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Different *Lactobacillus* populations dominate in "*Chorizo de León*" manufacturing performed in different production plants

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ABSTRACT

"*Chorizo de Léon*" is a high-value Spanish dry fermented sausage traditionally manufactured without the use of starter cultures, owing to the activity of a house-specific autochthonous microbiota that naturally contaminates the meat from the environment, the equipment and the raw materials. Lactic acid bacteria (particularly *Lactobacillus*) and coagulase-negative cocci (mainly *Staphylococcus*) have been reported as the most important bacterial groups regarding the organoleptic and safety properties of the dry fermented sausages. In this study, samples from raw minced meat to final products were taken from five different producers and the microbial diversity was investigated by high-throughput sequencing of 16S rRNA gene amplicons. The diverse microbial composition observed during the first stages of "*Chorizo de Léon*" evolved during ripening to a microbiota mainly composed by *Lactobacillus* in the final product. Oligotyping performed on 16S rRNA gene sequences of *Lactobacillus* and *Staphylococcus* populations revealed sub-genus level diversity within the different manufacturers, likely responsible of the characteristic organoleptic properties of the products from different companies.

1. Introduction

Fermentation is one of the oldest strategies employed for meat preservation. Manufacture of dry fermented sausage originated during Roman times in the Mediterranean area, where climatic conditions favors the ripening process, and their manufacture is still widespread in these countries, with great regional diversity within and between countries (Lucke, 2000; Comi et al., 2005). Traditional dry fermented sausages are manufactured without the addition of starter cultures and thus indigenous microbiota from the environment, the equipment and the raw materials is responsible of the fermentation, safety and organoleptic properties of the end products (Talon et al., 2007; Ortiz et al., 2014). Meat contamination naturally occurs during slaughtering of the animals and along manufacturing, but the development of a specific microbiota depends on their capability to overcome environmental conditions (such as low pH and water activity) and to compete against other populations (Hammes et al., 1990; Rantsiou et al., 2005). Lots of efforts have been done to investigate microbial populations involved in dry fermented sausages in Europe (Aquilanti et al., 2016) and two bacterial groups have been found to be the main responsible: lactic acid bacteria (LAB) and gram-positive coagulase-negative cocci (CNC). LABs (particularly *Lactobacillus* spp.) constitute the most abundant group at the end of ripening and are responsible of the "tangy" flavor (Cocolin et al., 2001; Aymerich et al., 2003). Moreover, thanks to the decrease of pH resulting from sugar fermentation, they guarantee the safety of the product (Benito et al., 2007). Under certain environmental conditions, some LAB can produce several antimicrobial metabolites including organic acids, diacetyl, hydrogen peroxide, CO_2 and bacteriocins (Gálvez et al., 2007). CNC (particularly *Staphylococcus* spp.) participate in the development of color and flavor by degrading free

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amino acids and inhibiting the oxidation of unsaturated free fatty acids (Talon et al., 2004; Talon and Leroy, 2006; Lebert et al., 2007). Species-level diversity of the microbiota involved in dry sausages ripening is more complex: *Lactobacillus sakei, L. curvatus* and *L. plantarum* have been reported in Southern Europe dry fermented sausages, along with *Staphylococcus xylosus, S. saprophyticus* and *S. equorum*, as LAB and CNC members, respectively (Mauriello et al., 2004; García Fontan et al., 2007a,b; Talon et al., 2007; Villani et al., 2007).

Chorizo is a dry fermented sausage with high regional diversity composed mainly by pork meat, pork fat, salt and Spanish paprika (sometimes garlic, additives and conservatives are also added) and is usually manufactured without the use of starter cultures (Santos et al., 1997; Benito et al., 2007; Martín et al., 2007). *Chorizo de León* is a type of *chorizo* produced in the north-west region of *Castilla y León* that has received the Distinctive Mark of Guarantee by the Spanish Office of Patents and Brands in 2012 under the Council Regulation (EC) No 40/94, due to its special manufacture and organoleptic properties. *Chorizo* is an important part of the Spanish meat industry and many studies have been developed during the last two decades to better understand the microbial composition involved in the ripening of this high-value product (Rovira et al., 1997; García-Varona et al., 2000; Aymerich et al., 2003, 2006; Martin et al., 2006; Benito et al., 2007; Fonseca et al., 2013). Nevertheless, none of the studies was focused on the microbial ecology of *Chorizo de León* trade mark. The use of high-throughput sequencing (HTS) technologies targeting the taxonomic relevant 16S rRNA gene previously amplified by PCR, has allowed researchers to an in-depth characterization of the taxonomic composition and relative abundance of the microbial communities from a sample, saving time and money compared to the classic microbiological approaches (Ercolini, 2013; van Hijum et al., 2013; Cocolin and Ercolini, 2015). The 16S amplicon sequencing methods have become the most widely used in food microbiology research during the last years (Ercolini, 2013; De Filippis et al., 2016a) and when combined with fruitful bioinformatics analysis pipelines they can provide in depth microbial ecology insights also at sub-genus diversity level (Eren et al., 2014, 2015; De Filippis et al., 2016b).

This study aims to an in depth investigation of the microbial populations involved in the *Chorizo de León* ripening process, from the raw meat to the final products. The microbial communities and their dynamics were studied by sequencing of 16S rRNA gene amplicons, whereas oligotyping (Eren et al., 2013) was used for a sub-genus resolution of *Lactobacillus* and *Staphylococcus* populations.

2. Material and methods

2.1. Chorizo de León manufacturing and sampling

Five *Chorizo de León* factories (from now on A, B, C, D and E) in the north-west region of Castilla y León (Spain) were selected to carry out the study. The factories used similar traditional manufacturing procedures, without the use of microbial starters. Main ingredients were common, including pork lean meat (80-75%) mixed with pork fat (20–25%), salt (16–18 g/kg), Spanish paprika (26–28 g/kg), garlic (5–6 g/kg), additives (sugar and phosphate) and preservatives (nitrate and nitrite, maximum 250 mg/kg). Products from two production lines were taken from manufacturer C, as they used sweet (C-1) or spicy (C-2) Spanish paprika. Meat and fat were minced at 0–2 °C and mixed with the rest of ingredients in a kneader. Once homogenized, the marinated meat was let to repose without breaking the cold line. Marinated meat was then stuffed into a natural casing (cow or pork intestine) and left to dry for 12–36 h. After this period, the fresh sausage was transferred to the ripening room, where temperature (5–8 °C), humidity (80–90% in the beginning, decreasing slowly until 70% at the end of the process) and aeration (2–4 m/s) were controlled. Smoking took place at the beginning or during ripening process in the ripening room, where the sausages remained up to 25–30 days. Common time points were chosen for sampling at the different factories, including minced meat, marinated meat, marinated meat at the end of the repose phase, fresh sausage, sausage at the half of the ripening process (days 15–21) and final product (20–30 days depending thickness of the natural casings). We also took samples at the beginning of smoking in manufacture C (for both process C-1 and C-2) and at the beginning and the end of smoking in manufacture E.

2.2. Total DNA extraction

For each sample point, 25 g of meat were homogenized in a stomacher bag with 225 mL of saline peptone (Oxoid). Fifty mL were collected and big debris were discarded by centrifugation at 200*g* for 5 min. Interphase (around 40 mL) was carefully transferred to a new tube containing 6 mL Triton X-100 (Sigma) and 1% (w/v) trypsine (Sigma), incubated in a horizontal shaker for 30 min at 37 °C and then centrifuged at 6000 rpm for 30 min at room temperature. *Pellet* was carefully washed twice with 1 mL of sterile PBS 1X and used for total DNA extraction by using QIAmp DNA Mini Kit (Qiagen) following manufacturer instructions. DNA concentration was quantified with Qubit® (Invitrogen).

2.3. 16S rRNA gene amplicon library preparation and sequencing

Microbial diversity was studied by pyrosequencing the amplified V3-V4 region of the 16S rRNA gene by using primers and PCR conditions previously reported (Klindworth et al., 2013). Samples multiplexing, library purification and sequencing was carried out as described in the "16S Metagenomic Sequencing Library Preparation" guide by Illumina.

Libraries were sequenced by Genomix4Life s.r.l. (Salerno, Italy) on a MiSeq platform (Illumina Italy s.r.l., Milan, Italy), leading to 250bp, paired-end reads.

2.4. Bioinformatics and data analysis

Quality of the 16S rRNA amplicon raw reads was evaluated by using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Bases with a Phred score below 20 within a 25-bp-long window were trimmed and reads shorter than 150 bp were discarded by using Prinseq (http://prinseq.sourceforge.net/). Paired-ends reads were then merged using FLASH (Magoč and Salzberg, 2011). Primers and barcodes were removed. Sequences were then analyzed using QIIME 1.9.0 software (Caporaso et al., 2010), with a pipeline previously described (De Filippis et al., 2014). In order to avoid biases due to different sequencing depths, all samples were rarefied to 27,000 reads per sample and singleton OTUs were discarded. Alpha (Good's coverage, Chao1 richness and Shannon diversity indices) diversity measures and Weighted UniFrac (Lozupone and Knight, 2005) distance matrices were obtained through QIIME. Reads assigned to *Lactobacillus* and *Staphylococcus* genera were extracted, and entropy analysis and oligotyping were carried out as described by developers (Eren et al., 2013). High-entropy positions were chosen to compute the oligotypes (-C option): 1, 2, 30, 76, 82, 93, 101, 102, 106, 107, 111, 119, 120, 123, 124, 125, 139, 191, 240, 241, 242, 243, 247, 249, 251, 252, 254, 261, 262, 263, 268, 275, 281, 285, 286, 301, 317, 318, 357, 362, 385, 386, 411, 412, 420 and 425 and 2, 67, 74, 105, 108, 112, 119, 123, 126, 228, 231, 241, 263, 275, 282, 307, 318, 326, 331, 350, 354, 383, 386,

396, 409, 411, 427, 412, 420 and 425 for *Lactobacillus* and *Staphylococcus* datasets, respectively. To minimize the impact of sequencing errors, oligotypes were required to be represented by at less 100 reads (-M option) and present in at less 10 samples (-s option). Representative sequences were queried against the NCBI nr database by using BLASTn, and the top hit was considered for taxonomic assignment. Plotting was carried out in R environment (https://www.r-project.org), by using *ggplot2*, *made4* and *factoextra* packages.

2.5. Nucleotide sequence accession number

All the sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (PRJNA386744).

3. Results

3.1. The microbial population involved in the ripening of Chorizo de León

Fig. 1A and B shows the relative abundance of the 13 most abundant genera in the different manufacturing steps from the different making companies and from the overall process, respectively. Microbial communities evolve from the minced meat to the beginning of the ripening process, where the microbial composition becomes less diverse and stable until the end of the process, with a progressive decrease of Chao1 and Shannon diversity indices (alpha diversity estimations are reported in the Supplementary Table S1). Differences in microbiota composition were observed among the five manufactures. *Pseudomonas* was the most abundant genera found in minced meat from all the manufactures, followed by *Carnobacterium* in manufacturers A and B or *Brochothrix* in manufacturers C1, C2, D and E. The microbial composition of the meat changed after the marinating process. The spoilage associated bacteria *Brochothrix* became the most abundant genus in manufacturers A, B and D. In manufacturer C, for both processes C1 and C2, *Staphylococcus* dominated, followed by *Lactobacillus*, while lower levels of *Brochothrix* were observed. On the contrary, *Bacillus* became the dominant genus in E after marinating. The microbial pattern did not change until the beginning of the ripening process after the sausage production. At the half of the ripening process, the microbial population was replaced by *Lactobacillus* in all manufacturers (Fig. 1A and B). *Lactobacillus* relative abundance was stable until the end of the process reaching abundances of 83.8% in *Chorizo de León* from manufacturer B and higher than 92.0% in those from manufacturers C-1, C-2 and D. Its abundance was lower (65.1 and 69.2%) in final products from manufactures A and E, where *Brochothrix* (22.1%) and *Staphylococcus* (19.7%) reached higher levels, respectively (Fig. 1A and B).

The differences between samples were evaluated by Weighted UniFrac phylogenetic metric (Fig. 2). Fresh minced meat from manufactures C, D and E showed similar microbiota and clustered closer. The microbial composition of the samples evolved from the minced meat (composed mainly by *Pseudomonas*) through the marinating and repose phases, where the microbiota from the different samples showed differences according to the making company. Nevertheless, samples from the late-ripening stages clustered together, showing a similar microbial evolution in *Chorizo de León* ripening (where *Lactobacillus* almost dominate) independently from the initial meat contamination (Fig. 2).

3.2. Sub-genus diversity analysis of the key genera Lactobacillus and Staphylococcus

Lactobacillus and *Staphylococcus* represent the most important bacterial populations in dry fermented sausages fermentation and ripening as well as in the development of their organoleptic and safety properties. Therefore, we oligotyped reads assigned to these genera in order to explore differences at sub-genus level, possibly associated to the different *Chorizo de León* manufactures. *Lactobacillus* was present in all the samples and its relative abundance ranged from 0.05 to 96.49% (Fig. 1A and B). The minimum abundance occurred in minced meat (0.52% average), increasing slightly during the marinating and repose steps, depending on the producing company. *Lactobacillus* population increased widely as the ripening process begun, remaining stable until the end of the process (81.89 and 83.35% average at the half of the ripening process and in *Chorizo de León* final products, respectively). *Staphylococcus* was also found in all the samples, although its mean relative abundance was lower and its distribution depended on the manufacturer. A total of 65 and 23 oligotypes were found for *Lactobacillus* and *Staphylococcus*, respectively. BLASTn identification of the oligotype representative sequences are reported in Supplementary Table S2, and the number of *Lactobacillus* or *Staphylococcus* oligotypes within each sample can be visualized in Supplementary Figs. S1A or S1B, respectively. BLASTn taxonomic assignment revealed five different *Lactobacillus* species and *L. sakei* was found as the most abundant specie (93.0% of all *Lactobacillus* oligotypes identified), followed by *L. fuchuensis* (2.3%), *L. plantarum* (2.2%), *L. curvatus* (2.0%) and *L. algidus* (0.5%). *Lactobacillus* oligotypes L1 and L2 (both identified as *L. sakei*) were the most abundant overall. Fig. 3A shows the relative abundance of *Lactobacillus* oligotypes in the half of the ripening process (inner circle) and in the final products (outer circle) in the different making companies. Although the most abundant *Lactobacillus* oligotypes (L1 and L2) were the same in most of the manufactures analyzed, differences in the oligotype pattern were observed (Fig. 3A). Final products from manufacture E showed L1 (33.4%) and L2 (15.6%) associated to high levels of L6 (*L. curvatus*, 14.0%) and L22 (*L. curvatus*, 6.2%), *Lactobacillus* oligotypes that were present at very low abundance in samples from the other factories. Finally, L5 (*L. fuchuensis*, 15.9%) was the second most abundant *Lactobacillus* oligotype in manufacture A, followed by L2 (15.5%) and L17 (*L. fuchuensis*, 8.2%). Fig. 3B shows the distribution of *Lactobacillus* oligotypes among the different samples. Minced meat and intermediate samples clearly clustered apart from samples at the half and the end of ripening, that showed a higher number of different *Lactobacillus* oligotypes (see Supplementary Fig. S1A). Some oligotypes appeared associated mainly to samples from a specific production plant. *Lactobacillus* oligotypes L5 and L17 (both *L. fuchuensis*) were found mainly in products from manufacturer A, whereas L6 and L22 (both *L. curvatus*) were associated to products from manufacturer E, that implies that 88.1% and 79.4% of all *L. fuchuensis* and *L. curvatus* found in this study, respectively, were present in those facilities.

Twenty-three oligotypes were found for *Staphylococcus*. BLASTn identification revealed five species, where *S. xylosus* was found to be the most abundant (84.5% of all *Staphylococcus* oligotypes), followed by *S. sciuri* (8.5%), *S. saprophyticus* (3.2%), *S. equorum* (1.9%) and *S. condimenti* (1.9%). Fig. 4 shows the relative abundance of the different *Staphylococcus* oligotypes across the samples. S1 and S2 (both *S. xylosus*) were the most abundant and represented almost 60% of all *Staphylococcus* oligotypes found. *Staphylococcus* was particularly abundant during marinating to fresh sausage stages of *Chorizo de León* manufacture in companies B, C-1 and C-2 (Fig. 1A and B). Surprisingly, the most abundant *Staphylococcus* oligotype found in E, the only manufacture where *Staphylococcus* was found

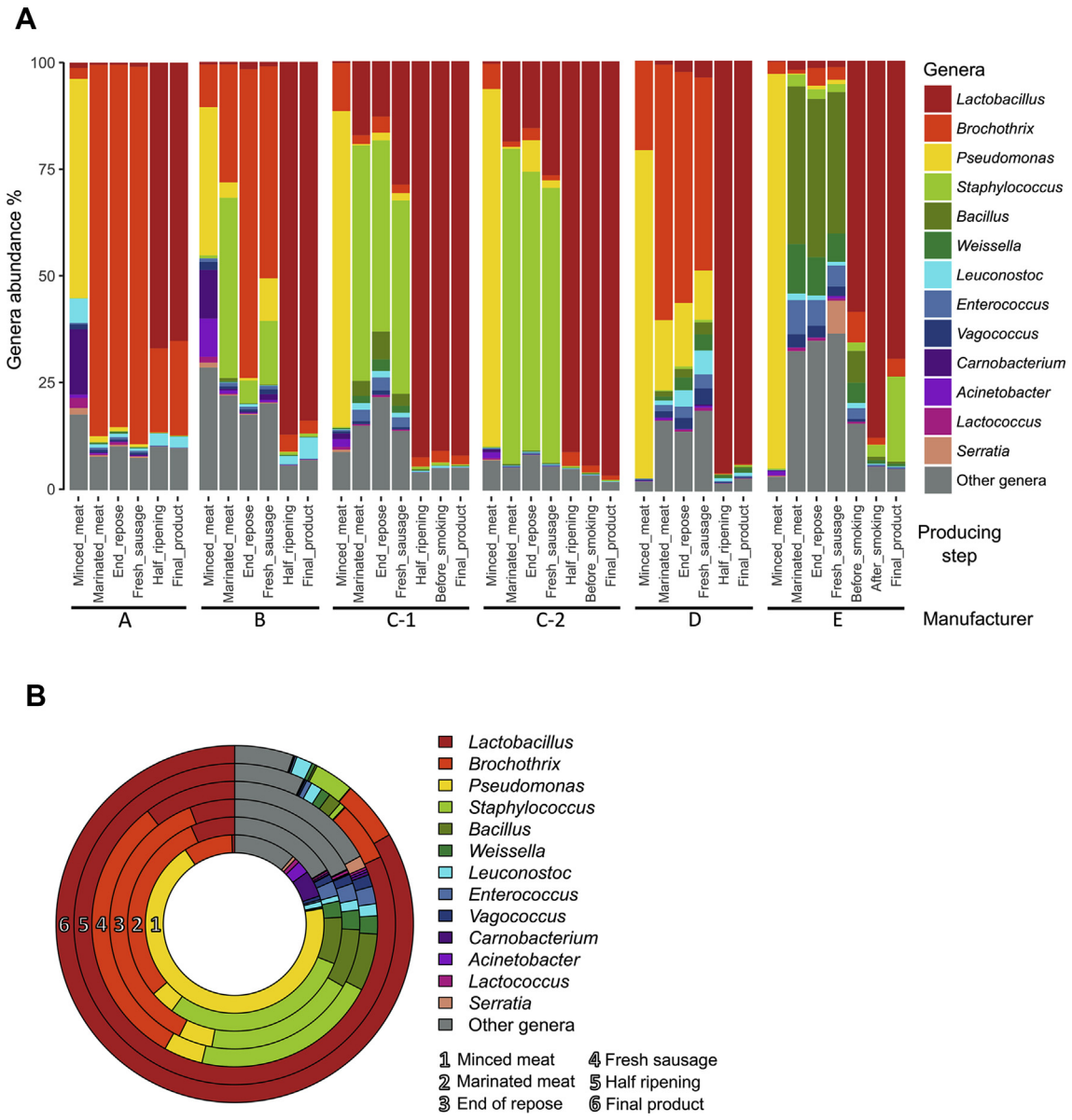

Fig. 1. Relative abundance (%) of the 13 most abundant genera found in the different manufacturers (**A**) or in the process overall (**B**). Circles and their numbers in **B** reveals the common manufacturing stage from the minced meat (inner circle) to the final products (outer circle).

at higher levels in the final product (19.7%, Fig. 1), was identified as *S. equorum* (>96% of *Sthaphylococcus* oligotypes in E).

4. Discussion

Fermentation of meat products is a practice that has been widely employed in European countries since Roman times (Lucke, 2000). Although similar manufacturing methods are used, fermented sausages differ widely between countries and also within the same country (Comi et al., 2005). Traditional meat fermentation is a complex biological phenomenon carried out by the desirable action of certain autochthonous microorganisms that come from the equipment, the environment and the raw material. The success of such bacteria in colonizing the food, relies on their ability to overcome adverse physicochemical factors (low temperature, low water activity, low pH, high salt content) and to outcompete other microorganisms (Rantsiou et al., 2005; Nieminen et al., 2012). First studies on microbial composition of fermented sausages date back to 1974 (Lucke, 1974). Most studies are mainly focused on LAB and CNC populations, as their behavior during ripening is important in establishing the sensorial, safety and nutritional properties of the final products, as well as in extending the shelf-life (Fonseca et al., 2013).

Several studies have investigated the Spanish dry fermented sausage (*chorizo*) microbial ecology (Aquilanti et al., 2016), but none focused on *Chorizo de León*.

HTS methods have changed the way of studying food microbial ecology, giving the researchers the opportunity of an in-depth characterization of the microbial ecology of different environments and to monitor the dynamics of potential pathogens, spoilage and fermentative microbes (Cocolin and Ercolini, 2015; De Filippis et al., 2016a).

Microbial and chemical changes are indicators of meat quality or freshness (Dainty, 1996; Nychas et al., 2008). The meat spoilers *Brochothrix* and *Pseudomonas* were found as dominant in the first stages of *Chorizo de León* manufacture (from minced meat along

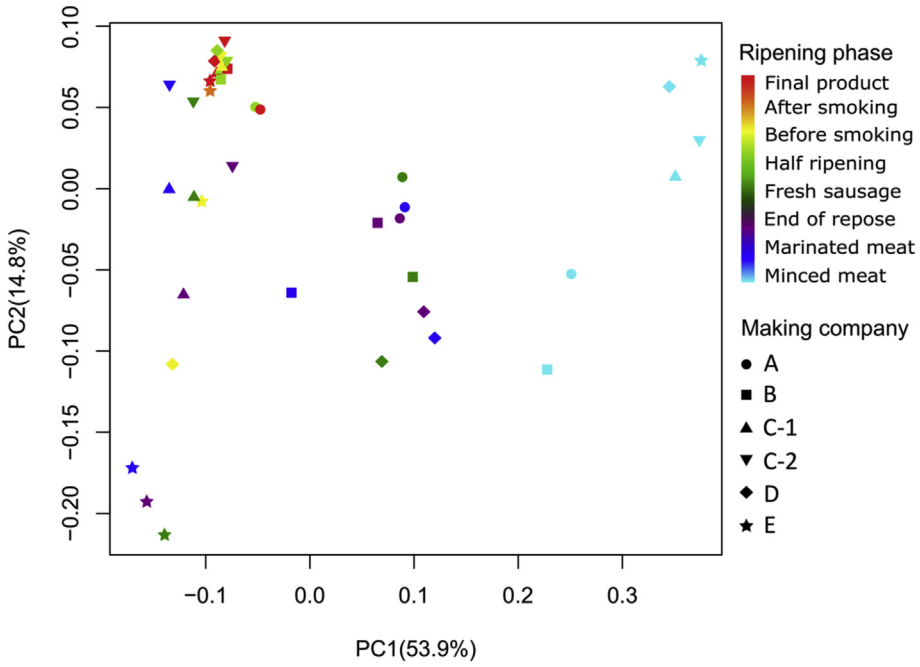

Fig. 2. Principal Coordinates Analysis (PCoA) shows similar microbial dynamics for the different manufacturers, where diverse microbial composition during the first manufacturing stages evolves along ripening to a microbiota dominated by *Lactobacillus* and so plots clustered together independently to the manufacturer. Plot shapes are specific for each making company and colors reveal the "*Chorizo de Léon*" manufacturing stage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

marinating to fresh sausage) and their abundance decreased as ripening process developed. These microorganisms are associated to meat spoilage during refrigerated storage (De Filippis et al., 2013) and may produce undesirable off-flavours (Ercolini et al., 2010; Casaburi et al., 2014, 2015). Moreover, they often withstand during early-fermentation stages, while decrease during ripening (Samelis et al., 1998; Comi et al., 2005; Drosinos et al., 2005; Rantsiou et al., 2005; Chevallier et al., 2006).

Similar microbial dynamics were observed along *Chorizo de León* manufacture in the different making companies. As *Chorizo de León* ripening proceeded, *Lactobacillus* became dominant in samples from all the manufactures and dominated in the final products. LAB presence during ripening is crucial, as they are responsible of some organoleptic properties and safety of the products (Benito et al., 2007). Sugar added during marinating is fermented to lactic acid, decreasing the pH and hindering the growths of low tolerant bacteria, while enhancing *Lactobacillus* growth (Hammes et al., 1990; Leroy and De Vuyst, 2004). *Lactobacillus* contributes to flavor due to their carbohydrate metabolism and the resulting production of lactic and acetic acids and volatile compounds (Molly et al., 1996). Natural casings (usually preserved in high salt and low temperature conditions) have been proposed as a source of well-adapted LAB, although its origin form the processing environment is still unclear (Bedia et al., 2011). Several *Lactobacillus* species (such as *L. sakei*, *L. plantarum* and *L. curvatus*) have been found to be present in natural casings and to widespread in the fermented sausages during ripening (Pisacane et al., 2015; Tremonte et al., 2017).

Investigation of *Lactobacillus* populations at sub-genus level was carried out in this study. Indeed, closely related species may harbor different genetic pools and therefore may be responsible of the unique properties of *Chorizo de León* from the different making companies. Oligotyping was previously shown to be effective in exploring sub-genus diversity in natural environments, human and animal gut, highlighting diversity associated to ecologically relevant factors (De Filippis et al., 2016b; Eren et al., 2013, 2014) and it has been recently found useful to characterize *Pseudomonas* spp. in meat and dairy environment (Stellato et al., 2017). In this case, the oligotyping allowed to reach a confident identification at species level within the genera *Lactobacillus* and *Staphylococcus*.

A succession of *Lactobacillus* oligotypes was observed during manufacturing and ripening and the oligotype pattern differentiated the final products from the different manufactures. *L. sakei* was the most abundant *Lactobacillus* species found overall and dominated the final products from all manufactures. Accordingly, other studies have also found it as dominant in other *chorizo* products (Aymerich et al., 2006; Fonseca et al., 2013). *L. sakei* is the most abundant LAB found in dry fermented sausages and its predominance over other LAB, such as *L. curvatus* or *L. plantarum*, is due to its superior competitiveness, which can be explained to its psychrotrophic and salt-tolerant nature and its specialized metabolic repertoire that is well adapted to the sausage environment, including the arginine deiminase pathway and the utilization of nucleosides (Chaillou et al., 2005; Rimaux et al., 2011; Ravyts et al., 2012). Talon et al. (2008) demonstrated that the acidification produced by the inoculation of *L. sakei* to the sausages favored their dehydration and improved the overall texture and consistency of the final products. Furthermore, *L. sakei* possess heme-dependent catalase activity (which is active in meat products since these substrates contain heamin) that hydrolyses hydrogen peroxide (produced by most LAB) and thus preventing rancidity and discoloration of the final products (Ammor and Mayo, 2007). *L. sakei* has been found to dominate fermented sausages and intra-species analysis may allow identifying strains with desirable properties (Cocolin and Ercolini, 2015). Oligotyping allowed the identification of different *L. sakei* oligotypes along *Chorizo de León* ripening, two of them being especially abundant in final products from all manufacturers. This finding is in agreement with a recent study that identified that different *L. sakei* biotypes dominating meat fermented sausages (Fontana et al., 2016).

Other *Lactobacillus* species where found in *Chorizo de León* in our

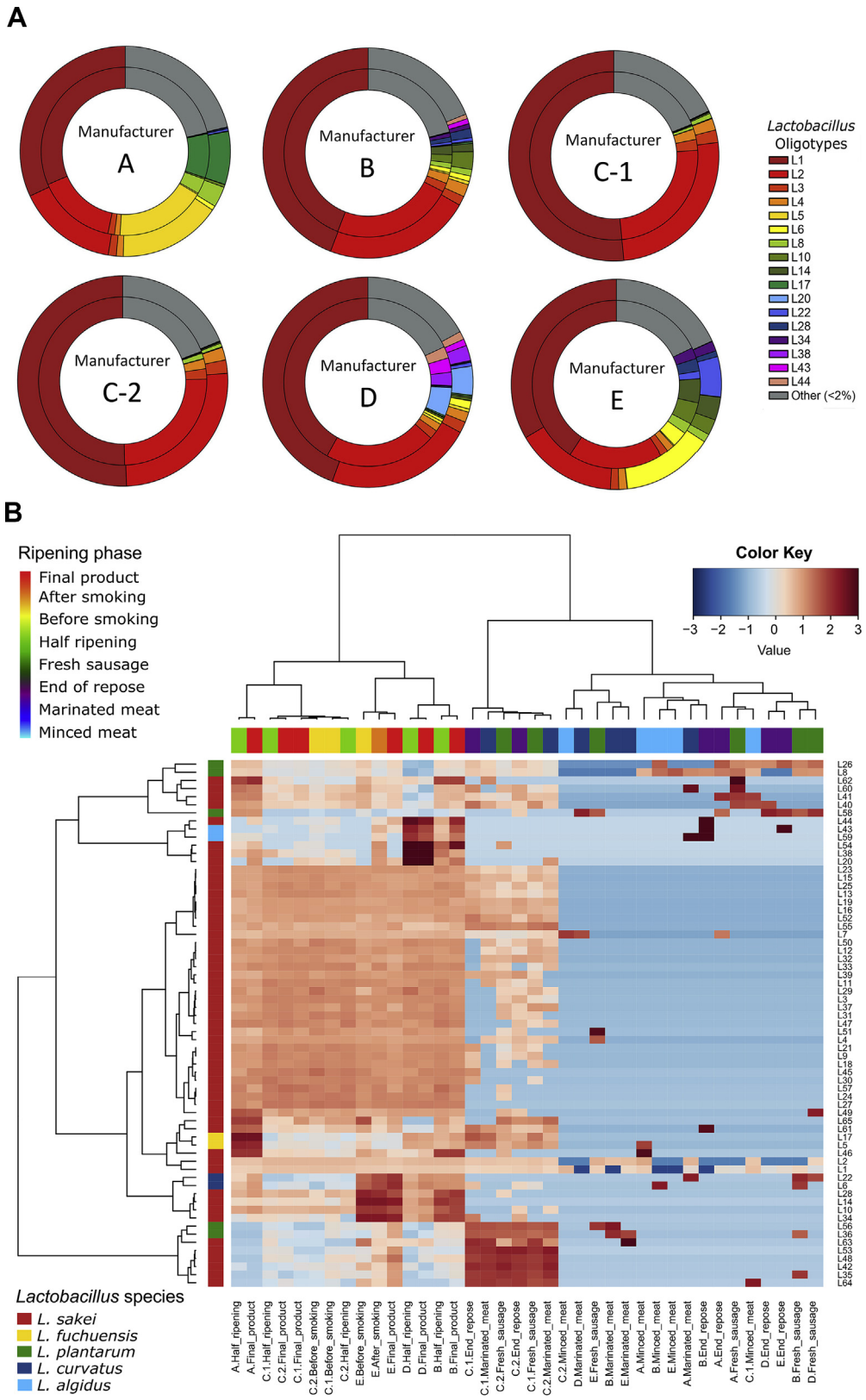

Fig. 3. A. Pie graph with the relative abundance of *Lactobacillus* oligotypes at the half of the ripening process (inner circle) and in the final "*Chorizo de Léon*" products (outer circle). **B**. Pseudo-heat map representing the distribution of *Lactobacillus* oligotypes (each row represent the 100% of each *Lactobacillus* oligotype) through the different samples (in columns). Manufacturing stage of each sample is represented in the horizontal bar over the figure. Species level of each *Lactobacillus* oligotype can be seen in the vertical bar in the left side of the figure.

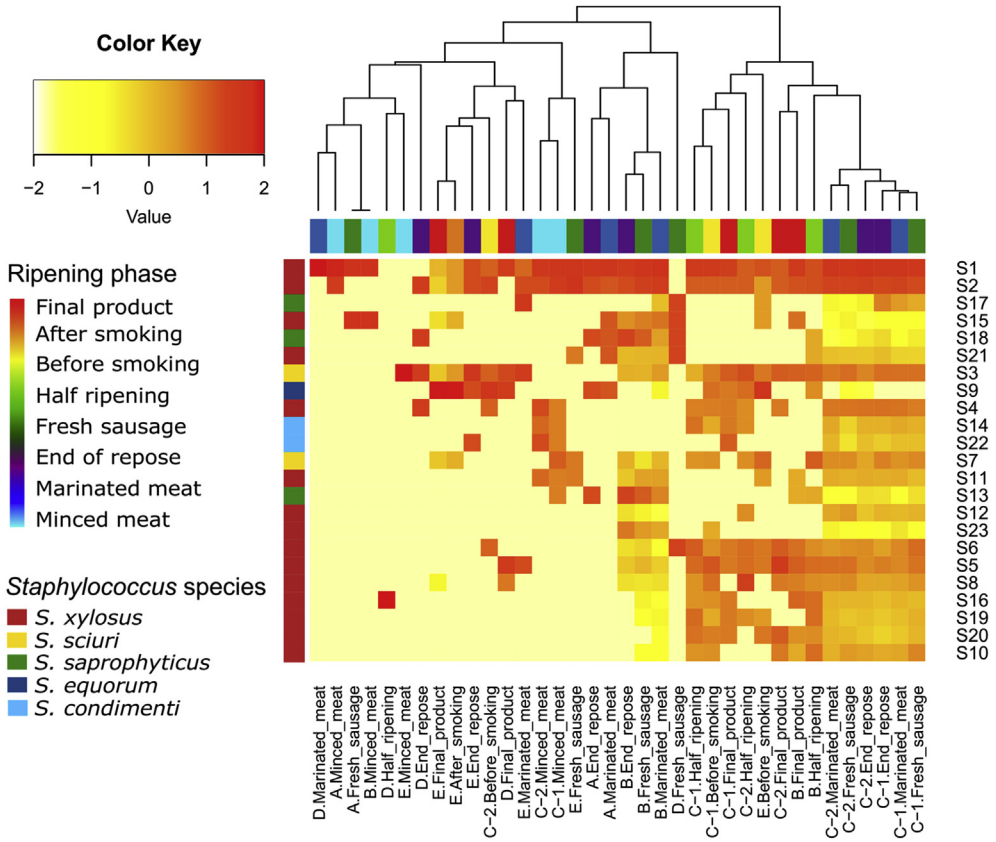

Fig. 4. Pseudo-heat map showing the distribution and relative abundance of *Staphylococcus* oligotypes (in rows) within the different samples (in columns). Manufacturing stage of each sample is represented in the horizontal bar over the figure. The vertical bar in the left side of the figure reveals the species identification of each *Staphylococcus* oligotype.

study, although in less proportion than *L. sakei*. *L. curvatus* and *L. plantarum* have been previously isolated from *chorizo* (Rovira et al., 1997), European (Drosinos et al., 2005; Rantsiou and Cocolin, 2006; Ammor and Mayo, 2007) and Argentinian (Fontana et al., 2005) sausages. *L. plantarum* is sometimes found in commercial starters for meat, but it is not always able to prevent spontaneous outgrowth of non-starter LAB with undesirable effects and may also give rise to a product with overacidity, which is not well perceived by the consumer (Garriga et al., 1996; Hugas and Monfort, 1997; Coppola et al., 2000).Oligotyping of *Staphylococcus* reads revealed *S. xylosus* as the most abundant specie (84.5% of all *Staphylococcus*) and that dominated during the marinating to the fresh sausage process in several manufacturers while decreased as the ripening process developed. This is in agreement with recent studies that identified a marked decrease of *S. xylosus* in dry fermented sausages during the ripening process as *L. sakei* increased (Połka et al., 2015). *S. xylosus* was identified as the most common specie in *chorizo*, Greek and Italian traditional sausages (García-Varona et al., 2000; Cocolin et al., 2001; Papamanoli et al., 2003; Blaiotta et al., 2004; Mauriello et al., 2004; Aymerich et al., 2006; Martin et al., 2006). Even *Staphylococcus* appear in a lesser extent than LAB, it plays a role in desirable sensory quality of sausages (Montel et al., 1998; Leroy et al., 2006). *Staphylococcus* species or strains differ in their ability to produce flavor compounds although it is not clear yet which species are best suited for flavor enhancement during fermented dry sausage production (Ravyts et al., 2009, 2010). For instance, the use of *S. xylosus* has been mentioned to yield rounder and less acidic taste sausages compared to *S. saprophyticus* and *S. equorum* (Samelis et al., 1998; Søndergaard and Stahnke, 2002). Nevertheless, *S. xylosus* may be unable to carry out the fermentation entirely, as the sausage fermentation conditions are not favorable for its growth (Ravyts et al., 2012). In this study, *Staphylococcus* was found mainly during the marinating process until the production of the fresh sausage. Interestingly, *S. equorum* was dominant in the final product of manufacture E. This specie has been found in *chorizo* (Fonseca et al., 2013) and in French fermented sausages (Corbière Morot-Bizot et al., 2006; Leroy et al., 2006). *S. equorum* may play an important role in the ripening process (Ravyts et al., 2012). Fonseca et al. (2013) suggested that this specie could have been underestimated in other studies due to the difficulties of in discriminating it from *S. xylosus*, especially when only phenotypical methods are used.

The microbial dynamics in *Chorizo de León* fermentation was clear from our study. The start of the drying process of the fresh sausage determined the beginning of the ripening and represented a change in the microbial populations: *Lactobacillus* became the most abundant genera and dominated in the final products. Evaluation of sub-genus diversity of *Lactobacillus* and *Staphylococcus* revealed differences in the microbiota associated to the manufactures studied, which can be responsible of the specific organoleptic properties of the sausages from each producer. Differences in sub-genus diversity patterns may be associated to a facility-specific resident microbiota. Many studies remark the importance of country- or house-specific microbiota in the development of traditionally manufactured products (Rantsiou et al., 2005; Lebert et al., 2007). The increasing knowledge on the microbiota involved in *chorizo* manufacturing process, as well as the differences associated to a facility-specific microbiota, may help in the development of specific starters, well adapted to the manufacturing

environment and thus capable of dominating the meat microbiota during production, in order to standardize the ripening process and to control the quality and safety of such appreciated products.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2017.09.009.

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