact of starter culture and/or HPP on the DFS. Results are shown in Fig. 2. The initial pH at day 0 in DFS without starter culture added and without HPP (A-INP), was  $6.0 \pm 0.1$ , which is in agreement with results from previous studies with south European DFS (Juárez-Castelán et al., 2019; Martín et al., 2021; Rodríguez-González, Fonseca, Centeno, & Carballo, 2020). The pH drop was more pronounced initially in sausages with starter ( $p < 0.05$ ), similar to what Latorre-Moratalla et al. (2007) found. However, at the end of the ripening process pH values were similar for all types of DFS, with a final pH of around 5.0, with the exception of sausages without starter but with HPP treatment

(A-IP) which showed significantly higher pH ( $5.4 \pm 0.1$ ,  $p < 0.05$ ) probably due to the partial inactivation effect of HPP on autochthonous LAB (Marcos, Aymerich & Garriga, 2005).

Water activity in A-INP sausages slowly decreased during ripening from an initial value of 0.98, reaching a value of  $0.88 \pm 0.01$  in the finished product. Other authors have reported similar values, reaching  $a_w$  of 0.84 after 30 days ripening (Juárez-Castelán et al., 2019). DFS with added starter culture and without pressurization (S-INP) reached lower  $a_w$  at the end of ripening ( $0.85 \pm 0.01$ ). For the HPP treated sausages (A-IP and S-IP), the decrease of  $a_w$  was faster, and lower values ( $0.82 \pm 0.01$  and  $0.80 \pm 0.01$ , respectively) were reached ( $p < 0.05$ ). This fact is probably due to the denaturation of proteins in HPP treated products, which favors desiccation (Marcos, Kerry, & Mullen, 2010).

#### 3.2. Evolution of indigenous microbiota and starter culture bacteria, as determined by culture-dependent analyses

Culture-dependent analyses were carried out to study the evolution of different bacteria in the sausages during the 36-day of ripening under the different conditions applied.

##### LAB.

The evolution of LAB throughout ripening was similar in all types of DFS (Fig. 3). This group became dominant in all batches, reaching  $8.9 \pm 0.2$  log CFU/g and  $8.5 \pm 0.2$  log CFU/g after 7 days of ripening in A-INP and A-IP, respectively. Higher ( $p < 0.05$ ) LAB counts ( $9.5 \pm 0.2$  log CFU/g) were observed at day 7 in sausages with starter culture (S-INP). In A-IP DFS, immediately after HPP treatment LAB slightly decreased ( $< 0.5$  log), while in S-IP DFS higher reductions were detected (1–1.5 log). Throughout the rest of the ripening, LAB counts slightly decreased and at the end of the process reached the lowest values, i.e.,  $8.7 \pm 0.4$  log and  $7.4 \pm 0.4$  log CFU/g in S-INP and S-IP DFS, respectively. These results show that the starter culture has good potential for growth in DFS but is somewhat less adapted to the stress conditions prevailing during ripening than the indigenous LAB population in DFS without starter. Contrary to our findings, Marcos, Aymerich & Garriga (2005) observed that when the same pressure (300 MPa; 10 min) was applied to DFS immediately after stuffing, the growth of LAB was delayed. On the other hand, Latorre-Moratalla et al. (2007) did not find a significant influence of a 200 MPa (10 min) HPP treatment applied on chorizo sausages just after stuffing on LAB progression during maturation.

A remarkable finding was the suppression of heterofermentative LAB in A-IP DFS compared to levels in A-INP DFS, which was detected not only by following the culture-dependent approach (1–1.5 log CFU/g reduction of heterofermentative LAB on HPP treated sausages) but also by the metataxonomic approach (Figs. 4-6; lower relative abundances of heterofermentative species (e.g., *Leuconostoc*) compared to homofermentative ones (e.g., *Latilactobacillus*)). Therefore, HPP has the capacity to reduce the heterofermentative activity and the accumulation of undesirable products (carbon dioxide, hydrogen peroxide, acetic acid, ethanol, etc.) and, as a consequence, could have a positive effect on the quality of the end products. Hence, our results evidence that the application of HPP after one week of ripening favors the development of a desirable microbiota leading to a better and sustained growth of homofermentative LAB.

##### Coagulase negative cocci.

Numbers of CNC (Fig. 3) were to a large degree stable throughout ripening and were marginally affected by HPP or the addition of the starter culture to DFS.

Initial levels were already high in the batter ( $6.5 \pm 0.2$  log CFU/g, day 0), probably due to a positive effect on CNC populations of the pre-ripening phase (Toldra, 2002) and showed a moderate but statistically significant ( $p < 0.05$ ) growth along ripening, reaching  $7.4 \pm 0.2$  log CFU/g by day 18. Then, they slightly decreased to  $7.0 \pm 0.4$  log CFU/g on the last day of production in A-INP sausages. The results show that CNC are tolerant to HPP and to the environmental conditions prevailing during DFS ripening (Leroy, Verluysen, & De Vuyst, 2006).

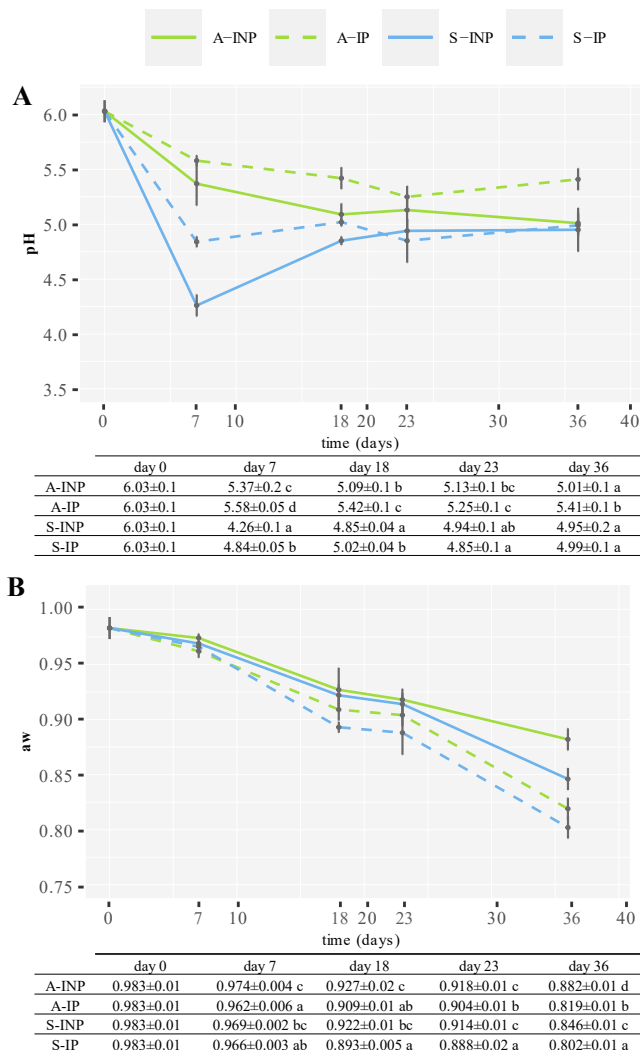
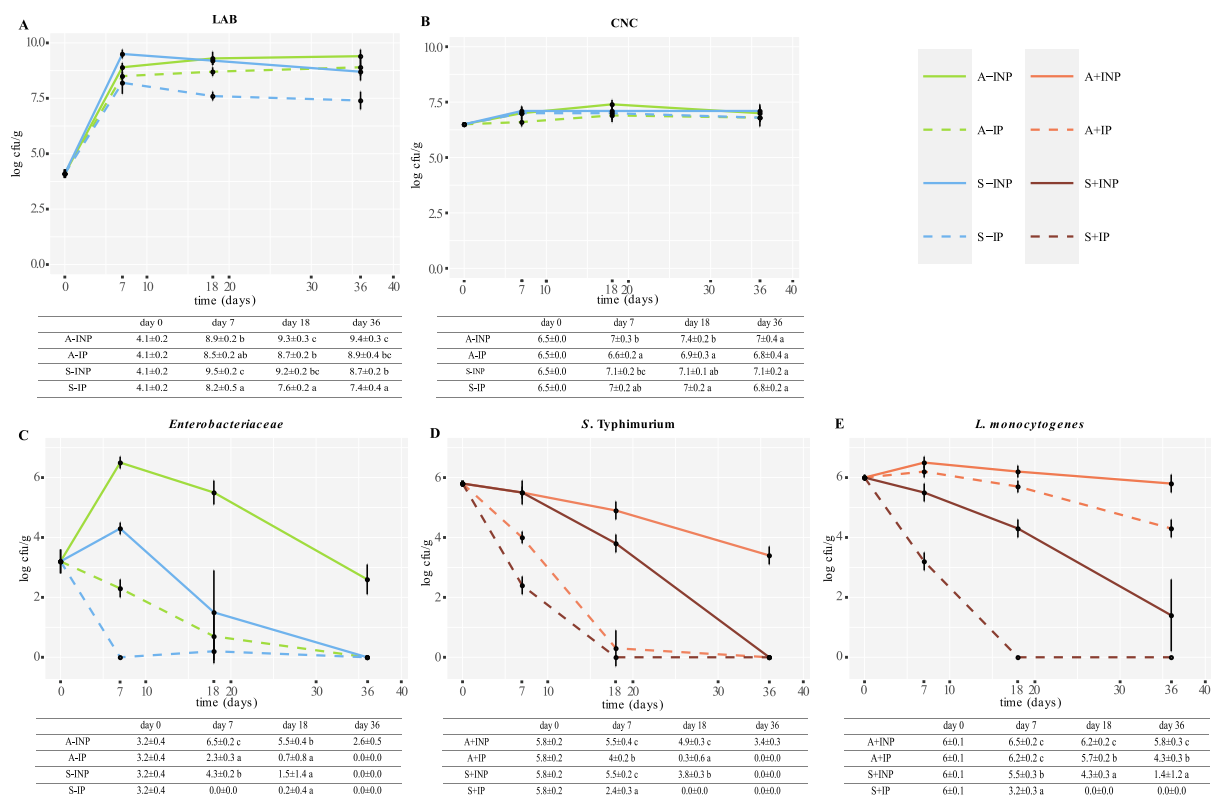
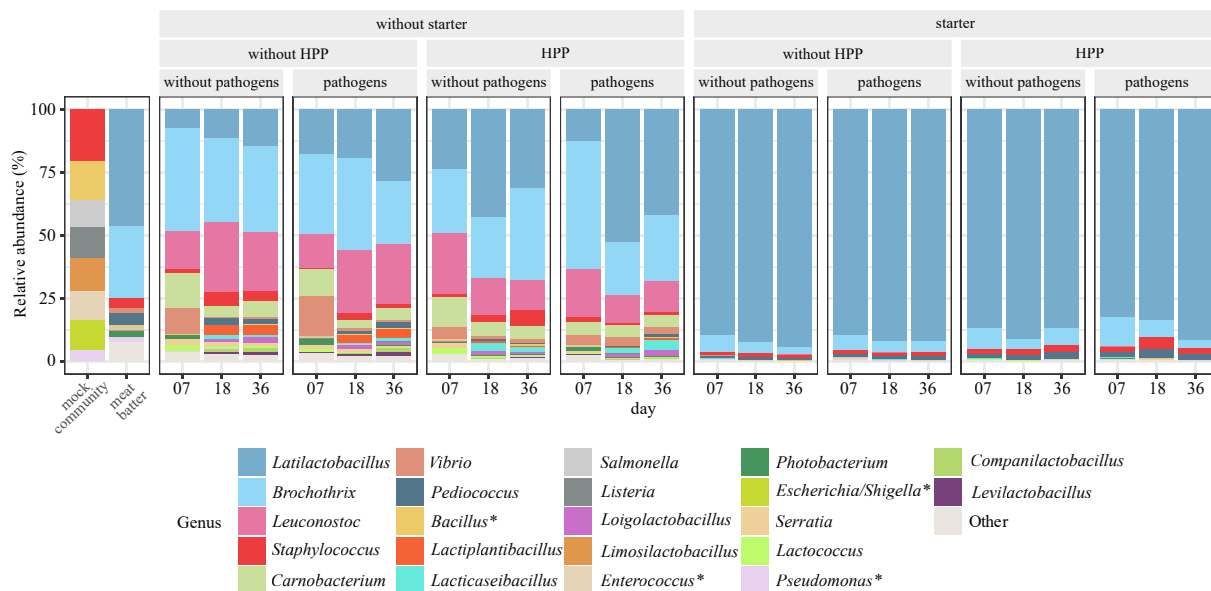


Fig. 2. Evolution of pH and  $a_w$  during the 36-days long ripening of DFS: green lines correspond to sausages with no starter culture added and blue lines to sausages with starter culture added; dashed lines correspond to HPP-treated sausages and straight lines to not HPP-treated sausages. Each bar shows the mean and the standard deviation of 6 replicates ( $n = 3 \times 2$ ). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** Evolution of microbial counts of (A) Lactic acid bacteria, (B) Coagulase Negative Cocci, (C) *Enterobacteriaceae*, (D) *Salmonella Typhimurium* and (E) *Listeria monocytogenes* during the 36-days long ripening of DFS. Green lines correspond sausages with no starter culture added and blue lines to sausages with starter culture added; orange lines correspond to sausages with no starter culture added but inoculated with both pathogens and brown lines correspond to sausages with starter culture added and inoculated with both pathogens; dashed lines correspond to HPP-treated sausages and straight lines to not HPP-treated sausages. Each bar shows the mean and the standard deviation of 6 replicates ( $n = 3 \times 2$ ). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Relative abundance of bacterial taxa at genus level for the 8 batches of DFS during the 36-days long ripening. (\*) Genera only present in the mock community.

**Enterobacteriaceae.**

Members of this group were clearly affected by intrinsic factors, starter culture activity and HPP treatment (Fig. 3). *Enterobacteriaceae* counts increased in A-INP DFS in the first week from  $3.2 \pm 0.4$  to  $6.5 \pm 0.2$  log CFU/g, taking advantage of existing favorable conditions ( $a_w$  and

pH). But from this point onwards numbers decreased, similarly to what has been previously reported in the investigations of Comi et al. (2005), and Drosinos et al. (2005), where they reported a decrease in their counts. Remarkably, this reduction along time was deeper and faster when HPP, the starter culture, or their combination were applied. HPP

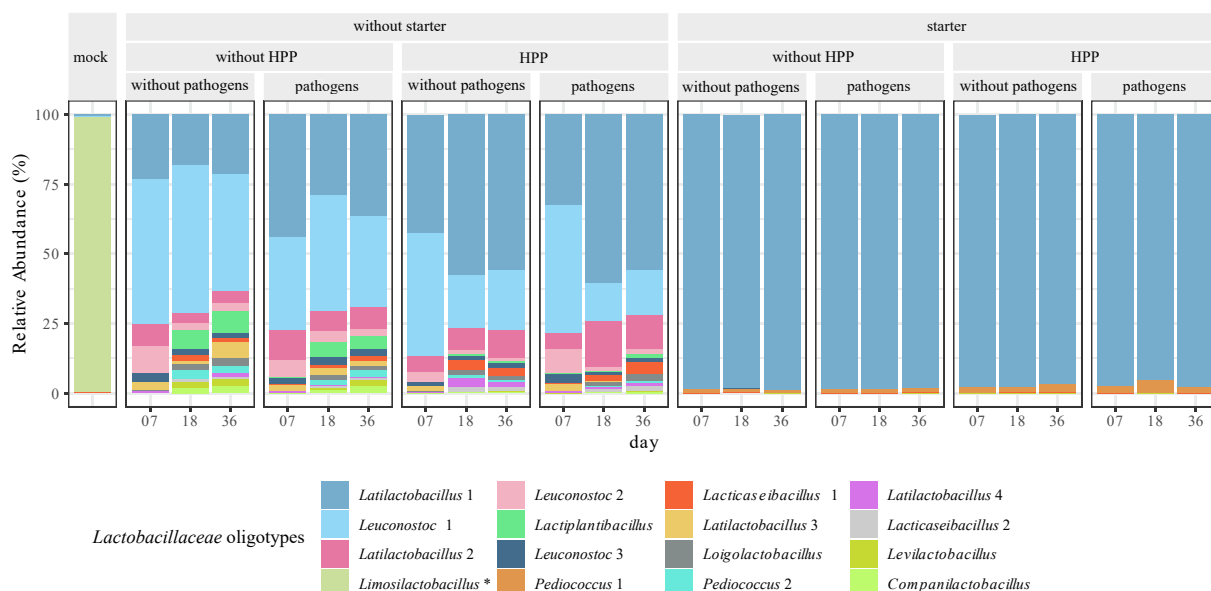


Fig. 5. Relative abundance of *Lactobacillaceae* oligotypes for the 8 batches of DFS during the 36-days long ripening. (\*) Oligotypes only present in the mock community.



Fig. 6. Relative abundance of members of the *Lactobacillaceae* family grouped by genus for the 8 batches of DFS during the 36-days long ripening. (\*) Genera only present in the mock community.

provoked a 4-log reduction in *Enterobacteriaceae* counts compared to unpressurized sausages at the same stage of production. The combined use of HPP and the starter culture caused a synergistic effect, with *Enterobacteriaceae* counts being below the detection limit (1.0 log CFU/g) immediately after HPP. At the end of the production process *Enterobacteriaceae* counts were  $2.6 \pm 0.5$  log CFU/g in A-INP DFS, while in the other DFS batches were under the detection limit (<1 log CFU/g).

Latorre-Moratalla et al. (2007) investigated the application on chorizo of starter cultures and high pressure (200 MPa, 10 min) just after stuffing and they found that HPP reduces growth of enterobacteria. However, they evidenced that the use of starter cultures is more effective in inhibiting enterobacteria than a 200 MPa HPP treatment. Omer et al. (2015) reported that the use of HPP (600 MPa, 6 min) for the processing of meat trimmings used to produce DFS (morr, salami) induced a 1.2 to 1.7 additional log reduction of *Enterobacteriaceae* counts, as compared to

sausages elaborated from untreated meat.

### 3.3. Evolution of *S. Typhimurium* and *L. monocytogenes*

Populations of *S. Typhimurium* and *L. monocytogenes* were counted by culture-dependent methods to evaluate the effect of the starter culture and/or HPP on their growth. The production process following standard conditions (A + INP sausages) resulted in an overall reduction of *S. Typhimurium* of 2.4 log CFU/g in the DFS, from initial counts of  $5.8 \pm 0.2$  log CFU/g (Fig. 3). Marcos, Aymerich & Garriga (2005) reported a lower reduction level of *Salmonella* during ripening, of just 1 log, in chorizo produced also without added nitrite and without starter culture.

The use of the starter culture accelerated the inactivation of *S. Typhimurium*, which was not detected at the end of ripening (<1 log CFU/g) in S + INP DFS. Therefore, the reduction was at least 2.4 log

higher than in *chorizo* with no starter culture added.

The application of HPP alone significantly increased the inactivation of *S. Typhimurium*, which was barely detected after 18 days of ripening, while at the end of production it remained undetected. Porto-Fett et al. (2010) showed that the application of 483 Mpa (12 min) or 600 Mpa (5 min) at the end of production reduced *S. Typhimurium* in Genoa salami for at least an additional 2.4 log, compared to control samples. Marcos, Aymerich & Garriga (2005) found that a pressure of 300 Mpa applied the day after stuffing led to a reduction of *Salmonella* by 2 log after 27 days of ripening, i.e. one log higher than in untreated sausages.

In S + IP DFS, *S. Typhimurium* counts were already under the detection limit after 18 days of ripening. Therefore, the combination of the starter culture and HPP showed a powerful, intense synergistic effect.

Regarding *L. monocytogenes*, numbers were stable along ripening in A + INP sausages. Thus, after a slight increase (ca. 0.5 log CFU/g) from initial counts of  $6.0 \pm 0.1$  log CFU/g, the ripening process very slightly reduced the counts to  $5.8 \pm 0.3$  log CFU/g in the final product. When the starter culture was used (S + INP), *L. monocytogenes* numbers declined steadily throughout ripening, with average values of  $4.3 \pm 0.3$  log CFU/g at day 18 and  $1.4 \pm 1.2$  log CFU/g in the finished sausages. Overall, in S + INP samples, *L. monocytogenes* reductions at the end of ripening were >4 logs higher than in A + INP sausages.

In other studies of DFS elaborated with starter cultures, *L. monocytogenes* reductions were also reported but in noticeable lesser level, ranging from below 1 log CFU/g to 2.2 log CFU/g (Cosansu, Geornaras, Ayhan, & Sofos, 2010; Kamiloğlu, Kaban, & Kaya, 2019; Zanette, Dalla Santa, & dos Santos Bersot, 2015). Consansu et al. (2010) reported a 3.3 log CFU/g reduction in the pathogen growth compared to the 1.37 log CFU/g reduction in the control when *P. acidilactici* was applied in the production of sucuk. Kamiloğlu, Kaban, & Kaya (2019) found a 2.74 log CFU/g reduction in the counts of *L. monocytogenes* when sucuk was produced with *L. plantarum*, whereas in the control sample only a 0.54 log CFU/g decrease was observed. Zanette, Dalla Santa, & dos Santos (2015) obtained a 1.7 log CFU/g reduction of the pathogen when either a bacteriocinogenic *L. plantarum* strain or a non-bacteriocin producer *L. plantarum* were added to sausages. The different strains, the inoculum concentrations, the ripening parameters and antagonistic interactions of the starter cultures with the indigenous microbiota are among the factors that may impact on the differences in findings between studies in the literature (Ducić et al., 2016). Overall, our results evidence that the starter culture employed has a strong capability for inhibiting *Salmonella* and *L. monocytogenes*.

When DFS without starter culture were HPP-treated, the inactivation observed for *L. monocytogenes* was limited. The immediate effect of HPP was minor, but from that point of ripening onwards there was a steady albeit limited reduction of *L. monocytogenes* counts, which reached  $4.3 \pm 0.3$  log CFU/g in the final product. This effect may be explained by sublethal injuries caused by HPP leading to a slow progressive inactivation during ripening (Jofré, Aymerich, Bover-Cid, & Garriga, 2010). Jofré et al. (2010) discussed that sublethal damaged cells can slowly recover from a HPP treatment, but when other hurdles are also present, for example, low pH or starter cultures, the cells are unable to cope with the stress and gradually die. Overall, this study shows that mild high-pressure treatments at 300 Mpa applied on A + IP sausages in the early stages of ripening induced an additional 1.5 log reduction of *L. monocytogenes* in the finished product, compared to counts obtained for A + INP sausages.

In the study by Marcos, Aymerich & Garriga (2005) *L. monocytogenes* counts in *chorizo* decreased by 1 log immediately after HPP treatment, then increased by 1.8 log and finally declined during the rest of ripening and reached a final level of 2 log CFU/g. According to the authors, HPP induced a temporary decrease in numbers of LAB; this slowed the pH drop and reduced the production of antilisterial factors, such as bacteriocins, and consequently enabled recovery of sub-lethally damaged *L. monocytogenes*. In other studies, reductions of *L. monocytogenes* in DFS

when HPP (600 Mpa) was applied to the final fermented product ranged from no reduction (Marcos, Aymerich, Garriga & Arnau, 2013), or <1 log reduction (Rubio, Bover-Cid, Martin, Garriga & Aymerich, 2013) to a 5 log reduction in treated products (Porto-Fett et al., 2010).

When the starter culture and HPP were combined (S + IP), *L. monocytogenes* decreased by 2–2.5 log on the day 7 of ripening. This abrupt decrease was maintained and the pathogen was not detected (<1 log CFU/g) from that point on. Such results indicate a clear synergism between HPP and the starter culture, and show that the combined approach assessed here is an effective strategy for the control of major foodborne pathogens in meat products.

#### 3.4. Evolution of indigenous microbiota and starter culture bacteria, as determined by culture-independent metataxonomic analyses

The relative abundance of the main 18 genera present in the *chorizo* is represented in Fig. 4. In DFS with starter culture, the most dominant genus was *Latilactobacillus*, with relative abundances ranging from 83.8 to 94.8 %. It was observed that the meat batter, sampled before adding the starter culture, already presented nearly 50 % dominance by *Lactilactobacillus* genus. The oligotyping of the reads revealed that an ASV corresponding to a single *Latilactobacillus* oligotype was responsible for > 95.5 % of the relative abundance of the family *Lactobacillaceae* in DFS with starter culture added, regardless of HPP treatment (Fig. 5). This ASV probably corresponds with the *L. sakei* strain added with the starter culture, which was clearly the dominant species and therefore showed a high adaptability to meat conditions during fermentation (Aquilanti, Garofalo, Osmani, & Clementi, 2016).

In DFS without starter culture, the spoilage bacterium *Brochothrix* was the most abundant genus in almost all the not pressurized samples, followed by *Leuconostoc* and *Latilactobacillus*. On the one hand, this result is in accordance with the findings of Quijada et al. (2018) on fresh-produced sausages, although they found variation between different manufacturers. *Brochothrix* has been frequently reported in a wide variety of meat products, since it is a very versatile bacterium, well-adapted to different environments (Dousset, Jaffrès, & Zagorec, 2016). On the other hand, Quijada et al. (2018) showed that after mid ripening (15–21 days) *Lactobacillus* (old classification) became the most abundant genus. In other similar study conducted by Juárez-Castelán et al., 2019, *Pseudomonas* (25 %), *Streptococcus* (23.7 %), *Acinetobacter* (15.3 %), *Bacillus* (14.3 %), and *Brochothrix* (12.2 %) were the genera mostly found in *chorizo* at time 0. According to the authors during the ripening process, *Lactobacillus* became the dominant genus, and, opposite to our findings in A-INP, *Brochothrix* accounted for approximately just 1 % of relative abundance, whereas in our study it was the dominant genus. Likewise, the dominance of *Leuconostoc* in Juárez-Castelán et al. (2019) is quite different to our findings, where it is one of the three most abundant genera of the DFS.

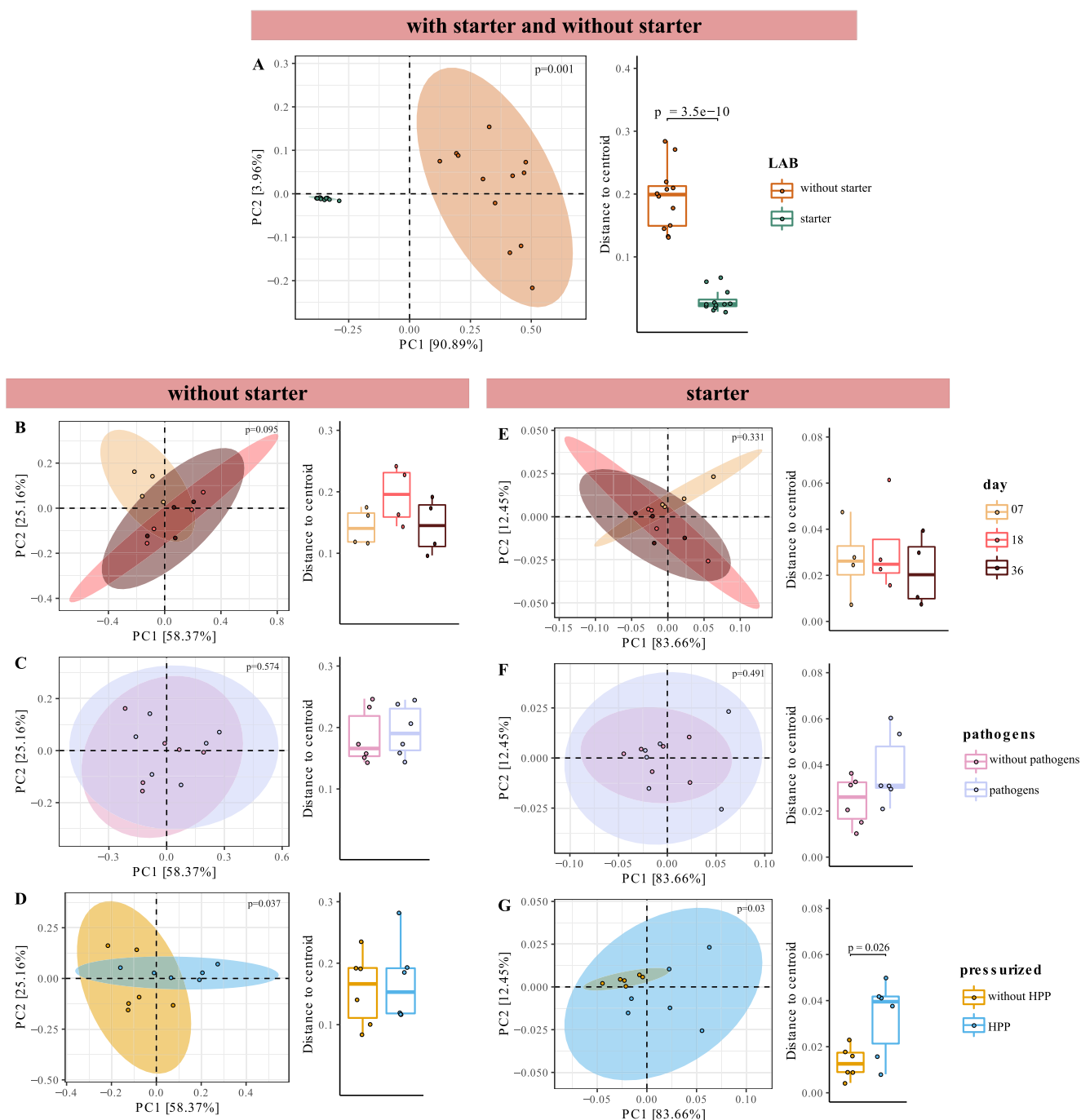
In contrast, in pressurized DFS without starter culture there was no general predominance of one single ASV in all the samples, although *Latilactobacillus*, *Brochothrix* and *Leuconostoc* were again the most abundant genera.

*Lactobacillaceae* oligotypes were much more diverse in DFS with no starter culture added than in those elaborated with starter culture. In fact, in DFS without starter, one *Latilactobacillus* and one *Leuconostoc* ASV accounted together for approximately 70 % of the total abundance of bacterial ASVs, and the rest comprised a wide variety of different oligotypes, including oligotypes from *Lactiplantibacillus*, *Lacticaseibacillus*, *Loigolactobacillus*, *Levilactobacillus* or *Companilactobacillus*. When all the *Lactobacillaceae* oligotypes were grouped by genus (Fig. 6) it was observed that *Latilactobacillus* and *Leuconostoc* accounted for >99 % of relative abundance on day 7 of ripening, while the rest oligotypes were more abundant as ripening progressed, finally accounting for up to 20 % of relative abundance. Again, the high abundance of *Latilactobacillus* is likely due to its good adaptation to the environmental conditions prevailing in fermented meat products (Janssens, Myter, De Vuyst, & Leroy,

2012; Montanari et al., 2018; Stavropoulou, Filippou, De Smet, De Vuyst, & Leroy, 2018; Van Reckem et al., 2019). Remarkably, the predominant *Lactobacillus* ASV in DFS with no starter culture added, was the one which also dominated in samples with starter culture added. This fact may be attributed to the phylogenetic proximity between strains, and/or to the limitations of the 16S rRNA gene amplicon sequencing approach, which is limited to the V3-V4 region of the 16S rRNA gene and can underestimate diversity (Stellato et al., 2017). However, Quijada et al. (2018) also reported that *L. sakei* was the most abundant species among the *Lactobacillus* genus in chorizo without

starter addition.

It is important to point out that spoilage bacteria frequently occurring in meat products, such as *Acinetobacter*, *Aeromonas*, *Pseudomonas* or *Psychrobacter*, were not found in this study. Nevertheless, *Brochothrix* was widely present, together with *Serratia* and *Photobacterium*, although the latter ones were found in less than a 1.4 % relative abundance. *Serratia* was barely occurring in DFS with starter culture added (<0.04 % relative abundance), meaning that spontaneously fermented sausages were more susceptible to the growth of this undesirable microorganism (Zhang et al., 2021). Thus, the use of the starter culture improved the



**Fig. 7.** Principal coordinates analysis (PCoA) representing the beta-diversity of the microbial communities in DFS. The centroid of each ellipse represents the group mean and the shape is defined by the covariance within each group. Distance to the centroid values were employed to evaluate the homogeneity of variances within each group. (A) Grouping of samples with or without starter culture added; (B, E) Grouping of samples without or with starter culture added, respectively, by ripening time; (C, F) Grouping of samples without or with starter culture added, respectively, by pathogens' inoculation status; (D, G) Grouping of samples without or with starter culture added, respectively, by HPP treatment status.



microbiological quality of DFS by competing with native undesirable microbes (Wang et al., 2021). The authors observed that when a starter culture (containing *L. sakei*, *Pediococcus pentosaceus*, *Staphylococcus xylosum* and *S. carnosus*) was added to Chinese Cantonese sausages, undesirable bacteria such as *Vibrio* spp., *Brochothrix* spp., *Yersinia* spp. and *Enterobacter* spp. were repressed, if compared to spontaneous fermented sausage.

The results of ordination and beta-dispersion analyses are represented in Fig. 7. Interestingly, the use of the starter culture had a significant influence on the taxonomic profile of samples (adonis,  $p = 0.001$ ) and explained 94.85 % of the variation observed. Indeed, samples with starter culture added grouped separately from samples with no starter culture added and showed a much lower beta-dispersion evidencing a more homogeneous taxonomic profile among samples. This finding is consistent with the results obtained by Wang et al. (2021), who found a clear separation between sausages with and without starter culture added. The authors also suggest that bacterial communities with starter culture were more stable than populations in samples without starter culture. This clear separation may be attributed to the very high abundance of the *Lactilactobacillus* strain included in the starter culture.

Given the important dissimilarities due to the use of the starter culture, beta-diversity analyses were performed independently for sausages with or without starter culture added. Reasonably limited inter-sample diversity associated with the ripening day or the artificial contamination with pathogenic microorganisms was found, both in DFS with or without starter culture added. This finding does not agree with those by other previous studies in which microbial communities were shown to fluctuate widely during ripening (Quijada et al., 2018; Wang et al., 2021). Quijada et al. (2018) found that microbial communities in the first stages of sausage production evolve throughout the ripening process until the final product. *Pseudomonas* spp. and *Brochothrix* spp. were highly found in the early stages while *Lactobacillus* spp. became dominant in the final product. On the other hand, HPP had a significant influence on the taxonomic profile of DFS (adonis,  $p = 0.03$  and  $p = 0.037$  for DFS with and without starter culture added, respectively). DFS with starter culture added and subjected to HPP were much more heterogeneous than those that were unpressurized. There is no clear explanation for this behavior as the influence of HPP applied at the early stages of ripening of DFS with or without starter culture added is being

assessed for the first time in the current study. However, this observation might be due to the fact that the starter culture applied was negatively affected by the HPP treatment to a certain extent, as it can be also seen through the slight reduction of LAB counts just after HPP. As a result, other less abundant indigenous microbial taxa likely had the opportunity to play a more prominent role. Moreover, although *Lactilactobacillus* remained dominant after HPP treatment, it is important to bear in mind that sequencing approaches target all the present DNA, no matter if bacteria are still alive or dead (Li et al., 2017).

The impact of ripening stage, inoculation with the starter culture or with pathogens and pressurization was also evaluated by assessing intra-sample alpha diversity indices. DFS without starter had significantly higher ASV richness (20–26 vs 12–19 ASVs per sample) and Simpson and Shannon indices than DFS with starter culture added. Thus, the use of the starter culture restrained bacterial diversity in DFS and produced a clear dominance of the starter culture, and especially of the *L. sakei* strain (Wang et al., 2021). Considering this inequality, samples with and without starter culture added were subsequently evaluated individually. No significant differences were observed among ripening days (Supplementary file 1) or upon inoculation of DFS with pathogenic microorganisms (Supplementary file 2), but some differences were detected with HPP (Fig. 8). In DFS without starter, pressurized samples were less diverse than not pressurized samples, while in DFS with starter culture, pressurized samples were more diverse than their not pressurized counterparts (see Shannon index). This observation can be again due to the inhibitory effect that the HPP treatment showed on starter LAB, which would facilitate the proliferation of other less abundant indigenous bacteria.

### 3.5. Sensorial analysis

A sensory analysis was performed to evaluate the consumer perception of DFS under the applied conditions (starter culture and/or HPP). The first triangular test aimed to assess whether differences in sensorial attributes were produced by the HPP treatment in DFS without starter (Table 1). Only 9 of the 16 panelists were able to differentiate between A-INP and A-IP, and therefore significant differences were not detected. Interestingly, the 9 panelists who distinguished between A-IP and A-INP DFS showed preference for the HPP-treated product ( $p < 0.01$ ). This result is in accordance with another study that aimed to

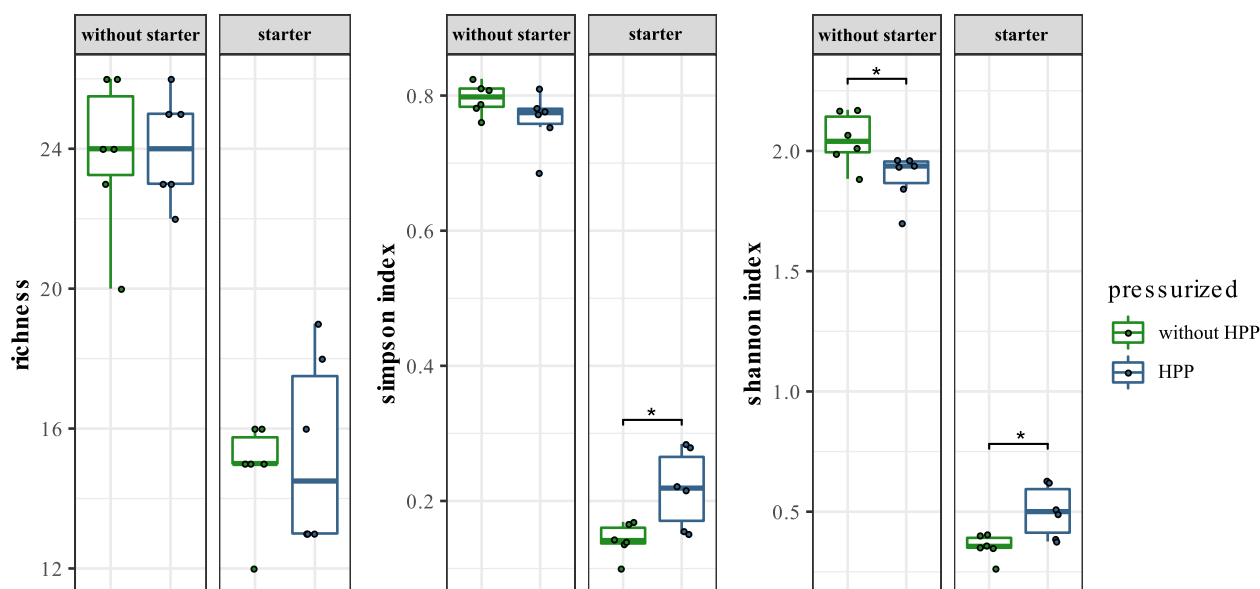


Fig. 8. Influence of HPP on the alpha-diversity of microbial communities (richness, Simpson index and Shannon index) in DFS. Green boxes correspond to not HPP-treated sausages and blue boxes to sausages treated with HPP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 1**

Results from the sensorial tests performed by the 16 member-panel: triangular test (correct judgements), preference test and attributes guiding the differentiation in the triangular test (attributes evaluation). Test 1 evaluates the effect of HPP treatment in samples with no starter culture added; test 2 evaluates the effect of HPP treatment in samples with starter culture added and test 3 evaluates the effect of the use of the starter culture in unpressurized DFS.

	Correct judgements	Preference	Attributes evaluation	
Test 1	9 (56 %)	9 of 9 for A-IP (100 %) $p < 0.01$	Aspect	2.00 ± 1.20
			Aroma	2.50 ± 1.31
			Taste	4.50 ± 0.53
Test 2	10 (63 %) $p < 0.05$	7 of 10 for S-IP (70 %)	Aspect	2.33 ± 1.32
			Aroma	1.44 ± 0.53
			Taste	3.80 ± 1.14
Test 3	9 (56 %)	6 of 9 for A-INP (66.67 %)	Aspect	1.89 ± 1.17
			Aroma	2.00 ± 1.12
			Taste	3.50 ± 1.20

investigate the effect of HPP (500 MPa) during 5 and 15 min on vacuum-packed cooked sausages (Mor-Mur & Yuste, 2003). Results showed that pressurized samples were found to be preferred in 50 % of the judgements in comparison to the control negatives, which were preferred in 18.8 % and 27.3 % respectively for 5 and 15 min treatment (Mor-Mur & Yuste, 2003). This preference is likely to be related to the fact that A-IP DFS showed the highest pH value after the 5 weeks of ripening. In addition, as it is shown in the metataxonomic analysis, HPP has a significant effect on the microbial composition of DFS. Thus, changes in microbes can lead to different profiles of volatile compounds, affecting the sensorial characteristics of the product (Ferrocino et al., 2018). When the second triangular test was carried out, differences between S-INP and S-IP samples were noticed ( $p < 0.05$ ) by 10 out of the 16 panelists. The differences observed could be due to the mild inhibitory effect of the HPP treatment on the starter culture. Despite the differences detected, no preference was significantly expressed for any of the two DFS. Nonetheless, 7 of the 10 panelists who differentiated the samples showed preference for the HPP-treated samples. Finally, the sensory panel did not notice any significant differences in the third triangular test, between A-INP and S-INP DFS, and no apparent preference was shown for any of the two samples either. Overall, the results of the sensorial analysis revealed that the application of HPP, the starter culture, or their combination did not lead to important changes in the quality attributes of DFS, and that, when panelists detected differences among samples HPP, treated sausages were generally preferred. When panelists detected differences among samples in the triangular tests, the major attribute responsible for the differences was taste, with an average score of  $4.5 \pm 0.5$ ,  $3.8 \pm 1.1$  and  $3.5 \pm 1.2$  out of a 5-point scale, respectively, for triangular test 1, test 2 and test 3. In conclusion, no adverse effects associated with the use of the starter culture, HPP or their combination were detected, demonstrating the feasibility of applying these techniques as an alternative to traditional production methods (Kingcha et al., 2012).

### 3.6. Color and texture measurements

The results of the main parameters of color ( $L^*$ ,  $a^*$ ,  $b^*$ ) measured on DFS after 15 days of storage are presented in Table 2. The  $L^*$  values of color (lightness) from DFS produced without or with starter culture added, regardless of whether HPP was applied or not, did not differ significantly ( $p > 0.05$ ). In the study of Marcos, Aymerich & Garriga (2005) the same level of high pressure, 300 Mpa, applied at the beginning of the ripening, increased  $L^*$  of finished products, compared to untreated control samples. The authors mentioned protein denaturation

**Table 2**

Evaluation of color parameters ( $L^*$ ,  $a^*$  and  $b^*$ ) and texture parameters (hardness, cohesiveness and springiness) after 15 days of storage for DFS elaborated with or without starter culture added, and with or without the HPP treatment of the sausages.

	A-INP	A-IP	S-INP	S-IP	
color	Lightness ( $L^*$ )	33.85 ± 4.37	33.11 ± 5.56	33.82 ± 5.12	33.2 ± 5.61
	Redness ( $a^*$ )	19.63* ± 2.29	16.35 ± 2.85	17.86 ± 2.96	16.32 ± 2.7
	Yellowness ( $b^*$ )	16.47 ± 3.97	15.28 ± 3.84	15.76 ± 3.55	14.35 ± 4.53
texture	Hardness	74.35 ± 16.87	67.93 ± 10.61	79.97 ± 22.51	86.66 ± 24.2
	Cohesiveness	0.32 ± 0.03	0.32 ± 0.06	0.28 ± 0.03	0.3 ± 0.04
	Springiness	0.39 ± 0.04	0.43 ± 0.04	0.41 ± 0.04	0.37 ± 0.19

Values represent mean and standard deviation ( $n = 3 \times 3$ ).

\* Differs significantly from other averages in the same row.

or loss of active pigments as possible explanations for an increased share of reflected light from HPP sausages. The application in our study of the same level of high pressure, but after the fermentation process has already started (7 days after stuffing), with an associated protein coagulation and partial loss of water redistributed within the food matrix, could be the reason for the stabilization of color and reflectance of light and, consequently, the absence of an increase in  $L^*$  values in HPP treated sausages. This was also stated by Bak et al. (2017). In addition, a high proportion of back fat (50 %) was included in the formulation of DFS in the study by Marcos, Aymerich & Garriga (2005), and an earlier investigation by Jimenez-Colmenero et al. (1997) observed that high fat content was associated with higher lightness values in sausages, regardless of whether they were pressurized or not. On the other hand, no increases of  $L^*$  were reported by Marcos et al. (2007) for whole sausages pressurized at the end of the ripening period with 400 Mpa (10 min), and Rubio et al. (2007) for sliced DFS pressurized at the end of the ripening period at 500 Mpa (5 min). These last two reports confirmed that changes in color induced by HPP are less pronounced in products with lower water content (Bajovic et al., 2012).

The redness value ( $a^*$ ) of DFS, which is mainly related to the chemical and physical state of myoglobin, was significantly higher ( $p < 0.05$ ) in not pressurized DFS with no started culture added (A-INP) than in the rest of DFS (Table 2). However, Marcos, Aymerich & Garriga (2005) and Latorre-Moratalla et al. (2007) reported that pressurization of fermented sausages at the beginning of the ripening process did not induce significant changes of  $a^*$ . Interestingly, in our study there was a positive relationship between this color parameter and DFS  $a_w$  ( $R^2 = 0.94$ ,  $P < 0.05$ ) so that the results of  $a^*$  are higher in batches with higher water activity. Therefore, it is likely that an earlier end of ripening for S-INP, A-IP and S-IP DFS, i.e. at a time when their  $a_w$  values are similar to those of A-INP sausages, could result in higher values of redness.

Regarding yellowness ( $b^*$ ), neither the application of high pressure nor the use of the starter culture induced changes in this color parameter ( $p > 0.05$ ) (Table 2). The same observation was made in other aforementioned studies (Marcos et al., 2007; Marcos, Aymerich & Garriga, 2005; Rubio, Martínez, García-Cachán, Rovira, & Jaime, 2007). In general, dry cured or fermented meat products, unlike fresh meat, do not suffer prominent color changes upon HPP treatment due to the resistance of nitrosylmyoglobin to HPP and the low  $a_w$  of DFS (Cava, García-Parra, & Ladero, 2020).

With respect to texture parameters, the influence of the starter culture and/or HPP on hardness, cohesiveness and springiness of chorizo sausages was analyzed after 15 days of storage (Table 2). No significant differences were observed among sausages regarding any of the texture parameters. This finding is in agreement with those by Essid and Hassouna (2013) on a Tunisian DFS. These authors showed no significant

differences between control samples and samples with starter culture (*S. xyloso* and *L. plantarum*). On the other hand, in the study performed by Marcos et al. (2007), values of chewiness, cohesiveness and springiness were higher in pressurized DFS (400 Mpa applied after 28 days of ripening) than in not pressurized samples. In the current study, changes induced by HPP in the texture parameters were not noticed by the sensory panel also, meaning that the effects, if any, were minor and will not be appreciated by consumers.

#### 4. Conclusions

The combination of HPP after one-week of ripening and the use of starter culture during production of DFS was demonstrated to have positive effects on the microbiological quality of the finished products with no negative changes in their sensorial quality. Regarding the inhibition of pathogens, these preservation strategies acted synergistically allowing for a successful control of *L. monocytogenes* and *S. Typhimurium*. In addition, the use of the starter culture and HPP also inhibited the development of spoilage bacteria during ripening and induced a faster lowering of  $a_w$ . Moreover, no adverse effects regarding other physico-chemical or sensory parameters were observed. On the contrary, panelists showed a higher preference in most cases for DFS subjected to HPP at the early stages of ripening. Altogether, it can be concluded that the combined use of starter cultures and HPP of sausages at the early stages of ripening has potential to be used as a preservation strategy in the manufacturing of *Chorizo de León* DFS.

Considering all results obtained from this study it can be assumed that a new approach of HPP lead to a final product of DFS with improved selected characteristics and consequently better competitiveness on the market. Potential industrial applicability is even more realistic in large scale manufacturing of dry fermented sausages, regarding costs.

#### CRedit authorship contribution statement

**Miroslav Dučić:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Coral Barcenilla:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **José F. Cobo-Díaz:** Data curation. **Mercedes López:** Supervision. **Avelino Álvarez-Ordóñez:** Supervision, Writing – review & editing. **Miguel Prieto:** Conceptualization, Writing – review & editing, Project administration, Supervision.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2022.112162>.

#### References

- Aquilanti, L., Garofalo, C., Osimani, A., & Clementi, F. (2016). Ecology of lactic acid bacteria and coagulase negative cocci in fermented dry sausages manufactured in Italy and other Mediterranean countries: An overview. *International Food Research Journal*, 23(2), 429–445.
- Bajovic, B., Bolumar, T., & Heinz, V. (2012). Quality considerations with high pressure processing of fresh and value added meat products. *Meat Science*, 92(3), 280–289. <https://doi.org/10.1016/j.meatsci.2012.04.024>
- Bak, K. H., Bolumar, T., Karlsson, A. H., Lindahl, G., & Orlien, V. (2019). Effect of high pressure treatment on the color of fresh and processed meats: A review. *Critical Reviews in Food Science and Nutrition*, 59(2), 228–252. <https://doi.org/10.1080/10408398.2017.1363712>
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13(7), 581–583. <https://doi.org/10.1038/nmeth.3869>
- Campus, M. (2010). High Pressure Processing of Meat, Meat Products and Seafood. *Food Engineering Reviews*, 2(4), 256–273. <https://doi.org/10.1007/S12393-010-9028-Y>
- Carrasco, J., García-Delgado, C., Lavega, R., Tello, M. L., Toro, M. D., Barba-Vicente, V., ... Preston, G. M. (2020). Holistic assessment of the microbiome dynamics in the substrates used for commercial champignon (*Agaricus bisporus*) cultivation. *Microbial Biotechnology*, 13(6), 1933–1947. <https://doi.org/10.1111/1751-7915.13639>
- Cava, R., García-Parra, J., & Ladero, L. (2020). Effect of high hydrostatic pressure processing and storage temperature on food safety, microbial counts, colour and oxidative changes of a traditional dry-cured sausage. *LWT*, 128, Article 109462. <https://doi.org/10.1016/J.LWT.2020.109462>
- Cosansu, S., Geornaras, I., Ayhan, K., & Sofos, J. N. (2010). Control of *Listeria monocytogenes* by bacteriocin-producing *Pediococcus acidilactici* 13 and its antimicrobial substance in a dry fermented sausage sucuk and in turkey breast. *Journal of Food and Nutrition Research*, 49(4), 206–214.
- Cui, S., & Fan, Z. (2019). Lactic Acid Bacteria and Fermented Meat Products. In W. Chen (Ed.), *Lactic Acid Bacteria* (pp. 211–226). <https://doi.org/10.1007/978-981-13-7283-4>.
- Doussot, X., Jaffrès, E., & Zagorec, M. (2016). Spoilage: Bacterial Spoilage. In *Encyclopedia of Food and Health* (pp. 106–112). <https://doi.org/10.1016/B978-0-12-384947-2.00649-8>.
- Ducic, M., Blagojevic, B., Markov, S., Velicanski, A., & Buncic, S. (2014). General patterns of background microbiota and selected bacterial pathogens during production of fermented sausages in Serbia. *Food Control*, 43, 231–237. <https://doi.org/10.1016/J.FOODCONT.2014.03.026>
- Ducic, M., Klisara, N., Markov, S., Blagojevic, B., Vidakovic, A., & Buncic, S. (2016). The fate and pasteurization-based inactivation of *Escherichia coli* O157, *Salmonella* Typhimurium and *Listeria monocytogenes* in dry, fermented sausages. *Food Control*, 59, 400–406. <https://doi.org/10.1016/J.FOODCONT.2015.06.014>
- Fadda, S., López, C., & Vignolo, G. (2010). Role of lactic acid bacteria during meat conditioning and fermentation: Peptides generated as sensorial and hygienic biomarkers. *Meat Science*, 86(1), 66–79. <https://doi.org/10.1016/J.MEATSCL.2010.04.023>
- Ferrocino, I., Bellio, A., Giordano, M., Macori, G., Romano, A., Rantsiou, K., ... Coccolin, L. (2018). Shotgun Metagenomics and Volatilome Profile of the Microbiota of Fermented Sausages. *Applied and Environmental Microbiology*, 84(3). <https://doi.org/10.1128/AEM.02120-17>
- Filippis, F. D., Parente, E., & Ercolini, D. (2017). Metagenomics insights into food fermentations. *Microbial Biotechnology*, 10(1), 91–102. <https://doi.org/10.1111/1751-7915.12421>
- Gormley, F. J., Little, C. L., Grant, K. A., de Pinna, E., & McLauchlin, J. (2010). The microbiological safety of ready-to-eat specialty meats from markets and specialty food shops: A UK wide study with a focus on *Salmonella* and *Listeria monocytogenes*. *Food Microbiology*, 27(2), 243–249. <https://doi.org/10.1016/J.FM.2009.10.009>
- Janssens, M., Myter, N., De Vuyst, L., & Leroy, F. (2012). Species diversity and metabolic impact of the microbiota are low in spontaneously acidified Belgian sausages with an added starter culture of *Staphylococcus carnosus*. *Food Microbiology*, 29(2), 167–177. <https://doi.org/10.1016/j.fm.2011.07.005>
- Jofré, Anna, Aymerich, Teresa, Bover-Cid, Sara, & Garriga, Margarita (2010). Inactivation and recovery of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* after high hydrostatic pressure treatments up to 900 MPa. *International Microbiology*, 13, 105–112. <https://doi.org/10.2436/20.1501.01.115>. In press.
- Juárez-Castelán, C., García-Cano, I., Escobar-Zepeda, A., Azaola-Espinosa, A., Álvarez-Cisneros, Y., & Ponce-Alquicira, E. (2019). Evaluation of the bacterial diversity of Spanish-type chorizo during the ripening process using high-throughput sequencing and physicochemical characterization. *Meat Science*, 150, 7–13. <https://doi.org/10.1016/J.MEATSCL.2018.09.001>
- Kamiloglu, A., Kaban, G., & Kaya, M. (2019). Effects of autochthonous *Lactobacillus plantarum* strains on *Listeria monocytogenes* in sucuk during ripening. *Journal of Food Safety*, 39(3), 1–6. <https://doi.org/10.1111/jfs.12618>

- Kingcha, Y., Tosukhowong, A., Zendo, T., Roytrakul, S., Luxanani, P., Chareonpornsook, K., ... Visessanguan, W. (2012). Anti-listeria activity of *Pediococcus pentosaceus* BCC 3772 and application as starter culture for Nham, a traditional fermented pork sausage. *Food Control*, 25(1), 190–196. <https://doi.org/10.1016/j.foodcont.2011.10.005>
- Komora, N., Maciel, C., Amaral, R. A., Fernandes, R., Castro, S. M., Saraiva, J. A., & Teixeira, P. (2021). Innovative hurdle system towards *Listeria monocytogenes* inactivation in a fermented meat sausage model - high pressure processing assisted by bacteriophage P100 and bacteriocinogenic *Pediococcus acidilactici*. *Food Research International*, 148, Article 110628. <https://doi.org/10.1016/J.FOODRES.2021.110628>
- Leroy, F., Verluuyten, J., & De Vuyst, L. (2006). Functional meat starter cultures for improved sausage fermentation. *International Journal of Food Microbiology*, 106(3), 270–285. <https://doi.org/10.1016/J.IJFOODMICRO.2005.06.027>
- Li, R., Tun, H. M., Jahan, M., Zhang, Z., Kumar, A., Fernando, D., ... Khafipour, E. (2017). Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. *Scientific Reports*, 7(1). <https://doi.org/10.1038/s41598-017-02516-3>
- Marcos, B., Aymerich, T., Dolores Guardia, M., & Garriga, M. (2007). Assessment of high hydrostatic pressure and starter culture on the quality properties of low-acid fermented sausages. *Meat Science*, 76(1), 46–53. <https://doi.org/10.1016/J.MEATSCI.2006.09.020>
- Marcos, B., Aymerich, T., & Garriga, M. (2005). Evaluation of High Pressure Processing as an Additional Hurdle to Control *Listeria monocytogenes* and *Salmonella enterica* in Low-Acid Fermented Sausages. *Journal of Food Science*, 70(7), 339–344. <https://doi.org/10.1111/J.1365-2621.2005.TB11477.X>
- Marcos, B., Aymerich, T., Garriga, M., & Arnau, J. (2013). Active packaging containing nisin and high pressure processing as post-processing listericidal treatments for convenience fermented sausages. *Food Control*, 30(1), 325–330. <https://doi.org/10.1016/J.FOODCONT.2012.07.019>
- Marcos, B., Kerry, J. P., & Mullen, A. M. (2010). High pressure induced changes on sarcoplasmic protein fraction and quality indicators. *Meat Science*, 85(1), 115–120. <https://doi.org/10.1016/J.MEATSCI.2009.12.014>
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal*, 17(1), 10–12. <https://doi.org/10.14806/EJ.17.1.200>
- Martín, M. J., García-Parra, J., Trejo, A., Gómez-Quintana, A., Miguel-Pintado, C., Riscado, A., ... Bernabé, R. R. (2021). Comparative effect of high hydrostatic pressure treatment on Spanish and Portuguese traditional chorizos and evolution at different storage temperatures. *Journal of Food Processing and Preservation*, 45(1), e15082.
- Montanari, C., Barbieri, F., Magnani, M., Grazia, L., Gardini, F., & Tabanelli, G. (2018). Phenotypic diversity of *Lactobacillus sakei* strains. *Frontiers in Microbiology*, 9(AUG). <https://doi.org/10.3389/fmicb.2018.02003>
- Mor-Mur, M., & Yuste, J. (2003). High pressure processing applied to cooked sausage manufacture: Physical properties and sensory analysis. *Meat Science*, 65(3), 1187–1191. [https://doi.org/10.1016/S0309-1740\(03\)00013-5](https://doi.org/10.1016/S0309-1740(03)00013-5)
- Omer, M. K., Prieto, B., Rendueles, E., Alvarez-Ordóñez, A., Lunde, K., Alvseike, O., & Prieto, M. (2015). Microbiological, physicochemical and sensory parameters of dry fermented sausages manufactured with high hydrostatic pressure processed raw meat. *Meat Science*, 108, 115–119. <https://doi.org/10.1016/J.MEATSCI.2015.05.002>
- Omer, M. K., Álvarez-Ordóñez, A., Prieto, M., Skjerve, E., Asehun, T., & Alvseike, O. A. (2018). A Systematic Review of Bacterial Foodborne Outbreaks Related to Red Meat and Meat Products. *Foodborne Pathogens and Disease*, 15(10), 598–611. <https://doi.org/10.1089/fpd.2017.2393>
- Porto-Fett, A. C. S., Call, J. E., Shoyer, B. E., Hill, D. E., Pshebniski, C., Cocoma, G. J., & Luchansky, J. B. (2010). Evaluation of fermentation, drying, and/or high pressure processing on viability of *Listeria monocytogenes*, *Escherichia coli* O157: H7, *Salmonella* spp., and *Trichinella spiralis* in raw pork and Genoa salami. *International Journal of Food Microbiology*, 140(1), 61–75. <https://doi.org/10.1016/J.IJFOODMICRO.2010.02.008>
- Quijada, N. M., De Filippis, F., Sanz, J. J., García-Fernández, M. D. C., Rodríguez-Lázaro, D., Ercolini, D., & Hernández, M. (2018). Different *Lactobacillus* populations dominate in “Chorizo de León” manufacturing performed in different production plants. *Food Microbiology*, 70, 94–102. <https://doi.org/10.1016/j.fm.2017.09.009>
- Rendueles, Eugenia, Omer, Mohamed K., Alvseike, Ole Arne, Alonso-Calleja, Carlos, Capita, Rosa, & Prieto, Miguel (2011). Microbiological food safety assessment of high hydrostatic pressure processing: A review. *LWT - Food Science and Technology*, 44(5), 1251–1260. <https://doi.org/10.1016/j.lwt.2010.11.001>. In press.
- Rodríguez-González, M., Fonseca, S., Centeno, J. A., & Carballo, J. (2020). Biochemical Changes during the Manufacture of Galician Chorizo Sausage as Affected by the Addition of Autochthonous Starter Cultures. *Foods*, 9(12), 1813. <https://doi.org/10.3390/FOODS9121813>
- Rubio, B., Martínez, B., García-Cachán, M. D., Rovira, J., & Jaime, I. (2007). The effects of high pressure treatment and of storage periods on the quality of vacuum-packed “salchichón” made of raw material enriched in monounsaturated and polyunsaturated fatty acids. *Innovative Food Science & Emerging Technologies*, 8(2), 180–187. <https://doi.org/10.1016/J.IJFSET.2006.09.005>
- Stavropoulou, D. A., Filippou, P., De Smet, S., De Vuyst, L., & Leroy, F. (2018). Effect of temperature and pH on the community dynamics of coagulase-negative staphylococci during spontaneous meat fermentation in a model system. *Food Microbiology*, 76, 180–188. <https://doi.org/10.1016/j.fm.2018.05.006>
- Stellato, G., Utter, D. R., Voorhis, A., De Angelis, M., Murat Eren, A., & Ercolini, D. (2017). A few *Pseudomonas* oligotypes dominate in the meat and dairy processing environment. *Frontiers in Microbiology*, 8(MAR). <https://doi.org/10.3389/fmicb.2017.00264>
- Van Reckem, E., Geeraerts, W., Charmpi, C., Van der Veken, D., De Vuyst, L., & Leroy, F. (2019). Exploring the Link Between the Geographical Origin of European Fermented Foods and the Diversity of Their Bacterial Communities: The Case of Fermented Meats. *Frontiers in Microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.02302>
- Wang, X., Zhang, Y., Sun, J., Pan, P., Liu, Y., & Tian, T. (2021). Effects of starter culture inoculation on microbial community diversity and food safety of Chinese Cantonese sausages by high-throughput sequencing. *Journal of Food Science and Technology*, 58(3), 931–939. <https://doi.org/10.1007/s13197-020-04607-y>
- Zanette, C., Dalla Santa, H., & dos Santos Bersot, L. (2015). Effect of *Lactobacillus plantarum* starter cultures on the behavior of *Listeria monocytogenes* during sausage maturation. In *Article in International Food Research Journal*. Retrieved from <http://www.ifrj.upm.edu.my>
- Zhang, Q. Q., Li, D., Zhang, W., Jiang, M., Chen, X. H., & Dong, M. S. (2021). Comparative analysis of the bacterial diversity of Chinese fermented sausages using high-throughput sequencing. *LWT*, 150. <https://doi.org/10.1016/j.lwt.2021.111975>