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Use of Indigenous Lactic Acid Bacteria for Industrial Fermented Sausage Production: Microbiological, Chemico-Physical and Sensory Features and Biogenic Amine Content

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Abstract: The use of starter cultures in the meat industry is common, even if the number of available commercial cultures is limited, inducing product standardisation and microbial diversity reduction. On the other hand, some artisanal products relying on spontaneous fermentation can represent a source of isolation of new interesting strains. In this work, four LAB strains derived from Mediterranean spontaneously fermented sausages were tested as new starter cultures for the industrial production of fermented sausages, in comparison to a commercial starter culture. The products obtained were analysed for physico-chemical parameters, microbiota, biogenic amines and aroma profile. A consumer test was also performed to evaluate product acceptability. The strains induced different acidification kinetics. LAB counts showed high persistence when *Latilactobacillus curvatus* HNS55 was used as the starter culture, while the addition of *Companilactobacillus alimentarius* CB22 resulted in a high concentration of enterococci (6 log CFU/g), 2 log higher than in other samples. Tyramine was detected at concentrations of 150–200 mg/kg, except for in the sample produced with *Lactiplantibacillus plantarum* BPF2 (60 mg/kg). Differences were observed in the aroma profile, with a high amount of 2-butanone found in the samples obtained with *Comp. alimentarius* CB22. These latter sausages also showed the lowest score in terms of acceptability. This study allowed us to select new LAB strains for fermented sausage starter cultures, increasing the product diversification.

Keywords: fermented sausages; starter culture selection; tyramine; volatile organic compounds

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1. Introduction

Fermented sausages are generally produced using minced lean and fat meat combined with salt, spices and additives (sugar, ascorbate, nitrates and nitrites). After stuffing into natural or synthetic casing, the meat mixture is subjected to fermentation and ripening processes, during which products achieve the final desired characteristics. For centuries, salt has been added as an important strategy for food preservation, as it lowers a_w. Moreover, its presence contributes to the textural properties of the product, allowing sol–gel system formation, as well as the development of its aroma profile [1]. However, food industries have started to reduce salt concentrations due to health concerns. This has lead to research into different strategies, such as the use of selected starter and bioprotective cultures, which are able to strictly guide the controlled fermentation process.

Microorganisms involved in meat fermentation exert an important role in both safety and technological features [2,3]. In this context, since the 1960s, the use of starter culture mixtures of lactic acid bacteria (LAB) (containing *Latilactobacillus sakei*, *Lat. curvatus*, *Lactiplantibacillus plantarum*, *Pediococcus acidilactici* and *P. pentosaceus*) and staphylococci (*Staphylococcus xylosus* and *Staph. carnosus*) has been introduced in the fermented meat

industry [4,5]. The addition of LAB aids fermentation through a rapid drop in pH in the first days of production, with a consequent reduction in the potential growth of pathogen and spoilage microorganisms. The use of different LAB species can determine different acidification kinetics and also affect technological parameters as well as sensory attributes [6]. Moreover, some studies have also investigated the addition of probiotic LAB strains as starter cultures to enhance the functional properties of the final product, thus generating health-related benefits for consumers [7]. On the other hand, the use of staphylococci promotes the formation of a typical aroma profile, avoiding colour defects, during the following ripening phase [8].

However, the availability of commercial starter cultures is limited to a small number of strains that can guarantee fermented sausage safety but, on the other hand, can also cause a reduction in their natural microbial biodiversity. These requirements may result in a standardisation of the products, with a loss in their recognisability and typicity linked to local cultural heritage [9]. In fact, despite the large diffusion of the use of starter cultures in the meat industry, the Mediterranean area is characterised by a wide array of artisanal fermented sausages, still obtained through spontaneous fermentation [10]. For this reason, in recent years, an increasing interest has been focused on the isolation of new autochthonous strains that can potentially be used as starter cultures to obtain fermented meat products characterised by peculiar sensory properties [11–13]. Moreover, new LAB strains have been selected, not only based on their technological properties but also for their antimicrobial potential. Indeed, they can be applied both as starter and bioprotective cultures, which are able to prevent the growth of undesirable microorganisms (pathogenic and spoilage microflora) through the production of specific metabolites with antimicrobial activity (i.e., bacteriocins), ensuring high-quality products with improved safety and prolonged shelf-life [14,15]. In this context, artisanal spontaneously fermented sausages can represent a relatively unexplored reservoir of microbial biodiversity, within which it is possible to find new microorganisms with relevant biotechnological properties, both for fermentation and bioprotection purposes.

The aim of this work was to investigate the potential of selected LAB strains, deriving from Mediterranean spontaneously fermented sausages [10], as new starter cultures, valorising microbial biodiversity and product differentiation while maintaining their safety features. In particular, strains belonging to different LAB species occurring in fermented sausages (namely *Latilactobacillus sakei*, *Latilactobacillus curvatus*, *Lactiplantibacillus paraplantarum* and *Companilactobacillus alimentarius*) were added as starter cultures during the production of Italian traditional sausages in an industrial plant. It is noteworthy that, to our knowledge, this is the first study in which a strain of *Comp. alimentarius*, a species not frequently isolated from fermented sausages (and found mainly in Spanish products), was tested as a starter culture. A control sample, obtained with the addition of the usual commercial starter culture, was also produced. The different fermented sausages were monitored during fermentation and ripening to evaluate the changes in physico-chemical parameters (pH, a_w and weight loss), the principal microbial groups and the formation of aroma profile. Moreover, biogenic amine (BA) content in the samples at the end of ripening was assessed. Finally, a consumer test was performed in order to evaluate the acceptability of the final products.

2. Materials and Methods

2.1. LAB Strains Used as Starter Cultures

The strains to be tested as starter cultures are listed in Table 1 and were chosen based on previous research, in which LAB were isolated from spontaneously fermented sausages collected in the Mediterranean area [10]. Starting from about 150 different biotypes, LAB strains were characterised by their safety, i.e., the absence of antibiotic resistance and the inability to produce biogenic amines [16–18]. The resulting safe LAB were further studied for some technological features [18,19], and the most promising strains, in terms of growth performances in different in vitro conditions (increasing NaCl concentration,

low temperature), were selected for this trial in a real system. Moreover, the strain Lpl. paraplantarum BPF2 was also chosen for its bioprotective features against Listeria monocytogenes, demonstrated both in vitro and in vivo (in Spanish fermented sausages), making this strain another promising candidate to increase product safety [17,20].

Table 1. Fermented sausage samples and LAB strains used as starters.

Sample Name	LAB Strain	Origin
Control	*Latilactobacillus sakei* TMX	Commercial starter
BPF2	*Lactiplantibacillus paraplantarum* BPF2	Spanish fermented sausage
HNS55	*Latilactobacillus curvatus* HNS55	Croatian fermented sausage
CB22	*Companilactobacillus alimentarius* CB22	Spanish fermented sausage
HZK39	*Latilactobacillus sakei* HZK39	Croatian fermented sausage

2.2. Fermented Sausage Production

The fermented sausages analysed in this study were produced by C.l.a.i. Soc. Coop. (Imola, Italy). The meat batter (40 kg) consisted of pork meat (about 83% lean meat from shoulders and 17% fat from throat) combined with salt (2.4% *w*/*w*), dextrose (0.2% *w*/*w*), black pepper (0.1% *w*/*w*), red garlic (0.03% *w*/*w*), ascorbic acid (0.08% *w*/*w*), KNO_3 (0.015% *w*/*w*) and $NaNO_2$ (0.02% *w*/*w*). The mixture was then divided into five different batches (approx. 10 kg each), inoculated with different LAB strains as starter cultures at an initial concentration of 7.5 log CFU/g (Table 1). Their performances were compared with those of a commercial starter culture traditionally used for the same product (Tera Meat® TMX-M, Teracell S.r.l., Cremona, Italy) inoculated in the same conditions.

Moreover, the same strain of *Staphylococcus xylosus*, isolated from a commercial starter culture, was inoculated in each batch at a concentration of 5 log CFU/g.

Aliquots of meat batter (approx. 500 g) were stuffed into a natural (beef middle) casing, with a diameter of about 6.5 cm and a length of about 30 cm. The fermentation and ripening process was carried out at a temperature ranging from 20 to 10 °C and at a relative humidity (RH) between 90 and 65% for 45 days. The obtained samples were evaluated during all the production process. The analysis was performed in triplicate (three independent sausages for each batch) immediately after the production and 5, 11, 20 and 45 days after the production.

2.3. Physico-Chemical Parameters

The samples were monitored during fermentation and ripening regarding physico-chemical parameters as reported for similar fermented meat products [21]. pH was measured using a pH-meter HD2105.2 (Delta Ohm, Padova, Italy) by weighing 10 g of sample, adding an equal amount of distilled water and then homogenising in a Lab Blender Stomacher (Seward Medical, London, UK) for 2 min. a_w values were measured with an Aqualab CX3-TE (Labo-Scientifica, Parma, Italy) in two different parts of the sausages: the external section (1.5 cm immediately below the casing) and internal section (centre of the sausages). Moreover, the samples were weighed to calculate the mean weight loss (%) with respect to the initial weight. All the analyses were performed in triplicate.

2.4. Microbial Counts

For each sample, the principal microbial groups were detected through plate counting. Briefly, after aseptically removing the casing, a slice of approx. 10 g of sample was diluted with 90 mL of 0.9% (*w*/*v*) NaCl sterile solution in a sterile bag and homogenised in a Lab Blender Stomacher (Seward Medical, London, UK) for 2 min. Subsequently, decimal dilutions were prepared and plated onto selective media (Oxoid, Basingstoke, UK): lactobacilli, coagulase negative cocci and yeasts and moulds were enumerated onto de Man–Rogosa–Sharpe Agar (MRS) supplemented with cycloheximide (200 mg/L), Mannitol Salt Agar (MSA) and Sabouraud Dextrose Agar (SAB) supplemented with chloramphenicol

col (200 mg/L), respectively, after 48 h of incubation at 30 °C. Enterococci were detected on Slanetz and Bartley medium (SL) incubated for 24 h at 42 °C; *Enterobacteriaceae* were detected on Violet Red Bile Glucose Agar (VRBGA) after incubation at 37 °C for 24 h. All media were provided by Oxoid (Basingstoke, UK). All these analyses were performed in triplicate (three different sausages).

2.5. Biogenic Amine Quantification

Biogenic amine (BA) content in the samples at the end of ripening was assessed through extraction and HPLC analysis. Briefly, 10 g of each sample was treated with a trichloroacetic acid (TCA) 5% w/v solution and the extracts were then subjected to dansyl chloride derivatisation (Sigma Aldrich, Gallarate, Italy), according to the method of Martuscelli et al. [22]. The samples obtained were injected into an HPLC Agilent Technologies 1260 Infinity with an automatic injector (G1329B ALS 1260, loop of 20 µL), equipped with a UV detector (G1314F VWD 1260) set at 254 nm. A C18 Waters Spherisorb ODS-2 (150 × 4.6 mm, 3 µm) column was used for the chromatographic separation, with a flow rate of 0.6 mL/min and the following gradient elution: acetonitrile/water 35:65 for 1 min, acetonitrile/water 55:45 reached in 5 min, acetonitrile/water 60:40 reached in 10 min, acetonitrile/water 90:10 reached in 8 min and maintained for 11 min, then initial acetonitrile/water ratio (35:65) restored in 5 min and maintained for 5 min.

The amounts of amines were expressed as mg/kg with reference to a calibration curve obtained through aqueous dansyl-chloride-derivatised amine standards of concentrations ranging from 10 to 200 mg/L (Sigma-Aldrich, Milano, Italy). The detection limit for all the amines was 3 mg/kg of the sample under the adopted conditions. All the analyses were performed in triplicate.

2.6. Aroma Profile

Volatile organic compounds of products were detected through gas chromatography–mass spectrometry coupled with the solid-phase microextraction technique (GC-MS-SPME) after 20 days and at the end of ripening. An Agilent Hewlett–Packard 7890A GC gas chromatograph coupled with an MS 5975 MSD detector (Hewlett–Packard, Geneva, Switzerland) was used. A known amount of 4-methyl-2-pentanol (Sigma-Aldrich, Steinheim, Germany) was added as an internal standard to 3 g of sample placed in a 10 mL sterilised vial, sealed by PTFE/silicon septa. The sample was heated for 10 min at 45 °C and then a fused silica SPME fibre covered with 75 µm carboxen/polydimethylsiloxane (CAR/PDMS StableFlex) (Supelco, Steinheim, Germany) was introduced into the headspace for 40 min. Adsorbed molecules were desorbed in the gas chromatograph for 10 min. The chromatographic conditions were those reported by Montanari et al. [23]. Volatile peak identification was carried out using the Agilent Hewlett–Packard NIST 2011 mass spectral library (Gaithersburg, MD, USA) [24]. All the analyses were performed in triplicate.

2.7. Consumer Test

To evaluate the sample acceptability, the final products were subjected to a consumer test with 40 untrained panellists, who were distributed into 55% female and 45% male. The age of consumers involved was between 21 and 70 years, distributed as follows: 21–30 years old (30%), 31–40 years old (25%), 41–50 years old (20%), 51–60 years old (12.5%) and 61–70 years old (12.5%). The samples were prepared as reported by García-López et al. [20] and analysed regarding four selected descriptors (colour intensity, odour intensity, aroma intensity and acceptability), using a 5-point hedonic scale (1 = do not like at all, 5 = like very much). A single session was organised, in which the different sausages were evaluated in a randomised order by the panellists.

2.8. Statistical Analysis

The data collected were statistically analysed through a one-way ANOVA procedure and significant differences in each sample at each sampling time were evaluated with the

LDS test ($p < 0.05$) and highlighted with different letters or asterisks. The influence of starter cultures on the product aroma profile at the end of ripening was also explored by principal component analysis (PCA). The data of the consumer test were analysed using the Mann–Whitney test to determine differences among the sausages obtained with different starter cultures ($p < 0.05$). All statistical analyses were carried out with Statistica 8.1 (StatSoft Italy srl, Vigonza, Italy).

3. Results and Discussion

3.1. Evolution of Physico-Chemical Parameters (pH, a_w and Weight Loss)

During fermentation and ripening, pH, a_w and weight losses were monitored. The results showed, in general, a pH decrease from approx. 5.85 to 5.35, with significantly different kinetics depending on the strains used as starter cultures (Figure 1). The behaviour of *Lat. curvatus* HNS55 and *Comp. alimentarius* CB22 after 5 days was similar to the control. Otherwise, in the sample obtained with *Lpl. paraplantarum* BPF2 and *Lat. sakei* HZK39, the acidification kinetics appeared significantly slower. In fact, the pH reached values of 5.40 at the end of fermentation, while the lowest pH (5.32) was measured after 11 days. After this phase, pH rapidly increased, especially in the HNS55 samples, which showed values significantly higher than the other products because of the proliferation of other microbial species besides LAB. In particular, this increase was due to mould growth, which was scarcely present at the beginning of the process but then proliferated, reaching values of about 5 log cfu/g after 10 days. It is known that this microbial group is able to degrade the lactic acid produced by LAB, thus increasing fermented sausage pH [25]. The final pH ranged from 5.47 to 5.57, depending on the inoculated strain.

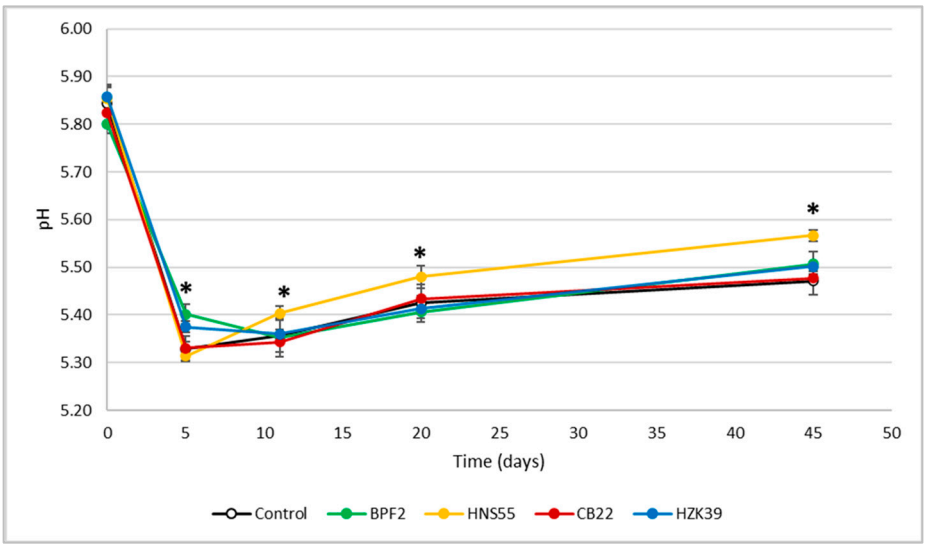

Figure 1. Evolution of pH during the production of the Italian fermented sausages (salami) obtained with the different LAB used as starter cultures (black: commercial starter; green: *Lactiplantibacillus paraplantarum* BPF2; yellow: *Latilactobacillus curvatus* HNS55; red: *Companilactobacillus alimentarius* CB22; blue: *Latilactobacillus sakei* HZK39). At each time, the presence of an asterisk indicates significant differences among the samples.

Regarding a_w, measurements were carried out in the inner and outer sections. The results of the inner part of the products showed similar kinetics among samples until 20 days after production (Figure 2a). During the following ripening period, differences among the samples, according to ANOVA, were observed. The HZK39 sample was characterised by lower final values with respect to the other products (0.918 vs. 0.922–0.928, respectively). The same considerations can be applied to the data about the outer part (Figure 2b), in which the HZK39 sample showed, again, a lower a_w value (0.901). The values observed are in agreement with similar Mediterranean products [24,25] and, together with the pH

drop (Figure 1), can be considered hurdles that can inhibit the most common spoilage (e.g., pseudomonads) or pathogenic bacteria, such as Salmonella and *Listeria monocytogenes* [25].

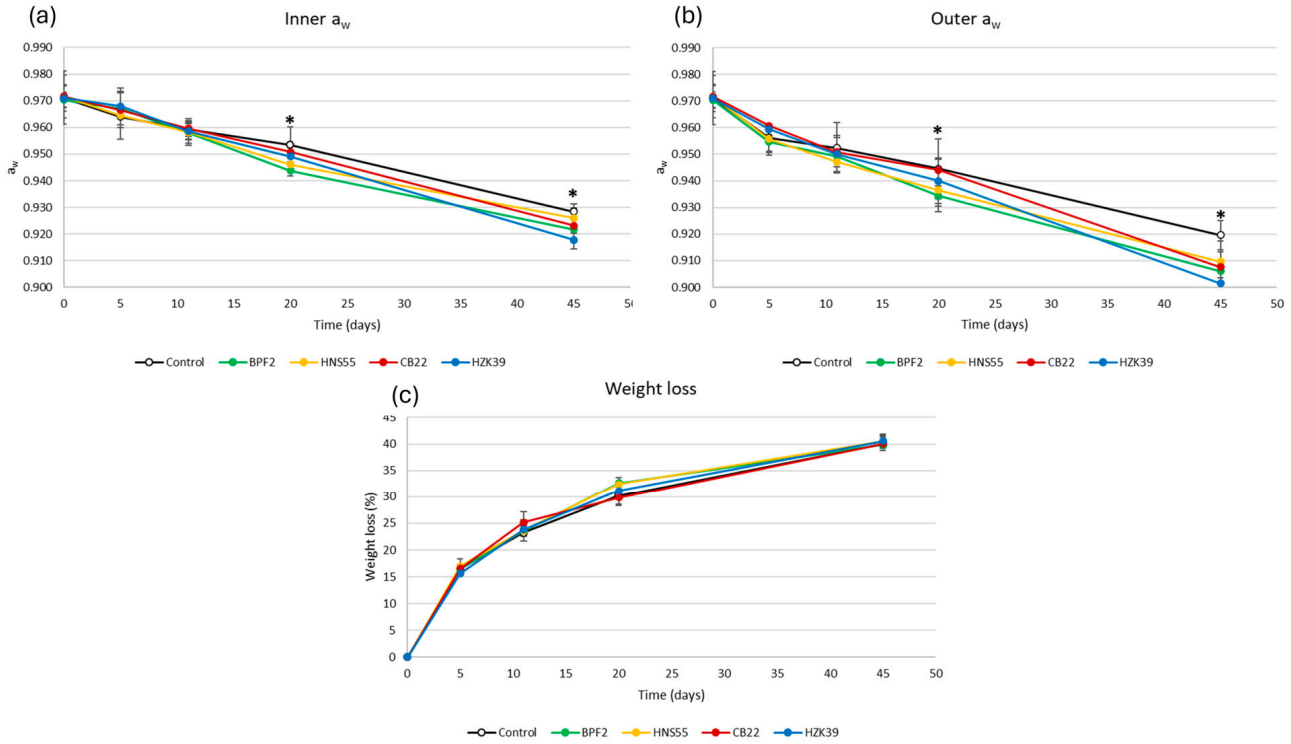

Figure 2. Inner a_w (**a**), outer a_w (**b**) and weight loss (**c**) monitored during fermentation and ripening of the fermented sausages obtained with the different LAB as starter cultures (black: commercial starter; green: *Lactiplantibacillus paraplantarum* BPF2; yellow: *Latilactobacillus curvatus* HNS55; red: *Companilactobacillus alimentarius* CB22; blue: *Latilactobacillus sakei* HZK39). At each time, the presence of an asterisk indicates significant differences among the samples.

The weight losses of the fermented sausages obtained with different starter cultures showed similar behaviour during the whole process, regardless of the used LAB strain, with final values of about 40% at the end of production (Figure 2c).

3.2. Microbial Characterisation

The principal microbial groups associated with fermented sausages were evaluated during fermentation and ripening, and the results are summarised in Table 2. Lactobacilli, including the starter cultures added at a concentration of about 7.5 log CFU/g, were characterised by a rapid growth in the control and the sample inoculated with *Lat. curvatus* HNS55, reaching values of about 9 log CFU/g after 5 days of production. Conversely, the growth kinetics in the first 5 days were significantly slower in the other samples and this difference was confirmed at 11 days after production. Concerning the evolution of LAB counts during ripening, the cell load was maintained in the samples obtained with the strain HNS55, while in the control, a progressive reduction was observed, reaching a final value of 7.62 log CFU/g, similar to the sausages in which HZK39 was used as a starter culture.

The strain *Lat. curvatus* HNS55 had already shown good technological features in terms of growth performances in laboratory conditions simulating stresses occurring during fermented sausage production such as increasing NaCl concentration and low temperature [16]. These features could explain its high persistence throughout the whole ripening process. The acidification behaviour reflected this massive LAB proliferation (Figure 1). The other tested strains showed slower growth kinetics, reaching concentrations of 8.0–8.4 log CFU/g at the end of fermentation. They were characterised, with respect to

the control, by a higher persistence during the whole ripening phase, except for the sample inoculated with *Lat. sakei* HZK39 (7.69 log CFU/g 45 days after production).

Table 2. Microbial counts (log CFU/g) detected in the traditional Italian fermented sausages obtained with the different LAB as starter cultures (control with commercial starter culture; *Lactiplantibacillus paraplantarum* BPF2; *Latilactobacillus curvatus* HNS55; *Companilactobacillus alimentarius* CB22; *Latilactobacillus sakei* HZK39) during fermentation and ripening. For each microbial group at each sampling time, differences among the samples (by row) according to ANOVA are indicated by the presence of different letters.

Microbial Group	Time (Days)	Control	BPF2	HNS55	CB22	HZK39
Lactobacilli	Meat batter			4.23 ± 0.63		
	0	7.79 ^{a}	7.72 ^{a}	7.60 ^{a}	7.41 ^{a}	7.67 ^{a}
	5	9.10 ^{a}	8.12 ^{bc}	9.07 ^{a}	8.37 ^{b}	8.01 ^{c}
	11	9.17 ^{a}	8.39 ^{b}	9.10 ^{a}	8.60 ^{c}	8.21 ^{b}
	20	8.73 ^{a}	8.43 ^{b}	8.97 ^{a}	8.45 ^{b}	8.06 ^{c}
	45	7.62 ^{a}	8.50 ^{b}	9.06 ^{c}	9.02 ^{c}	7.69 ^{a}
Staphylococci	Meat batter			3.57 ± 0.15		
	0	5.57 ^{a}	4.90 ^{b}	5.24 ^{a}	4.80 ^{b}	5.07 ^{b}
	5	5.50 ^{a}	4.70 ^{b}	5.20 ^{c}	4.95 ^{c}	5.01 ^{c}
	11	6.30 ^{a}	5.17 ^{b}	6.15 ^{a}	5.39 ^{b}	5.19 ^{b}
	20	6.00 ^{a}	5.82 ^{a}	6.21 ^{a}	5.48 ^{b}	5.27 ^{b}
	45	6.30 ^{a}	5.90 ^{b}	6.85 ^{c}	6.02 ^{b}	5.25 ^{d}
Enterobacteria	Meat batter			2.12 ± 0.07		
	0	2.01 ^{a}	2.04 ^{a}	1.98 ^{a}	2.01 ^{a}	2.00 ^{a}
	5	2.24 ^{a}	2.20 ^{a}	2.19 ^{a}	2.03 ^{a}	1.70 ^{a}
	11	1.92 ^{a}	1.87 ^{a}	1.87 ^{a}	2.02 ^{a}	1.72 ^{a}
	20	1.69 ^{a}	1.50 ^{a}	1.84 ^{a}	1.65 ^{a}	1.75 ^{a}
	45	2.02 ^{a}	1.24 ^{b}	2.10 ^{a}	2.42 ^{c}	1.59 ^{d}
Enterococci	Meat batter			2.09 ± 0.94		
	0	1.96 ^{a}	2.01 ^{a}	2.00 ^{a}	1.98 ^{a}	1.94 ^{a}
	5	2.47 ^{a}	2.14 ^{b}	2.69 ^{a}	4.44 ^{c}	2.82 ^{a}
	11	2.43 ^{a}	2.10 ^{a}	3.14 ^{b}	4.84 ^{c}	2.84 ^{b}
	20	2.29 ^{a}	1.93 ^{b}	4.33 ^{c}	5.56 ^{d}	3.51 ^{e}
	45	2.96 ^{a}	3.00 ^{a}	4.14 ^{b}	6.07 ^{c}	3.17 ^{a}
Yeasts	Meat batter			1.69 ± 0.30		
	0	2.10 ^{a}	2.05 ^{a}	2.03 ^{a}	2.09 ^{a}	2.10 ^{a}
	5	1.80 ^{a}	2.22 ^{a}	1.79 ^{a}	2.33 ^{a}	1.73 ^{a}
	11	3.22 ^{a}	3.12 ^{a}	3.30 ^{a}	2.38 ^{b}	2.91 ^{a}
	20	2.56 ^{ab}	2.90 ^{a}	3.06 ^{a}	2.39 ^{b}	3.00 ^{a}
	45	2.87 ^{a}	2.83 ^{a}	3.04 ^{a}	3.22 ^{a}	2.62 ^{a}
Moulds	Meat batter			<1 *		
	0	<1 ^{a}	<1 ^{a}	<1 ^{a}	<1 ^{a}	<1 ^{a}
	5	1.58 ^{a}	1.46 ^{a}	0.49 ^{b}	1.78 ^{ac}	2.14 ^{c}
	11	5.14 ^{a}	5.18 ^{a}	5.02 ^{a}	4.70 ^{b}	5.12 ^{a}
	20	4.44 ^{a}	5.11 ^{b}	4.70 ^{a}	4.62 ^{a}	4.61 ^{a}
	45	3.09 ^{a}	4.19 ^{b}	3.67 ^{c}	4.19 ^{b}	4.20 ^{b}

* below the detection limit (1 log CFU/g).

Regarding staphylococci, added in the meat batter as starter cultures (approx. 5 log CFU/g), their concentration was characterised by a lower increase during production. The final staphylococci cell load ranged between 5.25 and 6.85 log CFU/g with significant differences in relation to the tested strains. In particular, the control and HNS55 samples presented higher counts, i.e., 6.30 and 6.85 log CFU/g, respectively.

The concentration of enterobacteria detected in the meat batter was rather limited (approx. 2 log CFU/g) and their presence remained stable, except for in the BPF2 and HZK39 samples, in which a slight reduction was observed (0.4–0.8 log cycles), and in the case of CB22, which presented a significantly higher value in the final samples. The control of this microbial group, which can include both spoilage and pathogenic species (i.e., *Salmonella* and *Escherichia coli*), is crucial to assess the quality and safety of the final product. Moreover, some of them can produce biogenic amines, which can cause several adverse reactions in the consumers if accumulated in high amounts. However, the enterobacteria counts in the samples obtained through the inoculum of the tested strains were not alarming and in accordance with those found in other similar products [26].

Enterococci counts varied in relation to the inoculated LAB strain. In particular, the initial concentration of about 2 log CFU/g remained constant during fermented sausage productions in the control and in the BPF2 sample, with a final increase of 1 log cycle at the end of ripening. Conversely, in the other samples, the same microbial group increased significantly from the first phases, reaching values up to 6 log CFU/g in the products obtained with the strain *Comp. alimentarius* CB22. This level of enterococci is higher than that usually found in this kind of meat product [27,28], due also to the fact that this group needs to be monitored because it can raise safety concerns. In particular, besides their marked capability to produce biogenic amines, in particular tyramine, they showed the ability to acquire and transfer antibiotic resistance and the potential presence of virulence factors [29,30].

Finally, yeasts were characterised by similar behaviours in all the samples with a slight increase of about 1 log cycle during production (2.62–3.22 log CFU/g). Moulds, added onto the product surfaces to guide a proper drying process, showed similar growth kinetics, with a relevant increase in cell counts (up to about 5 log CFU/g) until the 11th day after production, followed by a progressive reduction to a concentration of about 2 log cycles during the remaining ripening period.

The LAB considered in these trials belonged to different species. The species *Lat. sakei* and *Lat. curvatus* are highly adapted to meat environments and are often part of commercial starter cultures because they are characterised by a high persistence in the sausages during ripening when the sugars added at the beginning are completely fermented [5,9]. From this perspective, it is noteworthy that in the sausages in which *Lat. sakei* strains were used (control and HZK39), the LAB counts at the end of ripening were the lowest. On the other hand, the raw material used for sausage manufacturing contains an autochthonous microbiota, which includes LAB. These microorganisms can multiply during ripening, even in the presence of starter cultures. It has been demonstrated that active LAB starter cultures can represent an important proportion of these bacteria, but other wild species can grow and affect (positively or negatively) the quality of the final product [20,21,27]. Concerning *Lpl. paraplantarum*, the strain BPF2, isolated from fermented sausage and characterised by the production of an effective anti-listeria bacteriocin [17], already demonstrated a good potential in driving sausage fermentation [20], even if, as in this case, the kinetics of LAB growth in the samples in which it was present appear to be slower compared with the other strains. Interestingly, *Comp. alimentarius* CB22 was not competitive against enterococci. This species, to our knowledge not yet used as a starter culture, is sporadically isolated from fermented sausages, and its major presence in this environment has been observed in Spanish products [10,31,32].

3.3. Biogenic Amine Determination

Biogenic amine content was analysed in the samples at the end of ripening. Histamine, putrescine, cadaverine and 2-phenylethylamine were below the detection limits (<3 mg/kg). Tyramine was the only biogenic amine present at concentrations ranging from 150 to 195 mg/kg in the samples obtained with the commercial starter and autochthonous HSN55, CB22 and HZK39 strains (Figure 3). The amounts of tyramine detected in the final products are not alarming and follow those reported for similar products [10,33].

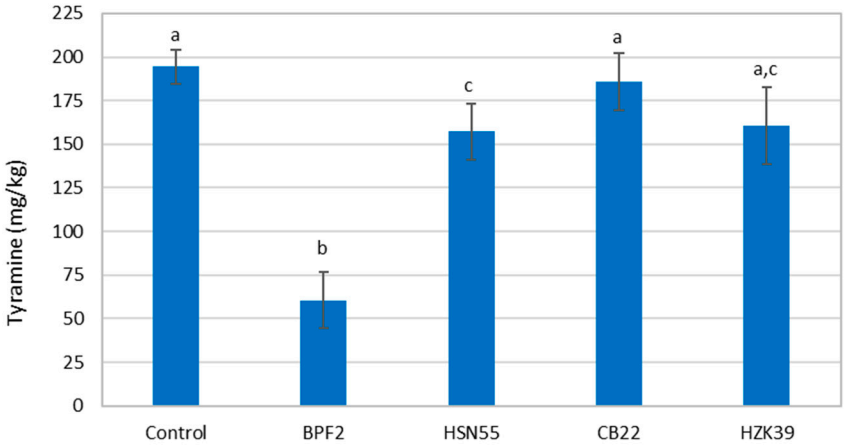

Figure 3. Tyramine content (expressed as mg/kg) in the fermented sausages obtained with the different LAB starter cultures (control with commercial starter culture; *Lactiplantibacillus paraplantarum* BPF2; *Latilactobacillus curvatus* HNS55; *Companilactobacillus alimentarius* CB22; *Latilactobacillus sakei* HZK39) at the end of ripening. The differences among the samples according to ANOVA are indicated by the presence of different letters.

These tyramine concentrations can be due to the presence of wild decarboxylase-positive strains in the raw materials and production environments, which are able to grow and compete with starter cultures and can affect the safety and quality of the final product [20,21,27]. On the other hand, *Lpl. paraplantarum* BPF2 was able to counteract the accumulation of tyramine, resulting in a final amount of about 60 mg/kg in these samples, which is significantly lower than the other tested strains (Figure 3). A similar behaviour of the strain BPF2 was also observed in salchichónes, a type of traditional Spanish fermented sausage, which was able to limit biogenic amine accumulation [20]. This phenomenon can be due to antagonistic activity against wild microflora, including tyraminogenic LAB.

3.4. Aroma Profile of Fermented Sausages

The fermented sausages after 20 days of ripening and at the end of production (45 days) were analysed using SPME-GC-MS to evaluate how the different LAB strains used as starter cultures could affect the aroma profile. The results are reported in Table 3.

Aldehydes, almost absent in the meat batter, increased during ripening, as expected. In particular, benzaldehyde and benzeneacetaldeyde, deriving from aromatic amino acid (tyrosine and phenylalanine) metabolism [34], at the end of ripening (45 days) were significantly higher (approx. 20% of the total volatile profile) in the samples obtained with the commercial starter culture (Control) and *Lpl. paraplantarum* BPF2, with respect to the others. The presence of aldehydes deriving from lipid oxidation was limited, since only small quantities of nonanal were detected. Usually, these compounds are found in larger amounts in Mediterranean fermented sausages; in particular, hexanal was detected in notable concentrations in similar Italian fermented sausages, in which other aldehydes were also accumulated [35]. The presence of remarkable contents of molecules belonging to this chemical class was also observed in Mediterranean spontaneously fermented sausages, in which the values were variable in relation to process parameters such as the presence of preservatives, the fat content, etc. [10,36]. The limited content of aliphatic aldehydes detected in the present study could be due to the low initial fat content (about 17%).

Regarding ketones, they generally increased during ripening, and in the final samples, significantly higher amounts were detected when *Comp. alimentarius* CB22 and *Lat. sakei* HZK39 were used as starter cultures (49.84% and 41.21%, respectively). This chemical group was mainly represented by acetone, 2-butanone and acetoin (3-hydroxy, 2-butanone). These molecules are generally always present in the volatile profile of fermented sausages [37,38], but their amounts and ratios can be significantly different, affecting the sensory properties of the final product. Indeed, high amounts of 2-butanone have been associated with olfactive

defects [27,35]. This molecule derives from LAB metabolism, starting from diacetyl through the action of a diol dehydratase [39]. At the end of ripening, its percentage was at least double in CB22 and HZK39 samples (25.16% and 19.81%, respectively) than in the other fermented sausages (less than 10% of the total volatile compounds detected).

Table 3. Aroma profile of fermented sausages obtained with different LAB starter cultures (control with commercial starter culture; *b*E-GC-MS after 20 days and at the end of ripening. Data are expressed as a percentage of the ratio between the peak area of each molecule and the peak area of the internal standard (4-methyl-2-pentanol). Results are the mean of three independent repetitions. For each molecule at each sampling time (20 or 45 days), differences among the samples according to ANOVA are indicated by the presence of different letters.

Compound (%)	20 Days					45 Days				
	Control	BPF2	HNS55	CB22	HZK39	Control	BPF2	HNS55	CB22	HZK39
Nonanal	0.68 ^{a}	0.36 ^{b}	0.40 ^{b}	0.39 ^{b}	0.32 ^{b}	0.93 ^{a}	0.74 ^{b}	0.87 ^{a}	1.07 ^{a}	1.10 ^{a}
Benzaldehyde	1.76 ^{a}	1.12 ^{b}	0.80 ^{c}	0.68 ^{c}	0.75 ^{c}	2.85 ^{a}	2.52 ^{a}	0.83 ^{b}	1.04 ^{b}	0.91 ^{b}
Benzeneacetaldehyde	10.40 ^{a}	3.84 ^{b}	1.45 ^{c}	0.92 ^{d}	1.04 ^{cd}	18.60 ^{a}	17.01 ^{a}	3.58 ^{b}	2.25 ^{c}	6.68 ^{d}
Aldehydes	12.83 ^{a}	5.31 ^{b}	2.65 ^{c}	1.99 ^{c}	2.11 ^{c}	22.38 ^{a}	20.27 ^{a}	5.28 ^{b}	4.37 ^{b}	8.70 ^{c}
Acetone	7.69 ^{a}	6.06 ^{b}	12.70 ^{c}	7.96 ^{a}	8.46 ^{a}	12.38 ^{a}	9.84 ^{b}	14.86 ^{c}	11.96 ^{a}	11.14 ^{a}
2-butanone	4.90 ^{a}	1.45 ^{b}	2.94 ^{c}	2.42 ^{c}	1.79 ^{b}	8.45 ^{a}	8.06 ^{a}	9.79 ^{b}	25.16	19.81 ^{c}
2-pentanone	2.23 ^{a}	2.50 ^{a}	3.58 ^{b}	4.16 ^{c}	2.63 ^{a}	2.77 ^{a}	4.85 ^{b}	3.55 ^{c}	3.83 ^{c}	2.48 ^{a}
Methyl isobutyl ketone	3.95 ^{a}	4.25 ^{a}	4.74 ^{b}	5.74 ^{c}	4.61 ^{b}	3.39 ^{a}	2.15 ^{b}	2.67 ^{b}	1.94 ^{b}	2.19 ^{b}
Acetoin	7.25 ^{a}	14.23 ^{b}	3.53 ^{c}	6.91 ^{a}	4.80 ^{c}	7.27 ^{a}	6.54 ^{a}	5.87 ^{b}	6.40 ^{a}	5.19 ^{b}
2-nonanone	0.93 ^{a}	0.04 ^{b}	0.19 ^{b}	0.27 ^{b}	0.03 ^{b}	1.04 ^{a}	0.00 ^{b}	0.32 ^{c}	0.54 ^{c}	0.40 ^{c}
Ketones	26.96 ^{a}	28.53 ^{a}	27.68 ^{a}	27.46 ^{a}	22.33 ^{b}	35.31 ^{a}	31.45 ^{b}	37.06 ^{a}	49.84 ^{c}	41.21 ^{d}
Isopropyl alcohol	6.82 ^{a}	0.58 ^{b}	0.73 ^{b}	0.72 ^{b}	0.65 ^{b}	0.92	1.12	1.19	1.02	1.21
Ethanol	8.03 ^{a}	5.21 ^{b}	4.49 ^{c}	3.97 ^{c}	2.98 ^{d}	1.77 ^{a}	3.86 ^{b}	3.42 ^{b}	2.21 ^{c}	2.86 ^{c}
2-hexanol	2.80 ^{a}	2.82 ^{a}	3.01 ^{a}	4.00 ^{b}	3.17 ^{a}	2.25 ^{a}	2.37 ^{a}	0.96 ^{b}	0.60 ^{b}	0.95 ^{b}
1-pentanol	0.67 ^{a}	0.29 ^{b}	0.42 ^{a}	0.55 ^{a}	0.57 ^{a}	0.35 ^{a}	1.52 ^{b}	0.59 ^{a}	0.92 ^{c}	0.53 ^{a}
3-buten-1-ol, 3-methyl-	0.41 ^{a}	0.62 ^{a}	0.66 ^{a}	0.75 ^{a}	0.55 ^{a}	0.30 ^{a}	0.26 ^{a}	0.39 ^{a}	0.26 ^{a}	0.30 ^{a}
2-buten-1-ol, 3-methyl-	0.71 ^{a}	0.54 ^{a}	0.89 ^{a}	0.77 ^{a}	0.56 ^{a}	0.48 ^{a}	0.37 ^{a}	0.42 ^{a}	0.26 ^{a}	0.24 ^{a}
1-octen-3-ol	0.71 ^{a}	0.35 ^{b}	0.37 ^{b}	0.62 ^{a}	0.37 ^{a}	0.28 ^{a}	0.74 ^{b}	0.40 ^{a}	0.78 ^{b}	0.40 ^{a}
Alcohols	20.15 ^{a}	10.40 ^{b}	10.56 ^{b}	11.37 ^{b}	8.85 ^{c}	6.35 ^{a}	10.25 ^{b}	7.38 ^{a}	6.04 ^{a}	6.49 ^{a}
Acetic acid	34.12 ^{a}	48.81 ^{b}	50.90 ^{b}	52.06 ^{b}	57.94 ^{c}	29.34 ^{a}	35.00 ^{a}	44.77 ^{b}	36.83 ^{a}	39.92 ^{ab}
Propanoic acid	0.31 ^{a}	0.41 ^{a}	0.77 ^{b}	0.52 ^{ab}	0.64 ^{b}	0.51	0.28	0.55	0.30	0.39
Butanoic acid	3.22 ^{a}	5.10 ^{b}	5.68 ^{b}	4.79 ^{c}	6.25 ^{d}	3.94 ^{a}	1.91 ^{b}	3.21 ^{a}	1.36 ^{c}	1.99 ^{b}
Hexanoic acid	0.69 ^{a}	0.96 ^{b}	1.16 ^{b}	1.06 ^{b}	1.11 ^{b}	0.69 ^{a}	0.35 ^{b}	0.81 ^{a}	0.60 ^{a}	0.58 ^{a}
Octanoic acid	1.72 ^{a}	0.48 ^{b}	0.60 ^{b}	0.75 ^{b}	0.77 ^{b}	1.49 ^{a}	0.49 ^{b}	0.94 ^{c}	0.65 ^{bc}	0.72 ^{bc}
Acids	40.06 ^{a}	55.76 ^{b}	59.11 ^{b}	59.18 ^{b}	66.71 ^{c}	35.97 ^{a}	38.03 ^{a}	50.28 ^{b}	39.75 ^{c}	43.61 ^{d}

Among alcohols, ethanol, isopropyl alcohol and 2-hexanol represented the principal compounds. A higher percentage of ethanol (about 8% of the total peak area) was detected in the control samples 20 days after production; then, its amount decreased in all the samples, reaching values between 1.77% and 3.86% at the end of ripening. Usually, ethanol is the main alcohol detected in Italian fermented sausages [23,40] where its concentrations are lower with respect to other Mediterranean fermented sausages, such as Spanish or Croatian ones [10]. Its presence can be attributed to several pathways and can result from the LAB metabolism of lactate [41].

Finally, acids were mainly characterised by the presence of acetic acid, as expected, resulting, together with ethanol, from LAB metabolism [41]. The percentage of this molecule was significantly lower in the control after 20 days of ripening with respect to the other samples (approx. 34% vs. 48–57%). This trend was observed also at the end of ripening, even if the differences were reduced.

To better highlight the difference between the aroma profile of samples, data were also elaborated through principal component analysis (PCA), which showed a good separation of the samples in relation to the ripening time, as expected, on the basis of Factor 1 (52.02%) and Factor 2 (26.57%) (Figure 4). It is worth noting that the control samples after 20 days and at the end of ripening (45 days) were located in the same quadrant of the graph. Samples obtained with autochthonous LAB strains are grouped in the second quadrants after 20 days of ripening, while they are in the right part of the graph at the end of ripening, separated from the other samples based on Factor 1.

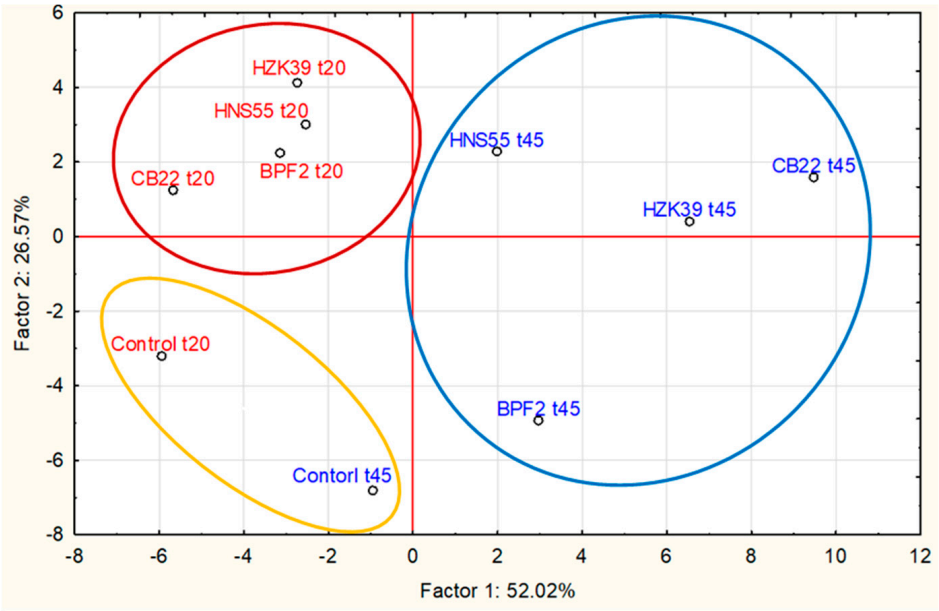

Figure 4. Principal component analysis (PCA) of the aroma profile of Italian fermented sausages obtained with different LAB strains as starter cultures (control with commercial starter culture; *Lactiplantibacillus paraplantarum* BPF2; *Latilactobacillus curvatus* HNS55; *Companilactobacillus alimentarius* CB22; *Latilactobacillus sakei* HZK39) after 20 days and at the end of ripening.

In Table 4, the factor coordinates of the most significant variables for Factor 1 and Factor 2 (representing 78.59% of the total variability) are also reported.

Table 4. Factor coordinates of the variables for the first two factors (representing 78.59% of the total variability), based on correlations.

Compound	Factor Coordinates of the Variables, Based on Correlations	
	Factor 1	**Factor 2**
Nonanal	0.1709	0.0474
Benzaldehyde	0.1108	−0.3169
Benzeneacetaldehyde	0.6580	−0.0887
Acetone	1.5160	−0.2415
2-butanone	4.2191	−0.1024
2-pentanone	0.4112	−0.0984
Methyl isobutyl ketone	−0.1700	−0.0860
Acetoin	0.3177	0.0267
2-nonanone	0.0333	−0.0016
Isopropyl alcohol	−0.0828	−0.0317
Ethanol	−0.0913	1.7874
2-hexanol	−0.2204	0.0148
1-pentanol	0.1322	0.1523
3-buten-1-ol, 3-methyl-	−0.0161	0.0509

Table 4. *Cont.*

Compound	Factor Coordinates of the Variables, Based on Correlations	
	Factor 1	**Factor 2**
2-buten-1-ol, 3-methyl-	−0.0330	−0.1103
1-octen-3-ol	0.0750	0.0474
Acetic acid	2.3249	−0.3169
Propanoic acid	0.0093	−0.0887
Butanoic acid	−0.2668	−0.2415
Hexanoic acid	−0.0017	−0.1024
Octanoic acid	0.0137	−0.0984

3.5. Sensory Analysis

Sensory analysis of the ripened samples was carried out by a consumer test with 40 untrained panellists considering four descriptors (colour intensity, odour intensity, aroma intensity and acceptability), in order to evaluate the acceptability of the obtained products. The mean score values for each attribute are reported in Figure 5. The BPF2 sample presented values similar to the control and was characterised by higher rates in terms of odour and aroma intensity. It was more appreciated than the control sample, together with the HZK39 and HNS55 samples, even if the latter was characterised by a low score regarding the other descriptors. Finally, the sample obtained with *Comp. alimentarius* CB22 as the starter culture was, in general, not appreciated; indeed, it showed the lowest score values for most of the tested descriptors and was judged the worst in terms of acceptability. This can be related to the high concentrations of molecules potentially responsible for spoilage phenomena such as 2-butanone [27].

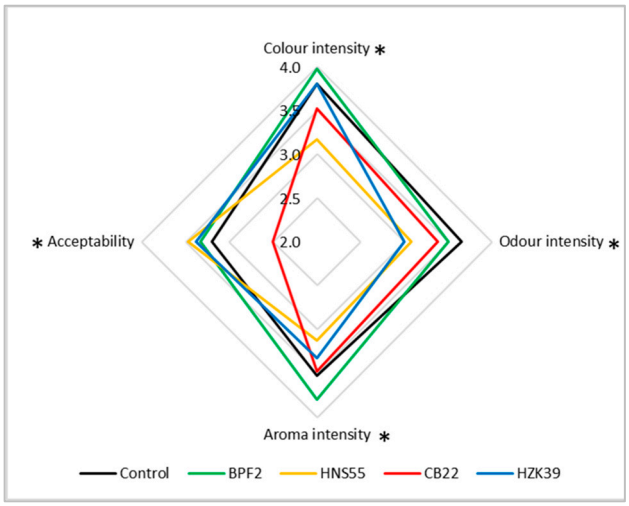

Figure 5. Spider plot reporting the sensory data attributed to fermented sausages obtained with different LAB starter cultures at the end of ripening (black: commercial starter; green: *Lactiplantibacillus paraplantarum* BPF2; yellow: *Latilactobacillus curvatus* HNS55; red: *Companilactobacillus alimentarius* CB22; blue: *Latilactobacillus sakei* HZK39). For each attribute, the presence of an asterisk indicates significant differences among the samples.

4. Conclusions

The technological parameters of the fermented sausages obtained with different starter cultures presented differences depending on the LAB strain used. In particular, the pH decrease was faster in the sample with *Lat. curvatus* HNS55, as well as in the control, with respect to the others. However, at the end of ripening, after the deacidification process, the control sample showed a lower pH value (5.47), while the HNS55 sample reached a value of 5.57. As expected, LAB were the main microbial group, since the different starter cultures were inoculated at a concentration of about 7.5 log CFU/g to guide the

fermentation. LAB behaviour in the fermented sausages obtained with *Lat. curvatus* HNS55 was similar to the control, probably because of the high adaption of this species to the environment (cell counts of about 9 log CFU/g 5 days after production), but differently from the control, a higher persistence during ripening was observed. The samples inoculated with *Comp. alimentarius* CB22, a species rarely isolated from meat products and typical of a specific ecological niche like Andalusian Spanish sausages, were characterised by high levels of enterococci, suggesting a low competitiveness of this strain against wild microflora. Conversely, *Lpl. paraplantarum* BPF2 seems to exert an antagonist activity against autochthonous microorganisms, reducing enterococci and enterobacteria counts, as well as limiting the content of tyramine, the only biogenic amine detected in these products. The use of different starter cultures also affected the aroma profile of products, mainly in terms of ketones (2-butanone, acetone and acetoin), ethanol and acetic acid, and these differences were reflected in the sensory profile of the products and were also perceived by panellists during the consumer test.

This trial, performed at the industrial level, demonstrated the ability of the tested strains to grow in this meat environment, with the strain *Lat. curvatus* HNS55 characterised by performances similar to the control (obtained with a commercial starter) but higher persistence over time. Moreover, between the tested strains, *Lpl. paraplantarum* BPF2 resulted in the most promising starter cultures due to its ability to reduce the accumulation of tyramine in the final product and its organoleptic impact on the sausages' aroma profile.

Therefore, this study allowed us to select LAB strains that could be potentially employed as new starter cultures in traditional fermented sausages, increasing product diversification.

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