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Abstract

Nitrite is widely used in meat products as multifunctional additive, combining flavour and colour curing properties with antioxidant and antimicrobial effects. However, nitrite may form reaction products (i.e. nitrosamine) potentially carcinogenic for humans. Meat industry, accomplishing consumers' demand of nitrite-free products, is seeking for natural alternatives to nitrite such as plant-based extracts. Three types of dry-fermented sausages were manufactured: NIT, containing 30 ppm of sodium nitrite; GSE, replacing nitrite with grape seed extract and olive pomace hydroxytyrosol; CHE, replacing nitrite with chestnut extract and olive pomace hydroxytyrosol. High-throughput sequencing was used to analyse microbial consortia, which were correlated with physical and chemical parameters. Prokaryotic community composition was similar among treatments with predominance of Staphylococcus xylosus and Lactobacillus sakei species, accounting together for 87% of the total community. However significant differences were observed for both OTUs presence/absence and relative abundance. Ten genera were differently abundant between treatments, a lower abundance of Photobacterium, a meat spoilage bacterium, was observed in nitrite-free samples. In conclusion, NGS analysis showed that prokaryotic community composition is similar in GSE and NIT dry-fermented sausages while CHE showed more differences in both composition and relative abundance of the different taxa.

Keywords	Local breed; Nitrite; Prokaryotic community; Natural extracts; Pig.

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ALIMENTARI, AMBIENTALI E FORESTALI

Florence, July 09th 2019

Dear Editor,

Please find enclosed the manuscript by Pini *et al*., "**Characterization of microbial community composition in Italian Cinta Senese dry-fermented sausages using natural extracts in place of sodium nitrite"** submitted for publication on *Food Microbiology*.

The manuscript here presented is an original work and it has not been submitted earlier to *Food Microbiology*.

The consumers' demand for products "free from" is constantly growing, opening several potential market opportunities. In particular, for meat products it is important to evaluate alternatives to nitrate and nitrite after World Health Organization has linked their consumption to increased cancer risk.

This study aimed to explore the use of two natural extracts (grape seed and chestnut extracts) as potential alternatives to sodium nitrite in natural dry-fermented salami. High-throughput sequencing was used to analyze microbiota composition in salami treated with nitrite or with the two natural extracts. The use of the two natural extracts do not drastically alter salami microbiota composition, however some differences were observed and discussed. On the basis of microbiological, physical and chemical parameters grape seed and chestnut extracts may be then considered good alternatives to the use of nitrite for curing salami.

Thank you for your consideration.

Yours sincerely,

Prof. Carlo Viti Ph.D

Highlights

- Nitrite-free samples, treated with grape seed and chestnut extracts, were characterized by lower pH levels probably due to a higher activity of *Lactobacillaceae*.

- Dry-fermented sausages microbiota characterization using NGS technology. *Staphylococcus xylosus* and *Lactobacillus sakei* are the most represented species.

- Prokaryotic communities of dry-fermented sausages treated with grape seed extracts, chestnut extracts or sodium nitrite showed differences for both OTU composition and relative abundance.

- In nitrite-free samples a lower abundance of the *Photobacterium* genus was observed.

Characterization of microbial community composition in Italian Cinta Senese dry-fermented sausages using natural extracts in place of sodium nitrite

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Abstract

Nitrite is widely used in meat products as multifunctional additive, combining flavour and colour curing properties with antioxidant and antimicrobial effects. However, nitrite may form reaction products (i.e. nitrosamine) potentially carcinogenic for humans. Meat industry, accomplishing consumers' demand of nitrite-free products, is seeking for natural alternatives to nitrite such as plant-based extracts.

Three types of dry-fermented sausages were manufactured: NIT, containing 30 ppm of sodium nitrite; GSE, replacing nitrite with grape seed extract and olive pomace hydroxytyrosol; CHE, replacing nitrite with chestnut extract and olive pomace hydroxytyrosol. High-throughput sequencing was used to analyse microbial consortia, which were correlated with physical and chemical parameters.

Prokaryotic community composition was similar among treatments with predominance of *Staphylococcus xylosus* and *Lactobacillus sakei* species, accounting together for 87% of the total community. However significant differences were observed for both OTUs presence/absence and relative abundance. Ten genera were differently abundant between treatments, a lower abundance of *Photobacterium,* a meat spoilage bacterium, was observed in nitrite-free samples.

In conclusion, NGS analysis showed that prokaryotic community composition is similar in GSE and NIT dry-fermented sausages while CHE showed more differences in both composition and relative abundance of the different taxa.

Keywords: Local breed, Nitrite, Prokaryotic community, Natural extracts, Pig

1. Introduction

Meat and processed meat products are important constituent of most of western diets. Since 1970's evidences for an association between meat consumption and the risk of some types of cancer began to emerge (Johnson, 2017), as well as for the onset of several cardiovascular diseases (Jiménez-Colmenero, 2007). In October 2015, the International Agency for Research on Cancer (IARC) under World Health Organization (WHO) classified processed meat as carcinogen (Group I) and red meat as probable carcinogen (Group 2A) (Jiang and Xiong, 2016). In processed meat products, nitrite and nitrate are used as curing agents. Nitrite (and nitrate, which is reduced to nitrite along curing) play a pivotal role in flavour development, in controlling lipid oxidation and food safety (Majou and Christieans, 2018; Perea-Sanz et al., 2018). Eventually, nitrite also positively affects colour. The reactive intermediate compounds derived from nitrite conversion, such as NO, bind to Fe^{2+} of myoglobin heme group, forming nitrosomyoglobin (Hammes, 2012). This compound is the characteristic red curing pigment and consumers consider it an essential organoleptic trait. Nitrite exerts its antioxidant activity in cured meat by forming the myoglobin-stable compounds and making the iron inaccessible for oxidation (Riazi et al., 2018). However, the main role of nitrite in processed meat products is linked to food safety, thanks to its bacteriostatic and bactericidal activity against pathogenic bacteria such as *Salmonella enterica* serovar *typhimurium, Listeria* spp., and *Clostridium botulinum* (Majou and Christieans, 2018). The mechanisms by which nitrite inhibits the growth of foodborne pathogens and food spoilage bacteria include oxygen uptake and oxidative phosphorylation interruption, formation of nitrous acid and NOs, and interruption of critical enzymes in bacterial metabolism such as aldolase (Lee et al., 2018). The major concern of nitrate/nitrite in food is related to the potential of nitrite to form cancerogenic N-nitroso compounds. Indeed, amines and amides are formed in the colon through bacterial metabolism of amino acids, and these can be N-nitrosated in the presence of nitrosylated haem derived from unabsorbed residues of red meat (Herrmann et al., 2015; Johnson, 2017; Meurillon and Engel, 2016). A

dramatic increase of consumer's demand for natural, fresh and minimally processed foods with fewer artificial additives, including preservatives has been observed (Majou and Christieans, 2018). Some attempts of curing without nitrite were made, but products resulted in very poor organoleptic and microbiological quality (Hammes, 2012). Recently, studies have focused on finding potential substitutes of nitrite and nitrate in plant extracts, that thanks to their high content of polyphenols can perform both antioxidant and antimicrobial activities (Jiang and Xiong, 2016; Shah et al., 2014; Shan et al., 2009). Several studies reported phenolic compounds diffuse into bacterial cells walls and interact with cytoplasmatic proteins, affecting Gram positive bacteria and, particularly, Gram positive cocci (Fasolato et al., 2016; Jayaprakasha et al., 2003; Riazi et al., 2018).

Bacterial fermentation of raw meat is fundamental for dry-fermented sausages production: lactic acid bacteria (LAB) decrease dry-fermented sausages pH thanks to hexose sugars fermentation to lactic acid; coagulase-negative cocci (CNC) are fundamental for lipolysis, proteolysis and free amino acids decomposition (Aquilanti et al., 2016; Cardinali et al., 2018). Thus, it is particularly important that additives used for dry-fermented sausages production do not alter the overall microbiota and, in particular, LAB and CNC groups. LAB and CNC species could be indigenous of food or added as starters; LAB includes many different genera belonging to the order *Lactobacillales*: *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus* and *Leuconostoc* (Makarova et al., 2006). In dry-fermented salami generally *Lactobacillus sakei* and *Lactobacillus curvatus* are predominant within LAB, *Staphylococcus xylosus* within CNC.

The aim of this work was to evaluate the effects of two different plant extracts mixtures as nitrite replacement on the prokaryotic community of Cinta Senese pigs dry-fermented sausages. Grape seed extract, chestnut extract and hydroxytyrosol extracted by olive pomace were chosen in function of their antimicrobial and antioxidant activity, as well as they are important regional productions, whose by-products are easily available (Aquilani et al., 2018). Moreover, Cinta Senese meat, a local breed with PDO, was employed to manufacture the dry-fermented sausages, in order

to address consumer's demand for high quality, regional-linked and healthier processed meat products.

2. Materials and Methods

2.1. Antioxidant mixtures

The natural antioxidants employed in the present studies were provided by Phytolab (Sesto Fiorentino, Florence, Italy). They consisted of grape seed and chestnut extracts, tocopherol and hydroxytyrosol (extracted by defatted olive pomace). The manufacturer provided the phenolic profile, total phenolic content and antiradical scavenging activity (EC50) of each extract (Aquilani et al., 2018). The grape seed and chestnut extracts were combined with the same amount of hydroxytyrosol and tocopherol to form two different mixtures; grape seed (GSE) and chestnut (CHE) mixtures (Aquilani et al., 2018).

2.2. Dry-fermented sausages manufacturing

In an industrial plant (Azienda Agricola Savigni, Pistoia, Italy), three different types of pork dry-fermented sausages were made. Sausage basis-mixture contained 20% of Cinta Senese backfat, 80% of Cinta Senese pork lean, which were minced and mixed with salt (23 g/kg), sucrose (35 g/kg) and black pepper (0.2 g/kg). The control batch, according the traditional recipe used by the manufacturer, was added with thirty ppm of sodium nitrite (E250), (NIT). In the second batch, 10 g/kg of GSE mixture were used to replace sodium nitrite, while 10 g/kg of CHE were added to the third batch. Sausages were weighed, dried at 28 °C and RH 85% for 4 days and then ripened 21 days (T 13 °C, RH 70%). At the end of ripening, six samples of each type were collected; pH, colour and processing loss were immediately measured. Samples were vacuum packed and stored at −80 °C.

2.3. Physical parameters and chemical composition

Instrumental colour parameters (L*, a* and b*) were determined by a Minolta Chromameter CR-200 (Tokyo, Japan) on cured samples, then Chroma ($\sqrt{(a^{*2}+b^{*2})}$) and Hue ($\tan^{-1}$ (b*/a*)) were

calculated. Similarly, chemical analysis to determine moistur (AOAC, 2012, ref: 950.46), protein content (AOAC, 2012, ref: 976.05), ash (AOAC, 2012, ref: 920.153) and ether extract (AOAC, 2012, ref: 991.36) were carried out. Fatty acids (FAs) of total lipids (Folch et al., 1957) were determined using a Varian GC-430 apparatus equipped with a flame ionization detector (FID) (Palo Alto, CA, USA) as reported by Sirtori et al. (2015). Methyl esters were identified by their retention time using an analytical standard (FAME Mix, C8-C22 Supelco 18,920-1AMP). Response factors based on the internal standard (C19:0) were used for quantification and results were expressed as mg/100g of sample.

2.4. Total DNA extraction from dry-fermented sausages

Total DNA was extracted using the DNeasy mericon Food Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Briefly, dry-fermented sausages samples were homogenized in a Waring blender three times for 1 min each at high speed with intermittent cooling on ice after each minute. DNA was extracted from 200 mg of homogenized sample. Extracted DNA was checked by agarose gel electrophoresis. DNA purity and quantity were measured using a ND-1000 Spectrophotometer (NanoDrop Technologies, Labtech, Ringmer, UK) and standardized to a concentration of 10 ng/µl.

2. 5. Illumina MiSeq sequencing and data processing

For each sample, the V3-V4 region of the 16S rRNA gene was amplified using primers Pro341f and Pro805R (Takahashi et al., 2014), barcodes were added to the forward primer. Amplicons for each library were purified and mixed in equal proportion. Illumina MiSeq v3 chemistry 300 base paired-end (PE) amplification and sequencing were performed at BMR genomics (Padova, Italy). Primer sequences were removed using Cutadapt (Martin, 2011). Reads quality was evaluated using DADA2 (Callahan et al., 2016), reads (R1 and R2) were then trimmed and filtered using the following parameters: truncLen=c(265,220), maxN=0, maxEE=c(2,2), truncQ=2. Reads were merged with FLASh v1.2.11 (Magoc and Salzberg, 2011) using the following parameters: -m 20, -

M 280, Phred score default of 33. Sequences shorter than 250 bp were filtered out with Prinseq-lite (Schmieder and Edwards, 2011). Chimeras were removed using USEARCH 6.1 (Edgar et al., 2011). De novo OTU picking was performed using Swarm (Mahe et al., 2014) within QIIME 1.9.1 (Caporaso et al., 2010) and Silva132 (Yilmaz et al., 2014) as the reference database. Representative sequences (most abundant) for each OTU were aligned, and an OTU table was constructed using sequences correctly aligned. Sequences identified as chloroplasts or mitochondria were removed. OTUs representing less than 0.005% of the total read abundance were discarded (Bokulich et al., 2013). Alpha diversity measures (number of observed OTUs, Chao1 value and Shannon index) were calculated within QIIME 1.9.1. Statistical analysis including Shapiro-Wilk test for normality, ANOVA, Kruskal-Wallis group test with false discovery rate ("fdr") *p*-value adjustment, Dunn test and Hellinger transformation were conducted in "R" version 3.5.1 (R Development Core Team, 2011). Permutational multivariate analysis of variance (PERMANOVA) and principal component analysis were conducted on a Hellinger transformed OTU table using the Dice and Bray-Curtis indices, with 9999 permutations, within PAST (Hammer et al., 2001). The 16S rRNA gene amplicon sequence data are available at the National Centre for Biotechnology Information Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra), SRA accession PRJNA552846.

3. Results and discussion

3.1. Effects on physical and chemical parameters

In the present study, pH scores (Table 1) are in line with those usually reported for natural dry-fermented sausages (between 5.3 and 6.2) (Aquilanti et al. 2016). Sodium nitrite replacement resulted in significantly lower pH, especially for CHE samples that showed the lowest score. Likely, the highest relative abundance of *Lactobacillaceae* in CHE samples, observed by phylogenetic analysis, had increased meat acidification. Similarly, also Lorenzo et al. (2013), observed that, at 20 days of ripening, grape seed extract and chestnut extract added sausages had the

lowest pH if compared to control (without any curing agent) or BHT-added products. Concerning the colour parameters, Chroma resulted highest in NIT samples, this was expected considering that it results from the combination of a* and b* parameters. In nitrite-added meat products, nitrite is reduced to its reactive intermediate compounds, such as NO, which binds to Fe^{2+} of myoglobin heme group and forms the nitrosomyoglobin complex (Hammes, 2012). Nitrosomyoglobin is the main red curing pigment of processed meat products and constitute a central sensory trait. Chemical composition was only slightly modified by natural antioxidants usage, indeed only ash showed a significant difference among experimental groups, with GSE samples having the lower content. The major differences in FAs profile (Table 2) of experimental groups were related to unsaturated fatty acids, which were lower in GSE samples. Consequently, also PUFA, PUFA n3 and PUFA n6 total amounts followed the same pattern. Since PUFA double bonds are the preferred substrates for oxidative reactions (Pateiro et al., 2015), these results suggest a greater extension of lipid oxidation phenomena in GSE samples, consistent with the greater EC_{50} of GSE compared to CHE.

3.2. Metagenomic analysis of prokaryotic communities associated with dry-fermented sausages

The composition of the bacterial communities associated with dry-fermented sausages treated with nitrite or with two different natural extracts were analysed. Illumina MiSeq v3 sequencing, which was performed on the variable region V3-V4 of 16S rDNA, produced a total of 1,266,646 sequences (ranging from around 50,270 to 106,165 sequences *per* library). Rarefaction curves showed a high sequencing coverage for all the samples (Fig. S1), allowing the identification of 131 OTUs with a range from 81 to 123 *per* sample (Fig. S2). The α-diversity was calculated using the number of OTUs observed, Chao1 value and Shannon diversity index. ANOVA did not show significant differences for α-diversity values in the three groups (Fig. S2).

Principal component analysis of microbial community profiles showed differences between the three groups in terms of OTUs presence/absence (Dice index) and relative abundance (Bray-Curtis index) (Fig. 1A and B; Table S1). With Dice index the three groups are clearly distinct, while with Bray-Curtis index the separation was more evident between CHE group and the other two groups

(Fig. 1B). Differences with both indices were statistically evaluated using PERMANOVA, which confirmed a clear separation between the three groups (Table S1).

3.3. Phylogenetic analysis of identified OTUs

Most of the sequences (99,53%) were identified at the genus level. The microbial communities associated with dry-fermented sausages were formed by at least 32 genera subdivided in 18 families (Fig. 1C), 10 orders, 4 classes and 4 different phyla (3 bacterial and 1 archaeal phylum). Bacterial communities were mostly represented by *Firmicutes* (96.2%) and in particular by two genera: *Staphylococcus* and *Lactobacillus* accounting for the 91.6% of the total prokaryotic community (63% and 28.6% respectively). OTUs denovo0 and denovo7 were the most represented OTUs (corresponding to 59.2 and 22.8% of total community respectively). Phylogenetic trees were constructed to ameliorate the classification of denovo0 and denovo7 OTUs (Fig. S3A and B). OTU denovo0 falls within *Staphylococcus xylosus* while denovo7 within *Lactobacillus sakei* (Fig. S3A and B). *Lactobacillus sakei* and *Staphylococcus xylosus* constituted the major part of LAB and CNC respectively and are commonly found in dry-fermented sausages manufactured in the Mediterranean area (Aquilanti et al., 2016). LAB and CNC are particularly important in natural fermented sausages as they drive the fermentation processes (Janssens et al., 2012). Lactic acid fermentation leads to meat acidification and protein coagulation (Aquilanti et al., 2016; Leroy and De Vuyst, 2005). CNS bacteria are required for the development of aroma thanks to their amino acid and lipid metabolism (Ravyts et al., 2009). *Staphylococcaceae* was the most represented family with values ranging from 58% to 67%; a high amount of *Staphylococcaceae* was not unexpected, indeed depending on the manufacturer has been already observed that the dominant group in dry fermented salami prokaryotic community could belong to *Staphylococcaceae* or *Lactobacillaceae* (Polka et al., 2015).

Within the order *Lactobacillalles,* five families showed significant variations among different treatments: *Carnobacteriaceae*, *Enterococcaceae, Lactobacillaceae, Leuconostocaceae* and

Streptococcaceae (Fig. 1C). For *Lactobacillaceae* the highest relative abundance was found in CHE sausages (37%), the lowest in GSE sausages (21%). For the other 4 LAB families, differences were observed also at genus level: *Carnobacterium* (*Carnobacteriaceae*; Fig. 2A), *Enterococcus* (*Enterococcaceae*; Fig. 2B), *Lactococcus* (*Streptococcaceae*; Fig. 2C), *Leuconostoc* and *Weissella* (*Leuconostocaceae*; Fig. 2D and E). No sequences belonging to *Enterococcus* genus were found in NIT sausages, in sausages treated with chestnut extracts (CHE) a small presence of *Enterococcus* was observed (0.001%), while GSE sausages contained the higher levels (0.02%). The presence of enterococci in raw meat could be due to intestinal or environmental colonisation. Indeed, these bacteria are able to survive and grow during fermentation and in absence of a competitive starter culture. In traditionally manufactured dry-fermented sausages, it is more likely to observe an increase of this genus (Giraffa, 2002; Hugas et al., 2003). Moreover, their fermentation activity may contribute to enrich dry-fermented sausages sensory traits (Hanchi et al., 2018). *Enterococcus* belongs to LAB, but its presence in dry-fermented sausages could be considered unacceptable (Holley et al., 1988). *Enterococcus* species are not among those bacteria classified as generally recognized as safe (GRAS) (Huys et al., 2013; Ogier and Serror, 2008) or recommended for the quality presumption of safety (QPS) list (Hazards et al., 2017). However, in the past years commensal and pathogenic enterococci strains have been clearly differentiated (Bonacina et al., 2017). Furthermore, several enterococci strains produce bacteriocins and other antimicrobial compounds, which may help in food preservation (Yang et al., 2014). Similarly, to *Enterococcus,* a higher relative abundance of *Lactococcus* and *Weissella* genera were observed in GSE dry-fermented sausages (Fig. 2H and I). In contrast *Carnobacterium* and *Leuconostoc* relative abundance was higher in NIT dry-fermented sausages (Fig. 2E and G). These bacteria are all LAB, *Weissella* and *Leuconostoc* are both obligate heterofermative *Leuconostocaceae*. The increased amount of different LAB in GSE and NIT could be due to the lower relative abundance of *Lactobacillaceae* in these two groups. The presence of these taxa is limited accounting between 0.06% (*Leuconostoc*) to 0.37% (*Lactococcus*). *Weissella* strains have been used for

biotechnological applications; anyway, it would be preferable to keep low their presence because some strains may act as opportunistic pathogens (Abriouel et al., 2015). *Weissella* sequences were classified in two species: *W. cibaria* (0.007%) and *W. hellenica* (0.12%), only *W. hellenica* showed significant variation in its relative abundance (data not shown). *W. hellenica* has probiotic activity thanks to the production of bacteriocin (Abriouel et al., 2015); e.g. strain D1501 is able to inhibit the growth of *Kurthia gibsonii*, *Staphylococcus aureus* and *Escherichia coli* and enhance safety and shelf-life of foods like tofu (Chen et al., 2014).

In 15 samples was observed the presence (below 0.01%) of Archaea sequences, all belonging to the genus *Halorubrum* (Fig. 3A). This is a red-pigmented Archaea able to thrive with high salt concentrations and it may be found in salt-fermented or salt-preserved food (Gibtan et al., 2018). Three different genera within *Gammaproteobacteria* were different: *Cobetia, Photobacterium* and *Pluralibacter* (Fig. 3C, D and E). No sequences belonging to *Pluralibacter* (*Enterobacteriaceae*) were detected in NIT samples while a low relative abundance of was observed in CHE and GSE samples (0.001 and 0.006% respectively). A higher relative abundance of the genus *Photobacterium* was encounter in NIT dry-fermented sausages (Fig. 2F). *Photobacterium* belongs to the *Vibrionaceae* family (Fig. 1C), several species within this genus are psychrophilic marine bacteria but could be also related with meat spoilage: *Photobacterium carnosum*, *Photobacterium phosphoreum* and *Photobacterium iliopiscarium* have been found in modified-atmosphere packages (MAP) unspoiled and spoiled meat (Hilgarth et al., 2018). In particular, high levels of *P. phosphoreum* were found associated to spoiled MAP raw pork meat (Nieminen et al., 2016). In dry-fermented sausages treated with natural extracts the relative abundance of *Photobacterium* genus was more than thirty times lower than in NIT ones, therefore the use of these additives may be interesting to reduce the potential spoilage bacteria amount. Among bacteria that may spoil dry-fermented sausages there is *Brochotrix thermosphacta,* a Gram-positive fermentative bacterium. It belongs to *Listeriaceae* family and it's phylogenetically close to *Listeria monocytogenes*

(Stanborough et al., 2017). The presence of *Brochotrix* was observed in all the three treatments ranging from 2.96% (NIT) to 3.45% (GSE), but no significant differences were found.

4. Conclusion

Two different natural extracts (grape seed and chestnut extracts) have been used in place of nitrites in dry-fermented natural sausages. Nitrite-free products showed lower pH and differences in Chroma, being less red and darker than nitrite-added samples. Moreover, fatty acids profile suggested that GSE extract had a lower antioxidant potential than sodium nitrite. *Lactobacillaceae* relative abundance was significantly higher in CHE than NIT and GSE, agreeing with the lower pH levels observed in these samples. Although the three groups showed significant differences, natural extract did not drastically alter the prokaryotic community and the other chemical/physical parameters indicating that these two extracts may be used as replacement for nitrites in dry-fermented sausages.

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Tables

Table 1. Physical and chemical parameter of dry-fermented sausages with grape seed extract (GSE) chestnut extract (CHE) or sodium nitrite (NIT). Different letters (a, b, c) within the same row indicate significant differences between treatments ($p < 0.05$).

	GSE	CHE	NIT	RMSE[c]	P[d]
Curing loss (%)	40.09	43.57	45.73	6.36	0.3283
pH	5.74 b	5.58 c	5.85 a	0.07	<0.0001
Chroma	15.45 b	14.70 b	17.37 a	2.31	0.0005
Hue	15.55	15.43	17.20	4.219	0.2743
Moisture	30.04	30.712	29.376	1.78	0.4486
Protein (g/100 g dm*)	45.32	46.26	46.26	1.09	0.2515
Fat (g/100 g dm)	45.97	44.93	44.73	1.00	0.1040
Ash (g/100 g dm)	7.83 b	8.25 a	8.24 a	0.27	0.0227

*** dry matter**

Table 2. Fatty acids profile (mg/100g) of dry-fermented sausages added with grape seed extract (GSE) chestnut extract (CHE) or sodium nitrite (NIT). Different letters (a, b, c) within the same row indicate significant differences between treatments ($p < 0.05$).

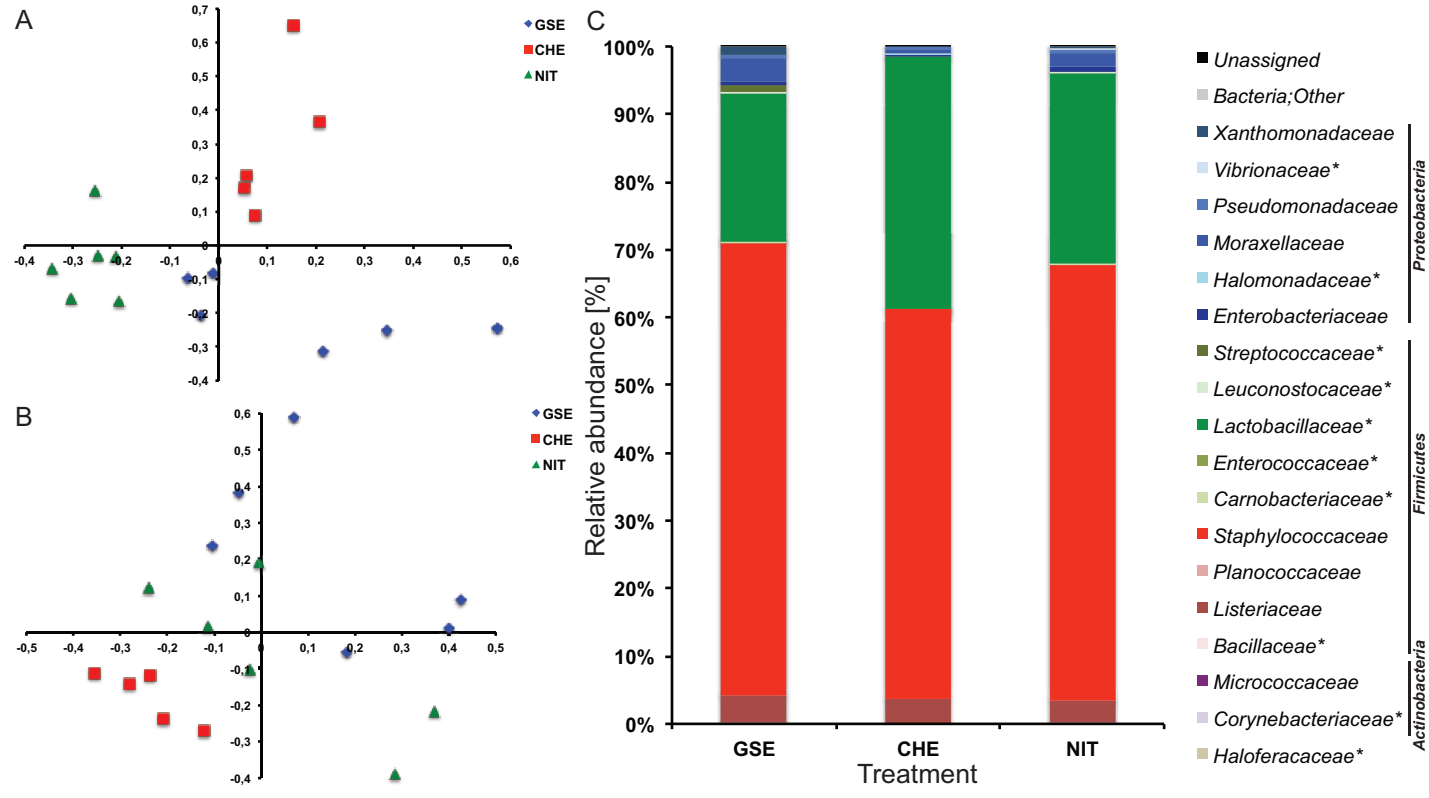
	GSE	CHE	NIT	RMSE[c]	P[d]
C 16:0	4.959	4.919	5.131	0.08	0.655
C 16:1	0.400	0.369	0.392	0.03	0.147
C 17:0	0.067 c	0.082 b	0.093 a	0.001	0.0001
C 17:1	0.05 b	0.056 a	0.06 a	0.005	0.0007
C 18:0	2.91 b	3.08 ab	3.24 a	0.264	0.125
C 18:1 n9	8.227	8.209	8.420	0.669	0.834
C 18:1 n7	0.615	0.571	0.589	0.043	0.242
C 18:2 n6	2.94 b	3.36 a	3.62 a	0.317	0.008
C 18:3 n3	0.17 c	0.23 b	0.27 a	0.22	<0.0001
C 20:0	0.04	0.044	0.046	0.005	0.488
C 20:1	0.009 a	0.008 ab	0.007 b	0.001	0.040
C 20:2 n6	0.139	0.158	0.154	0.017	0.137
C 20:3 n6	0.024	0.0241	0.025	0.003	0.523
C 20:3 n3	0.027 b	0.035 a	0.035 a	0.003	0.001
C 20:4 n6	0.101	0.102	0.104	0.006	0.791
C 22:4 n6	0.036 a	0.029 b	0.028 b	0.003	0.0001
C 22:5 n3	0.018 b	0.018 b	0.026 a	0.006	0.043
SFA	8.27	8.42	8.83	0.716	0.404
MUFA	9.52	9.44	9.69	0.763	0.851
PUFA n3	0.218 c	0.292 b	0.332 a	0.03	<0.0001
PUFA n6	3.245 b	3.672 a	3.927 a	0.341	0.011
PUFA	3.469 b	3.967 a	4.268 a	0.366	0.006

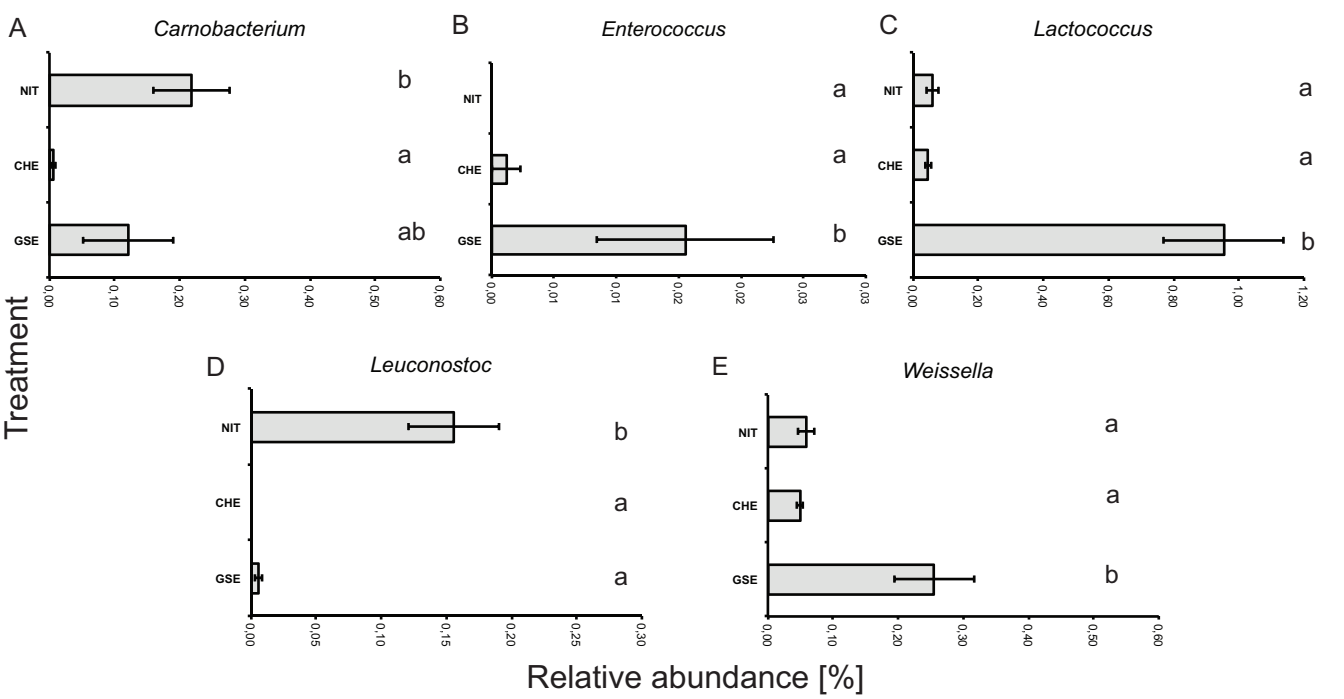
Figure legends

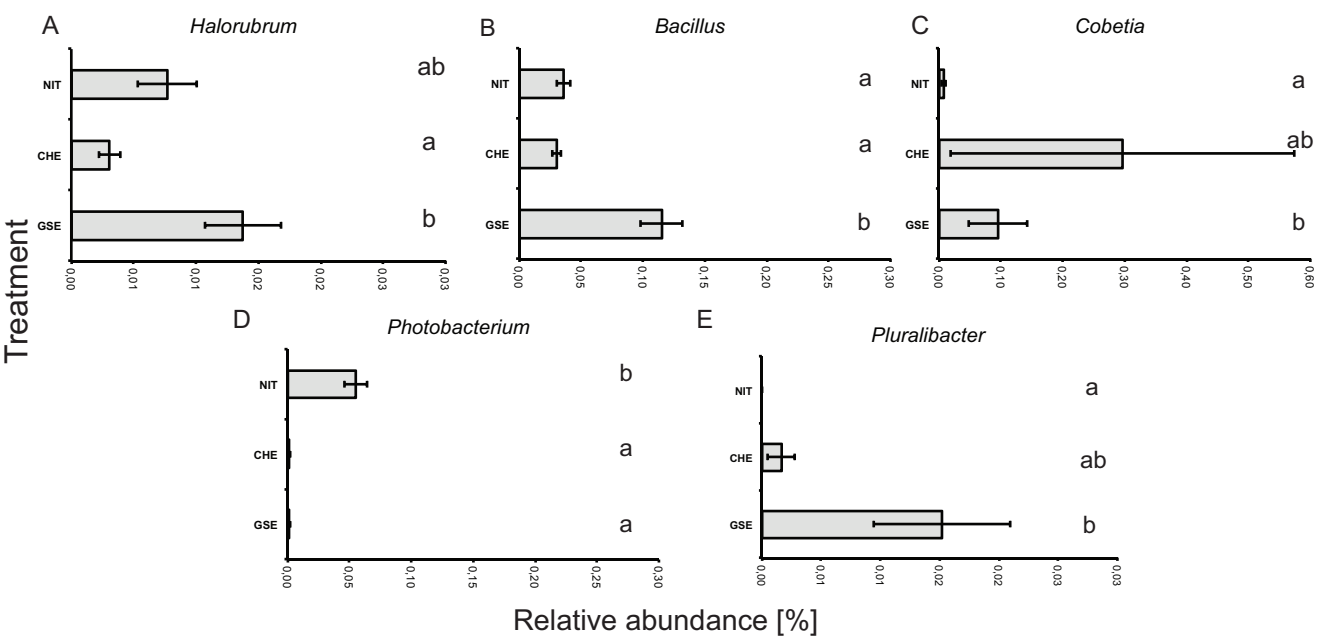
Figure 1. Dry-fermented sausages prokaryotic community. Prokaryotic communities are labelled respective to the treatment used (GSE = grape seed extract; CHE = chestnut extract; NIT = nitrite). A) ß-diversity, Principal coordinates analysis (PCoA) plot using the Dice index. B) ß-diversity, PCoA plot using the Bray-Curtis index. C). Prokaryotic community composition of dry-fermented sausages at family level. Stars indicate significant differences between treatments (Kruskal-Wallis, p (FDR) < 0.05).

Figure 2. Effect of the different treatments on lactic acid bacteria at genus level. Each bar is labelled respective to the treatment used (GSE = grape seed extract; CHE = chestnut extract; NIT = nitrite). A) *Carnobacterium,* B) *Enterococcus,* C) *Lactococcus,* D) *Leuconostoc* and E) *Weissella* (Kruskal-Wallis, p (FDR) < 0.05). Means sharing the same letter are not significantly different (Dunn test).

Figure 3. Prokaryotic genera influenced by treatment. Each bar is labelled respective to the treatment used (GSE = grape seed extract; CHE = chestnut extract; NIT = nitrite). A) *Halorubrum,* B) *Bacillus,* C) *Cobetia,* D) *Photobacterium* and E) *Pluralibacter* (Kruskal-Wallis, p (FDR) < 0.05). Means sharing the same letter are not significantly different (Dunn test).


A
GSE
CHE
NIT
B
GSE
CHE
NIT
C
Relative abundance [%]
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GSE
CHE
NIT
Treatment
Unassigned
Bacteria;Other
Xanthomonadaceae
Vibrionaceae*
Pseudomonadaceae
Moraxellaceae
Halomonadaceae*
Enterobacteriaceae
Streptococcaceae*
Leuconostocaceae*
Lactobacillaceae*
Enterococcaceae*
Carnobacteriaceae*
Staphylococcaceae
Planococcaceae
Listeriaceae
Bacillaceae*
Micrococcaceae
Corynebacteriaceae*
Haloferacaceae*
Proteobacteria
Firmicutes
Actinobacteria


A
Carnobacterium
B
Enterococcus
C
Lactococcus
D
Leuconostoc
E
Weissella
NIT
CHE
GSE
b
a
ab
a
a
b
a
a
b
b
a
a
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.00
0.01
0.01
0.02
0.02
0.03
0.00
0.20
0.40
0.60
0.80
1.00
1.20
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.00
0.10
0.20
0.30
0.40
0.50
0.60
Treatment
Relative abundance [%]


A
Halorubrum
B
Bacillus
C
Cobetia
D
Photobacterium
E
Pluralibacter
Treatment
NIT
CHE
GSE
ab
a
b
a
a
b
a
ab
b
b
a
a
a
ab
b
Relative abundance [%]

DECLARATION OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author.

Signed by all authors as follows:

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Carolina Pugliese

Carlo Viti

Supporting information

Table S1. QIIME taxa table of salami microbiota composition for each sample at phylum level.

Table S2. QIIME taxa table of salami microbiota composition for each sample at class level.

Table S3. QIIME taxa table of salami microbiota composition for each sample at order level.

Table S4. QIIME taxa table of salami microbiota composition for each sample at family level.

Table S5. QIIME taxa table of salami microbiota composition for each sample at genus level.

Figure S1. Sample-based rarefaction curves representing the number of observed OTUs at different sequencing depths (each point is the average of 10 iterations). Salami microbiota are labelled respective to the treatment used (GSE = grape seed extract; CHE = chestnut extract; NIT = nitrite).

Figure S2. Box-plots of bacterial α-diversity based on: A) Observed OTUs, B) Chao 1 value and C) Shannon index. Each box is labelled respect to the salami microbiota treatment used (GSE = grape seed extract; CHE = chestnut extract; NIT = nitrite).

Figure S3. Evolutionary relationships of the two most abundant OTUs. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. A) denovo0. B) denovo7.

Supporting methods

Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap test (10000 replicates) was performed (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

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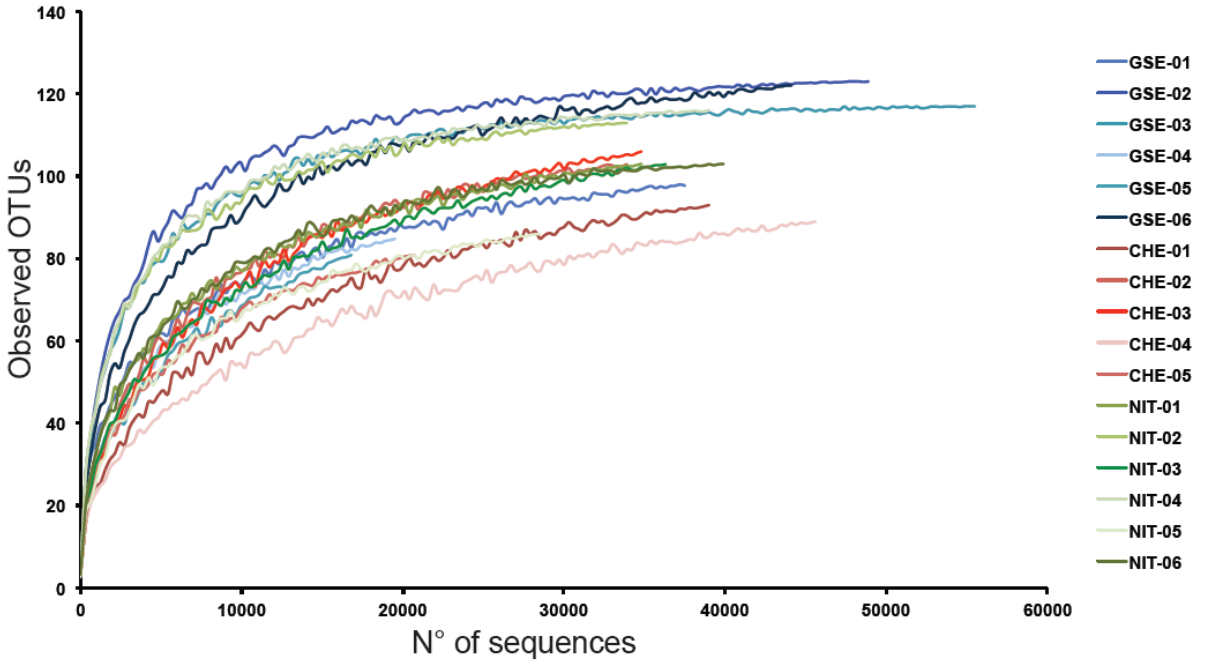

Figure S1. Sample-based rarefaction curves representing the number of observed OTUs at different sequencing depths (each point is the average of 10 iterations). Salami microbiota are labelled respective to the treatment used (GSE = grape seed extract; CHE = chestnut extract; NIT = nitrite).

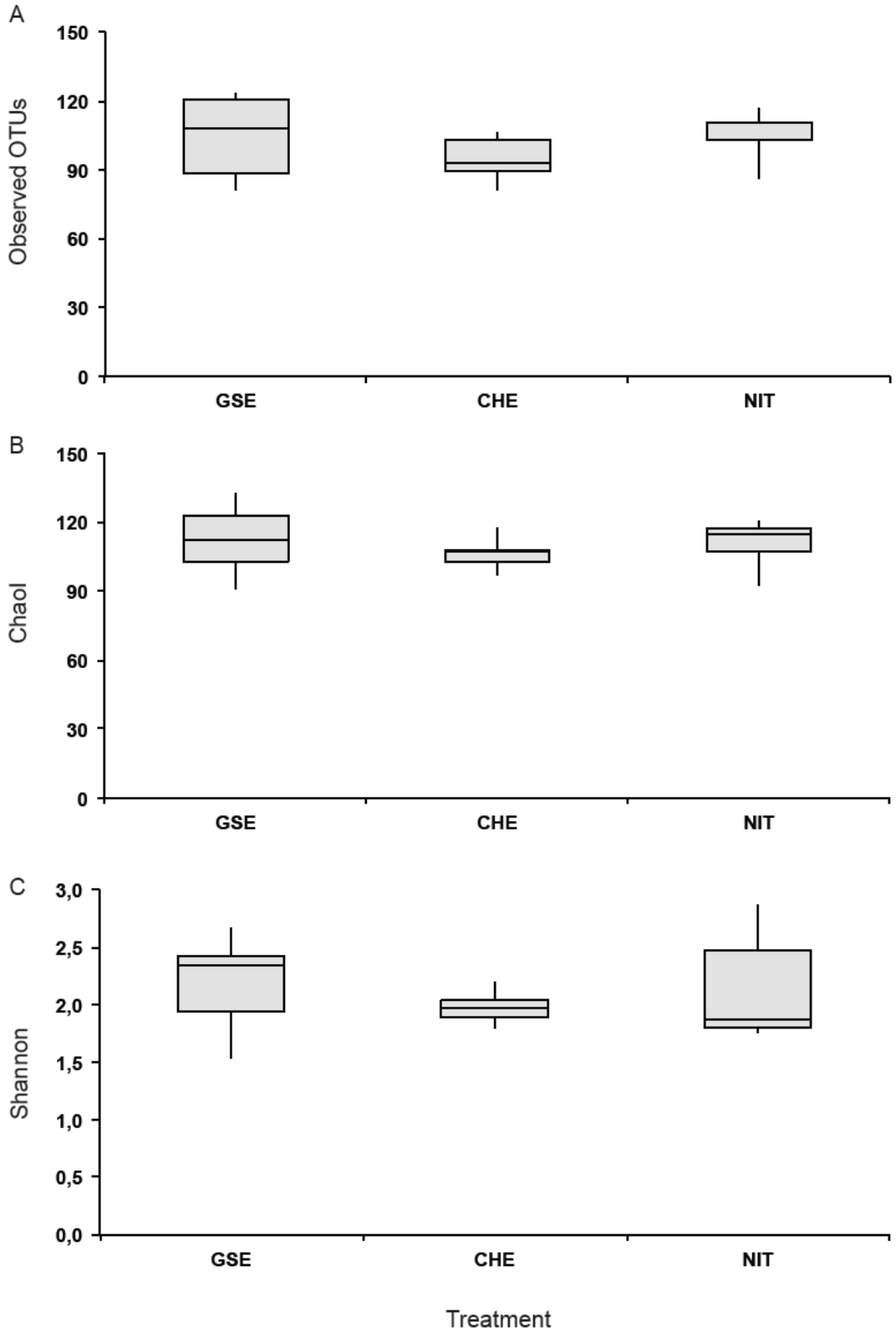

Figure S2. Box-plots of bacterial α-diversity based on: A) Observed OTUs, B) Chao 1 value and C) Shannon index. Each box is labelled respect to the salami microbiota treatment used (GSE = grape seed extract; CHE = chestnut extract; NIT = nitrite).

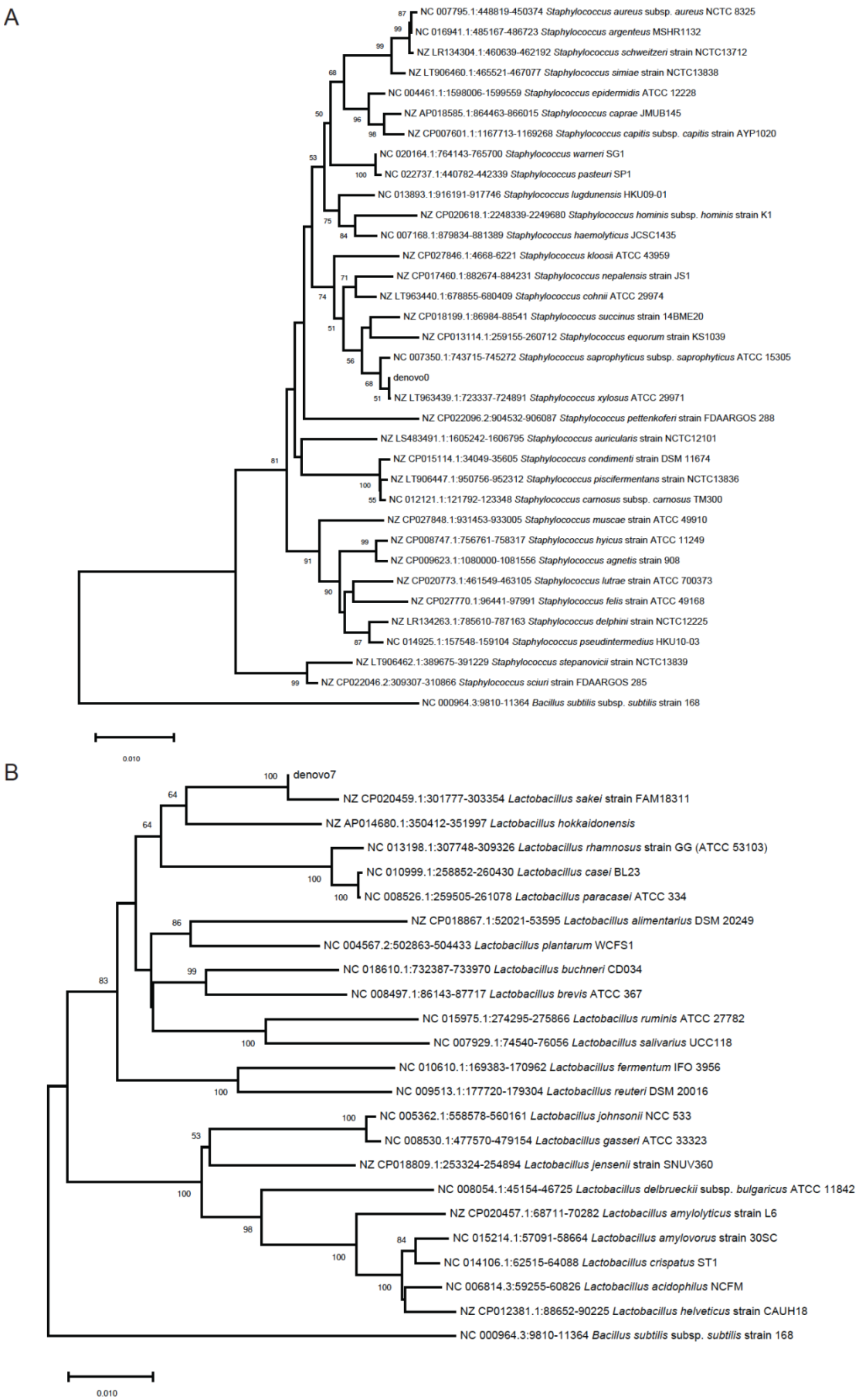
A
NC 007795.1:448819-450374 Staphylococcus aureus subsp. aureus NCTC 8325
NC 016941.1:485167-486723 Staphylococcus argenteus MSHR1132
NZ LR134304.1:460639-462192 Staphylococcus schweitzeri strain NCTC13712
NZ LT906460.1:465521-467077 Staphylococcus simiae strain NCTC13838
NC 004461.1:1598006-1599559 Staphylococcus epidermidis ATCC 12228
NZ AP018585.1:864463-866015 Staphylococcus caprae JMUB145
NZ CP007601.1:1167713-1169268 Staphylococcus capitis subsp. capitis strain AYP1020
NC 020164.1:764143-765700 Staphylococcus warneri SG1
NC 022737.1:440782-442339 Staphylococcus pasteuri SP1
NC 013893.1:916191-917746 Staphylococcus lugdunensis HKU09-01
NZ CP020618.1:2248339-2249680 Staphylococcus hominis subsp. hominis strain K1
NC 007168.1:879834-881389 Staphylococcus haemolyticus JCSC1435
NZ CP027846.1:4668-6221 Staphylococcus kloosii ATCC 43959
NZ CP017460.1:882674-884231 Staphylococcus nepalensis strain JS1
NZ LT963440.1:678855-680409 Staphylococcus cohnii ATCC 29974
NZ CP018199.1:86984-88541 Staphylococcus succinus strain 14BME20
NZ CP013114.1:259155-260712 Staphylococcus equorum strain KS1039
NC 007350.1:743715-745272 Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305
denovo0
NZ LT963439.1:723337-724891 Staphylococcus xylosus ATCC 29971
NZ CP022096.2:904532-906087 Staphylococcus pettenkoferi strain FDAARGOS 288
NZ LS483491.1:1605242-1606795 Staphylococcus auricularis strain NCTC12101
NZ CP015114.1:34049-35605 Staphylococcus condimenti strain DSM 11674
NZ LT906447.1:950756-952312 Staphylococcus piscifermentans strain NCTC13836
NC 012121.1:121792-123348 Staphylococcus carnosus subsp. carnosus TM300
NZ CP027848.1:931453-933005 Staphylococcus muscae strain ATCC 49910
NZ CP008747.1:756761-758317 Staphylococcus hyicus strain ATCC 11249
NZ CP009623.1:1080000-1081556 Staphylococcus agnetis strain 908
NZ CP020773.1:461549-463105 Staphylococcus lutrae strain ATCC 700373
NZ CP027770.1:96441-97991 Staphylococcus felis strain ATCC 49168
NZ LR134263.1:785610-787163 Staphylococcus delphini strain NCTC12225
NC 014925.1:157548-159104 Staphylococcus pseudintermedius HKU10-03
NZ LT906462.1:389675-391229 Staphylococcus stepanovicii strain NCTC13839
NZ CP022046.2:309307-310866 Staphylococcus sciuri strain FDAARGOS 285
NC 000964.3:9810-11364 Bacillus subtilis subsp. subtilis strain 168
0.010
B
denovo7
NZ CP020459.1:301777-303354 Lactobacillus sakei strain FAM18311
NZ AP014680.1:350412-351997 Lactobacillus hokkaidonensis
NC 013198.1:307748-309326 Lactobacillus rhamnosus strain GG (ATCC 53103)
NC 010999.1:258852-260430 Lactobacillus casei BL23
NC 008526.1:259505-261078 Lactobacillus paracasei ATCC 334
NZ CP018867.1:52021-53595 Lactobacillus alimentarius DSM 20249
NC 004567.2:502863-504433 Lactobacillus plantarum WCFS1
NC 018610.1:732387-733970 Lactobacillus buchneri CD034
NC 008497.1:86143-87717 Lactobacillus brevis ATCC 367
NC 015975.1:274295-275866 Lactobacillus ruminis ATCC 27782
NC 007929.1:74540-76056 Lactobacillus salivarius UCC118
NC 010610.1:169383-170962 Lactobacillus fermentum IFO 3956
NC 009513.1:177720-179304 Lactobacillus reuteri DSM 20016
NC 005362.1:558578-560161 Lactobacillus johnsonii NCC 533
NC 008530.1:477570-479154 Lactobacillus gasseri ATCC 33323
NZ CP018809.1:253324-254894 Lactobacillus jensenii strain SNUV360
NC 008054.1:45154-46725 Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842
NZ CP020457.1:68711-70282 Lactobacillus amylolyticus strain L6
NC 015214.1:57091-58664 Lactobacillus amylovorus strain 30SC
NC 014106.1:62515-64088 Lactobacillus crispatus ST1
NC 006814.3:59255-60826 Lactobacillus acidophilus NCFM
NZ CP012381.1:88652-90225 Lactobacillus helveticus strain CAUH18
NC 000964.3:9810-11364 Bacillus subtilis subsp. subtilis strain 168
0.010

Figure S3. Evolutionary relationships of the two most abundant OTUs. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. A) denovo0 B) denovo7.