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Running title: **Diameter and starter effects on fermented sausage characteristics**

ABSTRACT

Four batches of Milano-type dry fermented sausages were industrially produced to evaluate the effects of two different lactic acid bacteria starter cultures (*Lactobacillus sakei* and *Pediococcus pentosaceus*) and diameter on physico-chemical, microbiological and aroma characteristics during processing and in the final products. Values of a_w and pH were similar in the final sausages. Lactic acid bacteria counts were high and slightly influenced by the diameter, while higher concentrations of staphylococci were found in small sausages. The diameter had a strong influence on the formation of volatile organic compounds, as revealed by PCA analysis. The main differences regarded ketones (acetone, 2-butanone, 3-hydroxy-2-butanone and 2,3-butandione) and aldehydes (mainly hexanal). The influence of starter cultures was less evident, also due to the presence of indigenous *L. sakei* in the sausages inoculated with pediococci. Nevertheless, the choice of *L. sakei* or *P. pentosaceus* as starter culture showed a direct effect on some of the main process parameters (fermentation and acidification rate) and generation of volatile compounds. The results evidenced the need to optimize the process to increase starter culture performances.

Keywords: fermented meat, sausage size, aroma profile, starter cultures, ripening

1. Introduction

In Europe, the production of cured meats is widely diffused. In this framework, the production of fermented sausages has a great economic and cultural importance (Leroy et al., 2013). These products are various and the great differences could be related to several factors, such as raw material, mincing degree of meat batter, ingredients (salt concentration, nitrate/nitrite, spices and herbs, other additives), size (diameter and weight), type of casing and ripening conditions (temperature, relative humidity, use of moulds and/or smoke) (Leroy et al., 2013; Toldrá, 2006).

Recently, the pressure raised by new safety aspects (*i.e.* emerging pathogens and salt reduction) and by a general improvement of process efficiency (Leroy et al., 2013) determined the introduction of innovative processes in the fermented meat industries and stressed the need for the control of the microflora of the processing environments (Talon et al., 2007). In this period, the application of starter cultures, together with rigorous temperature and relative humidity conditions during ripening, are among the main tools adopted by industry to improve the quality and safety of its products (Bassi et al., 2015). By contrast, this could determine a standardization of the product characteristics, even if the wild ripening microbiota continues to influence the final features of sausages and, in particular, the flavor formation (Ravyts et al., 2012).

All these factors contribute to the formation of the "typical" characteristics, which are associated to each sausage typology. These characteristics concern physico-chemical (a_w and salt content, pH, acid content), textural (hardness, chewiness, moisture) and sensorial (aroma profile) features. The diameter of the sausages plays a relevant role in the formation of the desired characteristics. In fact, it affects many important events during ripening, in first instance the removal of water and the presence of oxygen inside the sausage, which, in turn, influence the biochemical activities of this crucial period (Anastasio et al., 2010; Incze, 2010). Volatile compounds responsible for the aroma profile are formed through the metabolism of lipids, proteins and end-products of the lactic fermentation (Carballo, 2012; Leroy et al., 2013;

Ordóñez et al., 1999). During maturation, these compounds are transformed into aroma molecules by the activity of enzymes, which can be endogenous or produced by microorganisms. Generally, the metabolism of lactic acid (derived from initial sugar fermentation) leads to the production of molecules, such as acetic and formic acids, ethanol, diacetyl and acetoin (von Wright and Axelsson, 2011). The products with major impact originated from the metabolism of amino acids, deriving from protein hydrolysis, are branched aldehydes and the relative acids and alcohols. The free fatty acids, deriving from lipids, can be converted into carbonyl compounds, such as aldehydes and methylketones, but also hydrocarbons and alcohols (Carballo, 2012; Ordóñez et al., 1999). These processes determine the characteristic product aroma profile and depend on complex interactions among several factors (Freiding et al., 2011; Incze, 2010; Leroy et al., 2013; Toldrá, 2006). Among these factors, the dimension of the fermented sausages and the use of starter cultures, consisting in lactic acid bacteria (LAB), staphylococci and moulds, play a key role.

Recently, the possible influence of diameter on the main characteristics and aroma profile in different Italian commercial fermented sausage typologies has been highlighted through a linear discriminant analysis (Montanari et al., 2016; Tabanelli et al., 2015). However, these products differed for several aspects (raw materials, mincing degree, process and ripening conditions) and only the ripened product were considered. Following the results obtained in these preliminary investigations, this study was aimed to the evaluation of the effect of the diameter of casing on the microbial population, some physicochemical parameters and the development of the aroma profile of sausages produced in an industrial environment. The same meat mixture were divided into two aliquots and inoculated with two different starter cultures. Each aliquot was stuffed in two synthetic casing having different diameter and length. The behavior of the microbial community was monitored during manufacture by culture-dependent and culture-independent methods and samples for aroma profile characterization were taken during fermentation and ripening.

2. Materials and methods

2.1. Sausages manufacture

The sausages used in this study were Milano-type dry fermented sausages manufactured in C.l.a.i. Soc. Coop. (Imola, Italy) using not frozen pork meat. Milano-type sausage were produced according to the diagram reported in Fig. 1. The meat was minced (3.5 mm) and mixed at approx. 0°C with the ingredients reported and the meat batter was divided in two batches. The first batch (named A) was inoculated with a defined commercial starter culture containing *Lactobacillus sakei* and *Staphylococcus xylosus* (Bactoferm Sm 181, Chr. Hansen, Parma, Italy). The second batch (named B) was inoculated with a starter composed of *Pediococcus pentosaceus* and the same staphylococcal strains of batch A (Bactoferm F1, Chr. Hansen). According to the indication of the producer, the two starter cultures were firstly suspended in water and then added to the meat mixture in order to have an initial concentration of lactic acid bacteria and staphylococci of approx. 6 log cfu/g.

The meat mixtures were stuffed in synthetic collagen casings having two different size, obtaining two type of sausages with different length and diameter (550 mm × 110 mm and 200 mm × 55 mm). The initial weight was about 5300 g for the big ones and 385 g for the small ones. A spore suspension of *Penicillium nalgiovense* (Kerry Ingredient, Ireland) was sprayed on the casings.

The meat batter added with ingredients and starter cultures was sampled immediately before casing (T0). Sausage samples were taken after 3 and 5 days from casing (T3 and T5). Finally, samples were taken at approx. 30% of ripening (T30%, corresponding to 38 days for large diameter sausages and 11 days for small diameter sausages), 60% of ripening (T60%, 69 and 17 days) and at the end of ripening (T100%, 105 and 28 days). The sampling times (and then, the percentage of ripening) were chosen based on the industrial usual ripening period required by the two different sausage sizes.

For each condition considered, sausages were produced from batch of approx. 600 kg.

All the results are expressed as mean of three independent samples (three different sausages), each of which analysed in triplicate.

2.2. pH, a_w, microbial counts

The pH and a_w of fermented sausages during fermentation and ripening were detected by using a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain) and an Aqualab CX3-TE (Labo-Scientifica, Parma, Italy), respectively.

After aseptically removing the casing, a slice of approx. 10 g of sausage was obtained. Then, the slices were 10-fold diluted with 90 ml of 0.9% (w/v) NaCl and homogenized in a Lab Blender Stomacher (Seward Medical, London, UK) for 2 min. Decimal dilutions were performed and plated onto selective media. Counts of lactobacilli were carried out by plating appropriate dilutions on MRS agar (Oxoid, Basingstoke, UK) incubated at 30°C for 48 h in anaerobic conditions. Staphylococci and enterococci were counted by surface-plating on Baird-Parker (added with egg yolk tellurite emulsion), and Slanetz and Bartley medium (Oxoid) incubated at 30°C for 48 h and 44°C for 24 h, respectively. For the yeast count, Sabouraud Dextrose Agar (Oxoid) plates, added with 200 mg/l of chloramphenicol, were used and incubated at 28°C for 72 h. Finally, *Enterobacteriaceae* were enumerated by pour plating in Violet Red Bile Glucose agar (Oxoid) at 37°C for 24 h, while *Pseudomonadaceae* counts were performed on Pseudomonas agar base (Oxoid), supplemented with Pseudomonas CFC Supplement (Oxoid) and incubated at 30°C for 48 h.

To isolate strains belonging to the species used in the two starter cultures, decimal dilutions of the starter cultures commercial products were prepared, plated on Brain Heart Infusion (BHI) Agar (Sigma-Aldrich) and incubated at 27°C. After 2 days, colonies showing cells of different morphology were selected and sub-cultivated in BHI broth to recover cell pellets for DNA isolation.

2.3. DNA extraction and molecular analysis

Extraction of genomic DNA from the pure starter cultures and the isolates was carried out by using the Wizard Genomic DNA Purification Kit (Promega Italia srl, Milan, Italy) according to manufacturer's instructions. The amplification of the 16S rRNA gene of the isolates was carried out according to Nejati *et al.* (2015) and the PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany). Isolates were identified using the EzBioCloud's Identify Service (Yoon et al., 2017).

For DGGE analysis, total DNA was directly extracted from each fermented sausage sample according to the procedure described by Torriani *et al.* (2008).Then, the DNA was used as a template to amplify fragments of approximately 200 base pairs (bp) of the V2–V3 hypervariable region of 16S rRNA gene with the primers HDA1-GC and HDA2 (Walter et al., 2000). PCR amplicons were separated by denaturing gradient gel electrophoresis (DGGE) using the D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Richmond, CA, USA) as described previously (Cocolin et al., 2001) with minor modifications. Briefly, a 30-60% gradient of urea and formamide was used, and the polyacrylamide gels were run at 60°C in 1×TAE buffer at 90 V for 10 min, and then at 50 V for 16 h. After electrophoresis, DGGE gels were stained for 10 min in a EuroSafe Nucleic Acid Stain (EUROCLONE S.p.A, Milan, Italy) solution (30 µl/300 ml deionized water), washed for 10 min in 300 ml deionized water and photographed under UV illumination.

DGGE excised band was re-amplified, purified with the Wizard SV gel and PCR clean-up system (Promega Corporation) and cloned with the cloning kit pGEMT-easy vector system (Promega Corporation). Recombinant plasmids were sequenced by Eurofins Genomics. Data were analyzed with EzBioCloud's Identify Service.

2.4. Analysis of lactic and acetic acids

For the extraction of organic acid, the method reported by Montanari *et al.* (2014) was followed. In particular, 10 g of each sample were added with with 40mL of 0.05mol/L sulfuric solution, homogenised for 10min by an Omni Mixer Homogenizer (Omni International,

Warrengton, VA, USA) and filtered through a 0.22 µm filter. The extracts analysis was performed by using an HPLC (PU-2089 Intelligent HPLC quaternary pump, UV–VIS multi-wavelength detector UV 2070 Plus, Jasco Corp., Tokio, Japan) and a manual Rheodyne injector with a 20 µl loop (Rheodyne, Rohnert Park, Calif., USA), equipped with a Bio-Rad Aminex (Bio-Rad Laboratories, Hertfordshire, UK) HPX-87H column (300x7.8 mm). The following conditions were used: mobile phase, 0.005M sulfuric acid; flow rate, 0.60 ml/min; temperature, 65°C; the UV detector was set at 210 nm. Chromatographic peaks were identified by comparing retention times with those of standards (Sigma-Aldrich, St. Louis, MO) and quantification was carried out by using the external standard method.

2.5. Aroma profile analysis

Volatile organic compounds of samples were analysed with gas-chromatography-mass spectrometry coupled with solid phase microextraction (GC-MS-SPME), using an Agilent Hewlett‑Packard 6890 GC gas-chromatograph and a 5970 MSD MS detector (Hewlett‑Packard, Geneva, Switzerland) equipped with a Varian (50 m X 0.32 mm X 1.2 µm) fused silica capillary column. In particular, samples (3 g) were placed in 10-mL sterilized vials, added with known amount of 4 methyl-2-pentanol (Sigma-Aldrich, Steinheim, Germany) as internal standard and sealed by PTFE/silicon septa. The samples were heated for 10 min at 45 °C and then a fused silica SPME fiber covered with 85 µm Carboxen/Polydimethylsiloxane (CAR/PDMS) (Supelco, Steinheim, Germany) was introduced into the headspace for 40 min. Adsorbed molecules were desorbed in the gas-chromatograph for 10 min. The conditions were the same reported by by Montanari *et al.* (2016).

Volatile peak identification was carried out by computer matching of mass spectral data with those of compounds contained in the Agilent Hewlett–Packard NIST 98 and Wiley vers. 6 mass spectral database. Moreover, for the most important compounds, the mass spectrum identification was confirmed by injection of the pure standards (Sigma-Aldrich, St. Louis, MO) in the same conditions.

For each type of fermented sausage, the volatile composition was expressed as ratio between each molecule peak area and peak area of the internal standard.

2.6. Statistical analysis

Three independent different sausages were analysed for each sampling time, each of which analysed in triplicate. The data were statistically analysed using the two-way ANOVA. The Tukey critical difference test was performed to determine differences between individual treatment means (diameter and starter cultures) and their interaction ($p < 0.05$).

As regards volatile profile, a Principal Component Analysis (PCA) was carried out.

All statistical analysis were carried out using Statistica 6.1 (StatSoft Italy srl, Vigonza, Italy).

3. Results and discussion

3.1. Chemico-physical parameters

The water losses during ripening determined a progressive diminution of a_w (Fig. 2). In the large diameter sausages (Fig. 2a), at the end of ripening, a_w was 0.912, corresponding to a weight loss of about 34%, without significant differences depending on the starter cultures. In the small sausages (Fig. 2b), the final a_w values differed significantly ($p < 0.05$) in relation to started cultures, with water losses of 31.6 and 33.4% for sausages inoculated with *L. sakei*/*S. xylosus* and with *P. pentosaceus/S. xylosus*, respectively.

The pH changes during ripening of the sausages are reported in Fig. 2c (large diameter) and 2d (small diameter). The sausages inoculated with *L. sakei*/*S. xylosus* showed a slower pH decrease during the fermentation step and the minimum pH reached was higher than the value recorded in the sausages in which *P. pentosaceus*/*S. xylosus* were used as starter culture. After the fermentation, the pH slightly increased and no differences were observed within the sausages with large diameter independently on the starter cultures (values of 5.2 at the end of ripening). By contrast, the small sausages presented statistically different pH values ($p < 0.05$)

at the end of ripening (about 5.2 in those fermented by *L. sakei*/*S. xylosus* and 5.0 using *P. pentosaceus*/*S. xylosus* as starter cultures).

The amount of lactic acid did not exactly reflect the behavior of pH in large diameter sausages. Indeed, the higher lactic acid concentrations were observed after the reaching of the minimum pH (Tab. 1) and, in particular, they were recorded in the 60% of ripening samples. After this ripening time, the lactic acid concentration decreased. This contradictory behavior can be explained by the complex phenomena, involving other meat constituents (mainly amino acid and proteins) which can produce molecules able to rise pH (such as ammonia or organic bases) and counteract the effects on pH of acid production by LAB. The delayed decrease of lactic acid can also found a reason in the time required to the hyphae of the moulds to penetrate inside the large diameter sausages and in the reduced oxygen availability (Spotti and Berni, 2007). In effect, in fermented sausages the increase of pH during ripening is mainly attributable to the respiration of lactic acid by moulds (Incze, 2010). The content of acetic acid was significantly higher ($p < 0.05$) in the sausages inoculated with *P. pentosaceus*/*S. xylosus* after 60% of ripening.

In the small diameter sausages lactic acid rapidly accumulated during the fermentation step (within the first 5 days), after which it only slightly increased. Significant differences ($p < 0.05$) were observed in relation to the starter cultures. In fact, the presence of *P. pentosaceus* determined higher lactic acid concentrations if compared with the sausages inoculated with *L. sakei*. In addition, in the small diameter sausages, acetic acid was accumulated in low amounts (less than 0.40 g/kg) in all the sausages, without significant differences between the starter cultures.

3.2. Microbial counts

The data of the microbial counts are reported in Table 1. The initial counts of LAB and staphylococci, added as starter cultures, were approx. 6 log cfu/g. Independently on the dimension of sausages and the starter culture used, the LAB counts reached their maximum

concentration (8.6-8.8 log cfu/g) after 3 days. At the different sampling times, LAB counts were not affected by diameter or starter cultures. After fermentation, a small reduction of LAB was observed (about 0.5 log units). However, the counts were always higher than 8 log cfu/g and showed a slight increase in the ripened sausages.

Staphylococci had a different behavior. While their concentration generally decreased during large diameter sausage ripening, this value increased in the small diameter sausages reaching counts higher than 8 log cfu/g at the end of ripening. Tabanelli et al. (2015) have already observed higher concentration of staphylococci at the end of ripening in small size Italian sausages.

The diffusion of oxygen inside the sausage is highly dependent on their diameter. Thus, the higher concentration of oxygen in the smaller sausages can favour the proliferation of staphylococci and modulate their metabolism with consequences on flavor formation (Sánchez Mainar et al., 2017). In fact, even if coagulase negative staphylococci are usually present in sausages in lower counts with respect to LAB, they contribute in significant way to the flavor development of sausages (Ravyts et al., 2012). In addition, the smaller size of the product favour the mould colonization and it has been demonstrated that the fungal growth can affect the qualitative and quantitative behavior of staphylococcal populations (Janssens et al., 2013). Finally, the not uniform water distribution during ripening inside the sausages (Cevoli et al., 2014) determines a higher salt concentration in the external part of sausages, which, in turn, provide a more competitive environment for staphylococci.

In this external part, the oxygen availability and salt concentration are higher in the small sausages, favouring staphylococci proliferation.

Yeasts had an increasing trend but they did not exceed the number of 5 log cfu/g in the final products. These values were higher than those observed in similar products (Tabanelli et al., 2012; Tabanelli et al., 2015) but were similar to those reported for sausage of southern Italy (Francesca et al., 2013; Gardini et al., 2001).

Noteworthy, enterococci showed concentrations depending on the starter culture. In fact, their number was significantly higher when *L. sakei*/*S. xylosus* was added. This difference was more evident in small diameter sausages. The presence of enterococci is often controversial in fermented foods, since this LAB are characterized by antibiotic resistance and transmission, presence of virulence factors and they are producers of tyramine (Franz et al., 2011; Paramithiotis et al., 2008; Bargossi et al., 2015). Their concentration in sausages is variable, depending on the quality of raw material, starter cultures and technological conditions adopted and should be controlled (Hugas et al., 2003).

The enterobacteria, present at concentration lower than 3 log cfu/g in the meat batter, were always under the detection limit in the successive samplings. Finally, pseudomonads were counted at a concentration of about 5 log cfu/g in the meat batter and their concentration decreased gradually during ripening. The presence of these microorganisms can have a negative impact because of their enzymatic potential (De Jonghe et al., 2011). In fact, even if their growth is inhibited by the salt concentration found in sausages, their survival during ripening can allow the activity of the proteases and lipases produced. In addition, it has been demonstrated that the activity of these enzymes can continue independently of the viability and integrity of pseudomonads cells (Chen et al., 2003).

3.3. DGGE analysis

Initially, to construct DGGE reference bands (Fig. 3), it was carried out an isolation of strains belonging to the species declared by the producer from the two commercial starter cultures, *i.e. S. xylosus* (*Sx1* and *Sx2*, from the starters SA and SB, respectively), *L. sakei* (*Ls*) and *P. pentosaceus* (*Pp*). 16S rRNA gene sequence analysis confirmed the identification of these new isolates, as the percentage of similarity to the quality-controlled 16S rRNA gene published sequences was more than 99 for all the strains (data not showed).

DGGE analysis of the PCR-amplified 16S rRNA gene fragments obtained from the pure cultures of isolates, the culture starters, and the samples collected during sausage production (just

before stuffing, at 5 days of fermentation, and at the end of ripening process) provided the fingerprints shown in Fig. 3.

Each isolate generated two or more DGGE bands, but a more intense reference band was always recognizable; this result can be correlated to the presence of multiple, heterogeneous ribosomal RNA operons in the genome, as previously indicated (Ercolini, 2004; Fasoli et al., 2003; Větrovský and Baldrian, 2013). In addition, the two *S. xylosus* isolates *Sx1* and *Sx2* showed the expected identical DGGE banding pattern. In accordance with these results, the DGGE profiles of the starters were clearly composed by the typical bands generated from each of the species declared. Therefore, the occurrence of certain microbial species was corroborated by direct DNA isolation from the two starters and PCR-DGGE analysis. The usefulness and reliability of DGGE in the identification of the species included in starter and probiotic cultures was amply demonstrated (Ercolini, 2004).

The bands of both the species present in each starter formulations were evident in the meat batters collected immediately after the starter addition. After 5 days of fermentation, the profiles of *L. sakei* and *P. pentosaceus* were detected as dominant in the sausages inoculated with the respective starters SA and SB, and their profiles persisted until the end of the ripening period. On the contrary, the bands corresponding to the species *S. xylosus* were very weak after 5 days of fermentation, but they became more intense at the end of the fermentation process in all the sausages, especially in the small sausages fermented by the starter SB (*P. pentosaceus/S. xylosus*). These results were in agreement with the microbial counts. The intensity of the bands on a DGGE gel can be considered a semi-quantitative measure to visualize the dominance of certain species in the sample over less dominant ones (Herbel et al., 2013). Thus, a limited quantification was also possible using this approach.

Interestingly, while *P. pentosaceus* was found only in the samples in which it was added as starter culture, the presence of *L. sakei* was assessed in all the sausages, including the samples inoculated with pediococci. This confirmed the affinity of this species for meat environments in which they can prevail independently of their addition as starter cultures (Bassi et al., 2015;

Drosinos et al., 2007). Further, some samples showed the presence of weak additional amplicons, probably associated with the indigenous bacteria of the meat. We were not able to obtain suitable sequence data after excision of these bands from the gels (data not shown), but this outcome suggested a probable contribution of some indigenous species to the fermentation process. As an example, the band *nd*, that was retrieved in the meat batters, still persisted during the sausages ripening, even if with decreasing intensity, and thus with minor relative abundance in the population. Finally, the profiles of the two kinds of sausages at the end of the ripening period did not contain additional distinct amplicons indicating a slight influence of the diameter in shaping the microbial community.

3.4. Aroma profile of sausages

The modifications of aroma profiles of the sausages after 5 days of fermentation and at the end of ripening are reported in Table 2. Other data regarding meat batter, 3 days of fermentation and samples taken during ripening period (30% and 60%) are reported in supplementary Table 1. The results are grouped according to their molecule chemical group and are reported as ratio between each peak area and the area of internal standard (4-methyl-2-pentanol), which was added in constant amount (33 mg/kg). In spite of the use of an internal standard, the use of SPME is considered in this paper as a qualitative tool to study the effect of the variables on the change of volatile profile during ripening. In other words, the study was not aimed to a quantification of the compounds in the sausages, but to evidence qualitative modification of their presence. In fact, the quantitative results obtained by this analytical technique depends on several factors, among which the type of fiber and the time and temperature of absorption are the most important, which can influence the amounts of each single molecules recovered in an important way (Lorenzo, 2014).

Terpenes and terpenoids, such as 3-carene, β-pinene, thujene, α- and β-phellandrene, β-myrcene, p-cymene, caryophyllene and limonene, deriving from spices (Montanari et al., 2016), were not considered and were omitted from the table. In fact, the use of whole or

coarsely ground pepper can cause a not uniform distribution of this spice in the samples and, consequently, a not uniform distribution of these volatile molecules. Similarly, the compounds derived from garlic, such as methyl allyl sulfide, diallyl sulfide, and dimethyldisulfide were not taken into consideration.

In the meat batter, the main compounds detected were hexanal, pentanal, 2-octenal and (*E*)-2-heptenal, deriving from the autoxidation of linoleic and linolenic acid, nonanal and octanal (deriving through the same pathway from oleic acid), as well as heptanal and 2-nonenal (Ordóñez et al., 1999). Also, small amounts of alcohol were detected (1-octen-3-ol, 1-pentanol) together with ketones (acetone, 2-6 dimethyl-4-heptanone).

Independently on the sausage size, the concentration of ketones remained quite constant until the 30% of ripening, while at 60% and at the end of ripening their presence was markedly increased. However, in the large diameter sausages this increment was mainly due to 2-butanone, especially in the samples inoculated with *L. sakei/S. xylosus*. In the small diameter sausages, instead, the increment of ketones was mainly due to the accumulation of acetone and 3-hydroxy-2 butanone (acetoin), especially in the samples inoculated with *P. pentosaceus/S. xylosus*. These samples had also high concentrations of 2,3-butanedione (diacetyl) and 3,5-octadien-2-one. Relevant amounts of 2-butanone have been already observed in some large diameter Italian sausages (Montanari et al., 2016).

The presence of methyl ketones in sausages can be the result of microbial β-oxidation of lipids, deriving from β-ketoacids produced during β-oxidation carried out by moulds and staphylococci (Lorenzo et al., 2016; Olivares et al., 2011; Ordóñez et al., 1999; Sunesen and Stahnke, 2003). 2-butanone can also be the results of LAB metabolism (Carballo, 2012; Routray and Mishra, 2011). It has been demonstrated that lactobacilli can produce 2-butanone starting from diacetyl through the action of a diol dehydratase, which can be successively followed by a dehydrogenase responsible for the corresponding alcohol formation (Speranza et al., 1997). Diacetyl can bring to the accumulation of 2-butanone (and consequently 2-butanol) in large size sausages and, alternatively, it can bring to the accumulation of 3-hydroxy-2 butanone

through its chemical reduction in smaller samples. This shift could be explained by the different conditions, which depends on the diameter of the sausages. Some authors observed a rapid formation of 2-butanone and 2,3-butanedione related to a fast acidification, but in their experiments the accumulation took mainly place only during the second half of ripening (Tjener et al., 2003).

The amounts of aldehydes decreased during ripening in all the sausages, independently on diameter and starter cultures. However, the diminution was drastic in the large diameter sausage and less pronounced in the small sausages. The decrease in both cases was mainly due to the diminution of hexanal but many other aldehydes, originating from lipid oxidation, followed the same trend. Hexanal and other aliphatic aldehydes presence depend firstly on the action of hydroperoxidases, which produce compounds with no consequences on flavor, but highly unstable and thus broken in by-products with organoleptic impact imparting herbal or floral notes (Ordóñez et al., 1999).

The concentration of hexanal was extremely variable in Italian sausages (Bianchi et al., 2007; Coloretti et al., 2014; Montanari et al., 2016; Summo et al., 2010; Tabanelli et al., 2012). Even if it was the major compound present in the meat batter before casing, its constant decrease during ripening seems to indicate that also the time of ripening could play a role on its final concentration and, in turn, on its contribution to the overall aroma of sausages. There are few data about the evolution of hexanal during ripening. Olivares *et al.* (2009) observed an increase of this aldehyde in Spanish sausages. Indeed, these sausages were more similar to the smaller ones considered here and were prepared with a mincing degree (10 mm) higher than that used here (3 mm). This technological aspect can explain the high hexanal level already observed in the meat batter in these trials. The same authors observed also an increase of 2-butanone and a diminution of the content of 2,3-butanedione, which was the principal molecule in the meat batter before casing.

In both sausage typologies, benzaldehyde concentration was high during fermentation (the first 5 days) and then decreased. This compound provides floral and almond notes (Lorenzo et

al., 2016; Singh et al., 2003) and does not originate from lipid oxidation, being the result of aromatic aminoacid metabolism (Smid and Kleerebezem, 2014). It was not detected in the larger sausages after 69 days (60% of ripening), while in smaller sausages its presence in the final products was about 1/4 of the maximum values reached at the beginning of fermentation.

Alcohols had a fluctuating behavior. In the larger sausages, they increased during the fermentation and then remained relatively constant; only in the ripened product their concentration drastically increased (two or three times the concentration previously recorded). In the small diameter sausages, alcohols reached their maximum level at the end of fermentation (5 days) after which decreased.

In the large diameter sausages at the end of ripening, the most relevant alcohols were 2-butanol (derived from the reduction of 2-butanone), ethanol and, to a lesser extent, 1-propanol. In the smaller sausages, hexanol prevailed followed by 1-octen-3-ol, 1-pentanol and ethanol. Noteworthy, in large sausages 1-hexanol (which derives from the reduction of hexanal) was present only in the sample at 30% of ripening. In general, the concentration of alcohols was higher in the samples inoculated with *P. pentosaceus/S. xylosus*. The presence of ethanol can be attributed to several pathways and together with acetic acid, may result from LAB metabolism of lactate (von Wright and Axelsson, 2011). Also, yeasts can contribute to the accumulation of alcohols (Flores et al., 2015). In addition 1-octen-3-ol, usually related to mould inoculation, can confer to sausages a marked mushroom odour and it is characterized by a very low odour threshold (Lorenzo and Carballo, 2015).

The presence of esters was limited, under the adopted conditions, to ethyl acetate, which was accumulated in the large diameter sausages especially at the end of ripening. Ethyl acetate amount can be related to the addition of wine as ingredient (Gardini et al., 2013), to the presence of acids (especially acetic acid, which is a constituent of esters) and to the presence of esterase producing microorganisms. Among microorganisms involved in sausage

fermentation, staphylococci are the main responsible for the formation of esters (Talon et al., 1998).

Acids were represented mainly by acetic acid, whose concentration constantly increased in the sausages with large diameter throughout all the ripening period. The use of *P. pentosaceus* as a starter culture component always resulted in higher acetic acid amounts. In the smaller sausages, the concentrations of this acid were lower and reached the maximum values in the samples taken after 17 days, after which drastically decreased. In the greater sausages, also butanoic acid (and propanoic acid, even if at a lesser extent) was found, while in the smaller products hexanoic acid was present with the same trend showed by acetic acid. Acetic acid, butanoic acid together with 1-octen-3-ol were described as potent odorants in dry fermented sausages (Corral et al., 2013; Olivares et al., 2011). It has also been demonstrated that bacteria involved in sausages fermentation can catabolise nucleosides, obtaining a mixture of acetic acid, formic acid and ethanol from their pentose moiety, as demonstrated in *L. sakei* (Rimaux et al., 2011) and coagulase-negative staphylococci (Janssens et al., 2014).

Compounds with a potential impact on organoleptic characteristics and deriving from amino acids, such as 3-methyl butanal and 2-methyl butanal, were not detected or detected in low amounts, even if in many similar Italian sausages has been found in more significant amount (Montanari et al., 2016; Summo et al., 2010; Tabanelli et al., 2012).

3.5. Principal Component Analysis

To better evidence the relationships between the formation of aroma profile during ripening in relation to the dimension of sausages and the starter culture used, a principal component analysis (PCA) was carried out on the correlation matrix based on the detected amounts of the 34 volatile compounds in the samples. In Fig. 4 the case coordinates on the sample score plot of the first two factors (PC1 and PC2) representing 54.5 and 14.5% of the total variability, respectively (Fig. 2a) and the PCA loadings plot of the volatiles on the same two factors (Fig. 2b) are reported.

The PC1 was highly positively correlated with all the aldehydes, with some ketones such as 2,3-octanedione and 1-octen-3-one, and with some alcohols (1-pentanol and 3-octen-1-ol) and with hexanoic acid. This principal component was highly negatively correlated 2-butanone, ethyl acetate, many alcohols (ethanol, isopropanol, 2-butanol, 1-propanol), and all the acids, with the exception of hexanoic acid. PC2 was highly negatively correlated with 1-hexanol and the major part of ketones (acetone, 3-hydroxy-2-butanone, 3,5-octadien-2-one and 2,3-butandione). This principal component showed generally low positive correlation with all the other volatile compounds. Depending on these values, the consequent grouping of the sausages is reported in Fig. 4a. Four distinct groups were observed. The meat batters were grouped at the origin of the axes, while the samples taken during the fermentation (within 5 days) were grouped in the first quadrant of the graph independently of size and starter cultures, mainly due to their content of aldehydes and 1-pentanol. Generally, the samples taken at 3 days after casing presented the highest concentration of aldehydes. In this cluster, only the small sausages, inoculated with *L. sakei*/*S. xylosus* after 11 days (30% of ripening) seems to be misplaced.

The other samples of the small sausages were grouped in the low part of the graph. The samples obtained using *P. pentosaceus*/*S. xylosus* as starter culture were characterized by a lower score along the y axis independently of the time of ripening.

The sausages with large diameter were mainly located in the 2nd quadrant reflecting their content of acids, ethyl acetate and 2-butanol and 2-butanone. As in the case of small diameter sausages, the final product was well separated from the others, but the effect of the starter cultures was not particularly evident.

4. Conclusions

The characteristics of the fermented sausages considered in this study were different in relation mainly to the sausages diameter. Even if they obviously required different ripening times, the final values of a_w and pH were similar. According to the PCA results, the composition

of the sausage aroma profile was rather uniform in the first day of fermentation, during which the sugar added was depleted by LAB. Nevertheless, the sausages were clearly separated in the successive step of ripening. The differences in the aroma profile composition can be attributable both to the longer ripening time and to the lower oxygen availability characterizing the large diameter sausages. The presence of the main microbial groups was less influenced by the diameter, as shown also by the PCR-DGGE analysis.

The influence of the starter cultures was less evident. This could also be related to the observed persistence of indigenous *L. sakei* in the sausages inoculated with pediococci, as revealed by PCR-DGGE. The limited effect of the two starter cultures could be also attributed to the adoption of the same profile of ripening conditions (chosen in accordance with the industrial practices) which does not allow the strains to exert their different physiological aptitudes. However, a higher number of staphylococci in the small size sausages was observed and this prevalence is relevant for the final aroma profile given the proteolytic and lipolytic activity of these microorganism, as well as their ability to produce aroma compounds in fermented meats. This underlines the needs for further technological studies able to optimize the starter culture performances in relation to the process in order to reach the desired features of the final product safeguarding the differentiation of the sausages which is an important cultural (before than economic) value for the Italian fermented meats.

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References

1. Anastasio, A., Draisci, R., Pepe, T., Mercogliano, R., Delli Quadri, F., Luppi, G., & Cortesi, M.L. (2010), Development of biogenic amines during the ripening of Italian dry sausages. *Journal of Food Protection,* 73(1), 114-118.

2. Bargossi, E., Tabanelli, G., Montanari, C., Lanciotti, R., Gatto, V., Gardini, F., & Torriani, S. (2015). Tyrosine decarboxylase activity of enterococci grown in media with different nutritional potential: tyramine and 2-phenylethylamine accumulation and tyrDC gene expression. *Frontiers in Microbiology*, Vol. 6, 259.

3. Bassi, D., Puglisi, E., & Cocconcelli, P.S. (2015). Comparing natural and selected starter cultures in meat and cheese fermentations. *Current Opinion in Food Science*, 2, 118-122.

4. Bianchi, F., Cantoni, C., Careri, M., Chiesa, L., Musci, M., & Pinna, A. (2007). Characterization of the aromatic profile for the authentication and differentiation of typical Italian dry-sausages. *Talanta*, 72(4), 1552-1563.

5. Carballo, J. (2012). The role of fermentation reactions in the generation of flavor and aroma of foods. In B. M. Mehta, A. Kamal-Eldin, R. Z. Iwanski (Eds.), *Fermentation, Effects on Food Properties*. Boca Raton: CRC Press. p 51-83.

6. Cevoli, C., Fabbri, A., Tabanelli, G., Montanari, C., Gardini, F., Lanciotti, L., Guarnieri, A. (2014). Finite element model of salami ripening process and successive storage in package. *Journal of Food Engineering*, Vol. 132, 14-20.

7. Chen, L., Daniel, R.M., & Coolbear, T. (2003). Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal*, 13(4), 255-275.

8. Cocolin, L., Manzano, M., Cantoni, C., & Comi, G. (2001). Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. *Applied and Environmental Microbiology*, 67(11), 5113-5121.

9. Coloretti, F., Tabanelli, G., Chiavari, C., Lanciotti, R., Grazia, L., Gardini, F., & Montanari, C. (2014). Effect of wine addition on microbiological characteristics, volatile molecule profiles and biogenic amine contents in fermented sausages. *Meat Science*, 96(3), 1395-1402.

10. Corral, S., Salvador, A., & Flores, M. (2013). Salt reduction in slow fermented sausages affects the generation of aroma active compounds. *Meat Science*, 93(3), 776-785.

11. De Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P., & Heyndrickx, M. (2011). Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. *Applied and Environmental Microbiology*, 77(2), 460-470.

12. Drosinos, E.H., Paramithiotis, S. Kolovos, G. Tsikouras, I., & Metaxopoulos I. (2007) Phenotypic and technological diversity of lactic acid bacteria and staphylococci isolated from traditionally fermented sausages in Southern Greece. *Food Microbiology,* 24, 260-270.

13. Ercolini, D. (2004). PCR-DGGE fingerprinting: Novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, 56(3), 297-314.

14. Fasoli, S., Marzotto, M., Rizzotti, L., Rossi, F., Dellaglio, F., & Torriani, S. (2003). Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *International Journal of Food Microbiology*, 82(1), 59-70.

15. Flores, M., Corral, S., Cano-García, L., Salvador, A., & Belloch, C. (2015). Yeast strains as potential aroma enhancers in dry fermented sausages. *International Journal of Food Microbiology*, 212, 16-24.

16. Francesca, N., Sannino, C., Moschetti, G., & Settanni, L. (2013). Microbial characterisation of fermented meat products from the Sicilian swine breed "Suino Nero Dei Nebrodi". *Annals of Microbiology*, 63(1), 53-62.

17. Franz, C.M.A.P., Huch, M., Abriouel, H., Holzapfel, W., & Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology*, 151(2), 125-140.

18. Freiding, S., Gutsche, K.A., Ehrmann, M.A., & Vogel, R.F. (2011). Genetic screening of *Lactobacillus sakei* and *Lactobacillus curvatus* strains for their peptidolytic system and amino acid metabolism, and comparison of their volatilomes in a model system. *Systematic and Applied Microbiology*, 34(5), 311-320.

19. Gardini, F., Suzzi, G., Lombardi, A., Galgano, F., Crudele, M.A., Andrighetto, C., Schirone, M., & Tofalo, R. (2001). A survey of yeasts in traditional sausages of southern Italy. *FEMS Yeast Research*, 1(2), 161-167.

20. Gardini, F., Tabanelli, G., Lanciotti, R., Montanari, C., Luppi, M., Coloretti, F., Chiavari, C., & Grazia, L. (2013). Biogenic amine content and aromatic profile of *Salama da sugo*, a typical cooked fermented sausage produced in Emilia Romagna Region (Italy). *Food Control*, 32(2), 638-643.

21. Herbel, S.R., Vahjen, W., Wieler, L.H., & Guenther, S. (2013). Timely approaches to identify probiotic species of the genus *Lactobacillus*. *Gut Pathogens*, 5, 27.

22. Hugas, M., Garriga, M., & Aymerich, M.T. (2003). Functionality of enterococci in meat products. *International Journal of Food Microbiology*, 88(2-3), 223-233.

23. Incze, K. (2010). Mold-ripened sausages. In F. Toldrá (Eds.), *Handbook of Meat Processing*. Ames: Wiley-Blackwell. p 363-378.

24. Janssens, M., Myter, N., De Vuyst, L., Leroy, F. (2013). Community dynamics of coagulase-negative staphylococci during spontaneous artisan-type meat fermentations differ between smoking and moulding treatments. *International Journal of Food Microbiology,* 166, 168-175.

25. Janssens, M., Van der Mijnsbrugge, A., Sánchez Mainar, M., Balzarini, T., De Vuyst, L., & Leroy, F. (2014). The use of nucleosides and arginine as alternative energy sources by coagulase-negative staphylococci in view of meat fermentation. *Food Microbiology*, 39, 53-60.

26. Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L., & Scholliers, P. (2013). Meat fermentation at the crossroads of innovation and tradition: a historical outlook. *Trends in Food Science & Technology*, 31(2), 130-137.

27. Lorenzo, J.M. (2014). Influence of the type of fiber coating and extraction time on foal dry-cured loin volatile compounds extracted by solid-phase microextraction (SPME). *Meat Science*, 96(1), 179-186.

28. Lorenzo, J.M., & Carballo, J. (2015). Changes in physico-chemical properties and volatile compounds throughout the manufacturing process of dry-cured foal loin. *Meat Science*, 99, 44-51.

29. Lorenzo, J.M., Gómez, M., Purriños, L., & Fonseca, S. (2016). Effect of commercial starter cultures on volatile compound profile and sensory characteristics of dry-cured foal sausage. *Journal of the Science of Food and Agriculture*, 96(4), 1194-1201.

30. Montanari, C., Bargossi, E., Lanciotti, R., Chinnici, F., Gardini, F., & Tabanelli, G. (2014). Effects of two different sourdoughs on the characteristics of Pandoro, a typical Italian sweet leavened baked good. *LWT- Food Science and Technology*, 59(1), 289-299.

31. Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F., & Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. *Food Chemistry*, 192, 736-744.

32. Nejati, F., Gatto, V., Castioni, A., Tebaldi, M., Babaei, M., Fracchetti, F., & Felis, G.E. (2015). Genetic diversity of enterococci from Iranian home-made artisanal dairy products. *Dairy Science & Technology*, 95(2), 151-165.

33. Olivares, A., Navarro, J.L., & Flores, M. (2009). Establishment of the contribution of volatile compounds to the aroma of fermented sausages at different stages of processing and storage. *Food Chemistry*, 115(4), 1464-1472.

34. Olivares, A., Navarro, J.L., & Flores, M. (2011). Effect of fat content on aroma generation during processing of dry fermented sausages. *Meat Science*, 87(3), 264-273.

35. Ordóñez, J.A., Hierro, E.M., Bruna, L., & de la Hoz, L. (1999). Changes in the components of dry-fermented sausages during ripening. *Critical Reviews in Food Science and Nutrition*, 39(4), 329-367.

36. Paramithiotis S., Kagkli D.-M., Blana V., Nychas G.-J.E., & Drosinos E.H. (2008). Identification and characterisation of *Enterococcus* sp. in Greek spontaneous sausage fermentation. *Journal of Food Protection*, 71, 1244-1247.

37. Ravyts, F., De Vuyst, L., & Leroy, F. (2012). Bacterial diversity and functionalities in food fermentations. *Engineering in Life Sciences*, 12(4), 356-367.

38. Rimaux, T., Vrancken, G., Pothakos, V., Maes, D., De Vuyst, L., & Leroy, F. (2011). The kinetics of the arginine deiminase pathway in the meat starter culture *Lactobacillus sakei* CTC 494 are pH-dependent. *Food Microbiology*, 28(3), 597-604.

39. Routray, W., & Mishra, H.N. (2011). Scientific and technical aspects of yogurt aroma and taste: a review. *Comprehensive Reviews in Food Science and Food Safety*, 10(4), 208-220.

40. Sánchez Mainar, M., Matheuse, F., De Vuyst, L., Leroy, F. (2017). Effects of glucose and oxygen on arginine metabolism by coagulase-negative staphylococci. *Food Microbiology*, 65, 170-178.

41. Singh, T.K., Drake, M.A., Cadwallader, K.R. (2003). Flavor of cheddar cheese: a chemical and sensory perspective. Comprehensive Reviews in Food Science and Food Safety, 2, 166-189.

42. Smid, E.J., & Kleerebezem, M. (2014). Production of aroma compounds in lactic fermentations. *Annual Review of Food Science and Technology*, 5, 313-326.

43. Speranza, G., Corti, S., Fontana, G., Manitto, P., Galli, A., Scarpellini, M., & Chialva, F. (1997). Conversion of meso-2,3-butanediol into 2-butanol by lactobacilli. Stereochemical and enzymatic aspects. *Journal of Agricultural and Food Chemistry*, 45(9), 3476-3480.

44. Spotti, E., & Berni, E. (2007). Starter cultures: Molds. In F. Toldrá (Eds.), *Handbook of Fermented Meat and Poultry*. Ames: Blackwell Publishing. p 171-176.

45. Summo, C., Caponio, F., Paradiso, V.M., Pasqualone, A., & Gomes, T. (2010). Vacuum-packed ripened sausages: evolution of oxidative and hydrolytic degradation of lipid fraction during long-term storage and influence on the sensory properties. *Meat Science*, 84(1), 147-151.

46. Sunesen, L.O., & Stahnke, L.H. (2003). Mould starter cultures for dry sausages-selection, application and effects. *Meat Science*, 65(3), 935-948.

47. Tabanelli, G., Coloretti, F., Chiavari, C., Grazia, L., Lanciotti, R., & Gardini, F. (2012). Effects of starter cultures and fermentation climate on the properties of two types of typical

Italian dry fermented sausages produced under industrial conditions. *Food Control*, 26(2), 416-426.

48. Tabanelli, G., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F., & Montanari, C. (2015). Physico-chemical and microbiological characterisation of Italian fermented sausages in relation to their size. *Journal of the Science of Food and Agriculture*, 96(8), 2773-2781.

49. Talon, R., Lebert, I., Lebert, A., Leroy, S., Garriga, M., Aymerich, T., Drosinos, E.H., Zanardi, E., Ianieri, A., Fraqueza, M.J., Patarata, L., & Lauková, A. (2007). Traditional dry fermented sausages produced in small-scale processing units in Mediterranean countries and Slovakia. 1. Microbial ecosystems of processing environments. *Meat Science*, 77, 570-579.

50. Talon, R., Chastagnac, L., Vergnais, L., Montel, M.C., & Berdagué, J.L. (1998). Production of esters by staphylococci. *International Journal of Food Microbiology*, 45(2), 143-150.

51. Tjener, K., Stahnke, L.H., Andersen, L., & Martinussen, J. (2003). A fermented meat model system for studies of microbial aroma formation. *Meat Science*, 66(1), 211-218.

52. Toldrá, F. (2006). Meat Fermentation. In Y. K. Hui (Eds.), *Handbook of Food Science, Technology and Engineering,* vol. 4. Boca Raton: CRC Press. p 1-12, ch. 181.

53. Torriani, S., Gatto, V., Sembeni, S., Tofalo, R., Suzzi, G., Belletti, N., Gardini, F., & Bover-Cid, S. (2008). Rapid detection and quantification of tyrosine decarboxylase gene (*tdc*) and its expression in gram-positive bacteria associated with fermented foods using PCR-based methods. *Journal of Food Protection*, 71(1), 93-101.

54. Větrovský, T., & Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS One*, 8(2), e57923.

55. von Wright, A., & Axelsson, L. (2011). Lactic acid bacteria: an introduction. In S. Lahtinen, A. C. Ouwehand, S. Salminen, A. von Wright (Eds.), *Lactic Acid Bacteria: Microbial and Functional Aspects*, 4th ed. Boca Raton: CRC Press. p 1-16.

56. Walter, J., Tannock, G.W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D.M., Munro, K., & Alatossava, T. (2000). Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primer. *Applied and Environmental Microbiology*, 66(1), 297-303.

57. Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J. (2017). Introducing EzBioCloud: A taxonomically united database of 16S rRNA and whole genome assemblies. *International Joutnal of Systematic and Evolutionary Microbiology* (in press).

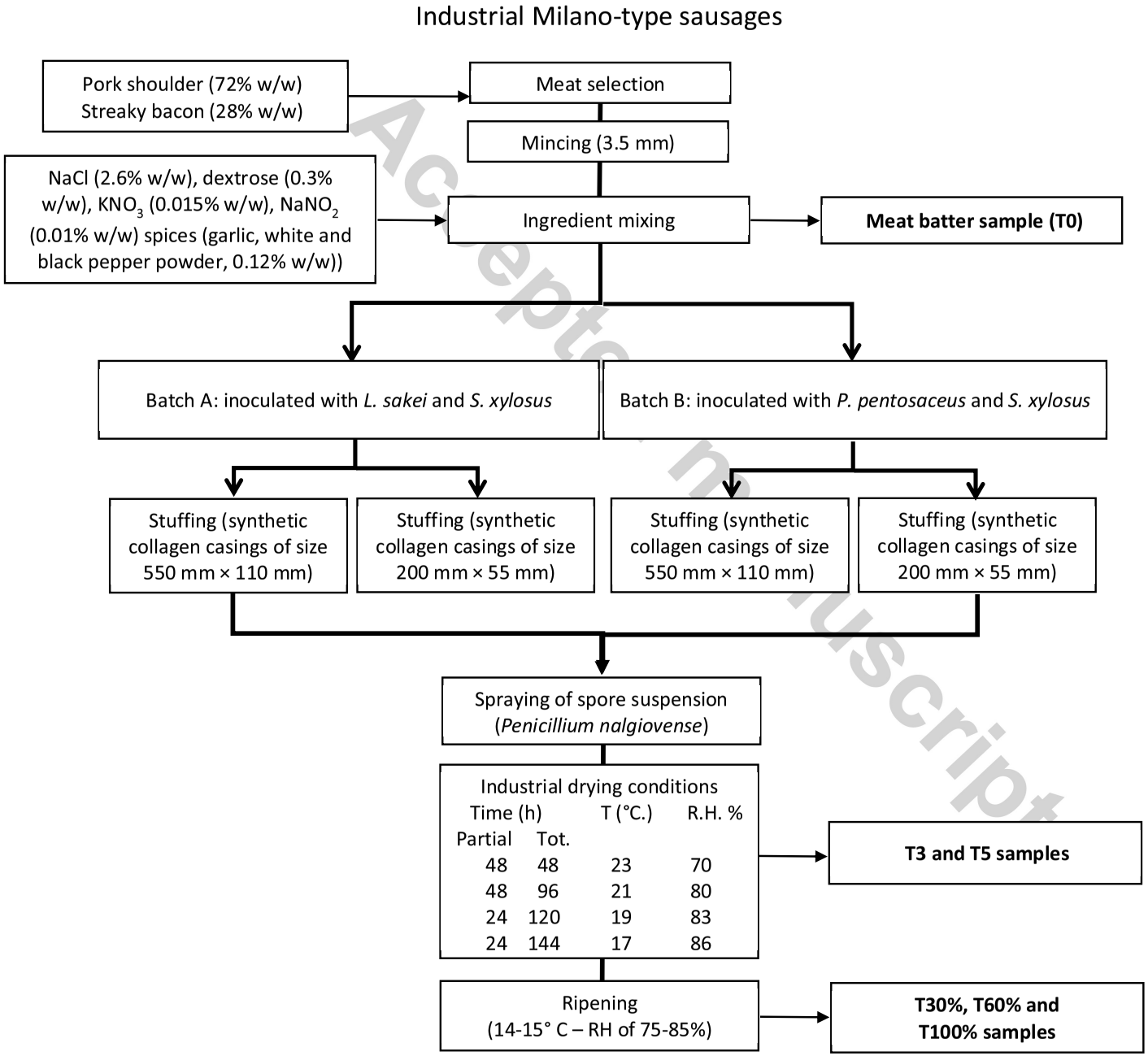
Fig 1. Flow sheet for the manufacture of the different Milano sausages and sample sampling points.

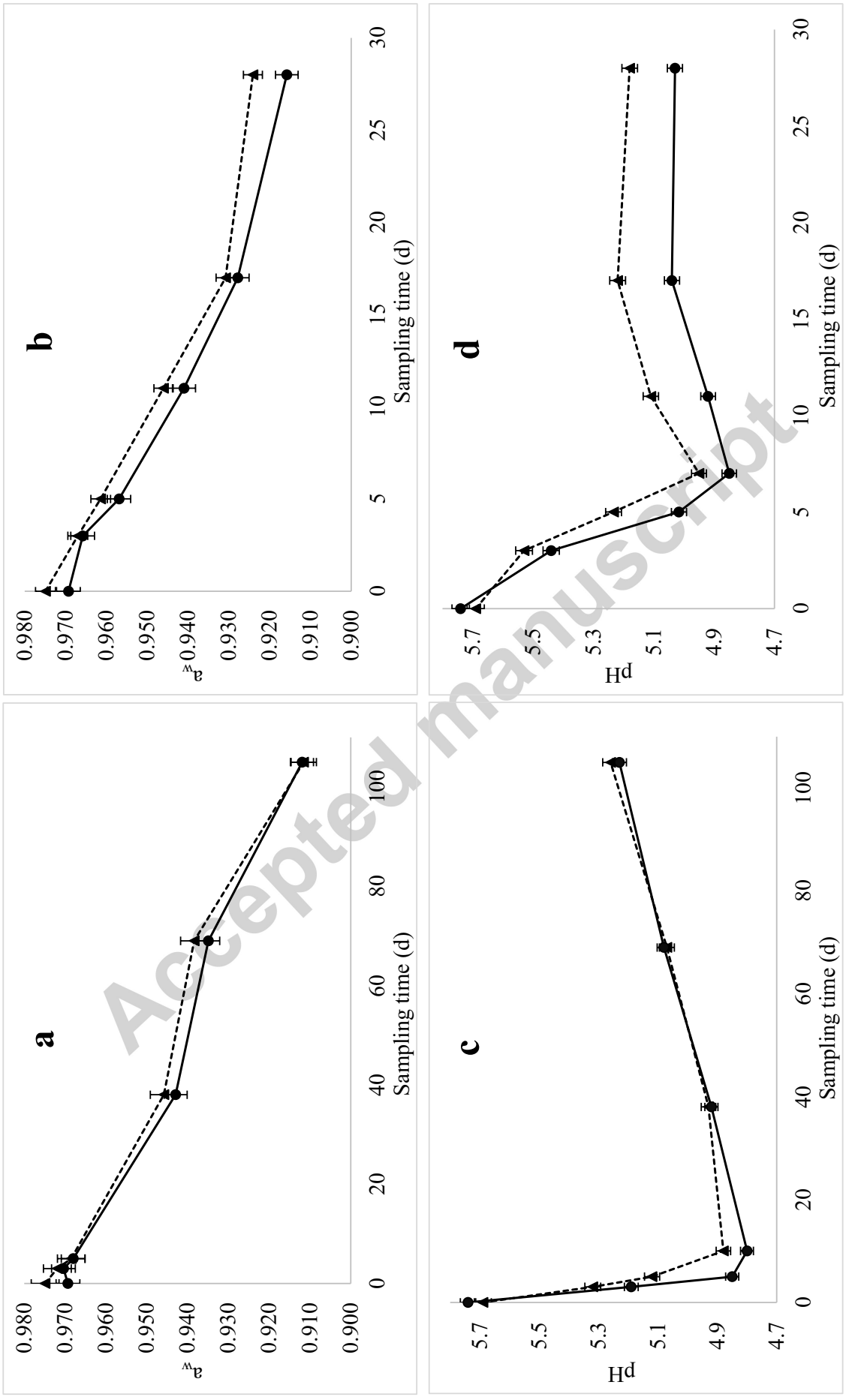
Fig. 2. a_w and pH changes during fermentation and ripening of the sausages inoculated with the two different starters: a, c) large diameter; b, d) small diameter. The dotted line with the triangles represents the inoculated samples with *L. sakei* and *S. xylosus* while the continuous line with circles represents the inoculated samples with *P. pentosaceus* and *S. xylosus*.

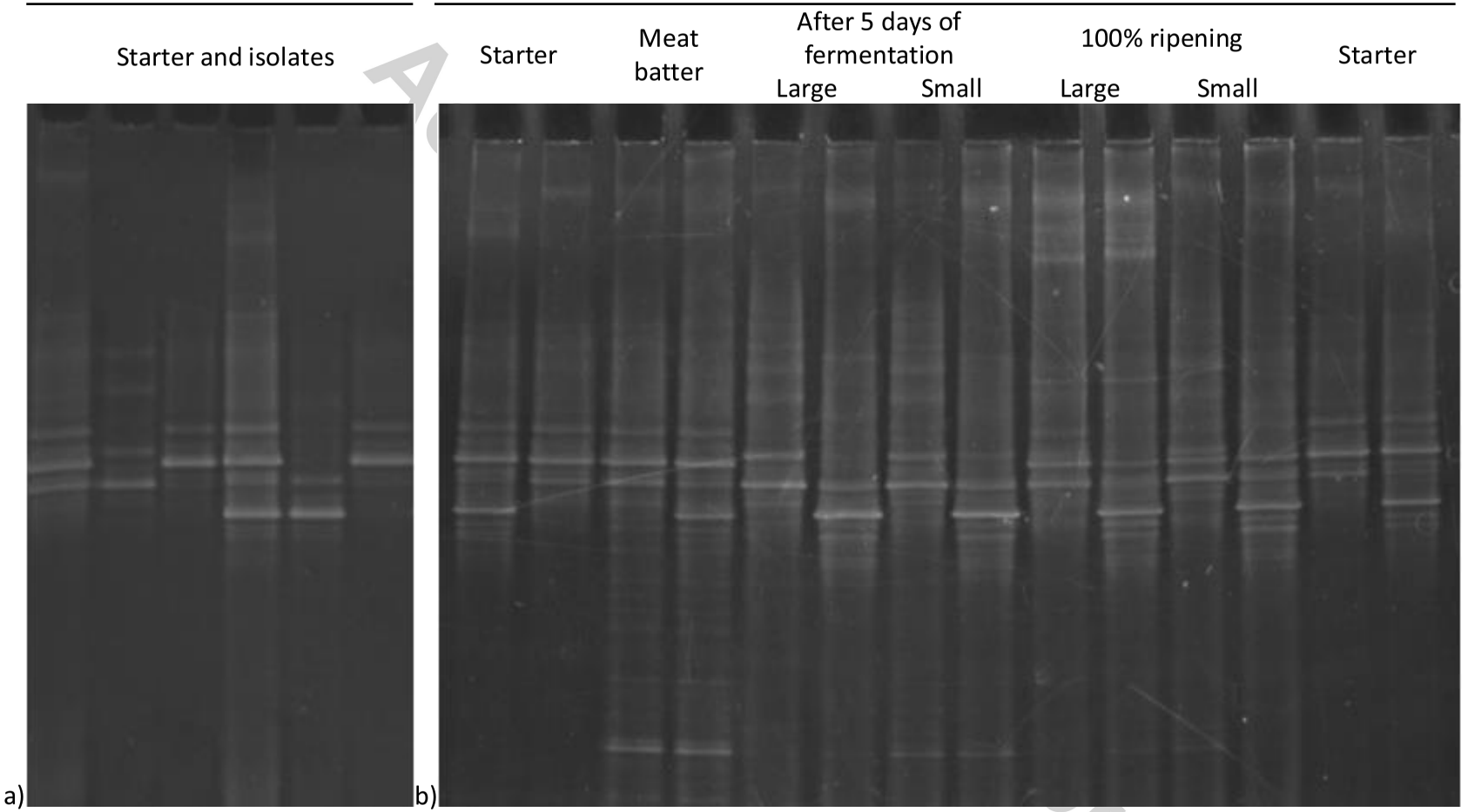
Fig. 3. DGGE profiles of the amplified 16S rRNA gene V2-V3 region obtained from starter cultures and reference isolates (a), starter cultures and samples taken during sausage production (b). Sx, Ls, Pp represent *Staphylococcus xylosus*, *Lactobacillus sakei* and *Pediococcus pentosaceus* isolates, respectively; lines SA and SB represent starter cultures A (*L. sakei/S. xylosus*, Bactoferm Sm 181, Chr. Hansen) and B (*P. pentosaceus/S. xylosus,* Bactoferm F1, Chr. Hansen), respectively; lines A and B represent samples collected during sausage production. Band nd was excised, cloned and subjected to sequencing.

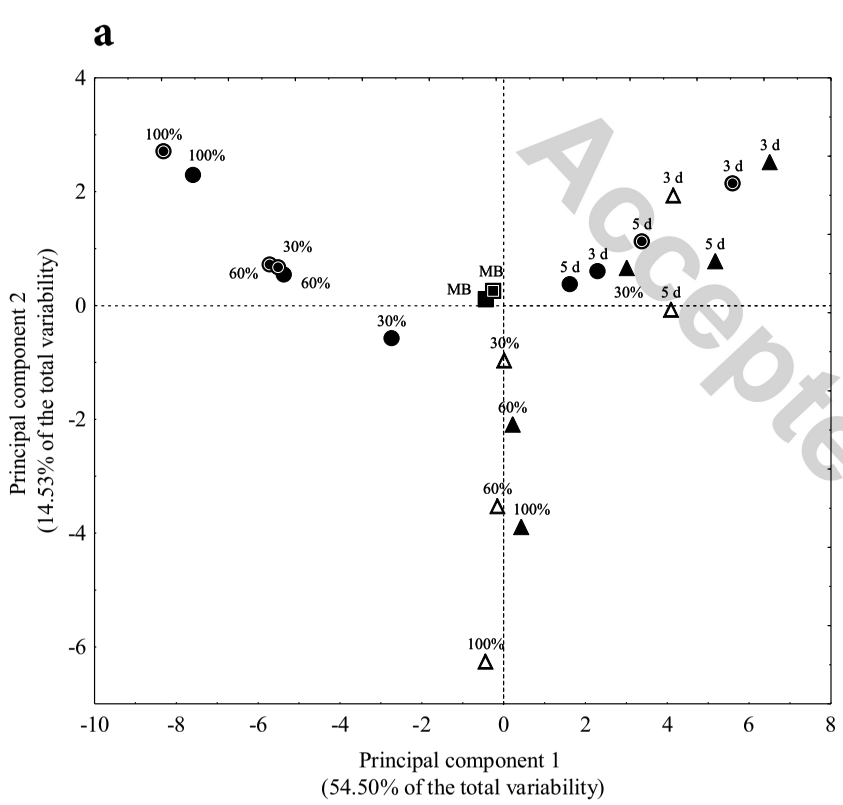
Fig. 4. a) PCA case coordinates for the first two factors of the meat batter and large and small diameter sausages during fermentation and ripening. Sample label: square: meat batter; circle: large diameter samples; triangle: small diameter samples; colorful label: inoculated with starter cultures A (*Lactobacillus sakei/Staphylococcus xylosus*); empty label: inoculated with

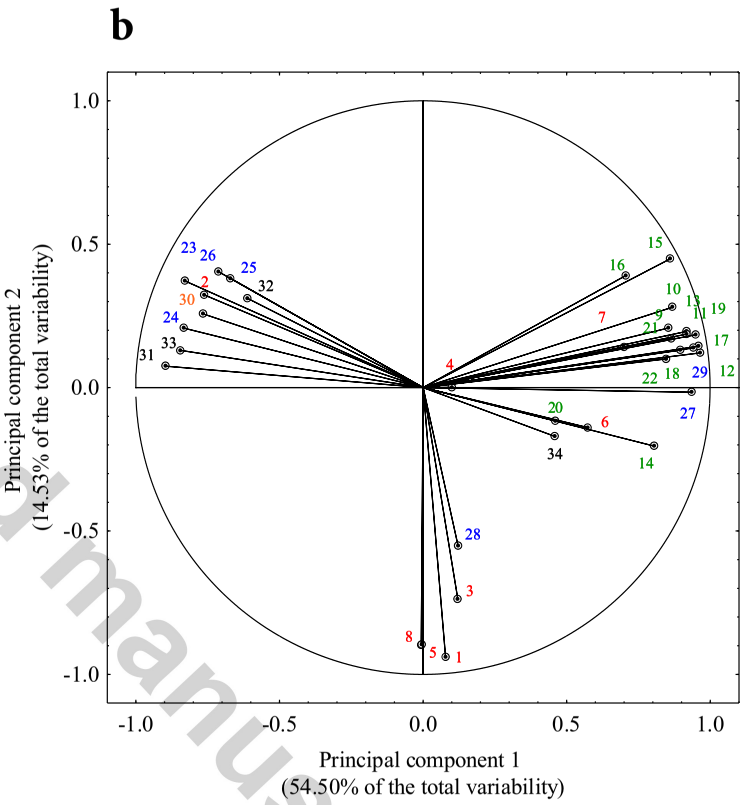
starter cultures B (*Pediococcus pentosaceus/Staphylococcus xylosus)*. b) PCA loading plot of the aroma compounds selected on the first two factors obtained from PCA.











1 Acetone
2 2-butanone
3 2,3-butanedione
4 2,6 dimethyl 4-heptanone
5 2-butanone, 3-hydroxy
6 1-octen-3-one
7 2,3 octanedione
8 3,5-octadien-2-one
9 Propanal
10 2-butanal
11 Pentanal
12 Hexanal
13 Heptanal
14 2-hexenal
15 Octanal
16 2-heptenal (E)
17 Nonanal
18 2-octenal (E)
19 Benzaldehyde
20 2-nonenal
21 2,4-nonadienal
22 2,4-dodecadienal
23 Isopropyl alcohol
24 Ethyl alcohol
25 2-butanol
26 1-propanol
27 1-pentanol
28 1-hexanol
29 1-octen-3-ol
30 Ethyl acetate
31 Acetic acid
32 Propanoic acid
33 Butanoic acid
34 Hexanoic acid

Table 1

Lactic and acetic acid concentration (g/kg) and microbial counts (log cfu/g), of the samples during fermentation and ripening. For each sampling time, different lower case letters represent statistically significant differences ($p < 0.05$) between samples according to Tukey test of the two-way ANOVA.

Sampling time[1]		Starter culture[2]	Lactic acid	Acetic acid	LAB	Coagulase negative staphylococci	*Enterobacter-iaceae*	Yeasts	Enterococci	*Pseudomona-daceae*
Meat batter (time 0)		A	4.45	<0.01[3]	6.31	6.03	2.56	2.15	2.20	5.04
		B	4.78	<0.01	6.33	6.35	2.84	1.91	2.19	4.98
After 3 days of fermentation	Large	A	7.47 [a]	0.08	8.84	6.00	<1[4]	4.13 [a]	4.23 [a]	5.56 [a]
		B	6.98 [b]	0.04	8.82	5.73	<1	4.65 [b]	<1[b]	4.03 [b]
	Small	A	7.93 [c]	0.10	8.62	5.93	<1	3.91 [a]	3.96 [c]	3.90 [b]
		B	7.79 [ac]	0.07	8.72	5.99	<1	2.99 [c]	<1[b]	4.09 [b]
After 5 days of fermentation	Large	A	8.41 [a]	0.15	8.39	4.53 [a]	<1	3.98 [a]	2.16 [a]	3.60 (±0.13)
		B	9.32 [b]	0.10	8.37	3.70 [b]	<1	4.32 [b]	1.63 [b]	3.61
	Small	A	9.08 [b]	0.11	8.14	4.51 [a]	<1	2.39 [c]	3.37 [c]	3.62
		B	10.36 [c]	0.15	8.16	5.15 [c]	<1	3.92 [a]	<1[d]	3.52
30% of ripening	Large	A	11.46 [a]	0.19	8.21	3.87 [a]	<1	4.79 [a]	3.97 [a]	3.22 [a]
		B	11.87 [a]	0.31	8.28	6.11 [b]	<1	4.96 [a]	<1[b]	3.57 [b]
	Small	A	8.88 [b]	0.21	8.14	7.17 [c]	<1	4.23 [b]	4.82 [c]	3.58 [b]
		B	10.23 [c]	0.26	8.26	7.23 [c]	<1	4.30 [b]	<1[b]	2.73 [c]
60% of ripening	Large	A	13.69 [a]	0.32 [a]	8.17	5.46 [a]	<1	4.52 [a]	3.56 [a]	<1[a]
		B	14.01 [a]	0.52 [b]	8.14	4.65 [b]	<1	4.58 [a]	3.27 [a]	2.30 [b]
	Small	A	8.86 [b]	0.29 [a]	8.58	8.01 [c]	<1	4.11 [b]	5.27 [b]	3.27 [c]
		B	10.07 [c]	0.35 [a]	8.47	7.95 [c]	<1	4.13 [b]	<1[c]	2.35 [b]
100% of ripening	Large	A	9.35 [a]	0.29 [a]	8.20	3.35 [a]	<1	3.64 [a]	2.11 [a]	<1[a]
		B	10.70 [b]	0.52 [b]	8.15	4.26 [b]	<1	4.07 [a]	<1[b]	<1[a]
	Small	A	9.46 [a]	0.35 [a]	8.51	8.81 [c]	<1	4.80 [b]	5.12 [c]	2.81 [b]
		B	10.61 [b]	0.31 [a]	8.45	8.27 [c]	<1	4.92 [b]	<1[b]	<1[a]

[1] 30%, 60% and 100% of ripening corresponded to 38, 69 and 105 days for large diameter sausages and 11, 17 and 28 days for small diameter sausages; [2] Starter cultures A (*Lactobacillus sakei*/*Staphylococcus xylosus*); starter cultures B (*Pediococcus pentosaceus*/*Staphylococcus xylosus*); [3] Under detection limit ((0.01 g/kg); [4] Under detection limit (1 log CFU/g). For two-way ANOVA the samples under the detection limit were set as 0 for organic acids and 1 for microbiological counts.

Table 2

Volatile compounds detected in the large and small diameter sausages during fermentation and ripening as determined by SPME-GC–MS. Data are expressed as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol). For each sampling time, different lower case letters represent statistically significant differences ($p < 0.05$) between samples according to Tukey test of the two-way ANOVA.

Volatile organic compounds	ID[1]	After 5 days of fermentation				End of ripening (T100%)			
		Large sausages		Small sausages		Large sausages		Small sausages	
		A[2]	B	A	B	A	B	A	B
Acetone	MS, RF	2.76 [a]	4.70 [b]	4.43 [b]	4.74 [b]	1.67 [a]	1.66 [a]	11.45 [a]	17.06 [b]
2-butanone	MS, RF	0.92 [a]	1.54 [b]	1.12 [a]	0.96 [a]	41.31 [a]	19.87 [b]	1.18 [c]	1.39 [c]
2,3-butanedione	MS, RF	-[3]	-	-	-	- [a]	- [a]	1.12 [b]	1.68 [c]
2,6-dimethyl-4-heptanone	MS	- [a]	1.53 [b]	0.46 [c]	- [a]	- [a]	- [a]	0.55 [b]	1.83 [c]
3-hydroxy-2-butanone	MS, RF	-	-	-	-	- [a]	- [a]	13.40 [b]	24.55 [c]
1-octen-3-one	MS	4.43 [a]	4.23 [a]	5.34 [b]	- [c]	- [a]	- [a]	2.51 [b]	2.38 [b]
2,3-octanedione	MS	1.51 [a]	2.85 [b]	2.90 [b]	1.94 [c]	-	-	-	-
3,5-octadien-2-one	MS	-	-	-	-	- [a]	- [a]	2.42 [b]	2.74 [b]
Total ketones		**9.62**	**14.85**	**14.25**	**7.65**	**42.99**	**21.52**	**32.63**	**51.63**
Propanal	MS, RF	0.80 [a]	6.01 [b]	10.11 [c]	10.22 [c]	- [a]	- [a]	2.52 [b]	2.68 [b]
2-butanal	MS, RF	0.85 [a]	0.71 [a]	1.26 [b]	1.15 [b]	- [a]	- [a]	0.38 [b]	- [a]
Pentanal	MS, RF	18.08 [a]	15.34 [b]	24.40 [c]	24.84 [c]	0.96 [a]	- [b]	9.27 [c]	8.10 [d]
Hexanal	MS, RF	169.07 [a]	180.86 [a]	230.21 [b]	231.42 [b]	5.20 [a]	5.57 [a]	113.59 [b]	94.44 [c]
Heptanal	MS	9.91 [a]	13.02 [b]	11.57 [ab]	13.27 [b]	1.40 [a]	0.81 [b]	6.64 [c]	6.60 [c]
2-hexenal	MS, RF	3.68 [a]	4.21 [a]	6.86 [b]	6.52 [b]	- [a]	- [a]	5.29 [b]	4.18 [c]
Octanal	MS, RF	14.71 [a]	19.10 [b]	23.29 [c]	22.39 [c]	- [a]	- [a]	1.78 [b]	0.86 [c]
2-(*E*)-heptenal	MS	7.24 [a]	17.38 [b]	7.16 [a]	- [c]	- [a]	- [a]	8.10 [b]	- [a]
Nonanal	MS, RF	14.61 [a]	28.97 [b]	25.24 [b]	20.03 [c]	2.16 [a]	1.51 [a]	15.17 [b]	14.53 [b]
2-(*E*)-octenal	MS	16.25 [a]	21.51 [b]	26.14 [c]	20.29 [b]	- [a]	- [a]	13.69 [b]	10.08 [c]
Benzaldehyde	MS, RF	10.44 [a]	15.53 [b]	16.63 [b]	14.66 [b]	- [a]	- [a]	5.17 [b]	4.47 [b]
2-(*E*)-nonenal	MS	7.64 [a]	9.88 [b]	9.79 [b]	7.79 [a]	- [a]	- [a]	4.03 [b]	3.31 [b]
2,4-nonadienal	MS	1.21 [a]	2.72 [b]	2.92 [b]	1.94 [ab]	- [a]	- [a]	1.76 [b]	1.4 [b]
2,4-dodecadienal	MS	- [a]	2.07 [b]	2.25 [b]	2.03 [b]	- [a]	- [a]	1.51 [b]	0.99 [c]
Total aldehydes		**274.48**	**337.31**	**397.82**	**376.54**	**9.72**	**7.89**	**188.92**	**151.64**
Isopropyl alcohol	MS, RF	-	-	-	-	1.73 [a]	1.24 [a]	- [b]	- [b]
Ethyl alcohol	MS, RF	1.62 [a]	3.51 [b]	1.06 [c]	0.98 [c]	7.61 [a]	12.38 [b]	2.66 [c]	1.72 [c]
2-butanol	MS, RF	-	-	-	-	39.74 [a]	36.71 [a]	- [b]	- [b]
1-propanol	MS, RF	-	-	-	-	2.73 [a]	3.54 [a]	- [b]	- [b]
1-pentanol	MS, RF	6.16 [a]	7.37 [b]	8.52 [c]	9.12 [c]	- [a]	- [a]	5.34 [b]	5.63 [b]
1-hexanol	MS, RF	- [a]	- [a]	8.51 [b]	32.25 [c]	- [a]	- [a]	8.40 [b]	18.56 [c]
1-octen-3-ol	MS, RF	13.42 [a]	12.05 [a]	17.56 [b]	17.18 [b]	- [a]	- [a]	11.01 [b]	6.41 [c]
Total alcohols		**21.2**	**22.93**	**35.65**	**59.54**	**51.81**	**53.86**	**27.4**	**32.33**
Ethyl acetate	MS, RF	0.21 [a]	0.36 [a]	- [b]	- [b]	1.65 [a]	3.21 [b]	- [c]	- [c]
Total esters		**0.21**	**0.36**	**-**	**-**	**1.65**	**3.21**	**-**	**-**
Acetic acid	MS, RF	- [a]	5.83 [b]	- [a]	2.52	57.1 [a]	65.6 [b]	10.36 [c]	14.97 [d]

Propanoic acid	MS, RF	-	-	-	-	1.12 [a]	4.01 [b]	- [c]	- [c]
Butanoic acid	MS, RF	-	-	-	-	4.88 [a]	4.88 [a]	- [b]	- [b]
Hexanoic acid	MS	3.86 [a]	1.47 [b]	5.78 [c]	4.35 [d]	- [a]	- [a]	2.03 [b]	- [a]
Total acids		**3.86**	**7.3**	**5.78**	**6.87**	**63.1**	**74.49**	**12.39**	**14.97**

[1]ID: Reliability of identification: MS, tentative identification by mass spectrum; RF: mass spectrum and retention time identical with a reference compound; [2] Starter cultures A (*Lactobacillus sakei*/*Staphylococcus xylosus*); starter cultures B (*Pediococcus pentosaceus*/*Staphylococcus xylosus*); [3] Not detected under the adopted conditions. For two-way ANOVA the samples under the detection limit were set as 0.