fermentation

MDPI

Article

Dynamics of Microbial Communities in Nitrite-Free and Nutritionally Improved Dry Fermented Sausages

Núria Ferrer-Bustins, Belén Martín, Mar Llauger, Ricard Bou, Sara Bover-Cid and Anna Jofré *

Food Safety and Functionality Program, IRTA, Finca Camps i Armet, E-17121 Monells, Spain
* Correspondence: anna.jofre@irta.cat; Tel.: +34-972630052

Abstract: Dry fermented sausage innovation trends are linked to consumer preferences for clean label and sodium-reduced foods. This study aims to evaluate the effect of the formulation and production process temperature on the dynamics of bacterial communities in fuet-type dry fermented sausages using metataxonomics. Six fuet batches were manufactured, including formulations without and with the addition of nitrifying salts (replaced or not by pork liver auto-hydrolysate as a colouring agent), processed at 3 to 12 °C, and a partial replacement of NaCl by KCl, processed at 12 °C. Fermentation was performed spontaneously or by a starter culture. Physicochemical characterisation and culture-dependent and independent bacterial analyses were performed at day 0, 4 and 12, at the end of ripening (a_w < 0.90) and after storage. Temperature was the most important factor determining the change in pH, a_w and lactic acid bacteria levels while the presence of a starter culture promoted a pH decrease. Metataxonomic analysis showed that low temperature processes and the absence of nitrifying salts allowed the growth of spoilage-related species, while sausages submitted to a mild temperature containing a starter culture and nitrifying salts showed less bacterial diversity. Liver auto-hydrolysate added putative probiotic species to the product. This study provides valuable information to manufacturers who want to innovate safely.

Keywords: metataxonomics; sodium reduction; meat fermentation; clean label; 16S rRNA; Zn(II)PPIX

Citation: Ferrer-Bustins, N.; Martín, B.; Llauger, M.; Bou, R.; Bover-Cid, S.; Jofré, A. Dynamics of Microbial Communities in Nitrite-Free and Nutritionally Improved Dry Fermented Sausages. *Fermentation* **2023**, *9*, 403. https://doi.org/10.3390/fermentation9040403

Academic Editor: Wanping Chen

Received: 31 March 2023
Revised: 16 April 2023
Accepted: 18 April 2023
Published: 21 April 2023

1. Introduction

Dry fermented sausages (DFS) are a traditional meat product appreciated for their organoleptic properties and sensory traits and are industrially produced through standardised but traditional and empirical processes and formulations [1]. Fuet is a Catalan low-acid and small-calibre DFS whose basic formulation consists of a mixture of lean pork and backfat added with salt (NaCl), pepper, carbohydrates (e.g., dextrose), ascorbate and nitrates/nitrites. Fuet is elaborated by stuffing meat batter in ca. 35–40 mm diameter natural casings, surface inoculation with mould spores (usually *Penicillium nalgiovense*) and ripening at 10–18 °C [2,3]. In some cases, the production process starts with a fermentation phase at 20–25 °C, promoting the rapid development of fermentative microbiota (endogenous LAB or inoculated starter cultures) and ensuring early acidification. Industrially, this typical Mediterranean DFS can also be formulated with starter cultures comprising lactic acid bacteria (LAB) and/or Gram-positive catalase-positive cocci (GCC+) whose main roles are to ensure controlled fermentation and to improve the sensory properties through its lipolytic and proteolytic activities, respectively [4,5].

The initial microbiota of DFS mainly depends on the microorganisms present in the meat and any other ingredient added. In the slaughterhouse, meat is contaminated with microorganisms from the gastrointestinal tract and external sources, such as hides, skin or slaughterhouse surfaces [6]. Subsequently, the microbiota of DFS is modulated by the combination of antimicrobial factors (hurdles) coming from additives and processing conditions that promote the growth of LAB/GCC+ and inhibit pathogenic and spoilage

microorganisms throughout the so-called hurdle technology [7]. The drying that occurs during the fuet ripening process results in a shelf-stable end product.

Nowadays, the meat industry is experimenting with new challenges since consumers are becoming more demanding regarding the quality, safety, and nutritional aspects of processed foods, wanting improved nutritional profiles (e.g., NaCl reduction), and the removal of additives, such as nitrifying agents (i.e., clean label products) [8]. Nitrifying agents have been described to play different roles in cured meat products, including antimicrobial effects, the outgrowth inhibition of pathogens, especially *Clostridium botulinum*, and aroma and colour formation [9,10]. However, the use of nitrates and nitrites in dry-cured meat products is controversial due to the potential formation of carcinogenic nitrosamines [11]. As a consequence, the use of alternative natural ingredients enhancing reddish colour formation, such as liver, with high capacity to form zinc protoporphyrin (Zn(II)PPIX), have been reported [12,13]. A reduction in dietary sodium intake is encouraged by health authorities to prevent cardiovascular diseases [14]. However, NaCl is an ingredient with a multifunctional role in both the technological aspect and food safety. Accordingly, sodium reduction in DFS requires its replacement by other salts, with KCl being the most frequently used [15,16]. Considering that nitrifying agents and NaCl concentration have an impact on the microbial growth [17] and the role microorganisms play on the quality and safety of DFS, the characterisation of microbial population dynamics is of upmost importance.

The study of complex microbial communities through classical microbiology techniques is time-consuming and has a limited taxonomic resolution. In contrast, a metataxonomic approach provides deep information of the microbiota present in foods by high-throughput sequencing (HTS) of the 16S rRNA gene [18]. In recent years, metataxonomics has been used to characterise the microbiota of different types of DFS and to study the dynamics of the bacterial communities of fermented meats as a function of the processing factors [19,20]. However, little is known about the influence of improved formulations and production processes on the bacterial composition of Mediterranean DFS by HTS.

In this work, metataxonomics was applied to evaluate the dynamics of microbiota during the production of fuet with innovative formulations, including a liver auto-hydrolysate ingredient rich in Zn(II)PPIX as a colouring substitute of nitrites and nitrates and a salt-reduced product.

2. Materials and Methods

2.1. Dry Fermented Sausage Manufacturing

Six batches of fuet-type DFS were manufactured. The basic formulation of the different batches consisted of meat batter containing pork mince (70:30, shoulder:belly proportion) and the following common additives (g per kg of meat): dextrose (2), maltodextrin (20), white pepper (3) and sodium ascorbate (0.5). The components of the six different formulations are detailed in Table 1. Specifically, batch 1 to 3 were formulated without nitrifying salts. Batch 1 and 2 included an innovative ingredient, based on auto-hydrolysed pork liver described as a rich source of Zn(II)PPIX [13,21] to improve the colour of DFS without nitrifying salts. In these batches, glucono-delta-lactone (GDL) was added to compensate for the pH increase produced by the liver auto-hydrolysate. In batch 4 to 6, nitrifying salts were added at concentrations usually applied to fuet. Additionally, batch 6 was formulated with an equimolar substitution of 50% of NaCl by KCl to obtain a sodium reduced product. Fermentation was led either by spontaneous LAB in batch 1 or by the bioprotective starter culture *Latilactobacillus sakei* CTC494 [22] added at an initial concentration of 6 log CFU/g in the rest of the batches. Meat batter was thoroughly mixed for 3 min at 4 °C (Mixer AVT-150, Castellvall, Girona, Spain) and stuffed (Junior continuous vacuum stuffer, SIA, Barcelona, Spain) into 36–38 mm diameter natural pig casings (Collelldevall, Girona, Spain). Sausages of 20 ± 5 cm in length were soaked in a *P. nalgiovense* solution (Danisco, France) and underwent different drying processes, as described in Table 1.

Table 1. Assessed dry fermented sausage formulations and process conditions.

Specific Additives/Ingredients (g/kg)					Process Conditions [3]		
Batch	**KNO_3/ $NaNO_2$**	**Liver Auto-Hydrolysate/GDL**	**NaCl/ KCl**	**Starter Culture [2]**	**Days**	**Temperature (°C)**	**Relative Humidity (%)**
1	na	300/3	20/0	na	20 2 18	3.4 ± 0.3 7.8 ± 2.1 12.5 ± 0.4	87.7 ± 8.6 90.0 ± 8.3 76.0 ± 8.6
2	na	300/3	20/0	+			
3	na	na [1]	20/0	+			
4	0.10/ 0.15	na [1]	20/0	+			
5	0.10/ 0.15	na [1]	20/0	+	27	12.4 ± 0.6	86.2 ± 7.6
6	0.10/ 0.15	na [1]	10/ 12.76	+			

[1] Instead of liver auto-hydrolysate, 300 g of water were added. [2] *Latilactobacillus sakei* CTC494 bioprotective starter culture. [3] Temperature and relative humidity values correspond to the mean ± standard deviation of the recorded profiles. Process conditions were the same for batches 1–4 (dynamic) and batches 5–6 (constant). na: not added; +: added.

Fuets without nitrifying agents added (batches 1 to 3) underwent cold ripening aiming to control the growth of *Clostridium botulinum*, both psychrotrophic (i.e., initially at temperature ca. 3 °C until a_w was <0.97) and mesophilic (at <10 °C until a_w was <0.94) [23,24] for 32–40 days. Batch 4 underwent the same ripening conditions in an independent drying chamber (separated from the batches 1, 2 and 3) to avoid nitric oxide cross-contamination through air. For batches 5 and 6, sausages were ripened at 12–13 °C for 27 days. Table 1 describes the temperatures and relative humidity (RH) recorded for each process. At the end of the process, DFS were vacuum packed in PA/PE bags (oxygen permeability of 50 $cm^3/m^2/24$ h and a low water vapor permeability of 2.8 $g/m^2/24$ h; Sistemvac, Estudi Graf S.A., Girona, Spain) and stored for 15 days at 4 °C [25].

For each batch, two independent manufacturing processes of DFS were performed on different days.

2.2. Microbial Counts, pH, a_w and Weight Loss

For the microbiological analysis of the DFS, first, the casing was removed aseptically and then ca. 25 g of chopped sausage was 10-fold diluted in a saline solution (0.85% NaCl and 0.1% Bacto Peptone; Beckton Dickinson, Franklin Lakes, NJ, USA), homogenised in a bag blender (Smasher®, bioMérieux, Marcy-l'Etoile, France) for 1 min and, if necessary, 10-fold serially diluted in a saline solution. LAB were enumerated in MRS (de Man, Rogosa and Sharpe; Merck, Darmstadt, Germany) agar plates incubated at 30 °C for 72 h under anaerobiosis using sealed jars with an AnaeroGen sachet (Oxoid Ltd., Altrincham, UK). GCC+ were enumerated in MSA (Mannitol Salt Agar; Oxoid™, Thermo Scientific™) plates incubated at 30 °C for 24 h.

The pH was measured with a puncture electrode 5232 and a portable pH meter PH25 (both from Crison Instruments S.A., Alella, Spain) and a_w with an Aqualab 3TE device (Decagon Devices, Inc., Pullman, WA, USA) at 25 °C.

All the analyses were performed in triplicate (three sausages randomly selected from the drying chamber) throughout the production process, which comprised day 0 (t0), 4 (t4), 7 (t7), 12 (t12) and 20 (t20), the end of ripening (tRip; day 32–40 for batches 1 to 4 and day 27 for batches 5 and 6) and at the end of the 15-day refrigerated storage (tStor), which was day 52–53 for batches 1 to 4 and day 40–41 for batches 5 and 6.

Five DFS from every batch located at different positions in the drying chamber were selected and labelled to follow the weight loss along the process. The weight loss (%) was calculated with respect to the initial weight of the sausage.

2.3. Monitoring of Starter Culture Implantation

To verify the implantation of the starter culture *L. sakei* CTC494, eight colonies per batch were isolated from MRS plates at t0, t4 and tRip, and were submitted to Enterobacteria Repetitive Intergenic Consensus (ERIC)-PCR with primers FW-ERIC R1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and RV-ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) [26] for typing under the conditions described in Rubio et al., 2014 [3].

2.4. DNA Purification, qPCR and High Throughput Sequencing

Samples processed for sequencing were those representing the microbial community of the meat batter used for the sausages production, the liver auto-hydrolysate and the DFS at the different times of the process: t0, t4, t12, tRip and tStor.

A volume of 200 mL of 10-fold diluted homogenates was filtered in sterile conditions with a nonwoven filter with a 22–25 µm diameter pore (475855-1R, Millipore Corp, Burlington, MA, USA). The filtered samples were centrifuged at 30.000× *g* for 15 min at 4 °C (Avanti® JXN-30, Beckman Coulter, Pasadena, CA, USA) in 35-mL capacity sterile centrifuge tubes (Nalgene, Rochester, NY, USA). The supernatant was discarded and up to 100 mg of pellet was recovered and stored at −20 °C until further processing.

DNA was extracted with the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany). The protocol followed was that described by the manufacturer. Mechanical lysis was performed through bead beating for 10 min at 30 Hz in a ball mill (MM200, Retsch, Haan, Germany). DNA purification and isolation steps were automated using the QIAcube sample preparation system (QIAGEN).

DNA was quantified spectrophotometrically (µDrop plate, Thermo Fisher Scientific, Waltham, MA, USA) and fluorometrically (Quant-iT^{TM} 1X dsDNA HS Assay Kit, Invitrogen, Merelbeke, Belgium) in a Varioskan^{TM} multiplate reader (Thermo Fisher Scientific, USA), and the concentration was adjusted to 5 ng/µL.

16S rRNA gene amplicons were obtained following the 16S rRNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The gene-specific sequences used in this protocol target the 16S rRNA gene V3 and V4 regions (459 bp) with the primers designed by Klindworth et al. (2013) [27]. Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. After 16S rRNA gene amplification, the multiplexing step was performed using Nextera XT Index Kit (FC-131-1096). After normalisation and pooling, libraries were sequenced using a 2 × 300 pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) on a MiSeq Sequencer according to manufacturer's instructions (Illumina, San Diego, CA, USA).

2.5. Bioinformatic Analysis

Sequencing reads were imported into the QIIME2 platform [28] and quality filtering, denoising, paired-ends joining and chimera depletion were performed using the DADA2 pipeline [29]. Taxonomic affiliations were assigned using the Naive Bayesian classifier integrated in the QIIME2 and SILVA database.

2.6. Statistical Analysis

Statistical differences regarding physicochemical parameters, weight loss and microbial counts between batches at each sampling time were assessed by one-way analysis of variance (ANOVA) and the Tukey–Kramer Honest Significant Difference (HSD) test (level of significance 0.05) using JMP v16.0.0 software (SAS Institute Inc., Cary, NC, USA).

Amplicon Sequence Variants (ASVs) table with the assigned taxonomy constructed in QIIME2 was exported to RStudio (v 1.4.1103) [30]. Alpha diversity metrics (including Shannon, Simpson evenness, Simpson dominance and Chao1 indexes) were calculated using "phyloseq" R package [31] after rarefaction of the samples (subsample without replacement) to a depth of 15,000 sequences per sample.

For the statistical analysis of the bacterial composition across samples, compositional data analysis methods [32] were used. OTU counts were normalised using the centred

log ratio (CLR) transformation after removing low-abundance OTUs (minimal proportion abundance: 0.5%) and including a pseudo-count using CodaSeq [32,33] and zComposition [34,35] R packages. Beta diversity was evaluated using principal component analysis (PCA) performed by plotting a singular value decomposition of the CLR-transformed data. Permutational Multivariate Analysis of Variance (perMANOVA) [36] was used to evaluate differences in beta diversity using the "RVAideMemoire" package. To identify treatment/time specific OTUs, the ANOVA-like different expression (ALDEx) was performed in the ALDex2 package [37,38].

3. Results and Discussion

3.1. Physicochemical Characteristics of DFS

Acidification and drying profiles of DFS depended on the sausage formulation and production process parameters (Table 1). The results of the physicochemical analysis, including pH, water activity (a_w) and weight loss, are reported in Table 2.

The initial meat batter pH ranged from 5.83 to 5.96. Subsequently, the pH profile was determined by the product formulation and process temperature. For batch 1, formulated without the *L. sakei* CTC494 starter culture and submitted to a low temperature fermentation and ripening process, the same pH was maintained throughout the production process. For batches 2, 3 and 4, formulated with the starter culture and following a low temperature process, a pH decrease was observed from t7, registering the minimum pH values at t20 (5.11 ± 0.04, 5.09 ± 0.02 and 5.13 ± 0.01, respectively). The pH drop did not show significant differences among these batches at t12, t20 and tRip. In contrast, batches 5 and 6, formulated with the starter culture and submitted to a mild temperature process, registered a fast and strong product acidification, with the lowest pH values at t7 (4.81 ± 0.03 and 4.83 ± 0.02, for batches 5 and 6, respectively, $p > 0.05$). *L. sakei* CTC494 was previously reported to show a higher capacity to reduce the pH and produce lactic acid in production processes at 21–23 °C than at 10–14 °C [2]. Often, low-acid DFS acidification is followed by a gradual increase in the pH due to proteolysis, a process generating small peptides and free amino acids and amines [4,39]. In this regard, pH increases up to 0.5–0.7 pH units (end product pH values of 5.3–5.5) were shown in the present study for batches 5 and 6, corresponding to batches with a higher temperature process (12 °C).

The a_w value of the initial meat batter was above 0.982 in all batches. At t12, the a_w of batches ripened at a low (3 °C) temperature (1, 2, 3 and 4) was 0.98, and was slightly lower (0.97, $p < 0.05$) in batches ripened at a mild (12 °C) temperature (5 and 6). Afterwards, batches 1, 2, 3 and 4 continued ripening at 3 °C until day 20 of the process, when a_w decreased below 0.97. Then, the ripening temperature was raised to 8 °C and after 2 days, when the a_w decreased below 0.94, a final ripening phase of 18 days at 12 °C was applied until the a_w was below 0.9. The overall process time for batches ripened at low temperature was 32–40 days, reaching final a_w values of 0.866, 0.846, 0.840 and 0.857 for batches 1, 2, 3 and 4, respectively ($p > 0.05$). On the other hand, the process for batches ripened at 12 °C (batches 5 and 6) lasted 27 days, and the final a_w ranged from 0.865 to 0.858 ($p > 0.05$). Weight loss decreased to values of 61–65% in the final products.

A_w is a key factor for the food safety of DFS, especially in the Mediterranean-type, which usually show an a_w below 0.92 [3,40]. A particular reference should be made to the DFS formulated without the addition of nitrifying salts as they require additional control measures (e.g., temperature, a_w/NaCl and pH) to guarantee inhibition of *Clostridium botulinum* growth and toxin production [24,41]. In the present study, process temperature was linked to product a_w (see details in Section 2.1), and no remarkable differences in a_w were observed between fuets formulated with and without nitrifying salts nor with the batch formulated with liver auto-hydrolysate.

Table 2. Weight loss (%), physicochemical parameters, lactic acid bacteria (LAB) and Gram-positive catalase positive cocci (GCC+) counts (in log CFU/g). Values are the mean ± standard deviation for the replicates. Specifically, for each sampling time, significant differences between batches are marked with different capital letters ($p < 0.05$). NA: Not applicable; ND: Non-determined.

Time	Batch [1]	Weight Loss	a_w	pH	LAB	GCC+
t0	1	NA	0.983 ± 0.001^{AB}	5.96 ± 0.04^{A}	5.01 ± 0.19^{B}	4.13 ± 0.19^{AB}
	2	NA	0.982 ± 0.000^{B}	5.93 ± 0.03^{A}	6.19 ± 0.00^{A}	4.38 ± 0.03^{A}
	3	NA	0.985 ± 0.001^{AB}	5.87 ± 0.11^{A}	6.26 ± 0.13^{A}	4.03 ± 0.01^{AB}
	4	NA	0.985 ± 0.001^{AB}	5.83 ± 0.12^{A}	6.08 ± 0.04^{A}	3.76 ± 0.09^{B}
	5	NA	0.985 ± 0.001^{AB}	5.84 ± 0.08^{A}	6.11 ± 0.04^{A}	4.04 ± 0.33^{AB}
	6	NA	0.985 ± 0.000^{A}	5.90 ± 0.05^{A}	6.06 ± 0.02^{A}	4.03 ± 0.15^{AB}
t4	1	2.49 ± 0.85^{C}	0.982 ± 0.000^{B}	5.94 ± 0.11^{A}	5.17 ± 0.03^{C}	3.84 ± 0.03^{A}
	2	2.70 ± 0.92^{C}	0.982 ± 0.000^{B}	5.95 ± 0.13^{A}	8.11 ± 0.16^{B}	4.13 ± 0.15^{A}
	3	3.68 ± 0.58^{C}	0.985 ± 0.001^{A}	5.90 ± 0.12^{A}	8.24 ± 0.17^{B}	3.97 ± 0.16^{A}
	4	12.45 ± 1.23^{A}	0.983 ± 0.001^{B}	5.90 ± 0.13^{A}	8.09 ± 0.28^{B}	3.70 ± 0.05^{A}
	5	6.37 ± 0.87^{B}	0.984 ± 0.000^{A}	5.11 ± 0.08^{B}	8.90 ± 0.11^{A}	3.40 ± 0.00^{A}
	6	6.00 ± 0.71^{B}	0.984 ± 0.000^{A}	5.09 ± 0.09^{B}	8.85 ± 0.03^{A}	3.40 ± 0.00^{A}
t7	1	6.87 ± 1.03^{C}	0.980 ± 0.000^{B}	5.95 ± 0.08^{A}	5.27 ± 0.07^{C}	4.14 ± 0.27^{A}
	2	8.05 ± 2.15^{C}	0.980 ± 0.001^{B}	5.74 ± 0.04^{B}	8.43 ± 0.16^{B}	4.38 ± 0.09^{A}
	3	9.74 ± 0.65^{BC}	0.983 ± 0.001^{A}	5.58 ± 0.02^{C}	8.47 ± 0.06^{B}	4.07 ± 0.26^{A}
	4	12.16 ± 1.27^{AB}	0.983 ± 0.000^{AB}	5.66 ± 0.14^{BC}	8.40 ± 0.02^{B}	3.95 ± 0.30^{A}
	5	13.21 ± 2.59^{AB}	0.983 ± 0.002^{AB}	4.81 ± 0.03^{D}	8.92 ± 0.04^{A}	3.52 ± 0.11^{B}
	6	13.46 ± 0.73^{A}	0.982 ± 0.001^{AB}	4.83 ± 0.02^{D}	8.97 ± 0.04^{A}	3.51 ± 0.12^{B}
t12	1	23.24 ± 2.06^{B}	0.976 ± 0.000^{A}	5.94 ± 0.09^{A}	5.48 ± 0.25^{D}	3.77 ± 0.29^{A}
	2	26.85 ± 3.47^{B}	0.976 ± 0.001^{A}	5.30 ± 0.01^{B}	8.77 ± 0.04^{ABC}	3.55 ± 0.01^{A}
	3	30.85 ± 1.86^{B}	0.979 ± 0.000^{A}	5.27 ± 0.02^{B}	8.54 ± 0.04^{C}	3.58 ± 0.21^{A}
	4	24.17 ± 1.70^{B}	0.979 ± 0.000^{A}	5.31 ± 0.06^{B}	8.63 ± 0.09^{BC}	3.40 ± 0.05^{A}
	5	44.45 ± 3.82^{A}	0.971 ± 0.003^{B}	4.85 ± 0.04^{C}	9.00 ± 0.16^{A}	2.76 ± 0.02^{B}
	6	46.36 ± 3.72^{A}	0.970 ± 0.003^{B}	4.90 ± 0.06^{C}	8.88 ± 0.05^{AB}	2.75 ± 0.37^{B}
t20	1	37.61 ± 2.36^{CD}	0.968 ± 0.004^{A}	5.85 ± 0.02^{A}	ND	ND
	2	41.38 ± 3.44^{CD}	0.967 ± 0.001^{A}	5.11 ± 0.04^{A}	ND	ND
	3	45.20 ± 2.10^{B}	0.967 ± 0.000^{A}	5.09 ± 0.02^{A}	ND	ND
	4	37.01 ± 2.28^{D}	0.970 ± 0.004^{A}	5.13 ± 0.01^{A}	ND	ND
	5	59.19 ± 2.22^{A}	0.939 ± 0.017^{AB}	5.30 ± 0.32^{A}	8.62 ± 0.10	2.73 ± 0.45
	6	59.86 ± 2.13^{A}	0.928 ± 0.007^{B}	5.59 ± 0.37^{A}	8.61 ± 0.07	2.53 ± 0.24
tRip	1	61.41 ± 1.81^{B}	0.866 ± 0.016^{A}	5.82 ± 0.00^{A}	8.25 ± 0.11^{B}	1.98 ± 0.73^{A}
	2	61.52 ± 1.37^{B}	0.846 ± 0.009^{A}	5.20 ± 0.05^{C}	8.34 ± 0.00^{AB}	1.27 ± 0.01^{A}
	3	64.75 ± 0.90^{A}	0.840 ± 0.031^{A}	5.20 ± 0.02^{C}	8.18 ± 0.00^{AB}	1.40 ± 0.12^{A}
	4	63.54 ± 1.25^{A}	0.857 ± 0.027^{A}	5.19 ± 0.01^{C}	8.17 ± 0.00^{B}	1.90 ± 0.08^{A}
	5	63.95 ± 1.47^{A}	0.865 ± 0.012^{A}	5.49 ± 0.17^{B}	8.32 ± 0.03^{AB}	1.90 ± 0.44^{A}
	6	64.24 ± 1.32^{A}	0.858 ± 0.013^{A}	5.54 ± 0.08^{B}	8.53 ± 0.00^{A}	2.88 ± 0.92^{A}
tStor	1	ND	ND	5.83 ± 0.00^{A}	8.02 ± 0.06^{B}	2.83 ± 0.16^{A}
	2	ND	ND	5.35 ± 0.01^{C}	8.21 ± 0.00^{AB}	2.31 ± 0.23^{AB}
	3	ND	ND	5.23 ± 0.01^{C}	8.22 ± 0.12^{AB}	2.24 ± 0.11^{AB}
	4	ND	ND	5.23 ± 0.08^{C}	8.20 ± 0.08^{AB}	1.97 ± 0.01^{B}
	5	ND	ND	5.32 ± 0.04^{C}	8.29 ± 0.00^{A}	2.25 ± 0.05^{AB}
	6	ND	ND	5.52 ± 0.12^{B}	8.30 ± 0.03^{A}	2.68 ± 0.24^{AB}

[1] Batch 1: With liver auto-hydrolysate at a low temperature; Batch 2: With liver auto-hydrolysate and the starter culture at a low temperature; Batch 3: With the starter culture at a low temperature; Batch 4: With the starter culture and nitrifying salts at a low temperature; Batch 5: With the starter culture and nitrifying salts at a mild temperature; Batch 6: With the starter culture, nitrifying salts, and sodium reduction at a mild temperature.

3.2. Culture-Dependent Microbial Dynamics

The levels of LAB and GCC+ of the fuets are reported in Table 2. For batches containing the *L. sakei* CTC494 starter culture, the LAB concentration at t0 was ca. 6 log CFU/g, whereas the initial LAB concentration for batch 1, without the starter culture, was 1 log CFU/g lower (5.01 ± 0.19 log CFU/g) ($p < 0.05$). The low temperature processed batches (2, 3 and 4) registered LAB values ca. 8 log CFU/g at t4 and reached maximum levels at t12, which slightly decreased until tStor. For batch 1, endogenous LAB growth was slow (0.5 log after 12 days at a low temperature), compared to other batches formulated with the starter culture, and stationary phase levels (8.25 ± 0.11 log CFU/g) were not registered until tRip. Consequently, the pH of batch 1 did not decrease and was maintained above 5.80 during the whole process. In contrast, for batches 5 and 6, ripened at 12 °C, the LAB grew the fastest and reached values ca. 9 log CFU/g at t4, maintained the population density until t20, and thereafter slightly decreased until tStor. Monitoring of the *L. sakei* CTC494 by ERIC-PCR showed 100% implantation in batches inoculated with the starter culture at t0, t4 and tRip.

The behaviour of GCC+ was similar in all batches of fuet, although the process was shorter for 12 °C-processed fuets. Initial GCC+ values were ca. 4 log CFU/g in all the batches and a progressive decrease was observed along the process time, reaching levels of 1.3–2.0 log CFU/g at tRip, without significant differences between batches ($p > 0.05$). During subsequent refrigerated storage, GCC+ slightly increased in batches 1 to 3 (a maximum of 1 log in batch 2) and was maintained in batches 4 to 6.

The initial increase in the LAB population in batches formulated with the starter culture highly depended on the process temperature and was not affected by the addition of the liver auto-hydrolysate, the removal of nitrifying salts or the NaCl reduction. *L. sakei* CTC494 is a psychrotrophic strain well adapted to the meat fermentation environment; it is able to rapidly grow and acidify DFS processed at both mild (12 °C) and low (3 °C) temperatures [2,22,42]. Given the importance of a rapid pH drop for food safety, the selection of starter cultures suitable for the conditions of the production process (e.g., low temperature) is a key aspect. GCC+ development is strongly modulated by the levels of LAB, which are able to grow at a lower pH and a_w and, thus, can impact the sensory characteristics of the final product given that flavour development is highly influenced by GCC+ proteolytic and lipolytic activities [43].

3.3. Diversity and Taxonomic Composition of the Bacterial Communities

A total of 17,894,929 high-quality reads were obtained after filtering and denoising, with an average of 101,101 sequences per sample. The rarefaction curve showed that the sequencing depth was sufficient to infer microbial composition.

Taxonomic assignment of ASV resulted in the identification of 800 taxa belonging to 14 different phyla, which was mainly represented by *Bacillota* (formerly *Firmicutes*), *Pseudomonadota* (formerly *Proteobacteria*) and *Actinomycetota* (formerly *Actinobacteriota*). A total of 171 different genera and 158 species (Figure 1, Supplementary Table S1) were identified.

Alpha diversity, which describes the intra-sample diversity, was evaluated through the Shannon index (Supplementary Table S2). Extended alpha diversity indexes (Simpson and Chao1) are also represented in Supplementary Table S2. Results show that batches formulated with liver auto-hydrolysate and/or without curing agents (batches 1, 2 and 3) had the highest microbial diversity during the first four days of ripening (i.e., Shannon index values of 2.84, 1.71 and 1.79, respectively), which decreased along the ripening process, especially in batches with the starter culture. Batch 1, formulated without the starter culture and with liver auto-hydrolysate, was the only one that maintained the index above 1.5 at tStor. Batch 2 registered the highest alpha diversity value at t0, which decreased during the process, reaching 0.66 at tStor. Liver auto-hydrolysate contributed to the presence of microorganisms that are not typically present in meat batters and, thus, increased the microbial richness of the samples. Batch 3 registered the highest Shannon

diversity index at t4 (1.79), which progressively decreased along the process, with a value of 0.80 at the end of storage. Alpha diversity decrease was related to the application of the starter culture. Starter cultures promote fermentation, ensure food safety, standardise product properties and shorten ripening times. However, they also decrease the microbial biodiversity of the fermented product [18,44]. Nitrified batches formulated with the starter culture (4, 5 and 6) had less diverse microbial communities, showing values $\leq$0.82 at t0 and $\leq$0.66 throughout the ripening process and storage, without differences between batches ($p > 0.05$). The combined application of nitrifying salts and the starter culture contributed to the reduction in the alpha diversity. The use of nitrifying agents has been described to cause acidic, oxidative and nitrative stresses to sensitive microorganisms [17], which could explain the microbial diversity reduction.

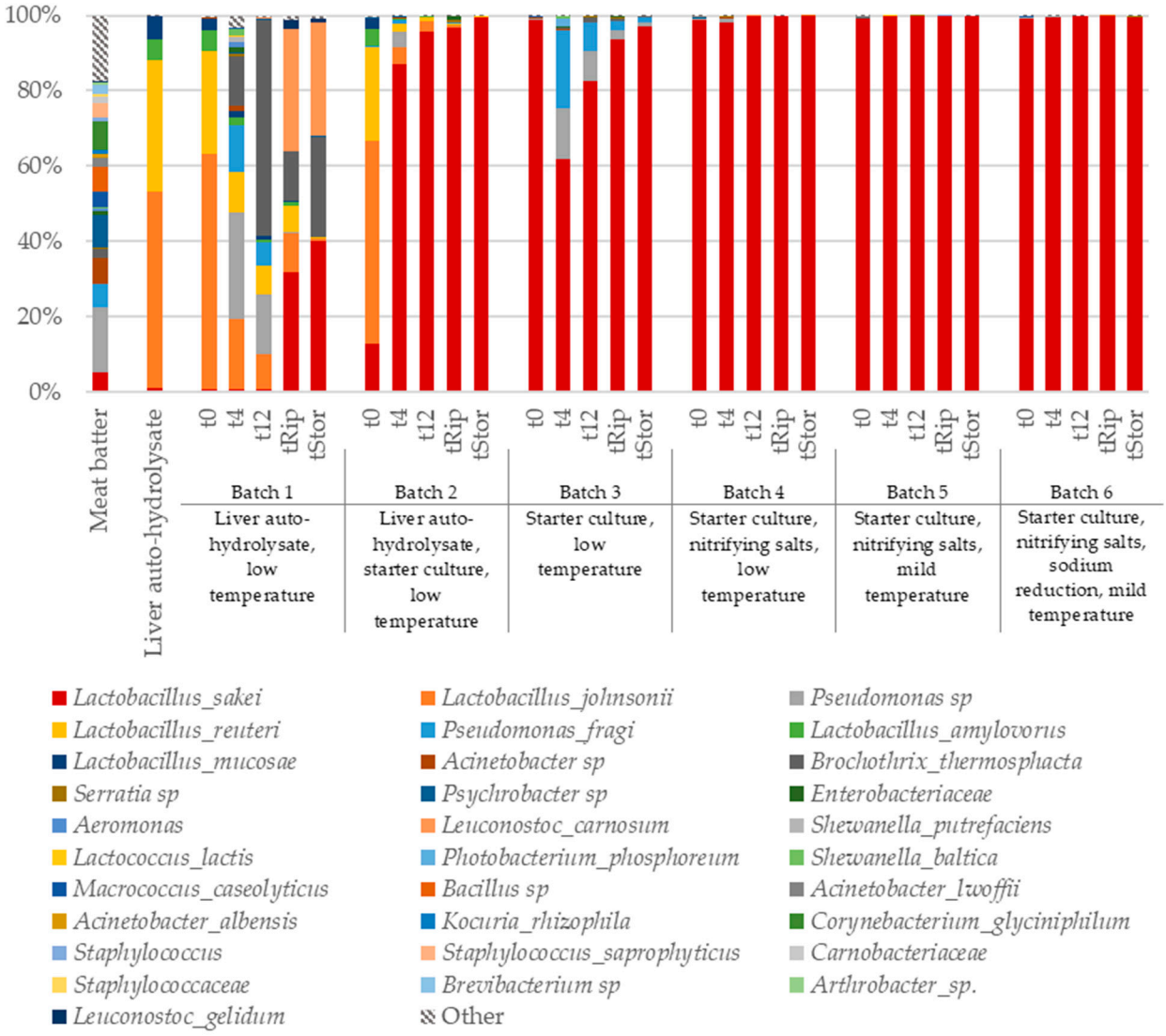

Figure 1. Taxonomic composition represented in the relative abundance (%) of microbial communities at the species level identified in the meat batter, liver hydrolysed ingredient and DFS batches (1, 2, 3, 4, 5 and 6) by time (days) 0, 4, 12, end of ripening and end of storage. Only species with an incidence above 1% in at least one sample are represented; the remaining are classified as "Other".

The bacterial relative abundances of DFS at the genus/species level were different depending on the type of fuet (i.e., formulation and production process) and sampling time (Figure 1, Supplementary Table S1). The initial meat batter was mostly characterised by the presence of *Pseudomonas* sp. (17.37%), *Psychrobacter* sp. (8.43%), *Corynebacterium glyciniphilum* (7.32%), *Acinetobacter* sp. (6.84%), *Bacillus* sp. (6.53%), *Pseudomonas fragi* (6.27%) and *L. sakei* (5.12%); all have been previously described in raw meat stored under refrigeration [6,45,46]. The psychrotrophic genus *Pseudomonas* sp. is the main spoilage bacteria of aerobically stored fresh meat kept at refrigeration temperatures since it can grow from 2 to 35 °C [6]. Specifically, the *P. fragi* species stands out for meat spoilage among the *Pseudomonas* spp. as meat can be considered its ecological niche [47]. *B. thermosphacta* can easily colonise the meat matrix since it can grow under aerobic and anaerobic conditions and has been classified as a fresh meat and cooked meat spoilage microorganism due to its off-odour metabolite production, mainly upon depletion of glucose [48]. Similar results were obtained in the meat batter of Fabriano-like fermented sausages, detecting *Pseudomonas* sp. and *B. thermospacta* [49]. In contrast, the pork liver auto-hydrolysate contained mainly LAB species, with the most abundant being *Lactobacillus johnsonii* (62.64%), *Limosilactobacillus reuteri* (34.89%), *Limosilactobacillus mucosae* (6.34%) and *Lactobacillus amylovorus* (5.43%). These species have been classified as putative probiotic [50], and are included in the EFSA QPS (Qualified Presumption of Safety) list [51].

At t0 (before fermentation), in batches 1 and 2 of DFS, the most abundant species were *L. johnsonii*, representing more than 50% of the total species, and *L. reuteri* (*ca*. 25%), both coming from the added liver auto-hydrolysate. The most abundant microorganisms found in the meat batter (*Pseudomonas* sp., *Psychrobacter* sp. and *C. glyciniphilum*) were detected at very low percentages (<0.14%). Batch 2 also showed 12.9% *L. sakei*, corresponding to the applied starter culture, as confirmed by ERIC-PCR. At t0, *L. sakei* was the most abundant species (>98%) in batches 3 to 6.

After 4 days at 3 °C, the batch 1 bacterial community changed as the relative abundance of *L. johnsonii* and *L. reuteri* decreased to 18.7% and 10.9%, respectively, and *Pseudomonas* sp., *B. thermosphacta* and *P. fragi* increased to 28.3%, 13.1% and 12.6%, respectively. At t12, there was still a high abundance of microorganisms coming from both the meat batter and the liver auto-hydrolysate (i.e., *B. thermosphacta* (57.6%), *Pseudomonas* sp. (15.9%), *L. johnsonii* (9.2%), *L. reuteri* (7.6%) and *P. fragi* (6.1%)). *Pseudomonas* sp. and *B. thermosphacta* have been classified as spoilage microorganisms mainly found in chilled fresh meat products [46,52] and were also present along the ripening process of sausages submitted to low temperatures. From t20 to tRip, when the process temperature was raised to 12 °C, an increase in the abundance of *L. sakei* (to 31.9%) and *Leuconostoc carnosum* (32.3%) was observed. In contrast, *B. thermosphacta* (13.1%), *Pseudomonas* sp. (0.2%) and *P. fragi* (0.1%) were decreased, and *L. johnsonii* and *L. reuteri* were maintained (10.3% and 7.0%, respectively). Those putative probiotic strains, *L. johnsonii* and *L. reuteri*, have been described to play a role in the intestines, increasing lipid absorption and stimulating host immunity against infectious agents, respectively [53]. By tStor, the most relevant species were *L. sakei* (40.1%), *L. carnosum* (30.3%) and *B. thermosphacta* (26.5%), while *L. johnsonii* and *L. reuteri,* decreased (<1%). *L. carnosum* is frequent in meat-based products and plays a controversial role as it can participate in the spoilage, affecting sensorial properties, or act as a bioprotective culture, through organic acid release or bacteriocin production [54]. Interestingly, specific strains of the putative probiotic species provided by the liver auto-hydrolysate (*L. reuteri* and *L. amylovorus*) have been proposed for incorporation, through microencapsulation, in fermented foods to ensure a desired level of probiotic microorganisms in the final product [55]. Moreover, other authors have concluded that the addition of LAB probiotic bacteria, such as *Lacticaseibacillus casei* (formerly *Lactobacillus casei)* and *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*), improve the quality of DFS [56].

Considering batches formulated with the starter culture, *L. sakei* CTC494 led the fermentation process and was maintained at high levels until the end of storage of fuets. *L. sakei* has been reported to be very competitive in meat fermentations [57] and, specifically for *L. sakei* CTC494, it has been described to grow in a wide range of temperatures and formulations [2,57,58], explaining the ability to dominate among other bacteria, even in DFS submitted to a low temperature ripening process [59]. At t4 in batch 2, *L. sakei* represented 86.9% of the total bacterial population; the abundance of *L. johnsonii* (4.7%) and *Pseudomonas* sp. (4.2%) was also remarkable. In batch 3, the main species was *L. sakei* (61.8%) followed by *P. fragi* (21.0%) and *Pseudomonas* sp. (13.4%). *L. sakei* was also the dominant species (>98%) in batches 4 to 6 from t4 until the end of storage. From t12, *L. sakei* dominance continued, representing 95% of the bacterial community in all batches except for number 3, which was formulated without nitrifying salts, whose relative abundance was 82.4%. In this batch, *Pseudomonas* sp. and *P. fragi* had a relative abundance of 8.2% and 7.6%, respectively, although they progressively decreased to 1.1% and 1.2%, respectively, at tStor. In parallel, *L. sakei* progressively increased from 82.4% (t12) to 97.2% (tStor). Strain typing by ERIC-PCR showed the competitiveness of the applied starter culture, *L. sakei* CTC494, being the dominant species until the end of ripening.

Beta diversity was studied through a compositional approach to analyse microbial abundance differences between batches. The PCA (Figure 2) based on ASVs table (Supplementary Table S1), showed samples from batches 1 and 2 clustered together and separated from the other batches at t0. The perMANOVA with Aitchison distances indicated a significant effect on the bacterial community between these two groups ($p < 0.05$). ALDEx results showed that the abundance of the main species of the liver auto-hydrolysate (i.e., *L. reuteri*, *L. mucosae*, *L. johnsonii*, *L. amylovorus*, *Lactobacillus salivarius* and *Lactobacillus delbrueckii*) were enriched in batches 1 and 2, while the abundance of *Bacillus* sp., *M. caseolyticus* and *L. sakei* was higher in batches 3 to 6.

During the fermentation and drying processes, microbial communities progressively changed (Figure 2), and from t12 until tStor, batches without curing salts (1, 2 and 3) showed significant beta diversity differences between them and with the rest of the batches ($p < 0.05$). No differences were found between batches containing nitrites and nitrates ($p > 0.05$); therefore, neither salt concentration nor temperature process exerted a significant effect on the bacterial community composition. Charmpi et al. [60] also reported no taxonomic differences among fermented meat with different salt concentrations. The ALDEx analysis showed some taxa being differentially abundant between batches 1 and 2. At t12, the abundance of *L. johnsonii*, *L. amylovorus* and *L. sakei* was increased in batch 2 while *Leuconostoc* spp., *C. divergens*, *B. thermosphacta* and *Pseudomonas* spp. had a significantly higher abundance in batch 1. After ripening and storage, only the abundance of *L. sakei* was increased in batch 2 when compared to batch 1, which maintained a significantly higher abundance of *B. thermosphacta*, *L. gelidum*, *L. carnosum* and *C. divergens*. During the whole process, the most important differences between batches with/without the liver auto-hydrolysate (i.e., batch 1 and 2 vs. batches 3–6) were the liver auto-hydrolysate associated species being significantly more abundant in batches 1 and 2, whereas batches 3 to 6 were enriched in *L. sakei*. When comparing batches containing nitrifying salts against batch 3 (no nitrite or nitrate added), the ALDEx analysis showed that *L. sakei* was enriched in batches 4 to 6 throughout the process and *P. fragi*, *Pseudomonas* sp. and *B. thermosphacta* were enriched in batch 3. It was shown in minced turkey meat that the application of nitrite lowered the relative abundance of both *Pseudomonas* spp. and *Brochothrix* spp. during storage at 4 °C [61].

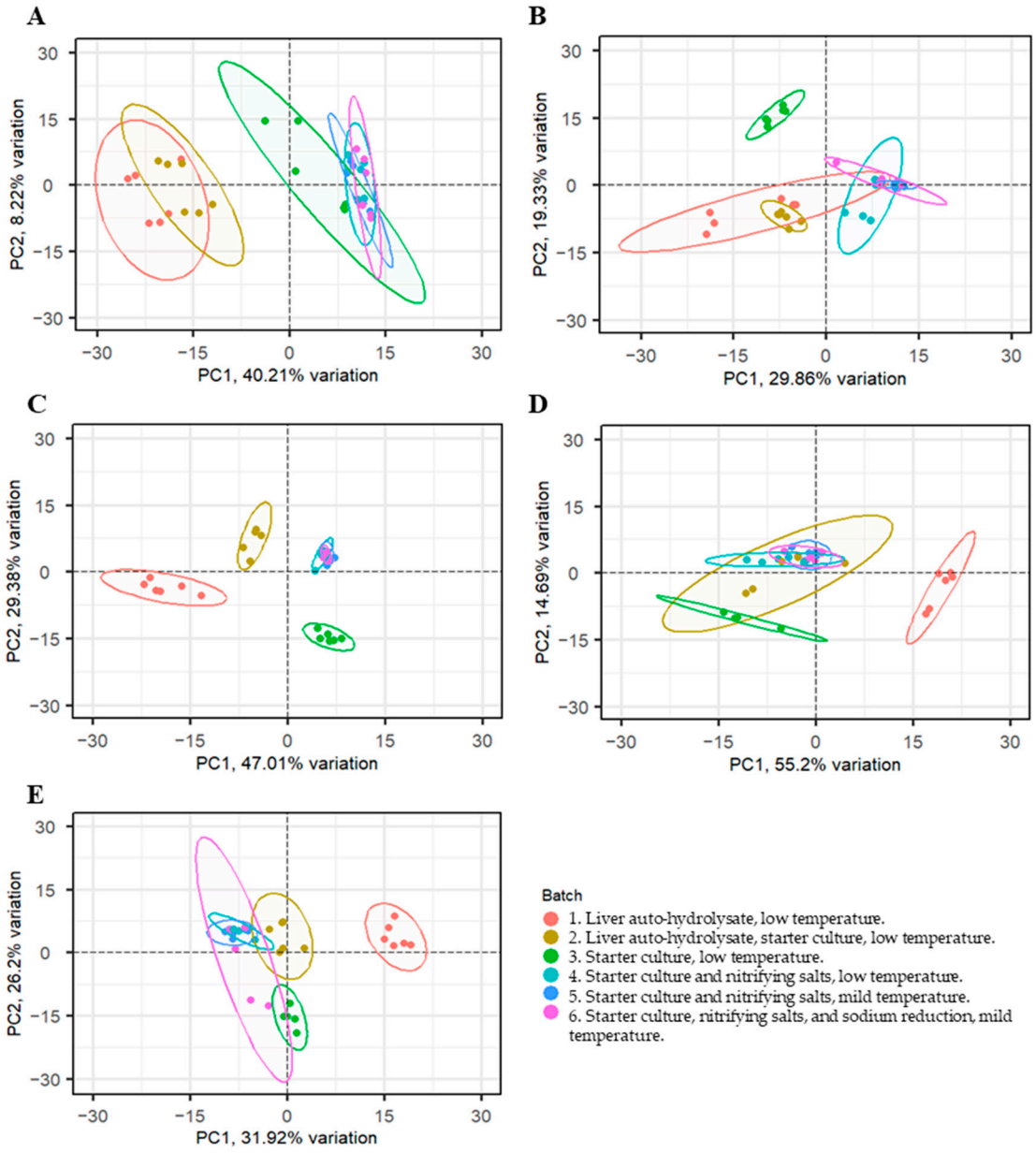

Figure 2. Principal component analysis plots representing the beta diversity analysis comparing every batch by time t0 (**A**), t4 (**B**), t12 (**C**), tRip (**D**) and tStor (**E**).

4. Conclusions

This research provides valuable microbiological information to DFS manufacturers who want to innovate safely. Nutritionally improved (low in sodium) and clean label (without nitrifying salts) formulations and low-temperature processes only cause minor shifts in the physicochemical characteristics of DFS when using a competitive starter culture to ensure product acidification. Accordingly, production processes requiring a low temperature to guarantee food safety can be used to produce DFS with similar characteristics to those fermented and ripened at mild temperatures. Irrespective of the formulation and process parameters, the microbial communities gradually change over time, decreasing their diversity due to the progressively harsher conditions that occur throughout fermentation and ripening. However, the factors that most impact the bacterial community composition are the addition of a starter culture and/or liver auto-hydrolysate, that provides putative probiotic species, and the presence of nitrate/nitrite salts that reduce the meat spoilage-

related microorganisms. Further studies are in progress to evaluate the sensory aspects of the newly developed fuet-type dry fermented sausages.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9040403/s1, Table S1: Taxonomic composition represented as the relative abundance (%) of all samples at the species level detected above 1% in at least one sample; Table S2: Alpha diversity indexes Shannon, Simpson and Chao1.

Author Contributions: Conceptualization, A.J., R.B., S.B.-C.; investigation: B.M., M.L., N.F.-B.; methodology, A.J., B.M., M.L., N.F.-B.; formal analysis, B.M. and N.F.-B.; writing—original draft preparation, N.F.-B.; writing—review and editing, A.J., B.M., M.L., N.F.-B., R.B., S.B.-C.; supervision, A.J.; project administration, A.J., R.B., S.B.-C.; funding acquisition, A.J., S.B.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been framed within the NG-sausaging (RTI2018-099195-R-I00) project funded by the Spanish Ministerio de Economía, Indústria y Competitividad and, the Consolidated Research Group (2017 SGR 1650) and the CERCA Programme from Generalitat de Catalunya. N. Ferrer-Bustins was the recipient of a doctoral fellowship from the Ministry of Science and Innovation (PRE2019-087847).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request.

Acknowledgments: Authors would like to thank Q. Arbonès for the sausages manufacturing, M. Viella and A. Morera for technical assistance and F. Sánchez-Molinero for his support in experimental design.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Garriga, M.; Aymerich, T. The microbiology of fermentation and ripening. In *Handbook of Fermented Meat and Poultry*; Toldrá, F., Hui, Y.H., Astiasarán, I., Sebranek, J.G., Talon, R., Eds.; John Wiley & Sons, Ltd.: Hoboken, NJ, USA, 2014; pp. 107–115.
2. Austrich-Comas, A.; Jofré, A.; Gou, P.; Bover-Cid, S. Assessing the impact of different technological strategies on the fate of *Salmonella* in chicken dry-fermented sausages by means of challenge testing and predictive models. *Microorganisms* **2023**, *11*, 432. [CrossRef] [PubMed]
3. Rubio, R.; Jofré, A.; Aymerich, T.; Guàrdia, M.D.; Garriga, M. Nutritionally enhanced fermented sausages as a vehicle for potential probiotic lactobacilli delivery. *Meat Sci.* **2014**, *96*, 937–942. [CrossRef] [PubMed]
4. Flores, M.; Toldrá, F. Microbial enzymatic activities for improved fermented meats. *Trends Food Sci. Technol.* **2011**, *22*, 81–90. [CrossRef]
5. Martín, B.; Garriga, M.; Hugas, M.; Bover-Cid, S.; Veciana-Nogués, M.T.; Aymerich, T. Molecular, technological and safety characterization of Gram-positive catalase-positive cocci from slightly fermented sausages. *Int. J. Food Microbiol.* **2006**, *107*, 148–158. [CrossRef] [PubMed]
6. Nychas, G.J.E.; Skandamis, P.N.; Tassou, C.C.; Koutsoumanis, K.P. Meat spoilage during distribution. *Meat Sci.* **2008**, *78*, 77–89. [CrossRef] [PubMed]
7. Leistner, L.; Gorris, L.G.M. Food preservation by hurdle technology. *Trends Food Sci. Technol.* **1995**, *6*, 41–46. [CrossRef]
8. De Araújo, P.D.; Araújo, W.M.C.; Patarata, L.; Fraqueza, M.J. Understanding the main factors that influence consumer quality perception and attitude towards meat and processed meat products. *Meat Sci.* **2022**, *193*, 108952. [CrossRef]
9. Marco, A.; Navarro, J.L.; Flores, M. The influence of nitrite and nitrate on microbial, chemical and sensory parameters of slow dry fermented sausage. *Meat Sci.* **2006**, *73*, 660–673. [CrossRef] [PubMed]
10. Tabanelli, G.; Barbieri, F.; Soglia, F.; Magnani, R.; Gardini, G.; Petracci, M.; Gardini, F.; Montanari, C. Safety and technological issues of dry fermented sausages produced without nitrate and nitrite. *Food Res. Int.* **2022**, *160*, 111685. [CrossRef]
11. De Mey, E. *N-Nitrosamines in Dry Fermented Sausages: Occurrence and Formation of n-Nitrosopiperidine*; KU Leuven: Leuven, Belgium, 2014; ISBN 978-90-8826-353-8.
12. Abril, B.; Sanchez-Torres, E.A.; Bou, R.; Benedito, J.; Garcia-Perez, J.V. Influence of pork liver drying on ferrochelatase activity for zinc protoporphyrin formation. *LWT* **2022**, *171*, 114128. [CrossRef]
13. Wakamatsu, J.I.; Murakami, N.; Nishimura, T. A comparative study of zinc protoporphyrin ix-forming properties of animal by-products as sources for improving the color of meat products. *Anim. Sci. J.* **2015**, *86*, 547–552. [CrossRef] [PubMed]
14. EFSA NDA Panel (EFSA Panel on Nutrition Novel Foods and Food Allergens); Turck, D.; Castenmiller, J.; de Henauw, S.; Hirsch-Ernst, K.I.; Kearney, J.; Knutsen, H.K.; Maciuk, A.; Mangelsdorf, I.; McArdle, H.J.; et al. Dietary Reference Values for Sodium. *EFSA J.* **2019**, *17*, e05778. [CrossRef] [PubMed]

15. Stollewerk, K.; Jofré, A.; Comaposada, J.; Arnau, J.; Garriga, M. The impact of fast drying (QDS Process®) and high pressure on food safety of NaCl-free processed dry fermented sausages. *Innov. Food Sci. Emerg. Technol.* **2012**, *16*, 89–95. [CrossRef]
16. Teixeira, A.; Domínguez, R.; Ferreira, I.; Pereira, E.; Estevinho, L.; Rodrigues, S.; Lorenzo, J.M. Effect of NaCl replacement by other salts on the quality of bísaro pork sausages (Pgi Chouriça de Vinhais). *Foods* **2021**, *10*, 961. [CrossRef]
17. Majou, D.; Christieans, S. Mechanisms of the bactericidal effects of nitrate and nitrite in cured meats. *Meat Sci.* **2018**, *145*, 273–284. [CrossRef]
18. Franciosa, I.; Alessandria, V.; Dolci, P.; Rantsiou, K.; Cocolin, L. Sausage fermentation and starter cultures in the era of molecular biology methods. *Int. J. Food Microbiol.* **2018**, *279*, 26–32. [CrossRef]
19. Belleggia, L.; Ferrocino, I.; Reale, A.; Haouet, M.N.; Corvaglia, M.R.; Milanović, V.; Boscaino, F.; Di Renzo, T.; Di Bella, S.; Borghi, M.; et al. Unravelling microbial populations and volatile organic compounds of artisan fermented liver sausages manufactured in central Italy. *Food Res. Int.* **2022**, *154*, 111019. [CrossRef]
20. Van Reckem, E.; Claeys, E.; Charmpi, C.; Sosa Fajardo, A.; Van der Veken, D.; Maes, D.; Weckx, S.; De Vuyst, L.; Leroy, F. High-throughput amplicon sequencing to assess the impact of processing factors on the development of microbial communities during spontaneous meat fermentation. *Int. J. Food Microbiol.* **2021**, *354*, 109322. [CrossRef]
21. Bou, R.; Llauger, M.; Albano, M.; Tenorio, E. Optimitzation of ZnPP-forming conditions in porcine livers using response surface methodology (RSM). In Proceedings of the 67th International Congress of Meat Science and Technology, Kraków, Poland, 23–27 August 2021.
22. Hugas, M.; Garriga, M.; Aymerich, M.T.; Monfort, J.M. Inhibition of *Listeria* in dry fermented sausages by the bacteriocinogenic *Lactobacillus Sake* CTC494. *J. Appl. Bacteriol.* **1995**, *79*, 322–330. [CrossRef]
23. ANSES. *Fiche de Description de Danger Biologique Transmissible Par Les Aliments: Clostridium Botulinum, Clostridium Neurotoxinogènes*; ANSES: Maisons-Alfort, France, 2019.
24. EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards). Opinion of the scientific panel on biological hazards on a request from the commission related to *Clostridium* spp. in foodstuffs. *EFSA J.* **2005**, *3*, 1–65. [CrossRef]
25. Serra-Castelló, C.; Bover-Cid, S.; Garriga, M.; Hansen, T.B.; Gunvig, A.; Jofré, A. Risk management tool to define a corrective storage to enhance *Salmonella* inactivation in dry fermented sausages. *Int. J. Food Microbiol.* **2021**, *346*, 109160. [CrossRef] [PubMed]
26. Versalovic, J.; Koeuth, T.; Lupski, J.R. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* **1991**, *19*, 6823–6831. [CrossRef] [PubMed]
27. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2013**, *41*, e1. [CrossRef] [PubMed]
28. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef] [PubMed]
29. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
30. R Core Team R. *A Language and Environment for Statistical Computing 2019*; R Foundation for Statistical Computing: Vienna, Austria, 2019; Available online: https://r-project.org/ (accessed on 30 March 2023).
31. McMurdie, P.J.; Holmes, S. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef]
32. Gloor, G.B.; Reid, G. Compositional analysis: A valid approach to analyze microbiome high-throughput sequencing data. *Can. J. Microbiol.* **2016**, *62*, 692–703. [CrossRef]
33. Gloor, G.B.; Macklaim, J.M.; Pawlowsky-Glahn, V.; Egozcue, J.J. Microbiome datasets are compositional: And this is not optional. *Front. Microbiol.* **2017**, *8*, 2224. [CrossRef] [PubMed]
34. Palarea-Albaladejo, J.; Martín-Fernández, J.A. ZCompositions—R Package for multivariate imputation of left-censored data under a compositional approach. *Chemom. Intell. Lab. Syst.* **2015**, *143*, 85–96. [CrossRef]
35. Martín-Fernández, J.-A.; Hron, K.; Templ, M.; Filzmoser, P.; Palarea-Albaladejo, J. Bayesian-multiplicative treatment of count zeros in compositional data sets. *Stat. Model.* **2014**, *15*, 134–158. [CrossRef]
36. Anderson, M.J. A new method for non-parametric multivariate analysis of variance. *Austral. Ecol.* **2001**, *26*, 32–46. [CrossRef]
37. Fernandes, A.D.; Macklaim, J.M.; Linn, T.G.; Reid, G.; Gloor, G.B. ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-Seq. *PLoS ONE* **2013**, *8*, e67019. [CrossRef] [PubMed]
38. Fernandes, A.D.; Reid, J.N.; Macklaim, J.M.; Mcmurrough, T.A.; Edgell, D.R.; Gloor, G.B. Unifying the analysis of high-throughput sequencing datasets: Characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* **2014**, *2*, 15. [CrossRef]
39. Aymerich, T.; Martín, B.; Garriga, M.; Hugas, M. Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. *Appl. Environ. Microbiol.* **2003**, *69*, 4583–4594. [CrossRef]
40. Ordöñez, J.A.; de la Hoz, L. Mediterranean products. In *Handbook of Fermented Meat and Poultry*; Toldrá, F., Ed.; Blackwell Publishing: Hoboken, NJ, USA, 2007; pp. 333–347.

41. Peck, M.W.; Stringer, S.C.; Carter, A.T. *Clostridium botulinum* in the post-genomic era. *Food Microbiol.* **2011**, *28*, 183–191. [CrossRef] [PubMed]
42. Zagorec, M.; Champomier-Vergès, M.C. *Lactobacillus sakei*: A starter for sausage fermentation, a protective culture for meat products. *Microorganisms* **2017**, *5*, 56. [CrossRef] [PubMed]
43. Ravyts, F.; Steen, L.; Goemaere, O.; Paelinck, H.; De Vuyst, L.; Leroy, F. The application of staphylococci with flavour-generating potential is affected by acidification in fermented dry sausages. *Food Microbiol.* **2010**, *27*, 945–954. [CrossRef]
44. Barbieri, F.; Tabanelli, G.; Montanari, C.; Dall'osso, N.; Šimat, V.; Možina, S.S.; Baños, A.; Özogul, F.; Bassi, D.; Fontana, C.; et al. Mediterranean spontaneously fermented sausages: Spotlight on microbiological and quality features to exploit their bacterial biodiversity. *Foods* **2021**, *10*, 2691. [CrossRef]
45. Doulgeraki, A.I.; Ercolini, D.; Villani, F.; Nychas, G.J.E. Spoilage microbiota associated to the storage of raw meat in different conditions. *Int. J. Food Microbiol.* **2012**, *157*, 130–141. [CrossRef]
46. Ferrer-Bustins, N.; Martín, B.; Bover-Cid, S.; Jofré, A. Caracterización de La Diversidad Microbiana En Productos Cárnicos Porcinos y Superficies de Instalaciones Con Métodos Dependientes e Independientes de Cultivo. In Proceedings of the XXVIII Congreso Sociedad Española de Microbiología, Online, 28 June–2 July 2021; p. 398.
47. Ercolini, D.; Casaburi, A.; Nasi, A.; Ferrocino, I.; Di Monaco, R.; Ferranti, P.; Mauriello, G.; Villani, F. Different molecular types of *Pseudomonas fragi* have the same overall behaviour as meat spoilers. *Int. J. Food Microbiol.* **2010**, *142*, 120–131. [CrossRef]
48. Danilo, E.; Federica, R.; Elena, T.; Paolo, M.; Francesco, V. Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. *Appl. Environ. Microbiol.* **2006**, *72*, 4663–4671. [CrossRef]
49. Cardinali, F.; Milanović, V.; Osimani, A.; Aquilanti, L.; Taccari, M.; Garofalo, C.; Polverigiani, S.; Clementi, F.; Franciosi, E.; Tuohy, K.; et al. Microbial dynamics of model Fabriano-like fermented sausages as affected by starter cultures, nitrates and nitrites. *Int. J. Food Microbiol.* **2018**, *278*, 61–72. [CrossRef] [PubMed]
50. Työppönen, S.; Petäjä, E.; Mattila-Sandholm, T. Bioprotectives and probiotics for dry sausages. *Int. J. Food Microbiol.* **2003**, *83*, 233–244. [CrossRef] [PubMed]
51. EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards); Koutsoumanis, K.; Allende, A.; Alvarez-Ordóñez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; de Cesare, A.; Hilbert, F.; et al. Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 10: Suitability of taxonomic units notified to EFSA until March 2019. *EFSA J.* **2019**, *17*, 1–79. [CrossRef]
52. Luong, N.D.M.; Coroller, L.; Zagorec, M.; Membré, J.M.; Guillou, S. Spoilage of chilled fresh meat products during storage: A quantitative analysis of literature data. *Microorganisms* **2020**, *8*, 1198. [CrossRef] [PubMed]
53. Casas, I.A.; Dobrogosz, W.J. Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. *Microb. Ecol. Health Dis.* **2000**, *12*, 247–285. [CrossRef]
54. Raimondi, S.; Nappi, M.R.; Sirangelo, T.M.; Leonardi, A.; Amaretti, A.; Ulrici, A.; Magnani, R.; Montanari, C.; Tabanelli, G.; Gardini, F.; et al. Bacterial community of industrial raw sausage packaged in modified atmosphere throughout the shelf life. *Int. J. Food Microbiol.* **2018**, *280*, 78–86. [CrossRef]
55. Muthukumarasamy, P.; Holley, R.A. Microbiological and sensory quality of dry fermented sausages containing alginate-microencapsulated *Lactobacillus reuteri*. *Int. J. Food Microbiol.* **2006**, *111*, 164–169. [CrossRef]
56. Wójciak, K.M.; Dolatowski, Z.J.; Kołożyn-Krajewska, D. Use of acid whey and probiotic strains to improve microbiological quality and sensory acceptance of organic fermented sausage. *J. Food Process Preserv.* **2015**, *39*, 539–547. [CrossRef]
57. Cocconcelli, P.S. Starter cultures: Bacteria. In *Handbook of Fermented Meat and Poultry*; Toldrá, F., Ed.; Blackwell Publishers: Hoboken, NJ, USA, 2007; pp. 137–145.
58. Hernández-Macias, S.; Ferrer-Bustins, N.; Comas-Basté, O.; Jofré, A.; Latorre-Moratalla, M.; Bover-Cid, S.; Vidal-Carou, M.D.C. Revalorization of cava lees to improve the safety of fermented sausages. *Foods* **2021**, *10*, 1916. [CrossRef]
59. Rocchetti, G.; Falasconi, I.; Dallolio, G.; Lorenzo, J.M.; Lucini, L.; Rebecchi, A. Impact of hurdle technologies and low temperatures during ripening on the production of nitrate-free pork salami: A microbiological and metabolomic comparison. *LWT* **2021**, *141*, 110939. [CrossRef]
60. Charmpi, C.; Van der Veken, D.; Van Reckem, E.; De Vuyst, L.; Leroy, F. Raw meat quality and salt levels affect the bacterial species diversity and community dynamics during the fermentation of pork mince. *Food Microbiol.* **2020**, *89*, 103434. [CrossRef] [PubMed]
61. Palman, Y.; De Leo, R.; Pulvirenti, A.; Green, S.J.; Hayouka, Z. Antimicrobial peptide cocktail activity in minced turkey meat. *Food Microbiol.* **2020**, *92*, 103580. [CrossRef] [PubMed]