MICROBIAL CHANGES AND AROMA PROFILE OF NITRATE REDUCED DRY SAUSAGES DURING VACUUM STORAGE

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12 Abstract

13 Slow fermented sausages with reduced ingoing amounts of sodium nitrate
14 (control, 15% and 25 % reduction) were stored under vacuum up to three months.
15 Changes in microbiology, chemical parameters and volatile compounds were studied.
16 Residual nitrate was not affected by vacuum storage and its reduction resulted in a
17 reduction of sausage redness. General microbial counts decreased during vacuum
18 storage, though nitrate reduction increased the growth of total mesophilic bacteria and
19 Gram positive cocci. Long storage time and 25% nitrate reduction affected microbial
20 activity and sausage aroma profile. Short vacuum storage times and moderate nitrate
21 reduction (15%) were related to compounds producing pleasant odours (3–hydroxy–2–
22 butanone, ethyl octanoate, ethyl–3–methylbutanoate and 2,3–pentanedione) and
23 cheesy/buttery odour (2,3–butanedione and ethyl–2–hydroxypropanoate). In contrast,
24 25% nitrate reduction increased compounds like heptanal (green, unpleasant odour) and
25 those related to unpleasant odours, methanethiol (rotten odour) and methional (cooked
26 potato).

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28 Keywords: nitrate; fermented sausage; storage; vacuum; flavor; health safety.

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1. Introduction

Consumers demands healthier meat products reduced in additives such as nitrite due to the generation of nitrosamines with carcinogenic potential (De Mey, De Maere, Paelinck, & Fraeye, 2015). However, nitrate and nitrite are used in fermented sausage manufacture as curing salts due to the nitrite effect on the control of *Clostridium botulinum* and its toxin production (Sindelar & Milkowski, 2011). Moreover nitrite influences several technological parameters like colour development, typical cured flavour and antioxidant effect (Honikel, 2008). In this term, the interest of producers is directed to the knowledge of the reasonable nitrite/nitrate reduction to operate with safety warrant and maintain the high organoleptic properties of traditional meat products. Recently, Christieans, Picgirard, Parafita, Lebert, & Gregori (2018) have demonstrated the impact of reducing the ingoing amount of nitrate/nitrite in dry fermented sausages manufacture and its effect on the growth of pathogens like *Salmonella* and *Listeria*. However, scientific studies should provide information not only regarding microbial risks but also on organoleptic properties like aroma and the changes that may be produced during the long shelf life of this type of products.

Different storage conditions are used depending on product type to extent their shelf-life while maintaining quality and safety. Dry fermented sausages can be kept unpackaged or packaged as whole or slices pieces under modified atmospheres or under vacuum conditions. Among these, vacuum packed is widely used to extend the shelf-life of dry sausages. Therefore, many studies have reported the changes observed during storage under vacuum conditions (Ansorena & Astiasaran, 2004; Dos Santos, Campagnol, Fagundes, Wagner, & Pollonio, 2015, 2017; Kim, Jo, Lee, Lee, Ahn, & Kang, 2012; Rubio, Martinez, Sanchez, Garcia-Cachan, Rovira, & Jaime, 2007, Rubio, Martinez, Garcia-Cachan, Rovira, & Jaime, 2008; Ščetar, Kovacic, Kurek, & Galic,

2013; Summo, Caponio, & Pasqualone, 2006, Summo, Caponio, Paradiso, Pasqualone, & Gomes, 2010, Summo, Caponio, Pasqualone, & Gomes, 2011; Zanardi, Dorigoni, Badiani, & Chizzolini, 2002), modified atmospheres (Rubio et al., 2007, 2008; Ščetar et al., 2013; Tabanelli, Montanari, Grazia, Lanciotti, & Gardini, 2013; Viallon et al., 1996; Zanardi et al., 2002;) and perforated packages (Lorenzo, Bedia, & Bañon, 2013; Bañon Serrano, & Bedia, 2014). Changes in pH, water activity (a_w), red colour (a*) and oxidation parameters (TBARS) during shelf-life have been reported. Moreover, microbiology counts show a general decrease, except for LAB (Kim et al., 2012; Rubio et al., 2007). Overall, sausage acceptability decreases during storage due to colour, aroma, and taste deterioration. The most common changes are the decrease in red intensity, ripened flavour and firmness and the increase in rancid aroma and hardness (Kim et al., 2012; Rubio et al., 2007; Summo et al., 2010; Zanardi et al., 2002) which is apparently accentuated by vacuum storage versus modified atmosphere (Rubio et al., 2008) and unpackaged storage (Summo et al., 2006).

In addition to the physicochemical, microbiological and sensory changes attributed to storage, several studies have dealt with the effect on volatile compounds responsible for ripened aroma (Summo et al., 2011; Tabanelli et al., 2013; Viallon et al, 1996). Viallon et al., (1996) described the variation in sausage volatile profile with packaging under modified atmosphere as an increase of compounds derived from carbohydrate and amino acid degradations. Recent studies revealed that microbial and endogenous enzyme activities during modified atmosphere packaging depended on the initial sausage water activity and, therefore, changes in the latter affected the aroma profile (Tabanelli et al., 2013). Regarding the effect of vacuum storage on aroma profile, differences in lipid oxidation compounds like aldehydes (Ansorena & Astiasaran, 2004) and a significant increase of volatile compounds derived from

carbohydrate and amino acid degradation reactions have been described in dry fermented sausages (Marco, Navarro, & Flores, 2006). Moreover, recent studies have shown a general increase in volatile compounds derived from lipid oxidation reactions (Summo et al., 2011; Dos Santos et al., 2015) and a decrease of those derived from spices under vacuum storage (Dos Santos et al., 2015). In summary, reported volatile changes during storage are highly dependent on sausage properties like a_w (Tabanelli et al., 2013), lipid profile (Ansorena & Astiasaran, 2004), curing agents (Marco et al., 2006) and salt substitutes (Dos Santos et al., 2015) in addition to packaging conditions like temperature and time (Ščetar et al., 2013).

The latest trends in fermented sausage composition are directed to the reduction of additives such as nitrifying agents (EFSA, 2010; FCEC 2016). Until now, only Hospital, Hierro, & Fernández, (2014) have studied the effect of nitrate and nitrite reduction in rapid fermented sausages on microbial evolution during 1 month of vacuum storage. These authors reported changes in microbial counts but the impact of nitrifying agents and storage conditions on aroma was not investigated. Furthermore, the possibility of the exclusive use of nitrates (250 ppm) without added nitrite in traditional slow ripened sausages such as "salchichón" and "chorizo" with maturation period of at least 30 days is indicated in a specific provision concerning nitrites and nitrates (EC Regulation no 1129/2011). Therefore, the aim of the present study is to determine the effect of vacuum storage and nitrate reduction on the aroma quality and microbial counts of slow fermented sausages manufactured with reduced sodium content.

2. Materials and methods

2.1 Dry fermented sausages manufacture

Three replicates of the experiment were performed. In each replicate, three different formulations of dry fermented sausages were manufactured: Control with 250 ppm sodium nitrate (C) and two formulations with a reduction of 15 % (RN15) and 25 % (RN25) of ingoing amounts of sodium nitrate. Lean pork meat (50 %) and pork fat (bellies boneless and skinless) (50%) were minced with the following ingredients (g/kg): lactose (20), dextrin (20), glucose (7), sodium chloride (NaCl) (20.25), potassium chloride (KCl) (6.75), sodium ascorbate (0.5), sodium nitrate at 250 ppm (C), 212.5 ppm (RN15) or 187.5 ppm (RN25) depending on the batch. A commercial starter culture (0.125) TRADI–302 containing *Lactobacillus sakei*, *Staphylococcus xylosus* and *Staphylococcus carnosus* (Danisco, Cultor, Madrid, Spain) was added. The mixture was stuffed into 95 mm diameter collagen casings (Fibran, S.A., Girona, Spain). After ripening for 62 d, dry sausages were vacuum packaged and stored at 18–20°C. For each of three replications, two sausages per batch were randomly taken after 1, 2 and 3 months of storage. In each sausage, colour was measured and a portion of 100 g was minced and used for moisture, water activity (a_w) and pH analyses. The remaining minced sausage was vacuum packed and frozen at –20ºC for physicochemical analyses (TBARS, lipid, protein and residual nitrite and nitrate). A slice of approximately 25 g was taken for microbial analyses. Several slices were wrapped in aluminium foil, vacuum packed and stored at –80ºC for volatile analysis.

2.2 Physicochemical analysis

pH was measured with a pH meter HI 99163 (Hanna Instruments Inc.) with an electrode including built–in temperature sensor and calibration was performed automatically at two points (4 and 7) using standard buffers. Water activity was measured with a Fast–lab water activity meter (Gbx, Romans sur Isère Cédex, France). Colour (CIE L*a*b* system) was analysed with a portable colorimeter (CR–400/410,

Konica Minolta Sensing Inc., Japan) with a fixed aperture (8 mm diameter diaphragm with optical glass) and measurements were made with a D65 iluminant and 0° viewing angle. Three colour measurements were made on each sausage. Moisture was determined by the dehydration method until constant weight (BOE, 1979).

Lipid content was determined by organic extraction (Folch, Lees & Stanley, 1957), lipid oxidation was evaluated using the thiobarbituric acid reactive substances test (TBARS) and protein content was determined by the Kjeldahl method as described in Olivares, Navarro, Salvador, & Flores, (2010). Residual nitrate and nitrite contents were extracted with hot water (Mohamed, Mubarak, Fawy, & El–Shahat, 2008) and determined using an enzymatic kit (Boehringer) (Arneth & Herold, 1988).

2.3 Microbiological analysis

Microbial counts were done on 25 g of dry fermented sausage. Samples were finely sliced, blended with 225 ml of buffered peptone water (Pronadisa, Spain) and homogenized in a Pulsifier (Microgen Biotech, Spain). Homogenates were used to prepare decimal dilutions which were spread on appropriate media plates. Microbial counts were determined on the following media: bacterial starter containing lactic acid bacteria (LAB) on MRS Agar (Scharlau, Spain) at 30 ºC for 3 days and Gram positive cocci on Mannitol Salt Agar (MSA) (CN–M) (Scharlau, Spain), at 30ºC for 3 to 5 days and Baird Parker Agar (BP) (CN–BP) (Pronadisa, Spain) at 37°C for 48 hours. Gram positive cocci isolates from BP were tested for coagulase activity (EN ISO 6888–1) using lyophilised rabbit plasma (Scharlau, Spain). Mesophilic bacteria (TMB) were determined on Plate Count Agar (Pronadisa, Spain) at 30 °C for 3 days, yeasts and moulds on Rose Bengal Agar with chloramphenicol (Scharlau, Spain) at 30°C for 5 to 7 days. Enterobacteriaceae were counted on Violet Red Bile Agar with Glucose (VRBG) (Pronadisa, Spain) at 37°C for 24 hours in anaerobiosis. Sulphite reducing clostridia

were determined from 1 ml homogenate sample inoculated in freshly prepared Lactose Sulfite Broth supplemented with sodium metabisulfite and ferric ammonium citrate (Pronadisa, Spain) dispensed into tubes with Durham gas collecting tubes and incubated in anaerobiosis (bioMerieux, Spain) at 46°C for 48 hours. Twenty–five ml of homogenated sample were used for enrichment of *Yersinia enterocolytica* in Sorbitol Peptone Broth and Bile Salts (PBS) (Pronadisa, Spain) at 25°C for 5 days and subsequently plated on Yersinia Selective Agar (YSA) (Pronadisa, Spain) at 30°C for 48 hours. Twenty–five ml of homogenated sample were used for enrichment of *Listeria* spp. in ½ Fraser and Fraser Broth supplemented with ferric ammonium citrate (Pronadisa, Spain) at 30°C for 24 and 48 hours, respectively. Dilutions of the *Listeria* enriched Fraser medium were inoculated onto Listeria Chromogenic Agar (Pronadisa, Spain) and incubated at 37°C for 24 hours.

The remaining homogenate was incubated at 37°C during 16–20 hours for Salmonella pre–enrichment. One millilitre of the incubated homogenate was used for enrichment of *Salmonella* in Rappaport Soy Broth (Pronadisa, Spain) at 41.5°C for 24 hours and Muller Kauffmann Broth Base w/Brilliant Green and Novobiocin supplemented with iodine and potassium iodide solution (MKTTN) (Pronadisa, Spain) at 37°C for 24 hours. Enriched cultures were plated on Xylose Lysine Desoxycholate Agar (XLD) (Pronadisa, Spain) and incubated at 37°C for 24 hours.

2.4 Volatile compound analysis

The analysis of headspace (HS) volatile compounds was carried out by solid phase micro extraction (SPME) with an 85 μm Carboxen/Polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, PA) using a gas chromatograph Agilent 7890 series II with a mass spectrometer detector MS 5975C, (Agilent, Palo Alto, CA) equipped with an autosampler (Gerstel MPS2 multipurpose sampler, Gerstel, Mülheim

an der Ruhr, Germany), as described Corral, Salvador, Belloch, & Flores, (2015) with minor modifications. Sausage sample (5 g) was weighed into a 20 ml headspace vial and 0.75 mg BHT was added. The vial was incubated at 37 °C for 30 min and volatile compounds were extracted by exposing the fibre to the headspace for 120 min at 37 °C. The fibre was desorbed in the injection port of the GC–MS for 5 min at 240 °C in splitless mode. The compounds were identified by comparison with mass spectra from the library database (Nist'05), by comparison to linear retention indices (Van Den Dool & Kratz, 1963) and using authentic standards. Quantification was based on the total extracted area (TIC). The results were expressed as abundance units (AU x 10^6).

2.5 Statistical analysis

Data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). The data was analysed using the linear mixed model and included nitrate reduction and storage time as fixed effects, and replicates as random effect. The interaction between fixed effects was tested, and it was not significant and was excluded from the model. The replication was not significant ($P>0.10$) for any of the traits. When significant effect of the treatment group was detected ($P < 0.05$), least squares means (LSM) were compared using Tukey test. Principal component analysis (PCA) was done to evaluate the relationships among sausage formulation (nitrate reduction), storage time and different parameters (pH, water activity, TBARS, protein and fat content, nitrate residual, colour, microbiota and volatile compounds).

3. Results

The results of the statistical analysis on physicochemical, microbiology and volatile compounds are shown in Tables 1 to 3 and supplementary tables have been included reporting the results of all nitrate groups at each storage time.

210 *3.1 Physicochemical analyses*

211 Physicochemical parameters were analysed taking into account the two factors
212 vacuum storage time and nitrate reduction (Table 1). During vacuum storage, pH and
213 water activity values suffered a significant decrease, as well as the redness parameter
214 (a*) which decreased significantly after 3 months of storage. In addition, lipid oxidation
215 values (TBARS) showed a significant increase after the second month of vacuum
216 storage that was maintained up to the third month of storage. About residual nitrite and
217 nitrate levels, residual nitrite was below the detection limits while residual nitrate was
218 not affected by vacuum storage.

219 Variation in sausage composition (protein and fat content) among nitrate batches
220 was attributed to variations in the trimming of the pork meat. Control sausages (C) had
221 the highest fat content. Sausages with the smallest ingoing amount of nitrate (RN25)
222 presented a slightly low pH value. Similarly, the redness parameter (a*) was lower in
223 reduced sausages (RN25) than in C batch. Regarding lipid oxidation, TBARS values
224 were lower in reduced nitrate sausages than in C sausage. Residual nitrate was lower in
225 nitrate reduced sausages and confirmed the reduced ingoing amount used in
226 formulation.

227 *3.2 Microbiology analyses*

228 The changes in microbiota are shown in Table 2. Total mesophilic bacteria
229 (TMB) and lactic acid bacteria (LAB) decreased a logarithm cycle ($p<0.001$) after three
230 months, whereas Gram positive cocci (CN–M and CN–BP) decreased between 1.5 and 2
231 logarithm cycles. Coagulase test on CN–BP cocci isolates (about 200 isolates) classified
232 all of them as coagulase negative suggesting that they are probably *Staphylococcus* from
233 the bacterial starter.

In the case of Enterobacteriaceae, yeast and moulds, sulphite reducing clostridia, *Salmonella* spp and *Yersinia enterocolytica* no counts were detected the whole vacuum storage period. *Listeria* counts were also negative in all samples except for one positive (blue–green) colony found in a LCA replicate of a RN25 sample at one month of vacuum storage. No positive colonies were found in the equivalent sample in successive months of vacuum storage. On the other hand, nitrate reduction (Table 2) produced a general increase in microbial counts, especially in case of Gram positive cocci, and for TMB only when nitrate was 15 % reduced.

3.3. Volatile compound analysis

Volatile compounds were analysed in the headspace of sausages by SPME–GC–MS. Fifty–three volatile compounds were identified and quantified (table 3) using the CAR/PDMS fibre. These volatile compounds were classified by their possible origin: microbiota activity (amino acid degradation (14), carbohydrate fermentation (9), lipid β–oxidation (3) and esterase activity reactions (6)), lipid oxidation reaction (20) and unknown origin (1). Figure 1 shows the abundance of volatile compounds groups according to storage time and nitrate reduction.

Volatile compounds derived from amino acid degradation were affected by storage time producing a decrease after 3 months of storage (Fig. 1a). This might be due to the significant decrease of benzene, 2–methyl–1–propanol, toluene, 3–methyl–1–butanol, and 2–methyl–1–butanol (Table 3). However, other compounds (2,6–dimethyl–pyrazine, methional and 3–methylbutanal) increased with vacuum storage time. In contrast, nitrate reduction did not affect the total abundance of volatiles derived from amino acid degradation, except for two compounds. An increase of 2,6–dimethyl–pyrazine and a decrease of benzene could be observed as nitrate concentration diminished.

Carbohydrate fermentation was the group who represented the highest proportion of volatile compounds throughout vacuum storage (70–75%). Among them, acetic acid and ethanol were the most abundant compounds. Volatile compounds from carbohydrate fermentation decreased significantly after 3 months of storage (Fig. 1b). Ethanol, acetic acid and 2,3–butanediol were less abundant after 3 months, while a reduction in butanoic acid was observed since the second month (Table 3). On the contrary, acetone and 2–butanone increased with vacuum storage. Regarding nitrate reduction, an increase in the compounds generated by carbohydrate fermentation was observed (Fig. 1b). Acetone, acetic acid and 2,3–butanediol were more abundant in RN25 sausages. In contrast, 2,3–butanedione and butanoic acid were more abundant in C batch.

Volatile compounds derived from esterase activity decreased after 3 months of storage (Fig. 1c). Ethyl acetate, ethyl butanoate, ethyl 2–hydroxypropanoate, ethyl–3–methylbutanoate and ethyl–2–methylbutanoate decreased at the third month of storage. However, nitrate reduction had not impact on production of these volatile compounds, except for ethyl octanoate which was less abundant in RN25 sausages (Table 3).

Regarding volatile compounds derived from lipid β–oxidation, only nitrate reduction produced a significant effect on the total abundance (Fig. 1d). The effect of vacuum storage time was only seen in few compounds such as 2–heptanone and 1–octen–3–ol which concentration increased and 2,3–pentanedione which showed the opposite effect (Table 3). Moreover, the highest reduction in nitrate (RN25) produced the decrease of 2–heptanone and 1–octen–3–ol.

In the same way, lipid oxidation volatile compounds increased with storage time (Fig. 1e). This is the case of pentane, butanal, pentanal, 1–pentanol, hexanal, 2–pentylfuran, and (E)–2–heptenal (table 3). However, several compounds decrease after 3

months of vacuum storage (propanal, 1–propanol, 2–hexenal and nonanal). Regarding the effect of nitrate content, only the highest nitrate reduction (RN25) produced a significant reduction of the total abundance (Fig. 1e). The strongest decrease in concentration was observed in pentane, heptane, octane, hexanal, hexanoic acid and octanal.

Carbon disulphide was identified as an unknown compound which increased with storage time and nitrate reduction (Table 3).

Among the 53 volatile compounds present in the sausages, 20 of them were identified as potential aroma contributors by gas chromatography–olfactometry (Perea–Sanz, Montero, Belloch, & Flores, 2018). These compounds contribute to specific aroma notes as indicated in Table 3. In order to examine the relationship of the chemical and microbiological parameters with the aroma compounds a principal component analysis (PCA) was performed (Fig. 2). Two principal components were able to explain the 55.34% of the total variability. PC1 accounts for 32.87% of the variability and distinguishes samples by vacuum storage time as seen by the time progression from right to left quadrant. First months of storage (1 and 2 months, right upper quadrant) were related to aromatic volatile compounds derived from microorganism metabolism (LAB and CN–BP, CN–M): carbohydrate fermentation (2,3–butanedione, 3–hydroxy–2–butanone and acetic acid), esterase activity (ethyl octanoate, ethyl butanoate, ethyl–2–hydroxypropanoate and ethyl–3–methylbutanoate), one compound from lipid β–oxidation (2,3–pentanedione) and amino acid degradation (dimethyl disulphide) as well as to aroma compounds derived from lipid oxidation (1–hexanol and heptanal). In contrast, longer vacuum storage times (3 months) were related to lipid oxidation volatile compounds (2–pentylfuran, 2–methyl–furan, octanal, and hexanal), lipid β–oxidation (1–octen–3–ol and 2–heptanone) and compounds from sulphur amino acid degradation

309 (methanethiol and methional) and one from carbohydrate fermentation (2–butanone).
310 PC2 accounts for 22.46% of the variability and distinguishes samples by nitrate content.
311 As can be observed, C and RN15 sausages are placed on the upper quadrant, and RN25
312 sausages on the bottom quadrant. In addition, C and RN15 sausages appeared related to
313 most of the aromatic volatile compounds analysed derived from microbial metabolism
314 and lipid oxidation reactions. However, volatile compounds derived from sulphur amino
315 acid degradation (methanethiol and methional) and 2–butanone were related to sausages
316 with 25% nitrate reduction (RN25).

317

318 ## 4. Discussion

319 During vacuum storage, dry fermented sausages underwent changes on
320 physicochemical and microbiological characteristics as reported by Kim et al., (2012)
321 and Rubio et al., (2007). The general decrease of microbial counts during vacuum
322 storage (Table 2) might have an impact on organoleptic sausage quality. This general
323 decline in microbial counts during vacuum storage appears to be the main consequence
324 of low pH and a_w, which act as hurdles for microbial growth (Leistner, 2000,
325 Christieans et al., 2018). The slight but continuous pH decrease observed at successive
326 months of storage (Table 1) may be due to the metabolic activity of LAB, which are still
327 active although to a lesser extent. Similar results were reported by Rubio et al. (2007) in
328 sliced sausages under vacuum storage and modified atmospheres, despite no changes in
329 a_w were seen. In agreement with our results, Bañon et al. (2014) and Tabanelli et al.
330 (2013) reported a general microbial growth inhibition possibly due to the a_w decrease.
331 Other authors have described few changes in microbial counts and no effect on pH
332 values (Hospital et al., 2014; Kim et al., 2012). On the contrary, an increase in sausage
333 pH under **vacuum conditions (Ščetar et al., 2013)**, modified atmospheres (**Ščetar et al.,**

334 2013; Tabanelli et al., 2013) and perforated packages (Bañon et al., 2014) has been
335 demonstrated in other studies.

336 Regarding sausage colour, a redness decrease (Table 1) was reported in sausages
337 storage under vacuum (Summo et al., 2006, 2010) and in entire sausages stored in
338 perforated packages (Bañon et al., 2014). In fact, several authors indicated that vacuum
339 packaged produce less redness intensity than modified atmosphere packaging (Rubio et
340 al., 2008; Zanardi et al., 2002) or perforated packaging (Summo et al., 2006). On the
341 contrary, other authors indicated an increase in redness (a*) under vacuum packed
342 storage (Kim et al. 2012; Rubio et al., 2008).

343 Concerning lipid oxidation, different results have been reported during vacuum
344 storage of dry fermented sausages. Rubio et al. (2008) observed a decrease on lipid
345 oxidation value in agreement with our results (table 1), while others did not observe
346 changes (Summo et al., 2010). However, many studies have reported an increase in lipid
347 oxidation values during vacuum storage (Dos Santos et al., 2017; Kim et al., 2012;
348 Summo et al., 2006, 2010; Ščetar et al., 2013; Zanardi et al., 2002) and under modified
349 atmosphere (Ščetar et al., 2013; Zanardi et al., 2002;). Different patterns of lipid
350 oxidation can be explained by different ingredients, such as spices with antioxidant
351 activity (Yashin, Yashin, Xia & Nemzer, 2017), in addition to the manufacture process.
352 Moreover, the low specificity of the TBARS test contributes to the observed differences
353 since malonaldehyde is an unstable molecule and could react with other compounds
354 present in the meat matrix (Janero, 1990).

355 The general decrease observed in microbial counts is in agreement with previous
356 studies (Bañon et al., 2014; Rubio et al., 2007). Despite the decrease in LAB and Gram
357 positive cocci inoculated with the bacterial starter, pH decreased slightly during storage

358 suggesting the existence of bacteria metabolic activity. The low pH and a_w effectively
359 prevented growth of pathogenic bacteria as *Salmonella* spp., *Listeria* spp., Gram
360 positive coagulase positive cocci and *Clostridium* spp. even in RN25 sausages (Bañon
361 et al., 2014). Therefore, our results suggest that no apparent risk regarding microbial
362 safety can be attributed to sausages stored in the conditions utilised in our study.

363 Microbial growth is related to volatile compounds production through their
364 metabolism. LAB generate volatile compounds from amino acid degradation and
365 carbohydrate fermentation reactions together with staphylococci, which also generate
366 ethyl esters with fruity notes through their esterase activity (Flores & Olivares, 2015).
367 During vacuum storage a general decrease of volatile compounds derived from
368 microbial activity was observed (Fig. 1). Similar results under vacuum storage were
369 reported by Summo et al. (2011). These authors found a decrease of volatile compounds
370 derived from carbohydrate fermentation during its shelf–life under vacuum storage, in
371 addition to an increase of volatile compounds derived from lipid oxidation (Fig. 1). On
372 the contrary, Dos Santos et al. (2015) observed an increase of volatile compounds
373 derived from amino acid degradation and carbohydrate fermentation in addition to those
374 from lipid oxidation. Differences between studies can be due to different sausage
375 manufacture process, use of spices and smoking process (Summo et al., 2011).

376 Nitrate residual content was not affected by the storage time under vacuum
377 although the residual concentration detected declined between 44 to 51% respect to the
378 initial amount measured in the minced meat in C, RN15 and RN25 sausages (Perea–
379 Sanz et al., 2018). The absence of nitrate reduction during vacuum storage could be due
380 to a low nitrate reductase activity available during storage due to the low Gram positive
381 cocci counts (Table 2) and the pH value close to 5.0 that inhibit this activity (Sanchez
382 Mainar & Leroy, 2015). Nevertheless, the reduction of nitrate ingoing amounts in

383 fermented sausages produced changes in the production of volatile compounds although
384 nitrate reduction did not affect directly microbial growth but affected microbial
385 metabolism (Perea–Sanz et al., 2018). Nitrate reduced sausages had less nitrite available
386 and therefore, lowest antioxidant activity, but the highest oxidation reactions were
387 detected in control sausages due to its high fat content (Olivares et al., 2010). This fact
388 is in accordance with volatile compounds derived from lipid oxidation and lipid β–
389 oxidation, which were in high abundance in control sausages. Moreover, reduced nitrite
390 antimicrobial activity in nitrate reduced sausages may be the reason for high Gram
391 positive cocci counts (CN–M and CN–BP) as observed by Hospital et al. (2014) after
392 thirty days of vacuum storage. The higher counts of Gram positive cocci detected in
393 nitrate reduced sausages (RN15 and RN25) would be responsible for the high amount of
394 volatile compounds derived from carbohydrate fermentation. Similarly, the increment in
395 the generation of volatile compounds from amino acid degradation and ester compounds
396 observed in nitrate reduced sausages would be the result of high counts of
397 Staphylococci (Flores & Olivares, 2015), as LAB were insignificantly affected by
398 nitrate reduction.

399 Changes in volatile compounds produced by vacuum storage of slow fermented
400 sausages (Table 3) affected the aroma profile of the product (Fig. 2). Under vacuum
401 storage, several authors observed a decline of the characteristic sausage aroma and
402 quality as reported by Kim et al., (2012), Rubio et al., (2007) and Summo et al., (2006).
403 Packaging under modified atmosphere altered the sausage volatile profile and produced
404 a more intense "raw meat" aroma and a less distinct "dry sausage" aroma (Viallon et al.,
405 1996). The effect on the volatile profile was related to the increase in ethanol, diacetyl,
406 acetoin and restriction of acetic acid, 1,3–butanediol and 2,3–butanediol (Viallon et al.,
407 1996). Moreover, packaging under vacuum storage produced a limited number of lipid

408 oxidation compounds as reported by Viallon et al., (1996), in opposition to the results
409 observed in Figure 2. The present results demonstrate the relationship of
410 microbiological and physicochemical characteristics and the effect of factors, vacuum
411 storage and nitrate reduction, on sausage aroma. The compounds with pleasant and
412 sweet aroma (3–hydroxy–2–butanone, ethyl octanoate, ethyl–3–methylbutanoate and 2,3–
413 pentanedione) and with cheesy/buttery odour (2,3–butanedione and ethyl–2–
414 hydroxypropanoate) were related to short vacuum storage times and to control and 15%
415 reduced nitrate sausages. In contrast, the characteristic "dry sausage" aroma loss might
416 be the result of the increase of volatile compounds such as heptanal (green, unpleasant
417 odour) and compounds related to unpleasant odours, methanethiol (rotten odour) and
418 methional (cooked potato) (Perea–Sanz et al., 2018). In summary, small nitrate
419 reductions of 15 % did not produce a significant effect on aroma profile in slow
420 fermented sausages in contrast to the more negative effect produced by a reduction of
421 25% nitrate.

422

423 ## 5. Conclusion

424 Vacuum storage and reduced amounts of ingoing nitrate influenced the shelf–life
425 of slow fermented sausages in terms of microbial and organoleptic characteristics.
426 Microbial growth was affected mainly by vacuum storage and to a lesser extent by
427 nitrate content, leading to changes in the profile of volatile compounds. On the one
428 hand, vacuum storage time produced a decrease in volatile compounds derived from
429 amino acid degradation, carbohydrate fermentation and esterase activity after three
430 months under vacuum. On the other hand, the reduction of ingoing nitrate amounts
431 caused a decrease of volatile compounds derived from lipid oxidation and β–oxidation

reactions. These changes affected the production of key aroma compounds and sausage aroma. More studies are necessary to elucidate the mechanism involved in the effect of nitrate reduction during vacuum storage in slow fermented sausages to determine the appropriate sausage shelf–life.

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553 FIGURA LEGENDS

554 Figure 1. Abundance of volatile compounds (Au x 10^6) according to storage time (1, 2
555 or 3 m of vacuum storage) and nitrate reduction (C: control batch 250 ppm sodium
556 nitrate; RN15: 15% reduction 212.5 ppm; RN25: 25% reduction 187.5 ppm). Different
557 letters in each group indicate significant differences: *** P<0.001, ** P<0.01, * P
558 <0.05. ns: P>0.05. Volatile compounds grouped according to origin: derived from
559 bacterial metabolism (a: amino acid degradation; b: carbohydrate fermentation; c:
560 esterase activity, d: lipid β-oxidation reactions) and chemical reactions (e: lipid
561 oxidation).

562 Figure 2. Loadings of the first two principal components (PC1-PC2) of the analysed
563 parameters (physicochemical and microbiological parameters and aroma volatile
564 compounds) in dry fermented sausages based on nitrate content: C: control batch 250
565 ppm sodium nitrate; RN15: 15% reduction 212.5 ppm; RN25: 25% reduction 187.5
566 ppm, and vacuum storage (1, 2 and 3 m). Abbreviations are indicated in tables 1 and 2.

Table 1. Effect of vacuum storage time and nitrate reduction on physicochemical parameters of dry fermented sausages. Values are presented as least squares means

	Vacuum storage time			Nitrate reduction			P_t[2]	P_n	RMSE[3]
	1m	2m	3m	C[1]	RN15	RN25			
pH	5.09 a	5.03 b	4.96 c	5.05 a	5.04 a	5.00 b	***	**	0.05
Aw	0.887 a	0.883 b	0.876 c	0.883	0.881	0.881	***	ns	0.01
Moisture (%)	40.3 b	42.4 a	42.3 a	40.6 b	41.9 a	42.5 a	***	**	1.38
Protein (% dm)	55.4 ab	56.4 a	54.0 b	51.7 c	55.5 b	58.5 a	**	***	2.00
Fat (% dm)	30.7 b	33.8 a	33.8 a	36.4 a	32.3 b	29.6 c	***	***	2.01
L*	48.1	48.1	47.8	48.4	48.2	47.5	ns	ns	1.27
a*	18.3 ab	18.6 a	17.9 b	18.5 a	18.4 ab	17.9 b	*	*	0.68
b*	6.7 b	7.1 a	7.3 a	7.2 a	7.2 a	6.7 b	***	***	0.36
TBARS[4]	0.99 a	0.75 b	0.74 b	1.02 a	0.86 b	0.62 c	***	***	0.18
NO_3 (ppm dm)	197.8	190.2	196.1	235.7 a	185.7 b	162.6 b	ns	***	35.0

[1]C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm). [2]*Pt*: *P* value of storage time effect and *Pn*: *P* value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. ns: $P > 0.05$. [3]RMSE: root mean square error. [4]TBARS expressed as µg malonaldehyde/g dm.

Table 2. Effect of vacuum storage time and nitrate reduction on microbial counts (log cfu/g) of dry fermented sausages. Values are presented as least squares means

	Culture medium	Vacuum storage time			Nitrate			P_t[2]	P_n	RMSE[3]
		1m	2m	3m	C[1]	RN15	RN25			
Total mesophilic bacteria (TMB)	PCA[4]	7.6 a	7.5 a	6.5 b	7.1 b	7.3 a	7.2 ab	***	*	0.2
Lactobacillus (LAB)	MRS	6.6 a	6.5 a	5.6 b	6.2	6.3	6.2	***	ns	0.2
Gram positive cocci (CN-M)	MSA	3.7 a	2.0 b	1.6 b	1.4 b	2.9 a	2.9 a	***	***	0.7
Gram positive cocci (CN-BP)	BP	4.1 a	3.4 b	2.6 c	3.2 b	3.5 a	3.4 a	***	***	0.2

[1]C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm). [2]*Pt*: *P* value of storage time effect and *Pn*: *P* value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P<0.001$, ** $P<0.01$, * $P<0.05$. ns: $P>0.05$. [3]RMSE: root mean square error [4] PCA: Plate Count Agar, MRS: Man Rogosa Sharpe agar, MSA: Mannitol Salt Agar, BP: Baird Parker Agar.

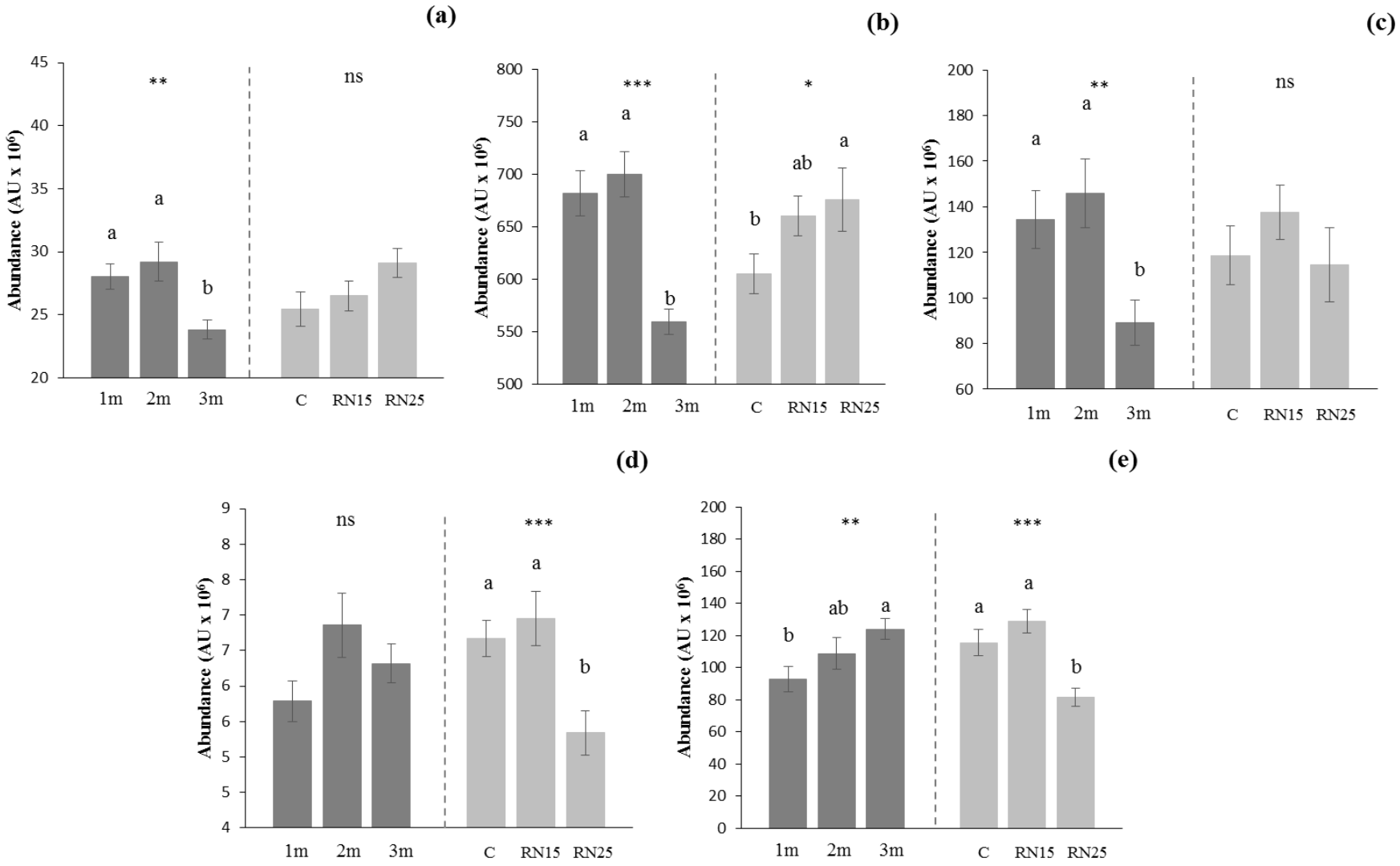
Table 3. Effect of vacuum storage time and nitrate reduction on volatile compounds generated (expressed as AU x 10^6) in dry fermented sausages. Values are presented as least squares means

				Vacuum Storage time			Nitrate					
	LRI[1]	RI[2]	Aroma[6]	1m	2m	3m	C[3]	RN15	RN25	P_t[4]	*Pn*	RMSE[5]
Amino acid degradation												
2-Methylpropanal	594	a		0.49	0.48	0.61	0.51	0.59	0.50	ns	ns	0.17
Benzene	676	a		0.19 a	0.17 a	0.13 b	0.19 ab	0.18 a	0.11 b	**	***	0.04
2-Methyl-1-propanol	683	a		1.44 a	0.68 b	0.99 b	0.87	1.25	0.99	***	ns	0.48
3-Methylbutanal	691	a		2.23 b	3.98 a	4.20 a	3.44	3.36	3.77	***	ns	0.87
Dimethyl disulfide	773	a	Toasted, garlic	0.45	0.55	0.39	0.41	0.47	0.49	ns	ns	0.15
Toluene	788	a		3.44 a	3.78 a	2.64 b	3.55	3.09	3.22	***	ns	0.73
3-Methyl-1-butanol	795	a		10.87 a	9.89 a	5.81 b	9.12	8.64	8.96	***	ns	2.41
2-Methyl-1-butanol	797	a		2.11 a	1.86 a	1.11 b	1.68	1.67	1.72	***	ns	0.51
2,6-Dimethylpyrazine	945	a		1.97 b	2.65 a	2.29 b	1.79 c	2.23 b	2.88 a	***	***	0.41
Methional	968	a	Cooked potato	0.65 b	1.31 a	1.17 a	0.88	0.86	1.31	*	ns	0.39
Benzaldehyde	1020	a		1.23	1.39	1.40	1.24	1.34	1.43	ns	ns	0.37
Benzeneacetaldehyde	1110	a		0.81	0.86	0.80	0.77	0.87	0.84	ns	ns	0.17
Phenol	1114	a		2.56	2.45	2.38	2.41	2.45	2.53	ns	ns	0.29
Methanethiol	473	a	Rotten	0.99	1.14	1.11	1.01	1.10	1.13	ns	ns	0.3
Carbohydrate fermentation												
Acetaldehyde	466	a		5.10	5.56	4.64	5.36	5.03	4.91	ns	ns	1.1
Ethanol	507	a		291.12 a	309.72 a	228.76 b	263.73	300.37	265.50	**	ns	66.11
Acetone	529	a		5.40 b	7.37 a	7.40 a	5.41 b	6.28 b	8.47 a	**	***	1.84
2,3-Butanedione	627	a	Cheese, butter	1.25	1.13	1.01	1.33 a	0.90 b	1.16 ab	ns	*	0.46
2-Butanone	631	a	Fruity, butter	1.99 b	3.13 a	3.43 a	2.93	2.44	3.18	***	ns	0.9
Acetic acid	718	a	Vinegar	290.55 a	300.94 a	247.70 b	260.05 b	278.82 ab	300.31 a	***	*	38.54

3-Hydroxy-2-butanone	781	a	Sweet, fruity	21.67		18.23		15.52		21.74		15.36		18.31		ns	ns	8.36
2,3-Butanediol	888	a		72.17	a	70.39	a	45.03	b	53.98	b	62.97	ab	70.64	a	***	**	12.61
Butanoic acid	896	a		8.51	a	5.97	b	3.23	c	7.09	a	5.18	b	5.44	b	***	***	0.68
Esterase activity																		
Ethyl acetate	635	a		112.65	a	122.35	a	69.68	b	94.63		109.45		100.62		***	ns	28.73
Ethyl butanoate	832	a	Fruity	9.95	a	9.58	a	6.57	b	8.84		9.54		7.71		***	ns	2.41
Ethyl-2-hydroxypropanoate	867	a	Fruity, sweet	9.54	a	9.70	a	6.09	b	7.45		9.31		8.57		***	ns	2.2
Ethyl-2-methylbutanoate	878	a		4.31	a	4.46	a	2.73	b	3.54		4.09		3.87		***	ns	1.16
Ethyl-3-methylbutanoate	882	a	Fruity, sweet	9.17	a	10.67	a	5.67	b	7.76		9.13		8.63		***	ns	3.17
Ethyl octanoate	123	a	vegetable, fruity	5.07		5.10		4.14		4.60	b	5.93	a	3.78	b	ns	***	1.48
Lipid β-oxidation																		
2,3-Pentanedione	745	a	Sweet, candy	2.17	ab	2.47	a	1.66	b	2.19		2.19		1.92		**	ns	0.65
2-Heptanone	935	a	Rancid, fruity	1.59	b	1.99	a	2.09	a	1.97	a	2.01	a	1.70	b	***	**	0.31
1-Octen-3-ol	1033	a	Mushroom	2.31	b	2.77	a	2.75	ab	2.75	a	2.92	a	2.16	b	*	***	0.55
Lipid oxidation																		
Pentane	500	a		3.40	b	5.28	a	4.49	ab	4.63	a	5.40	a	3.14	b	**	***	1.43
Propanal	524	a		0.73	ab	0.91	a	0.66	b	0.76	ab	0.93	a	0.61	b	*	**	0.22
Hexane	600	a		1.77		1.85		1.61		1.86	b	2.30	a	1.07	c	ns	***	0.5
1-Propanol	612	a		2.32	a	1.37	b	1.02	b	1.09	b	2.36	a	1.26	b	*	**	0.8
2-Methylfuran	616	a	Green, garlic	0.14		0.20		0.17		0.20		0.17		0.15		ns	ns	0.06
Butanal	622	a		0.06	b	0.13	a	0.15	a	0.14	a	0.11	ab	0.08	b	***	***	0.04
Heptane	700	a		15.36		16.81		17.45		19.65	a	20.18	a	9.77	b	ns	***	4.67
Pentanal	739	a		2.62	c	4.43	b	5.68	a	4.74	a	4.91	a	3.07	b	***	***	0.92
Octane	800	a		22.36		21.94		24.11		26.15	a	28.54	a	13.71	b	ns	***	6.95

1-Pentanol	827	a		2.20	b	3.20	a	2.97	a	3.07	a	3.38	a	1.92	b	**	***	0.87
Hexanal	842	a	Fresh cut grass	25.90	b	33.44	ab	40.19	a	35.03	a	40.30	a	24.20	b	***	***	9.41
2-Hexenal	907	a		0.24	a	0.21	a	0.14	b	0.18		0.22		0.20		***	ns	0.05
1-Hexanol	924	a	Oxidized fat	6.70		7.11		5.35		6.17		7.34		5.65		ns	ns	2.08
Heptanal	941	a	Green	7.61		7.92		7.28		5.69	b	8.21	a	8.92	a	ns	***	2.15
Decane	1000	a		0.40		0.43		0.42		0.39		0.45		0.42		ns	ns	0.09
2-Pentylfuran	1010	a	Garlic, onion	1.42	b	1.65	ab	1.75	a	1.60	ab	1.82	a	1.40	b	*	***	0.32
(E)-2-Heptenal	1013	a		0.15	b	0.24	a	0.20	ab	0.19		0.20		0.19		**	ns	0.07
Octanal	1049	a	Orange, sweet	3.30		3.25		3.42		3.23	ab	3.77	a	2.98	b	ns	*	0.81
Hexanoic acid	1079	a		3.46		3.59		3.67		3.76	a	4.10	a	2.85	b	ns	***	0.83
Nonanal	1151	a		5.70	a	5.34	ab	4.83	b	4.59	b	5.70	a	5.58	b	*	***	0.92
Unknown compound																		
Carbon disulfide	537	a		3.65	b	6.58	a	4.12	b	4.16	b	4.56	b	5.65	a	***	**	1.18

[1]LRI: Linear retention index of the compounds eluted from the GC-MS. [2]RI: Reliability of identification: a, identification by mass spectrum, coincidence with the LRI of an authentic standard; b, tentatively identification by mass spectrum.[3]C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm). [4]*Pt*: *P* value of storage time effect and *Pn*: *P* value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P<0.001$, ** $P<0.01$, * $P<0.05$. ns: $P>0.05$. [5]RMSE: root mean square error. [6]Compounds detected as aroma active compound by GC-olfactometry (Perea-Sanz et al., 2018).


(a)
(b)
(c)
(d)
(e)
Abundance (AU x 10⁶)
1m
2m
3m
C
RN15
RN25
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ns
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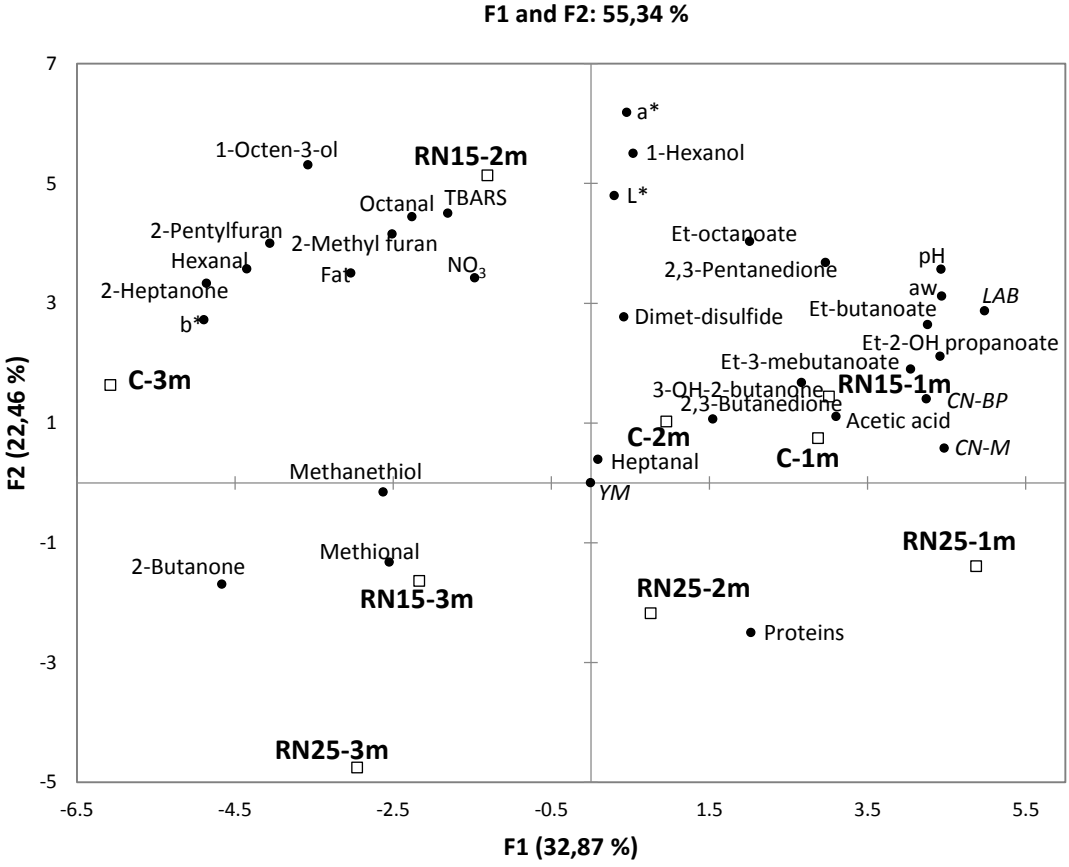
F1 and F2: 55,34 %
F2 (22,46 %)
F1 (32,87 %)
7
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-1
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a*
1-Hexanol
L*
1-Octen-3-ol
RN15-2m
Octanal
TBARS
2-Pentylfuran
2-Methyl furan
Hexanal
Fat
NO3
2-Heptanone
b*
C-3m
Et-octanoate
2,3-Pentanedione
pH
aw
LAB
Dimet-disulfide
Et-butanoate
Et-2-OH propanoate
Et-3-mebutanoate
3-OH-2-butanone
RN15-1m
CN-BP
2,3-Butanedione
Acetic acid
C-2m
C-1m
CN-M
Heptanal
Methanethiol
YM
RN25-1m
Methional
2-Butanone
RN15-3m
RN25-2m
Proteins
RN25-3m