Counteracting the effect of reducing nitrate/nitrite levels on dry fermented sausage aroma by *Debaryomyces hansenii* inoculation

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Abstract

The reduction of ingoing amounts of nitrate and nitrite in dry fermented sausages was studied together with the impact of *Debaryomyces hansenii* inoculation on aroma generation. Three different formulations of sausages were manufactured: control (C), reduced in nitrate and nitrite ingoing amounts (R) and reduced R inoculated with *D. hansenii* (RY). Changes in physicochemical and microbiological parameters, volatile compounds and aroma were investigated at different drying times. Nitrite/nitrate reduction did not seem to affect microbial growth but affected their metabolic activity. Moreover, nitrite/nitrate reduction decreased lipid oxidation and generation of derived volatile compounds. Yeast inoculation limited lipid oxidation and prevented nitrite oxidation. Sausage aroma profile was positively affected by *D. hansenii* inoculation which contributed to the generation of potent aroma compounds like ethyl ester compounds and 3-methylbutanal. Long drying time impacted sausage aroma profile as well as yeast metabolism. Yeast inoculation counteracted the negative influence of nitrite/nitrate reduction due to its antioxidant capacity, aroma generation and hindered nitrite oxidation.

Keywords: Nitrate; Nitrite; *D. hansenii*; Fermented sausage; Volatile compounds; Aroma

1. Introduction

The use of nitrite and nitrate as curing agents in dry fermented sausages have important technological functions. Nitrite has antioxidant and antimicrobial activities being the most important its activity against the pathogen *Clostridium botulinum* and control of toxin production. Furthermore, nitrite facilitates the generation and stabilization of the typical colour and enhances cured flavour (Honikel, 2008; Sindelar & Milkowski, 2011). Nitrate acts as nitrite reservoir in dry cured products with a long ripening time (Sanchez Mainar & Leroy, 2015). However, the controversy about the adequate amounts of nitrite used as additive in meat products processing is under concern (CODEX, 2019). Nitric oxide, from nitrite, can react with biogenic amines present in meat under heating conditions to form N-nitrosamines, with carcinogenic potential (De Mey, De Maere, Paelinck & Fraeye, 2015). Therefore, the actual trend is to reduce the use of nitrite and nitrate in cured meat products (EFSA, 2010), although nitrite is considered beneficial for improving the microbial safety of the products. Up to now, several alternatives to nitrite addition have been studied. Among them, plant derivatives are one of the most used, followed by organic acids, bacteria and bacteriocins (Alahakoon, Jayasena, Ramachandra & Jo, 2015). However, these alternatives do not provide all nitrite functions in dry cured fermented sausages. Nitrate and nitrite reduction below their allowed maximum levels in European legislation (150 mg/kg of sodium nitrate and 150 mg/kg of sodium nitrite, (Regulation (EC) no 1333/2008) have safety and sensory consequences. The reduction up to a 50 % of the allowed limits affects growth of pathogenic microorganisms (Hospital, Hierro & Fernandez, 2014), volatile compounds production (Hospital, Hierro, Stringer & Fernandez, 2016) and aroma profile (Perea-Sanz, Montero, Belloch & Flores, 2018). A recent study (Christieans, Picgirard, Parafita, Lebert & Gregori, 2018) has shown that the use of a mixture of sodium nitrate and sodium nitrite at a concentration of 80 mg/kg, was able to control the growth of *Listeria monocytogenes* and *Salmonella typhimurium*. Nevertheless, the importance of nitrite presence at the beginning of the process to control these pathogens was remarked due to the slow conversion of nitrate into nitrite (Christieans, et al., 2018).

The consequences of the use of reduced nitrate and nitrite mixtures on aroma profile of dry cured meat products have been scarcely studied. The inoculation of yeasts such as *Debaryomyces (D.) hansenii* with potential to generate desirable aromas in dry fermented sausages is a suitable alternative. This yeast has been shown to produce esters and sulfur compounds as cured aroma contributors to the aroma profile of dry fermented sausages (Cano-Garcia, Belloch & Flores, 2014; Perea-Sanz, Peris, Belloch & Flores, 2019a). The production of aroma compounds by *D. hansenii* strains is influenced by the concentration of curing agents (Flores, Moncunill, Montero, López-Díez & Belloch, 2017), as well as by the availability of specific amino acids used as volatile precursors (Perea-Sanz et al., 2019a). A recent study

regarding the production of volatile sulphur compounds that contribute to meat aroma notes by *D. hansenii* strains revealed that one yeast strain may have an impact on dry fermented sausage aroma due to its ability to express a gene directly related to sulfur compound production (Perea-Sanz et al., 2019a). Consequently, the aim of the present study is to evaluate the effect of the inoculation of a *D. hansenii* strain on dry fermented sausages manufactured with reduced ingoing amounts of nitrite and nitrate and its effect on aroma production during the ripening time.

2. Materials and methods

2.1. Dry fermented sausages manufacture

Three different formulations of dry fermented sausages were manufactured. Control (C), containing sodium nitrite at 150 ppm and potassium nitrate at 178 ppm, and two formulations with a 47 % reduction of both nitrite and nitrate (80 and 95 mg/kg, respectively) ingoing amounts (R) as proposed by Christeians et al. (2018), but one of them inoculated with *D. hansenii* strain L1 (RY). Three replicates of the experiment were performed. Pork's lean meat and belly fat (boneless and skinless) were purchased from a local producer (Cárnicas La Cope, Torrente, Spain). Lean (50% lean pork meat) and fat (50% pork belly) were ground through a 10 mm diameter minced plate and mixed with the following ingredients: 20 g/kg lactose, 20 g/kg dextrin, 6 g/kg glucose, 20.25 g/kg sodium chloride (NaCl), 6.75 g/kg potassium chloride (KCl), 0.5 g/kg sodium ascorbate and 1.5 g/kg black pepper. Commercial starter culture TRADI-302 (Danisco, Cultor, Madrid, Spain) containing *Lactobacillus sakei*, *Staphylococcus xylosus* and *Staphylococcus carnosus* was added to the meat (0.125 g/kg). In the three replications, each formulation was supplemented with the different ingoing amounts of nitrate/nitrite mixtures. Then, *D. hansenii* L1 was inoculated using 10 ml saline suspension containing approximately 6×10^9 cells/g. This yeast suspension was added to 6 kg of meat batter to reach a final concentration of 10^6 cells/g. To homogenize all ingredients into the meat batter, a mixer-kneading machine provided with rotating blades was used. Formulations were kept at 3–5°C for 24 h to rest after yeast inoculation and then, stuffed into 95 mm diameter collagen casings (Fibran, S.A., Girona, Spain), being the final weight of each sausage approximately 500 g. All sausages were submitted to a slow ripening process and dried at 10 °C and 75–90 % relative humidity (RH). In order to control the ripening process, temperature and RH of the ripening chambers were continuously recorded. A total of 9 batches (3 x 3) were produced. One sausage from each formulation was weighed regularly to control weight losses. Also, one sausage per formulation was used to control the pH by introducing a pH meter HI 99163 (Hanna Instruments Inc., Hoonsocket, USA) into the sausage centre as described by Olivares, Navarro, Salvador & Flores (2010). Sausages from the different formulation and replicates were sampled for analyses at 0, 60 and 83 d of process. At 0 d, approximately 200 g of the minced meat

mixture were collected for analysis. After 60 d of ripening (44-45 % weight loss), three sausages from each formulation and replicate (3 x 3 sausages) were randomly chosen for analyses. The remaining sausages were ripened for 23 additional days (50% weight loss), and afterwards they were collected as above. Slices were taken for microbial analyses (25 g) and other slices for volatile analysis were vacuum packed and stored at -80 °C. Sausage colour and physicochemical analysis (moisture, water activity a_w , pH, TBARS, protein and residual nitrite and nitrate content) were measured on minced sausage.

2.2. Physicochemical parameters measurement

Measurements of pH, a_w , weight loss, colour (CIE L*, a*, b*), moisture, and protein was done as described in Perea-Sanz et al. (2018). Lipid autoxidation was measured by the thiobarbituric acid reactive substances (TBARS) method and results expressed as μg malonaldehyde (MDA)/g in dry matter (Perea-Sanz et al., 2018). Residual nitrate and nitrite contents were extracted by Carrez precipitation and determined after zinc reduction and Griess reaction as reported in Merino (2009). Measurements were expressed as mg/kg of sodium nitrite and potassium nitrate in dry matter.

2.3. Microbiological analysis

Microbial counts of lactic acid bacteria (LAB), gram positive coagulase negative cocci (GCN), yeasts and moulds and enterobacteria were carried out as described in Perea-Sanz et al. (2018).

2.4. D. hansenii L1 monitoring by M13 RAPD PCR

Fifteen yeast colonies were isolated (Perea-Sanz et al., 2018) from each sausage batch (C, R and RY) at the three ripening times (0, 60 and 83 d). Colonies were tested for purity and freshly isolated colonies selected and cultured overnight at 25 °C in 5 ml of GPY (glucose 2%, peptone 0.5% and yeast extract 0.5%). DNA extraction and M13 minisatellite PCR amplification were carried out as described in Cano-García, Flores & Belloch. (2013). PCR products were separated by electrophoresis on 2 % agarose gel in 1X TAE buffer at 90 V for 3 h, stained with Red safe nucleic acid staining solution 20000x (Intron biotechnology, Korea) and visualized under UV light. DNA fragment sizes were determined using 100 bp DNA ladder (Invitrogen, USA).

2.5. Volatile compound analysis

Analysis of volatile compounds was carried out by headspace (HS) solid-phase microextraction (SPME) with an 85 μm carboxen/polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, USA) using a gas chromatograph (Agilent HP 7890 series II, Hewlett-Packard, Palo Alto, USA) with a quadrupole mass detector (HP 5975C, Hewlett-Packard, Palo Alto, USA) and equipped with an autosampler (MPS2 multipurpose sampler (Gerstel, Germany). In summary, 5 g of sausage supplemented with 0.75 mg BHT to avoid oxidation, were placed into a headspace

vial and incubated at 37 °C for 30 min. The extracted volatile compounds were adsorbed in the fibre for 90 min at 37 °C and desorbed in the injection port of the GC–MS for 5 min at 240 °C in splitless mode. The volatile compounds were separated using a DB-624 capillary column (30 m, 0.25 mm i.d., film thickness 1.4 µm, (J&W Scientific, Agilent Technologies, USA)) using the conditions described by Corral, Salvador, Belloch & Flores. (2015). The MS interface temperature was set to 240 °C. The compounds were identified in full scan mode and by comparison with mass spectra from the library database (Nist'05), with linear retention indices (Van Den Dool & Kratz, 1963) and with authentic standards. The quantitation was done in SCAN mode using either total or extracted ion current (TIC or EIC) on an arbitrary scale and expressed as abundance units (AU) x 10⁵.

2.6. Olfactometric analysis

The analysis of aroma compounds was carried out in a gas chromatograph (Agilent 6890, USA) equipped with a FID detector and a sniffing port (ODP3, Gerstel, Mülheim an der Ruhr, Germany) as described in Corral et al. (2015). Each assessment was carried out on 5 g of sausage using the detection frequency method (Pollien et al., 1997). The olfactometry analysis was done in sausages ripened for 60 d. Five trained panellists evaluated the odours from the GC-effluent. A total of 12 assessments were carried out. Aroma compounds were identified by comparison with mass spectra, using linear retention indices of authentic standards injected in GC–MS and GC-O and by the coincidence of the assessor's descriptors with those in the Fenaroli's handbook of flavour ingredients (Burdok, 2002).

2.7. Statistical analysis

Data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). Data were analysed using the linear mixed model including sausage formulation and ripening time as fixed effects, and replicates as random effect. The interaction between fixed effects was tested and evaluated as not significant, therefore it was excluded from the model. When a 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 plotted to evaluate the relationships among sausage formulations and parameters (physicochemical, microbiological and aroma compounds) measured at each sampling time.

3. Results

3.1. Physicochemical characteristics of the sausages

Measurements of physicochemical parameters from samples of the three formulations at the three sampling times are shown in Table 1. At the initial time (0 d), the three formulations were very similar regarding all parameters except nitrite and nitrate concentration. Residual nitrite

differences between C and reduced R and RY formulations was 45-52%, whereas residual nitrate differences were about 31-33%. After 60 d of ripening, no residual nitrite levels were detected in any formulation, while residual nitrate levels in R and RY respect to C were 40 % and 46 %, respectively. These differences in residual nitrate concentrations were maintained after 83 d of ripening. During the ripening time, residual nitrate decreased around 17 %, 36 % and 48 % within C, R and RY sausages, respectively. Colour parameter a^* increased after 60 d of ripening. Lipid oxidation (TBARS) evolution was different among the formulations. C and R showed the same trend, an initial increase at 60 d and a slow decline after 83 d, while RY sausages did not show lipid oxidation maintaining low TBARS values during the ripening time. In addition, the highest pH value was reached at 60 d in RY sausages. These differences between RY and the other two formulations could be observed also in water activity and moisture at 83 d of ripening time.

3.2. Microbiological counts

Results on microbial counts are shown in Fig. 1. *Enterobacteriaceae* were not found in any sausage batch at any sampling time (data not shown). LAB counts (Fig. 1A) increased from 0 d to 60 d and decreased slightly at 83 d. Significant differences between LAB counts were found at 0 d in RY respect to C and R batches, but at 60 and 83 d LAB counts were indistinguishable between batches. GCN counts (Fig. 1B) decreased constantly from 0 d and no significant differences were found among sausages at any ripening time. Yeast counts (Fig. 1C) were lower than 10 cfu/g in C (2 colonies) and R (5 colonies) sausages at 0 d, whereas the RY showed a yeast count in accordance with *D. hansenii* L1 inoculation. Yeast counts increased from 0 d to 60 d in all sausages and decreased slightly from 60 to 83 d, however counts in RY were higher than C and R.

3.3. *D. hansenii* monitoring by M13 RAPD-PCR along sausage ripening

The implantation of the inoculated *D. hansenii* strain on RY sausages, as well as its presence in C and R sausages, was followed by comparison of M13 minisatellite PCR patterns of yeast isolates with the inoculated *D. hansenii* L1 strain (Fig. 2). M13 patterns of yeast isolates from RY sausages at any ripening time were identical to the pattern of the inoculated *D. hansenii* L1 strain. On the other hand, several yeast isolates from C sausages (Figure 2A) and R sausages (Figure 2B) showed M13 patterns different from the pattern of *D. hansenii* L1 strain. In C sausages, only one isolate at 60 d and in R batch 5 isolates at 60 d and 6 isolates at 83 d were found with identical pattern of the inoculated *D. hansenii* L1 strain.

Table 1. Effect of nitrite/nitrate reduction and *D. hansenii* inoculation on physicochemical parameters of dry fermented sausages.

	C ¹						R						RY						RMSE ²	<i>P</i> _t ³	<i>P</i> _f
	0d	60d	83d	0d	60d	83d	0d	60d	83d	0d	60d	83d	0d	60d	83d	0d	60d	83d			
pH	5.79	a	4.85	d	4.94	bc	5.79	a	4.86	d	4.94	bc	5.80	a	4.99	b	4.92	c	0.05	***	**
aw	0.973	a	0.894	b	0.809	d	0.973	a	0.897	b	0.807	d	0.969	a	0.899	b	0.837	c	0.006	***	***
Moisture %	64.15	a	42.44	b	29.69	d	64.19	a	42.28	b	29.77	d	63.48	a	41.92	b	32.58	c	1.18	***	ns
Protein (% dm)	49.42	d	52.89	cd	54.14	bc	50.71	cd	58.08	a	53.23	bcd	49.97	d	56.22	ab	53.25	bcd	2.81	***	ns
L*	59.81	a	52.85	b	44.76	cd	59.91	a	52.21	b	44.34	d	60.26	a	53.45	b	46.64	c	1.71	***	ns
a*	13.79	c	16.81	a	14.40	bc	14.49	bc	16.76	a	14.24	bc	12.64	d	17.04	a	14.78	b	0.51	***	ns
b*	11.00	a	6.07	b	5.11	c	11.28	a	5.67	b	5.07	c	11.72	a	6.03	b	5.59	bc	0.47	***	*
TBARS⁴	0.59	d	3.62	a	2.21	b	0.48	d	2.23	b	1.27	c	0.47	d	0.63	d	0.47	d	0.32	***	***
NaNO₂ mg/kg dm	217.40	a	0.00	c	0.00	c	102.32	b	0.00	c	0.00	c	119.10	b	0.00	c	0.00	c	11.20	***	***
KNO₃ mg/kg dm	533.05	a	396.77	bc	442.44	b	363.81	c	235.32	d	233.20	d	364.66	c	212.67	d	188.39	d	42.37	***	***

¹C: control batch; R: 47% reduction in nitrite and nitrate; RY: 47% reduction in nitrite and nitrate and *D. hansenii* inoculated. ²RMSE: root mean square error.

³*P*_t: *P* value of time effect, *P*_f: *P* value of formulation 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. ⁴TBARS expressed as µg malonaldehyde/g dm.

Figure 1. Changes in microbial populations during the ripening of dry fermented sausages in Control (●), R (□) and RY (▲) formulations. Lactic acid bacteria (LAB) (a), Gram positive coagulase negative cocci (GCN) (b) and yeasts and moulds (c). Different letters in the same ripening time indicate mean differences at *** P < 0.001 among sausages.

Figure 2. Electrophoretic patterns of minisatellite M13 PCR amplification of yeast isolates at 0, 60 and 83 d of ripening in C (A), R (B) and RY (C) formulations. The arrow indicates bands of isolated yeast in C and R sausages matching the pattern of the inoculated strain L1. Lane M is the 100 bp ladder. Lane L1 in panel C represents the pattern of the inoculated *D. hansenii* strain.

3.4. Volatile compounds generation

Eighty volatile compounds were identified and quantified in the HS of dry fermented sausages using a CAR/PDMS fibre (Table 2). The volatile compounds were classified by their most probable origin. Within the bacterial metabolism origin, four subgroups were done: amino acid degradation (17), carbohydrate fermentation (8), esterase activity (10) and β -oxidation (1)), from the chemical reaction origin two subgroups: lipid oxidation reaction (22) and thiamine degradation (2)); and finally, from spices (17) and from unknown origin (3). Among the 80 volatiles identified, 61 compounds were confirmed while 11 were tentatively identified.

The RY sausages showed the highest concentration of volatile compounds from amino acid degradation at both drying times. The most abundant compounds were 3-methyl-1-butanol, 2-methyl propanoic acid and 2-methyl and 3-methyl butanoic acids, whereas dimethyl disulphide, toluene, 3-(methylthio)propanal, benzeneacetaldehyde, 2,6-dimethylpyrazine and phenol were the less abundant. On the other hand, methanethiol and 3-methylbutanal were the most abundant compounds found in C and R sausages. Comparison between C and R sausages revealed that nitrite and nitrate reduction produce significant differences only in two compounds; 3-(methylthio)propanal and benzeneacetaldehyde which were more abundant in C sausages. During the ripening period between 60 and 83 d, the total volatile compounds derived from amino acid degradation diminished in RY sausages. This decrease was significant in methanethiol, 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, and 3-methyl-1-butanol concentrations. Only benzeneacetaldehyde increased after the 60 d of ripening in RY sausages. Three volatile compounds derived from amino acid degradation were identified and only traces were detected of 2-acetyl-2-thiazoline, 2-acetyl-1-pyrroline and dimethyl trisulfide.

The sum of carbohydrate fermentation volatiles was significantly higher in C and R sausages than in RY sausages at both drying times. The most abundant compounds in C and R sausages respect to RY sausages were 2,3-butanedione, 2-butanone, acetic acid and 3-hydroxy-2-butanone. On the contrary, acetaldehyde and ethyl alcohol were present in significant high proportions in RY sausages. During the ripening time between 60 and 83 d, the total abundance of volatile compounds derived from carbohydrate fermentation increased exclusively in C sausages due to the increase in acetic acid.

Ethyl acetate was the most abundant volatile compound derived from esterase activity in all sausage types, followed by ethyl-3-methylbutanoate in RY sausages. The total abundance of volatile compounds derived from esterase activity was significantly higher in RY sausages than in C and R sausages at both drying times. This difference was observed in all ester compounds except for ethyl pentanoate and ethyl hexanoate. In addition, 3-methylbutyl acetate and ethyl-2-methylpropanoate were exclusively present in RY sausages. Nitrate and nitrite reduction in R

produced a decrease in two ester compounds, ethyl hexanoate and ethyl 2-hydroxypropanoate at 60 d although the additional drying time increased its abundance.

Among volatile compounds derived from chemical origin only those derived from lipid oxidation were analysed being the most abundant hexanal in all sausages. These compounds were the lowest in RY sausages at both ripening times. This was due to a significant decrease of most volatiles except for 1-propanol. The last step of ripening from 60 to 83 d, caused a decrease in the total abundance of volatile compounds derived from lipid oxidation although pentanoic and octanoic acids increased during this period.

Only two volatile compounds from thiamine degradation (2-methyl-3-furanthiol and methyl-2-methyl-3-furyldisulfide) were identified as traces. Finally, volatile compounds derived from spices were abundant in all sausage formulations but most of them were not affected by either formulation or drying time.

Table 2. Effect of nitrite/nitrate reduction and *D. hansenii* inoculation on volatile compounds generated in dry fermented sausages (expressed as abundance (AU) of total ion current (TIC) or area of the target ion shown in parenthesis as AU×10⁵).

[illegible]

Table 2. Continued.

	LRI ¹	RI ²	C ³		R		RY		RMSE ⁴	P _t ⁵	P _f
			60 d	83 d	60 d	83 d	60 d	83 d			
Carbohydrate fermentation			5631.62 b	7405.09 a	6280.08 ab	6877.28 ab	3473.90 c	3777.01 c	983.47	**	***
Acetaldehyde	467	a	25.14 b	20.89 b	18.13 b	16.98 b	73.09 a	28.18 b	13.21	***	***
Ethyl alcohol	507	a	887.72 b	739.85 b	670.60 b	854.35 b	2294.16 a	2192.11 a	151.08	ns	***
Acetone	529	a	69.34 a	47.78 ab	61.17 ab	42.92 b	49.51 ab	54.99 ab	13.49	**	ns
2,3-Butanedione	627	a	129.85 a	129.93 a	160.14 a	119.13 a	19.36 b	9.69 b	27.37	ns	***
2-Butanone	631	a	72.68 a	48.17 b	79.28 a	43.59 b	28.79 b	26.80 b	13.22	***	***
Acetic acid	717	a	2805.24 b	4435.95 a	3122.49 b	3822.32 ab	813.68 c	1343.73 c	651.28	***	***
3-Hydroxy-2-butanone	781	a	1586.48 a	1914.66 a	2109.15 a	1890.61 a	150.70 b	46.42 b	400.29	ns	***
Butanoic acid	891	a	55.16 de	67.85 bc	59.12 cd	87.39 a	44.60 e	75.09 b	6.81	***	***
Esterase activity			404.73 b	364.80 b	415.44 b	423.13 b	1077.97 a	1134.48 a	112.28	ns	***
Ethyl acetate	635	a	229.96 bc	178.56 c	283.17 b	199.47 bc	430.45 a	462.49 a	58.33	ns	***
Ethyl 2-methylpropanoate (71)	788	a	ND b	ND b	ND b	ND b	30.70 a	31.56 a	4.13	ns	***
Ethyl butanoate	831	a	29.65 b	29.57 b	27.13 b	32.82 b	69.91 a	77.54 a	7.21	ns	***
Ethyl 2-hydroxypropanoate	867	a	40.96 bc	46.99 c	26.86 d	51.52 c	81.60 b	143.68 a	10.39	***	***
Ethyl 2-methylbutanoate (57)	878	a	2.41 b	3.26 b	2.60 b	5.20 b	31.95 a	32.78 a	5.84	ns	***
Ethyl 3-methylbutanoate	882	a	33.96 b	55.91 b	32.89 b	94.84 b	387.96 a	386.12 a	84.17	ns	***
3-Methylbutyl acetate (70)	907	a	ND b	ND b	ND b	ND b	1.00 a	1.03 a	0.10	ns	***
Ethyl pentanoate	928	a	21.53 a	12.56 bc	13.65 b	10.93 bcd	8.44 cd	6.76 d	2.82	***	***
Ethyl hexanoate	1030	a	45.43 a	37.45 ab	28.48 b	27.88 b	32.58 b	36.30 ab	6.71	ns	***
Ethyl octanoate (88)	1230	a	0.83 c	0.50 c	0.66 c	0.46 c	3.37 b	4.10 a	0.28	ns	***

Table 2. Continued.

	LRI ¹	RI ²	C ³		R		RY		RMSE ⁴	P _t ⁵	P _f
			60 d	83 d	60 d	83 d	60 d	83 d			
β-Oxidation											
1-Octen-3-ol	1032	a	30.49 a	29.09 a	25.23 a	24.13 a	10.04 b	12.43 b	5.00	ns	***
Chemical origin											
Lipid oxidation			3441.38 a	2502.57 b	1768.25 bc	1192.48 cd	539.38 d	603.53 d	499.39	**	***
Pentane	500	a	31.95	17.41	28.03	28.22	32.05	35.80	11.09	ns	ns
Hexane	600	a	8.28	5.01	8.38	7.40	9.35	7.43	2.62	*	ns
1-Propanol	312	a	2.02 c	1.99 c	1.95 c	1.74 c	21.69 a	13.16 b	1.66	***	***
Butanal	622	a	3.68 a	2.41 b	1.91 bc	1.51 bcd	0.77 d	1.22 cd	0.62	ns	***
Heptane	700	a	9.49	6.94	9.76	8.63	6.92	7.06	3.20	ns	ns
Pentanal	739	a	136.60 a	88.75 b	69.27 bc	49.72 bcd	19.23 d	31.16 cd	24.34	*	***
Octane	800	a	135.27 a	128.26 ab	120.72 abc	102.54 abc	74.20 c	82.53 bc	29.58	ns	***
Propanoic acid	811	a	14.80 bc	21.55 a	12.39 c	18.80 ab	12.72 c	15.09 bc	2.77	***	**
1-Pentanol (70)	827	a	4.45 a	2.57 b	3.83 a	2.02 bc	1.82 bc	1.15 c	0.65	***	***
Hexanal	842	a	2774.14 a	1969.97 b	1223.18 c	768.59 cd	201.53 d	270.65 d	410.63	**	***
1-Hexanol	924	a	81.87 a	39.79 ab	80.92 ab	37.78 bc	30.23 c	11.20 c	24.98	***	***
Heptanal (70)	941	a	17.36 a	17.18 ab	13.11 ab	8.69 b	ND b	ND b	5.62	ns	**
Pentanoic acid (60)	980	a	4.00 ab	4.96 a	3.29 b	4.23 ab	1.08 c	1.30 c	0.73	**	***
2-Pentylfuran	1010	a	25.20 a	25.22 a	15.42 b	17.78 b	3.79 c	4.28 c	3.51	ns	***
(Z)- 2-Heptenal	1012	a	10.45 a	8.20 ab	5.90 bc	5.65 c	1.89 d	ND	1.47	*	***
Octanal (56)	1049	a	7.14 a	6.27 a	5.71 ab	3.71 bc	3.02 c	3.12 c	1.33	*	***
Hexanoic acid (60)	1077	a	22.26 a	25.95 a	19.09 a	22.42 a	5.27 b	6.88 b	3.98	0.038	***
(E)-2-Octenal	1117	a	13.03 a	11.01 ab	7.45 bc	7.76 bc	1.37 d	5.17 cd	2.25	ns	***
Nonanal	1151	a	122.97	95.75	122.58	75.31	97.86	87.20	30.58	**	ns

Table 2. Continued.

	LRI ¹	RI ²	C ³		R		RY		RMSE ⁴	P _t ⁵	P _f
			60 d	83 d	60 d	83 d	60 d	83 d			
(E)-2-Nonenal	1224	a	3.69 a	3.59 a	2.36 b	2.15 b	1.11 c	1.92 bc	0.49	ns	***
Octanoic acid	1267	a	11.02 d	18.10 a	11.42 cd	16.30 ab	12.29 bcd	16.16 abc	2.76	***	ns
Nonanoic acid (60)	1359	a	1.70	1.70	1.56	1.53	1.51	1.03	0.96	ns	ns
Thiamine degradation											
2-Methyl-3-furanthiol	896	a	tr	tr	tr	tr	tr	tr			
Methyl-2-methyl-3-furyldisulfide	1221	a	tr	tr	tr	tr	tr	tr			
Spices			3105.15	3210.12	3087.19	2804.85	3299.25	2941.91	376.00	ns	ns
O-Xylene (91)	917	a	0.84	0.72	0.70	0.67	0.69	0.59	0.14	ns	ns
Styrene (104)	919	a	0.57 c	0.78 c	0.51 c	0.64 c	3.17 a	2.53 b	0.19	ns	***
Unidentified terpene	934	c	165.77	141.31	168.37	138.55	160.73	141.74	20.13	***	ns
β-Phellandrene	988	b	460.31	419.40	486.90	407.33	490.25	440.91	66.65	*	ns
β-Myrcene (93)	1003	a	29.80	33.77	29.68	28.42	33.01	29.16	4.26	ns	ns
Unidentified terpene	1019	c	113.30	121.94	120.30	105.53	126.23	110.98	19.27	ns	ns
3-Carene	1022	a	571.43	630.26	555.16	515.62	638.08	540.43	100.34	ns	ns
Unidentified terpene	1034	c	51.50 ab	48.03 b	55.76 ab	48.49 b	61.38 a	55.69 ab	6.19	*	**
D-limonene	1046	a	1081.58	1170.91	1063.91	1004.14	1133.01	1022.63	129.31	ns	ns
Unidentified terpene	1075	c	68.52	68.29	68.15	61.41	83.39	66.98	17.45	ns	ns
Unidentified terpene (93)	1099	c	0.99	0.98	0.80	0.81	0.90	0.78	0.14	ns	ns
Terpinolene (93)	1101	a	3.70	3.86	3.75	3.31	4.08	3.48	0.50	ns	ns
Unidentified terpene	1121	c	15.05 ab	19.32 a	14.83 ab	17.49 ab	14.48 b	19.14 a	2.57	***	ns
Unidentified terpene	1366	c	26.89 a	26.61 a	22.58 ab	20.12 b	23.74 ab	21.74 ab	3.62	ns	**
Unidentified terpene	1377	c	8.24	9.49	6.77	7.60	7.66	8.29	2.50	ns	ns
Unidentified terpene	1407	c	71.50 ab	77.51 a	64.64 ab	59.95 b	66.96 ab	64.48 ab	9.45	ns	**
Caryophyllene	1470	a	435.18	436.96	424.39	384.77	451.48	412.34	49.79	ns	ns

Table 2. Continued.

	LRI ¹	RI ²	C ³		R		RY		RMSE ⁴	P _t ⁵	P _f
			60 d	83 d	60 d	83 d	60 d	83 d			
Unknown origin			27.74 b	29.19 c	34.36 ab	39.25 a	63.34 ab	64.12 ab	13.80	ns	***
2-Methyl-1-propene (41)	459	b	4.87 a	4.08 ab	2.80 bc	2.13 c	1.42 c	1.61 c	0.88	ns	***
Carbon disulfide	537	a	21.97 b	23.95 b	30.58 b	36.01 b	66.95 a	61.37 a	13.87	ns	***
4-Methylphenol (107)	1197	a	0.90 b	1.16 a	0.98 b	1.11 a	0.97 b	1.14 a	0.07	***	ns

¹LRI: Linear retention index of the compounds eluted from the GC–MS using a DB-624 capillary column. ²RI: Reliability of identification: a, identification by mass spectrum and by coincidence with LRI of authentic standard; b, tentative identification by mass spectrum; c: unknown. ³C: control batch; R: 47% reduction in nitrite and nitrate; RY: 47% reduction in nitrite and nitrate and *D. hansenii* inoculated. ⁴RMSE: Root mean square error. ⁵P_t: *P* value of ripening time effect, P_f: *P* value of formulation effect. Different letters in the same row indicate significant differences at *** *P* < 0.001, ** *P* < 0.01, * *P* < 0.05. ns: *P* > 0.05. ⁶tr: traces, not quantified.

3.5. Aroma characteristics of the sausages

Gas chromatography-olfactometry (Table 3) revealed the presence of 29 aroma active zones which were identified except for 1 odour zone attributed to an identified terpene. The most important aroma compounds with the highest DF (detection frequency) values ($DF > 10$) were 2,3-butanedione, acetic acid, hexanal, ethyl 2-methylbutanoate, 1-hexanol, 2-acetyl-1-pyrroline, methional, dimethyl trisulfide and 3-carene. The assessors were able to detect five odour zones that corresponded to compounds 2-methyl-3-furanthiol, 2-acetyl-1-pyrroline, dimethyl trisulfide, 2-acetyl-2-thiazoline and methyl-2-methyl-3-furyldisulfide which were not analysed by mass spectra due to their presence as trace levels.

The relationship between the instrumental variables and the ingoing amounts of nitrate and nitrite and *D. hansenii* inoculation at each ripening time, were examined by a principal component analysis (Figure 3). Comparisons were done using the parameters (physicochemical, microbiological parameters and aroma compounds) measurements at 60 (Fig. 3 A and B) and 83 d (Fig. 3 C and D) of ripening. Regarding results at 60 d (Fig. 3A), the first two principal components were able to explain the 72.09 % of the total variance. PC1 accounted for 56.34 % of the variance and distinguished sausages by *D. hansenii* inoculation, placing C and R sausages on the left quadrant and RY sausages on the right quadrant. Sausages inoculated with *D. hansenii* RY, were associated with higher water activity, pH, yeast and moulds and colour parameters. Regarding aroma compounds, RY sausages were related to those derived from esterase activity, as well as 3-methylbutanal and methanethiol resulting from amino acid degradation. On the other hand, C and R sausages were related to an increase in TBARS and aroma compounds derived from lipid oxidation, β -oxidation and carbohydrate fermentation, as well as methional resulting from amino acid degradation. PC2 represented 15.76 % of the variance corresponding to the variability among replicates.

Regarding results at 83 d of drying (Fig. 3 C and D), two principal components were able to explain the 70.40 % of the total variance. PC1 accounted for 56.21 % of the variance and distinguished sausages by *D. hansenii* inoculation and nitrate and nitrite reduction, placing C sausages on the left quadrant, R sausages on the centre and RY sausages on the right quadrant. Sausages inoculated with *D. hansenii* RY, were again related to higher water activity, yeast and moulds, colour parameters and aroma compounds derived from esterase activity. Moreover, C sausages were again related to TBARS and aroma compounds derived from lipid oxidation, while the R sausages were related to GCN counts and aroma compounds derived from amino acid degradation and carbohydrate fermentation. PC2 represented 14.19 % of the variance corresponding to the variability among replicates.

Table 3. Odour active compounds identified in dry fermented sausages.

Compounds	IRL GC-O¹	IRL std GC-O²	RI ³	GC-O description	DF ⁴
Methanethiol	474	471	a	Rotten, unpleasant	9
1-Propanol	611	614	a	Acid, fermented	4
2-Butanone	628	632	a	Wet, fresh, sweet	3
2,3-Butanedione	633	638	a	Cheese, butter, dairy, fruit	11
3-Methylbutanal	690	691	a	Sweet, green, spicy	5
Acetic acid	701	700	a	Acid, fermented, vinegar	11
3-Hydroxy-2-butanone	779	777	a	Cardboard, green	3
Ethyl 2-methylpropanoate	783	789	a	Sweet, fruity	4
Ethyl butanoate	824	825	a	Sweet, fruity, fresh	5
Hexanal	835	836	a	Green, fresh cut grass, fatty	12
Ethyl 2-hydroxypropanoate	861	859	a	Fresh, floral, acid	3
Ethyl 2-methylbutanoate	872	872	a	Pineapple, sweet, acid, unpleasant,	10
Ethyl 3-methylbutanoate	875	876	a	Sweet, fruity, unpleasant, acid,	7
2-Methyl-3-furanthiol	899	897	a	Fatty, medicinal, unpleasant, sulfur	3
1-Hexanol	925	919	a	Fatty, rancid, rotten fruit, unpleasant	12
Heptanal	942	939	a	Cured, rancid, unpleasant	6
2-Acetyl-1-pyrroline	962	963	a	Roasted, fried corn, roasted nuts, Cooked potatoes, vegetable, meaty,	11
Methional	969	969	a	sulfur	11
β-Myrcene	1002	1003	a	Green	3
Dimethyl trisulfide	1011	1009	a	Spicy, pungent, sulfur, rotten, vegetable	11
3-Carene	1021	1026	a	Mushrooms, earthy, green, fresh	12
1-Octen-3-ol	1032	1028	a	Green, fresh, mushrooms	5
Octanal	1046	1047	a	Sweet, citrus, floral	7
Hexanoic acid	1068	1064	a	Cheese, unpleasant, floral fresh	3
Unidentified terpene	1092		b	Spicy, fresh, floral	3
Nonanal	1150	1151	a	Fresh, herbaceous, green, unpleasant Toasted, fried corn, caramel, toasted	7
2-Acetyl-2-thiazoline	1180	1178	a	corn, bread	8
4-Methylphenol	1198	1190	a	Unpleasant, rotten, sulphur	6
Methyl-2-methyl-3-furyl disulfide	1225	1225	a	Meaty, unpleasant, wet wood, fermented, rotten	9

¹Linear retention index of the compounds eluted from the GC-FID-O using a DB-624 capillary column.

²Linear retention index of standard compounds in the GC-FID-O.

³Reliability of identification: a, identification by mass spectrum, coincidence with LRI of an authentic standard and by coincidence of the assessors's descriptors with those described in the Fenaroli's handbook of flavour ingredients (Burdok, 2002); b, tentative identification by mass spectrum.

⁴Detection frequency value.

Figure 3. Continued.

4. Discussion

Physicochemical changes observed were similar to the ones reported for this type of product (Olivares et al., 2010; Perea-Sanz et al., 2018) (Table 1 and Fig. 1). The reduction in the ingoing amounts of nitrate and nitrite was confirmed. The lowest nitrate reduction observed during drying in C sausages respect to R and RY sausages may be due to the generation of nitrate from nitrite oxidation (Honikel, 2008). Moreover, other authors have shown that the use of nitrate and nitrite mixtures increases the amount of residual nitrate (Gratacós-Cubarsí et al., 2013). The lowest residual nitrate detected in inoculated sausages (RY) could be explained by the inhibition of nitrite oxidation by *D. hansenii* (Cano-Garcia et al., 2014).

Nitrate and nitrite reduction did not affect pH value however, Aquilani et al. (2018) and Gonzales-Barron et al. (2015) observed a small increase in pH values due to the absence of nitrate and/or nitrite in dry fermented sausages. These differences among studies can be due to differences in formulations as well as differences in process manufacture like long drying times that may cause a pH increase. In RY sausages, *D. hansenii* consumption of organic acids and ammonium production (Flores, Corral, Cano-García, Salvador & Belloch, 2015) increased pH at the end of the ripening.

Regarding lipid oxidation, a decrease in TBARS in R sausages was observed in previous studies (Perea-Sanz et al., 2018). However, this is not in agreement with the reported antioxidant ability of nitrite (Zanardi, Ghidini, Battaglia & Chizzolini, 2004). Similar reductions in TBARS were observed in nitrite reduced Pastrima although they were related to the stage of processing (Aksu, Erdemir & Çakici, 2016). The chemistry of nitrate and nitrite in cured meats is a complex subject (Majou & Christieans, 2018). The nitric oxide generated from nitrite can react with neutrophil-derived superoxide present in muscle tissues and generate peroxynitrite (ONOO⁻), which has prooxidant activity and antimicrobial effects (Brannan, Connolly & Decker, 2001). Thus, in fermented sausages with high ingoing amounts of nitrate and nitrite, peroxynitrite formation might increase oxidation events including lipid oxidation. The nitrite prooxidant effect of peroxynitrite is directly related with its antimicrobial effect which is more harmful against Gram positive anaerobic bacteria, like *C. botulinum* (Majou & Christieans, 2018). On the contrary, the antioxidant capacity of *D. hansenii* limits lipid oxidation processes as shown by its great antioxidant capacity (Corral et al., 2015) during the entire drying time and, moreover, prevents nitrite oxidation as observed by the lowest residual nitrate content in RY.

On the other hand, the reduction of the ingoing amounts of nitrate and nitrite did not have effect on the microbial counts. LAB counts at both drying times were stable as reported by Christieans et al. (2018), and GCN and yeasts and moulds counts (Fig. 1A, 1C) were comparable to those found by Cano-García et al. (2014). On the contrary, Hospital et al. (2015) reported an increase

in GCN related to nitrite and nitrate reduction in dry-fermented sausages and a similar effect was observed in Pastrima (Aksu et al., 2016). Similar findings were reported by Gonzales-Barron et al. (2015) in dry-fermented sausages, although the presence in the formulation of polyphosphate, which produced a pH rise effect, was the main factor affecting the growth of acid sensitive microorganisms. In our formulations C and R, the absence of differences in pH due to the similar LAB growth corroborated that the reduction of nitrate and nitrite levels did not affect GCN growth. Regarding the absence of *Enterobacteriaceae*, this indicated that the process was carried out with good hygiene practices, which are important to assure microbial safety. The inoculation of *D. hansenii* in RY sausages was confirmed by the yeasts and moulds counts during the whole process and presence of M13 RAPD PCR pattern of *D. hansenii* L1 (Fig. 2). The presence of *D. hansenii* L1 patterns in yeasts isolated from C and R sausages could be due to yeast cross contamination in the drying chamber. Nevertheless, the cell load (Figure 1) is very different between samples, leading to a scarce impact of these isolates in R and C sausages.

The main mechanisms involved in generation of volatile compounds with aroma impact on fermented sausages are from microbial origin, chemical oxidation (Flores & Olivares, 2015) and thiamine degradation (Flores, 2018). Contrary to Hospital et al. (2015), total volatile compounds derived from amino acid degradation were not affected by the reduction of ingoing amounts of nitrate and nitrite. Besides, amino acid derived volatile compounds were in high abundance in sausages inoculated with *D. hansenii* strain L1 (Table 2) due to the yeast strain amino acid degradation capacity (Perea-Sanz et al., 2019a). On the other hand, our results indicated that *D. hansenii* might have consumed acetic acid and produced ethanol in contrast to what happened in C and R sausages (Table 2) (Cano-García et al., 2014). Esterase activity in dry-fermented sausages is carried out by *Staphylococcus* and *Debaryomyces* species (Sthanke, 1994; Flores, Dura, Marco & Toldra, 2004). Accordingly, *D. hansenii* inoculation in RY sausages increased production of esters compounds (Flores et al., 2004). Moreover, *D. hansenii* produced a strong decrease in lipid oxidation volatile compounds in agreement with the low TBARS values and the high antioxidant capacity of this yeast (Corral et al., 2015). Nitrate and nitrite reduction and *D. hansenii* did not affect volatile compounds derived from black pepper, which was used as spice in the formulations.

Generation of volatile compounds derived from amino acid degradation reactions in C and R sausages was not altered from 60 to 83 d (Table 2). However, RY sausages showed a decline in volatile compounds derived from amino acid degradation indicating a probable transformation of these into other compounds. The abundance of volatile compounds derived from lipid oxidation decreased in C and R sausages due to the further drying (Perea-Sanz et al., 2018).

Concerning volatile compounds with direct impact on the sausage aroma profile (Table 3) at 60 d of ripening, the reduction of the ingoing amounts of nitrate and nitrite produced few aroma differences in contrast to the highest effect produced by *D. hansenii*, inoculation. The yeast prevented oxidation reactions and generation of aromas derived from lipid oxidation (Flores et al., 2004) which may produce unpleasant rancid odour notes in the sausages. In addition, it was responsible for the production of ester compounds which provided fruity and sweet odours to the sausages (Cano-García et al., 2014; Corral et al., 2015). The decrease on carbohydrate fermentation aromas caused by *D. hansenii* inoculation could be explained by the yeast consumption of acetic acid (Table 2). In general, *D. hansenii* inoculation decreased acid notes detrimental to sausage aroma (Flores, 1997). On the other hand, at 83 d of ripening the effect of nitrate and nitrite reduction between C and R on aroma profile is more clear (Figure 3C and D) (Perea-Sanz, Montero, Belloch & Flores, 2019b) and *D. hansenii* yeast contributed to fruity notes (ester compounds) and prevented oxidation events. In addition, the relationship between nitrate/nitrite reduced fermented sausages (R) and the aroma compounds derived from carbohydrate fermentation (acetic acid, 2-butanone, 2,3-butanedione, 3-hydroxy-2-butanone, Figure 3D) may be explained by the GCN metabolic activity, although no differences in GCN growth were observed between nitrate/nitrite reduced and no-reduced sausages.

5. Conclusions

Nitrite and nitrate reduction in fermented sausages did not affect microbial growth but decreased lipid oxidation and generation of derived volatiles. Microorganisms metabolic activity caused differences in the aroma profile at the longest drying time. The nitrite prooxidant and antimicrobial effects were confirmed in C sausages. *D. hansenii* inoculation limited lipid oxidation and increased generation of volatile compounds derived from amino acid degradation and esterase activity. The antioxidant capacity of *D. hansenii* during the entire drying time prevented nitrite oxidation as demonstrated by the lowest residual nitrate levels in RY sausages. In summary, yeast inoculation counteracted the negative impact of nitrite and nitrate reduction on aroma due to its antioxidant capacity during drying time, aroma production and hindered nitrite oxidation.

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