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Bee products as an alternative for the preservation of nitrate and nitrite-reduced dry fermented sausages

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ABSTRACT

Food regulations are becoming increasingly restrictive on the use of nitrate and nitrite as additives in meat products, so different alternatives are being investigated to replace their functions. In this study, we tested the antioxidant activity of mixtures of bee products to partially replace nitrate and nitrite in dry fermented sausages. Two combinations of chestnut honey, propolis and royal jelly were added to sausages at 2% concentration, together with 0 and 75 mg/kg of nitrate and nitrite. TBARs and volatile profile analysis revealed a lower intensity of oxidation phenomena in sausages prepared with bee products, but these differences were not perceived by the tasters in the sensory analysis. Only instrumental differences in the a* colour parameter were observed when nitrate and nitrite were not used in the formulation. The addition of bee products did not affect the typical microbiota (lactic acid bacteria and gram-positive catalase-positive cocci) of sausages. The combination of 2% bee products and 75 mg/kg of nitrate and nitrite may be useful to obtain dry fermented sausages with a sensory quality and oxidation stability similar to a standard product containing the maximum amounts of these additives allowed in the European Union (150 mg/kg each).

1. Introduction

People's concern about food quality and safety has risen, hence ingredients are being highly monitored by consumers. In this way, there is an increasing demand for food with less or even no chemical additives, and the use of natural compounds as an alternative is in the spotlight. As a result, the food industry is looking for new substances to replace current preservative and antioxidant additives by, if possible, other compounds of natural origin.

Nitrate and nitrite are legally accepted as preservatives in the manufacturing of processed meat. Although there are other sources in the human diet, such as many vegetables (Karwowska & Kononiuk, 2020), food safety authorities are encouraging the reduction of the use of nitrate and nitrite as additives because of their role in the formation of nitrosamines (EFSA, 2023). In this way, new Regulation (EU) 2023/2108 on the use of nitrate and nitrite sets a reduction of the maximum ingoing amounts of these additives in different foods by 2025. However, due to the function of nitrite as antimicrobial, antioxidant, and in colour and flavour development, it is not easy to find a single compound to replace nitrate and nitrite.

Fermented meat products are the result of the combination of different reactions and hurdles that contribute to the quality and safety of the product. Their properties rely on the development of a typical microbiota of lactic acid bacteria (LAB) and gram-positive catalase-positive cocci (GCC+) that are essential for acidification and colour development, respectively, and also contribute to flavour. Fermented sausages also suffer an intense water loss during ripening, yielding to a low a_w, which together with the low pH and the presence of nitrite gives a stable and safe product (Vignolo et al., 2010). Nitrite also inhibits undesirable microorganisms, both spoilage and pathogenic, and modulates the oxidative reactions that take place during ripening and that are essential for the development of the typical cured meat flavour (Honikel, 2008). Therefore, the substitution of nitrite by any other ingredient requires an approach that does not disrupt this complex balance. Nitrite is a particularly relevant hurdle during the fermentation stage, when other hurdles have not yet been fully established. Pathogens such as

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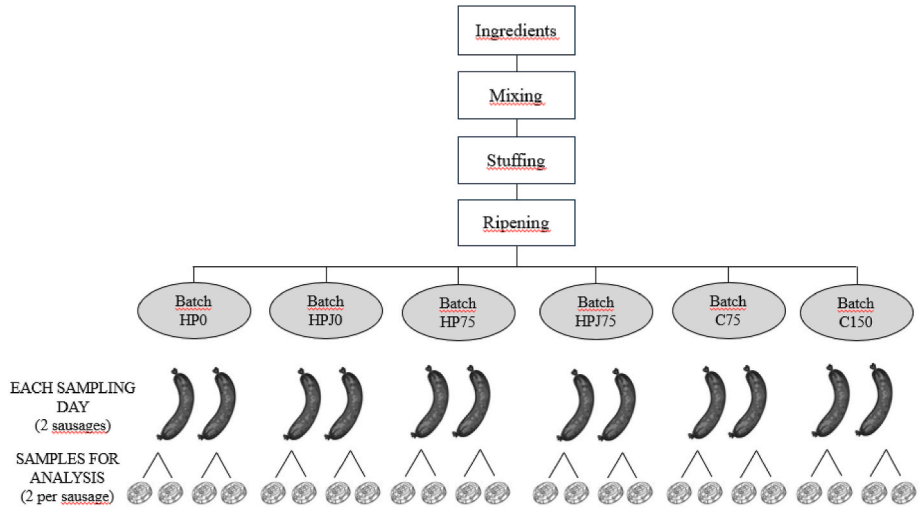

Fig. 1. Experimental design of a sausage manufacture. Two independent manufactures were carried out.

Clostridium botulinum, *Listeria monocytogenes* and *Salmonella* spp. have been reported to grow in nitrite-free sausages, which indicates that the complete removal of this additive is not possible for safety reasons unless other strategies are considered. In this sense, it has been stated that at least an ingoing amount of 75 mg/kg of nitrite should be used to control the above-mentioned pathogenic bacteria (Christieans et al., 2018; Hospital et al., 2012, 2014; Keto-Timonen et al., 2012).

Regarding the antioxidant activity of nitrite in cured meats, its reduction or removal could lead to the development of off-flavours due to an increase in the concentration of products derived from lipid oxidation causing rancidity. This issue could be addressed by the use of natural alternatives. Honey and other bee products have been used for their bioactive properties since ancient times. Currently, their anti-inflammatory, antiproliferative, immunomodulatory, antimicrobial and antioxidant activities are being thoroughly characterized. Honey, propolis and royal jelly are rich in flavonoids, phenolic acids, organic acids and peptides, which are responsible for their antioxidant and antimicrobial activities (Durazzo et al., 2021; Stefanis et al., 2023). Therefore, these ingredients could be of potential interest for their use in processed meats as nitrite substitutes. In addition, the search for alternative uses of bee products, beyond their nutritional and health values, benefits beekeeping, which is highly relevant from an environmental sustainability perspective (Durazzo et al., 2021).

In our previous studies, chestnut honey, propolis and royal jelly have revealed interesting antioxidant properties (Sánchez-Martín et al., 2022), that could be of interest for replacing, at least partially, nitrite in meat products. The aim of this work was to evaluate the *in situ* effectiveness of mixtures of those bee products in combination with reduced amounts of curing salts to obtain fermented sausages with optimal sensory quality and shelf life.

2. Materials and methods

2.1. Microorganisms

Ligilactobacillus fermentum, ceded by Dr. Juan Miguel Rodríguez (UCM), was used as LAB starter. As GCC + starters, we used *Staphylococcus xylosus* S-SX, provided by Christian Hansen (Hoersholm, Denmark), and *Staphylococcus carnosus* CECT 4491, supplied by the Colección Española de Cultivos Tipo (CECT, Valencia, Spain).

2.2. Bee products

Chestnut honey and propolis were obtained from beekeepers in the central region of Spain, and royal jelly was obtained from a supplier in France. Chestnut honey was selected because it has shown great antioxidant potential in comparison to other varieties of honey due to a high content of phenolic compounds (Combarros-Fuertes et al., 2019). Propolis tincture was prepared in 70% ethanol to optimize the extraction of antioxidant components.

Two combinations of bee products were used, which were selected according to previous results on their antioxidant properties (Sánchez-Martín et al., 2022): A) 90% chestnut honey+10% propolis (H+P), and B) 80% chestnut honey+10% propolis+10% royal jelly (H+P+J).

Table 1
Formulations of the experimental dry fermented sausages.

Batch	H+P (%)	H+P+J (%)	Dextrose (%)	$NaNO_2$ (mg/kg)	KNO_3 (mg/kg)	Sodium ascorbate (mg/kg)
HP0	2	0	0	0	0	250
HP75	2	0	0	75	75	250
HPJ0	0	2	0	0	0	250
HPJ75	0	2	0	75	75	250
C75	0	0	0.5	75	75	250
C150	0	0	0.5	150	150	500

H+P: 90% chestnut honey + 10% propolis.
H+P+J: 80% chestnut honey + 10% propolis +10% royal jelly.

2.3. Sausage manufacture

Two independent sausage manufactures were carried out in different days. In each manufacture, six batches of salchichón, a typical Spanish dry fermented sausage, were prepared. The experimental setup is shown in Fig. 1.

Pig leg meat (70%) and pork backfat (30%) were chopped in a mincer to 6 mm particle size. Then, lactose (3%), NaCl (2.5%) and ground black pepper (0.25%) were added to the mixture, together with *L. fermentum* at a concentration of 10^7 cfu/g, and *S. xylosus* and *S. carnosus*, at 10^6 cfu/g each. Afterwards, different concentrations of potassium nitrate, sodium nitrite, sodium ascorbate, dextrose and the selected bee product mixtures were added as shown in Table 1. The concentration of the bee product mixtures added to the batter (2%) was determined in preliminary experiments to assess their compatibility with the technological and sensory properties of sausages. The amounts of nitrate and nitrite added corresponded to: 1) a control standard sausage manufactured with the maximum ingoing amounts (150 mg/kg) currently allowed by Regulation (EC) 1333/2008; 2) a 50% reduction

(75 mg/kg) in batches with and without bee products (this amount of nitrite is the minimum that has been reported to exert sufficient antimicrobial activity); 3) batches without nitrate/nitrite and added with bee products as control for oxidation activity.

The different mixtures were stuffed into 45 mm collagen casings to obtain sausages of approximately 200 g. Potassium sorbate (20%) was sprayed on the surface to prevent mould growth. Fermentation was carried out at 22 °C and 90% relative humidity (RH) for 48 h; then conditions were changed to 19 °C and 88% RH (24 h) and 15 °C and 86% RH (24 h); finally sausages were dried for 24 days at 12 °C and 85% RH.

Two sausages were sampled per batch in each manufacture. Typical microbiota, pH and a_w were analysed on days 0, 3 and 28 of ripening. Peroxide value, colour, texture, sensory properties and volatile profile were analysed at the end of ripening (28 days). TBARs were assessed at the end of ripening and after 15 days storage. Each analysis was carried out in duplicate.

2.4. Microbiological analysis

Ten grams of sausage were homogenized with 90 ml of saline solution in a Stomacher bag for 2 min. Serial 10-fold dilutions were prepared. LAB were enumerated on double layer pH 5.5 MRS agar (Pronadisa, Madrid, Spain) incubated at 32 °C for 48 h. GCC+ were enumerated on MSA agar (Pronadisa) at 32 °C, 48 h. Double layer VRBG agar (Pronadisa) was used to count *Enterobacteriaceae* after incubation at 37 °C, 24 h.

In addition, the following microorganisms were also analysed at the end of ripening in the batches subjected to sensory analysis. For the detection of sulphite-reducing clostridia, 1 ml of the sausage homogenate (previously heated at 80 °C for 10 min) was mixed with SPS melted agar (Pronadisa) in glass tubes covered with a layer of sterile paraffin to maintain anaerobic conditions during incubation at 37 °C for 24 h. PALCAM agar (Oxoid, Basingstoke, UK) was used to detect the presence of *Listeria* spp. following incubation at 37 °C, 48 h. SS and XLD agar (Oxoid) were used to detect *Salmonella* spp., following incubation at 37 °C, 24 h. The results were expressed as cfu/g of sausage.

2.5. Determination of lipid hydroperoxides

Primary lipid oxidation was evaluated according to Salcedo--Sandoval et al. (2015) with some modifications. Two grams of the sample were mixed with 25 ml of chloroform/methanol (1:1, v:v) and 0.5 ml of a 0.19 M BHT ethanolic solution to avoid further oxidation. The mixture was homogenized for 2 min using an Ultra-Turrax T18 blender (Ika, Staufen, Germany), while maintaining tubes in a water/ice bath. After adding 6 ml of distilled water, samples were centrifuged at 3000×*g* for 30 min at 5 °C. Two hundred microliters of the lower layer, containing chloroform/methanol, were recovered using disposable Pasteur pipettes, and vortexed with 2.8 ml of methanol/1-butanol (2:1, v:v). Then, 15 μl of a ferrous iron solution and 15 μl of 3.94 M ammonium thiocyanate were added. The ferrous iron solution was prepared by combining 0.132 M barium chloride and 0.144 M ferrous sulphate, allowing the resulting salt to precipitate for 20 h, followed by filtration of the aqueous phase through 0.45 μm pore size filters (Filter-Lab, Barcelona, Spain). The reaction solutions were left in the dark for 20 min, and afterwards absorbance was measured at 510 nm using a spectrophotometer (U-2000 Hitachi, Tokyo, Japan). The hydroperoxide concentration was determined using a standard curve of cumene hydroperoxide (0–20 μM), and the results were expressed as mmol of cumene hydroperoxide/kg of sample.

2.6. Determination of thiobarbituric acid reactive substances (TBARs)

Secondary oxidation products were assessed by a TBARs method adapted from Salcedo-Sandoval et al. (2015). Two grams of sample were homogenized with 8 ml of 5% trichloroacetic acid (TCA) and 0.5 ml of a 0.19 M BHT ethanolic solution for 2 min using an Ultra-Turrax T18 blender, while maintaining tubes in a water/ice bath. The resulting homogenates were filtered through Whatman paper No. 1, and the outcome filtrates were adjusted to a volume of 10 ml with 5% TCA. Seven hundred microliters of the filtrates were mixed with 0.7 ml of 20 mM thiobarbituric acid and allowed to react in a 100 °C water bath for 30 min. Absorbance was then measured at 532 nm using a U-2000 Hitachi spectrophotometer. A standard curve of 1,1,3, 3–tetramethoxypropane was prepared, and results were expressed as mg of malondialdehyde (MDA)/kg of sample.

TBARs were assessed at the end of ripening and in vacuum-packaged slices stored at 20 °C under LED (4000 K) light exposure for 15 days, to induce oxidation. The slices were turned upside down every 3 days.

2.7. Colour analysis

The CIELAB colour space was used to obtain the colour parameters L^* (lightness), a^* (redness) and b^* (yellowness). Sausages were cut into slices (1 cm thick) using an electric meat slicer equipped with a circular blade. Measurements were taken immediately after slicing at two randomly selected spots using a tristimulus colorimeter (ChromaMeter CR-400, Konica Minolta Sensing, Osaka, Japan).

2.8. Texture analysis

A texture profile analysis (TPA) was performed on sausage cylinders of 1 cm high and 3 cm diameter using a Texture Analyser TA.XT2i (Stable Microsystems, Surrey, England) equipped with the Texture Expert software. Casings were removed before analysis. A double compression cycle test, with an elapsed time of 5 s from the end of the first to the beginning of the second cycle, was applied up to 50% compression of the original portion height using an aluminium cylinder probe P/25 (25 mm diameter) and at room temperature (22 °C). Force-time deformation curves were obtained with a 25 kg load cell applied at a crosshead speed of 2 mm/s. The following parameters were determined (Bourne, 1978): hardness (*N*), as the maximum force required to compress the sample; springiness, as the ability of the sample to recover its original shape after the deforming force ceased; adhesiveness (*N* x *s*), as the work necessary to overcome the attractive forces between the surface of the sample and the probe surface; cohesiveness was calculated as the ratio between the area underneath the second compression and the first compression curves; and finally chewiness (*N*) was calculated as the product of springiness, cohesiveness and hardness.

2.9. Sensory analysis

A panel of 14 tasters conducted a rank order test in an ISO normalized tasting room (ISO, 1981), as described by Fernández et al. (2020). The panel was recruited from the staff of the Food Technology Department, who are familiar with the sensory quality of meat products. The panel assessed colour, odour, taste and texture in the products at the end of ripening. The batches subjected to sensory evaluation were those that included nitrate and nitrite in the formula: HP75, HPJ75, C75 and C150. Prior to sensory analysis, these batches were screened for the presence of sulphite-reducing clostridia, *Salmonella* spp. and *Listeria* spp.

2.10. Volatile compound analysis

The headspace volatiles were extracted from 4 g of ground fermented sausage using solid-phase microextraction (SPME) followed by GC-MS. The sample was transferred to a headspace glass vial (15 ml) sealed with a PTFE/silicone septum (Supelco, Bellefonte, PA, USA) and placed on a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA). After equilibration at 40 °C for 30 min, volatile compounds were adsorbed using a carboxen/polydimethylsiloxane (CAR/PDMS) fiber (1 cm, 85 μm; Supelco) during 30 min while the sample was kept at 40 °C.

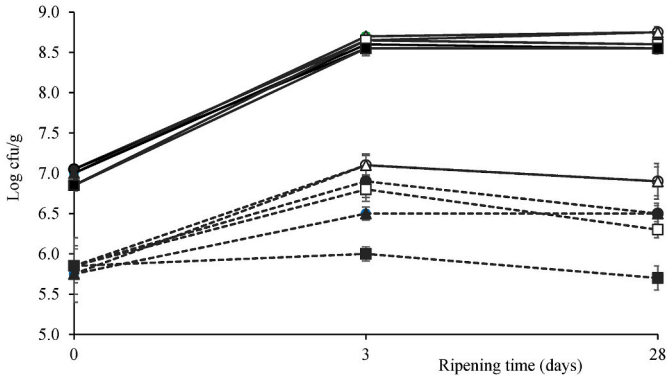

Fig. 2. Changes in lactic acid bacteria (——) and gram-positive catalase-positive cocci (GCC+) (------) numbers during ripening. (○) Batch HP0; (●) Batch HP75; (△) Batch HPJ0; (▲) Batch HPJ75; (□) Batch C75; (■) Batch C150. Error bars correspond to the standard deviation of the mean.

Next, compounds were desorbed in a gas chromatograph Agilent 8890 fitted to an Agilent 7000C triple quadrupole mass spectrometer. Separation was performed on a 5MS/silphenylene polysiloxane fused silica capillary column (60 m × 0.25 mm i.d., 1 μm film thickness, Quadrex Corporation, Woodbridge, CT, USA). The GC oven program was as follows: 40 °C for 3 min, to 280 °C at 4 °C/min and held for 5 min. A C8–C20 n-alkane standard solution (Sigma, St. Louis, MO, USA) was analysed under the same conditions to obtain the linear retention index (LRI) values for each component. Compounds were identified by first comparing their mass spectra with those of authentic standards and/or those contained in the NIST20 (National Institute of Standards and Technology, MD, USA) Mass Spectral Library, and wherever possible identities were confirmed by comparison of the LRI values with either those of commercial standards or published values (Acree & Arn, 2004; Kondjoyan & Berdagué, 1996). The area of each peak was integrated using Mass Hunter workstation software (Agilent Technologies). The results were expressed in area units x 10^{-5}. Analyses were performed in duplicate.

2.11. Statistical analysis

Data were analysed using Statgraphics Centurion 19 (Statpoint Technologies, Warrenton, VA, USA). One-way ANOVA was used to compare mean values of the different analyses obtained with the different combinations of nitrate/nitrite and bee products. Significant differences between pairs of means were assessed by Tukey test setting a 95% confidence level.

To rule out the effect of the level of nitrate/nitrite and bee products on TBARs, the General Linear Model (GLM) procedure contained in SAS (9.1, SAS Inst. Inc., Cary, NC, USA) was used.

For sensory analysis, the significance level of data obtained in the rank order test was determined by Friedman's rank addition according to the model proposed by Joanes (1985) and to the tables of multiple comparison procedures for analysis of ranked data (Christensen et al., 2006).

3. Results and discussion

3.1. Physicochemical and microbiological characterization

The use of honey, propolis and royal jelly did not affect the typical changes in pH and a_w during ripening. The initial pH was 5.5 and reached final values of 4.7–4.8, while a_w decreased from 0.97 to 0.88–0.89 in all batches (data not shown). These values are within the levels considered as indicators of an appropriate extent of drying in European dry sausages (Incze, 2004).

In relation to the typical microbiota, no differences were found between batches for LAB (Fig. 2). On the contrary, significant differences ($p<0.05$) were observed for GCC+, which are known to contribute to both the typical colour and flavour development of fermented sausages (Aquilanti et al., 2016). At the end of ripening, the control batch with 150 mg/kg of nitrate/nitrite (C150) showed the lowest counts, being numbers 1.2 log cfu/g lower in comparison to the batches manufactured with bee products and without curing salts (HP0 and HPJ0). On the other hand, the batches with 75 mg/kg of nitrate/nitrite (C75, HP75 and HPJ75) showed approximately 0.5 log cfu/g lower GCC+ numbers than the batches added with bee products and without nitrate/nitrite (Fig. 2). The presence of lower numbers of GCC+ in sausages with higher amounts of nitrate/nitrite has been previously reported, and could be explained by the oxygen tension-related growth characteristics of staphylococci and their ability to reduce nitrate/nitrite (Hospital et al., 2012). In any case, the final counts of GCC+ in all batches (5.7–6.9 log cfu/g) were in the range of those reported as typical in fermented sausages (Aquilanti et al., 2016). The addition of bee products did not interfere with the typical microbiological phenomena during sausage ripening. On the other hand, *Enterobacteriaceae* levels were below the limit of detection (0.9 log cfu/g) in all batches, so the hygienic quality of sausages was not affected by the use of bee products.

Finally, no sulphite-reducing clostridia, *Salmonella* spp. or *Listeria* spp. were detected at the end of ripening in the batches that were subjected to sensory analysis.

3.2. Lipid oxidation

Table 2 shows the peroxide values and the TBARs index obtained in the samples. Peroxide values ranged between 121 and 135 mmol hydroperoxide/kg. Similar concentrations have been reported by Magrinyà et al. (2009) in dry fermented sausages. No significant differences ($p>0.05$) were found among batches. Therefore, the addition of bee products did not affect primary oxidation, neither did nitrate/nitrite decrease in the formula, leading to similar values to control samples (C75 and C150). Peroxides are odourless and do not contribute directly to aroma, but they participate in secondary reactions, acting as

Table 2
Parameters related to lipid oxidation of dry fermented sausages manufactured with different concentrations of bee products and additives **(n = 4)**.

Batch	End of ripening		15 Days of storage
	Hydroperoxides (mmol/kg sample)	TBARs (mg MDA/kg sample)	TBARs (mg MDA/kg sample)
HP0	121.3 ± 5.38	0.51 ± 0.15c	2.13 ± 0.76b
HP75	123.2 ± 6.50	0.43 ± 0.10cd	3.41 ± 0.53b
HPJ0	129.3 ± 9.10	0.20 ± 0.12cd	3.93 ± 0.60b
HPJ75	135.1 ± 8.43	0.11 ± 0.05d	4.04 ± 0.91b
C75	131.3 ± 11.90	3.06 ± 0.44a	9.41 ± 1.03a
C150	132.2 ± 8.70	1.87 ± 0.89b	8.87 ± 1.27a
***p*-value**	**0.5931**	**0.0000**	**0.0004**

TBARs: Thiobarbituric acid reactive substances.
Results are expressed as mean ± standard deviation.
a,b,c,d: Different letters in the same column indicate significant differences ($p<0.05$).

Table 3
Effect of the level of nitrate/nitrite and bee products on TBARs, as determined by GLM.

Time	End of ripening		15 Days of storage	
Factor	F-value	*p*-value	F-value	*p*-value
A: Bee products	143.49	<0.0001	36.51	0.0004
B: Nitrate/nitrite	19.74	0.0023	0.99	0.4247
Interaction				
A *vs* B	0.00	0.9859	1.09	0.3359

Table 4
L^*, a^* and b^* values (mean ± SD) of dry fermented sausages manufactured with different concentrations of bee products and additives **(n = 4)**.

Batch	L^*	a^*	b^*
HP0	44.42 ± 2.51	5.18 ± 2.05b	5.64 ± 0.05
HP75	42.43 ± 2.75	9.79 ± 0.02a	6.25 ± 1.25
HPJ0	43.52 ± 4.69	6.03 ± 1.51b	6.38 ± 1.45
HPJ75	43.16 ± 3.77	9.93 ± 0.95a	7.01 ± 1.22
C75	39.56 ± 1.01	10.08 ± 1.04a	5.91 ± 0.74
C150	41.98 ± 3.32	9.48 ± 1.10a	5.99 ± 1.33
***p*-value**	**0.7400**	**0.0248**	**0.8548**

L^*: lightness, a^*: redness, b^*: yellowness.
a,b: Different letters in the same column indicate significant differences ($p<0.05$).

precursors for the formation of odour active compounds. A certain degree of oxidation is necessary for the typical flavour of fermented sausages, and nitrate/nitrite and other antioxidants play an important role in keeping oxidation under control.

Regarding TBARs, values at the end of ripening were lower in the sausages manufactured with bee products, and the lowest concentration corresponded to the combination of honey, propolis and royal jelly with a reduced amount of curing salts (HPJ75). After inducing oxidation during 15 days of storage, TBARs increased noticeably in all batches, but those manufactured with bee products showed significantly lower values ($p<0.05$) than the control sausages with nitrate/nitrite. The addition of bee products showed a higher effect on TBARs than nitrate/nitrite at both sampling times (Table 3). No interaction was observed between both ingredients.

Our results are in line with our previous studies (Sánchez-Martín et al., 2022), in which bee products, and particularly propolis, showed interesting antioxidant properties *in vitro* that have also been reflected in the products manufactured in this study. Other authors have reported the antioxidant effect of propolis in meat products. Vargas-Sánchez et al. (2014) observed an increased oxidative stability in beef patties added with 2% propolis and stored 8 days at 2 °C in the dark, showing significantly lower TBARs values in comparison to control samples. On the other hand, Bernardi et al. (2013), when investigating the addition of propolis (0.14 g/kg), both free and microencapsulated, to salami stored at 25 °C in the dark, observed that propolis performed as well as 500 mg/kg of sodium erythorbate to control oxidation during 90 days of storage. In our study, the amount of propolis added to sausages was 2 g/kg.

Table 6
Sum of ranks of the sensory evaluation of dry fermented sausages manufactured with different concentrations of bee products and additives.

Batch	Colour	Odour	Taste	Texture
HP75	38	36	32	37
HPJ75	40	37	33	34
C75	26	31	38	36
C150	36	34	37	33

Honey was the main component of the bee product mixtures added to our sausages. The antioxidant activity of honey has also been reported in meat products. In this way, Antony et al. (2006) observed a 10%-60% reduction of TBARs values in sliced cooked turkey rolls formulated with 5% and 15% of dry honey and stored under refrigeration during 11 weeks, in comparison to control samples made without honey. The antioxidant activity of honey and propolis is attributed to their high phenolic content and their free radical scavenging ability (Choi et al., 2006; Stefanis et al., 2023; Valencia et al., 2012), allowing them to control the formation of aldehydes and other oxidation products.

3.3. Colour analysis

The CIELAB colour parameters of the different sausages are shown in Table 4. No significant differences ($p>0.05$) were found for L^* and b^*, while some differences were found in the batches manufactured with bee products and without curing salts for a^*, due to a lower redness. In any case, the CIELAB values were typical of dry fermented sausages, and the inclusion of bee products at the concentrations tested did not affect colour development, rather the slight differences observed were due to the absence of nitrate/nitrite. These differences were not perceived in the sensory analysis. The nitrite amounts added in our study, even to the nitrite-reduced sausages, are above those considered sufficient for a normal colour development (Sindelar & Milkowski, 2011).

3.4. Texture profile

The texture parameters of the different batches are shown in Table 5. No significant differences ($p>0.05$) were found for any of the TPA parameters among batches. The values reported in the literature for sausage texture are variable, since they depend on different factors, among them the type of sausage, drying extent, fat content and composition, and storage time (Herrero et al., 2007; Rubio et al., 2007). Some authors have reported differences in sausage texture due to the amount of nitrite used, such as Tomovic et al. (2020), which observed a decreased cohesiveness and chewiness in sausages prepared without nitrite. Among the mechanisms through which nitrite may increase sausage firmness it has been reported that nitrous acid can react with sulfhydryl groups resulting in disulfide formation and cross-linking between proteins (EFSA, 2017). Other factors that may influence texture could be bacterial growth and acidification. As it can be seen in Fig. 2, LAB growth was similar in all batches, as well as the final pH (4.7–4.8). Regarding GCC+, numbers were moderately reduced by nitrate/nitrite

Table 5
Texture Profile Analysis (TPA) parameters of dry fermented sausages manufactured with different concentrations of bee products and additives **(n = 4)**.

Batch	Hardness (N)	Adhesiveness (N x s)	Cohesiveness	Springiness	Chewiness (N)
HP0	57.6 ± 4.1	−1.16 ± 0.20	0.31 ± 0.04	0.61 ± 0.06	10.9 ± 1.1
HP75	65.1 ± 2.1	−1.02 ± 0.45	0.38 ± 0.01	0.70 ± 0.19	17.1 ± 3.6
HPJ0	77.7 ± 10.7	−0.68 ± 0.36	0.37 ± 0.02	0.70 ± 0.10	20.7 ± 6.6
HPJ75	63.5 ± 9.6	−1.24 ± 0.11	0.31 ± 0.01	0.64 ± 0.02	12.5 ± 1.4
C75	68.4 ± 7.8	−1.31 ± 0.05	0.36 ± 0.04	0.59 ± 0.02	14.6 ± 2.6
C150	78.6 ± 7.5	−1.19 ± 0.33	0.40 ± 0.05	0.67 ± 0.12	21.8 ± 8.6
***p*-value**	**0.1466**	**0.2900**	**0.1293**	**0.7415**	**0.2146**

Results are expressed as mean ± standard deviation.

Table 7
Volatile compounds (area units x 10^{-5}) identified in the headspace of dry fermented sausages manufactured with different concentrations of bee products and additives **(n = 4)**.

LRI[a]	Compound	Batch			
		HP75	HPJ75	C75	C150
	Amino acid catabolism	**2977a**	**3156a**	**1312b**	**954c**
554	2-Methylpropanal	621a	605a	150b	143b
654	3-Methylbutanal	468a	432a	405a	257b
662	2-Methylbutanal	110b	145a	109b	49c
730	3-Methyl-3-butenol	1090a	1205a	133b	21c
744	3-Methyl-2-butenal	64b	85a	16c	18c
751	2-Methyl-2-butenal	123b	161b	210a	197a
973	Benzaldehyde	500a	523a	288b	269b
	Carbohydrate fermentation	**13304a**	**14165a**	**10181b**	**7500c**
503	2-Propanone	332a	256b	73c	99c
601	2,3-Butanedione (*diacetyl*)	32a	46a	16b	14b
604	2-Butanone	275a	226 ab	172c	201bc
649	Acetic acid	12399a	13302a	9742b	7011c
711	3-Hydroxy-2-butanone (*acetoin*)	94b	182a	21c	33c
785	2,3-Butanediol	172	153	157	142
	Lipid oxidation	**1355c**	**1709c**	**35355a**	**27121b**
500	Pentane	16c	24c	1304a	1055b
524	2-Propanol	27a	39a	11b	30a
560	1-Propanol	nd	nd	178	nd
604	2-Methylfuran	205b	219b	251b	499a
655	1-Butanol	nd	nd	144	nd
687	1-Penten-3-ol	nd	nd	483b	587a
696	Pentanal	285d	400c	1968b	3882a
701	2-Ethylfuran	nd	nd	953	1040
716	2,3-Pentanedione	nd	nd	24b	92a
765	1-Pentanol	nd	nd	101	113
769	Methylbenzene (*toluene*)	94c	115c	295a	192b
792	1-Octene	nd	nd	131	115
800	Octane	nd	nd	3367a	1452b
805	Hexanal	293d	426c	10686b	12609a
813	2-Octene	42b	33b	109a	113a
878	Hexanol	nd	nd	6550a	333b
892	2-Butylfuran	nd	nd	86a	44b
893	2-Heptanone	nd	nd	220a	151b
905	Heptanal	59c	75c	2078a	1796b
940	2-Heptenal	nd	nd	318	284
974	1-Heptanol	nd	nd	1073a	151b
982	1-Octen-3-ol	221c	249c	1460a	1123b
988	3-Octanone	nd	nd	22a	8b
994	2-Pentylfuran	nd	nd	955a	244b
1010	Octanal	83c	108c	1010a	584b
1062	2-Octenal	nd	nd	174a	56b
1079	1-Octanol	nd	nd	539a	137b
1110	Nonanal	29c	19c	866a	430b
	Microbial esterification	**3335a**	**3428a**	**1031c**	**1310b**
615	Ethyl acetate	3097a	3273a	960c	1165b
818	Ethyl 2-hydroxypropanoate	238a	155b	31c	19c
901	Ethyl pentanoate	nd	nd	nd	20
1002	Ethyl hexanoate	nd	nd	41b	106a
	Miscellaneous	**20762a**	**21844a**	**20639a**	**17764b**
503	Ethanol	2178a	1879b	904c	1014c
934	α-Pinene	1007a	1073a	559b	458b
991	β-Myrcene	312	479	354	423
978	β-Pinene	1959a	2133a	1897a	998b
1016	3-Carene	7926b	8402a	7992b	5938c
1021	o-Cymene	448a	518a	313b	193c
1031	Limonene	5474b	5742b	7002a	7115a
1034	β-Phellandrene	154	151	141	189
1340	δ-Elemene	95b	135a	137a	156a
1467	β-Caryophyllene	1210	1332	1342	1280
	Total volatiles	**41733c**	**44302c**	**68518a**	**54649b**

nd: not detected.
a,b,c,d: Values in the same row with different letters are significantly different ($p<0.05$).
[a] Linear retention index on a 5MS/silphenylene polysiloxane column.

addition (Fig. 2). The contribution of GCC+ to texture, through proteolytic phenomena, has been reported to be very limited, being proteolysis mainly attributed to endogenous proteases (Hierro et al., 2015).

3.5. Sensory analysis

Table 6 shows the results on the sensory quality of the sausages manufactured with bee products and reduced levels of curing salts, in comparison to the standard (C150) and the nitrate/nitrite-reduced (C75) sausage formulation. No significant differences were found among batches for any of the parameters, which confirmed that the addition of mixtures of chestnut honey, propolis and royal jelly at the levels set in our study did not affect the sensory quality of the product, as it had been observed in preliminary experiments. In the above-mentioned study by Bernardi et al. (2013), salamis added with 0.14 g/kg of either free or microencapsulated propolis showed lower sensory scores for taste and aroma. Our results are not in agreement with the observations by Bernardi et al. (2013), since the tasters could not differentiate among batches with and without propolis, which we added in higher amounts. Maybe the presence of honey and royal jelly could have helped to mask the bitterness and astringency of propolis. Encapsulation is an alternative when propolis is used alone, with the purpose of mitigating its unpleasant taste (El-Sakhawy et al., 2023). It appears from our results that the combination with other bee products might overcome its sensory limitations.

3.6. Volatile profile

Table 7 shows the headspace volatile profile of sausages manufactured with bee products and reduced levels of curing salts, together with those of standard (C150) and reduced (C75) nitrate/nitrite formulation. Fifty-five volatile substances were identified and semiquantitatively characterised as area units $\times 10^{-5}$. Compounds have been classified according to their principal origin. Significant differences ($p<0.05$) were found in the total content of volatiles between those batches added with bee products and those that did not. In batches prepared with bee products, volatile compounds derived from carbohydrate fermentation were one of the main groups, accounting for 32% of the total compounds. The most abundant volatiles in this group were acetic acid, together with ethanol; the latter has been included in the miscellaneous group since, in bee product batches, part of it comes from propolis extract. These substances originate from sugar fermentation activity of both starters, LAB and GCC+. It should be noted that batch C150 showed the lowest value, which could be due to the lowest counts of GCC+ in these sausages and also to its formulation, that included approximately half as much sugars than those prepared with bee products. Carbohydrate fermentation volatiles have been reported as the main group present in the headspace of sausages in other studies (Hospital et al., 2015; Perea-Sanz et al., 2018).

Volatile compounds derived from lipid oxidation were significantly affected by the presence of bee products in the formula, with HP75 and HPJ75 showing significantly ($p<0.05$) lower levels, representing around 3%-4%. These differences could be attributed to the antioxidant properties of bee products and are in accordance with the low TBARs values detected in both batches. However, these compounds accounted for 50%-52% of total volatiles in sausages prepared without bee products. It is worth noting that these differences were not reflected in the scores given by the tasters in the sensory analysis (Table 6). It appears that the extent of lipid oxidation in the batches formulated with bee products was sufficient to yield adequate amounts of aliphatic aldehydes, such as hexanal, pentanal and octanal, as well as 2-methylfuran, among other lipid derived compounds with an important role in aroma of dry sausages (Bleicher et al., 2022). Besides, HP75 and HPJ75 batches contained significantly higher levels of branched aldehydes and 3-methyl-3-butenol, compounds that have been correlated with the typical ripened aroma of cured meat products (Ruiz et al., 1999).

Branched aldehydes and alcohols derive from the catabolism of branched amino acids leucine, valine and isoleucine, and, as it has been mentioned, they have great impact on the aroma of cured meats. In fermented products, these compounds derive from the activity of starter cultures, GCC+ and LAB. In our study, the standard sausages (C150) showed the lowest concentration of this group of compounds. It has been reported that nitrite inhibits the growth of GCC+, and this could explain, first, the differences observed between sausages prepared with 150 and 75 mg/kg of nitrite. Batch C150 showed 0.7 log cfu/g lower numbers of GCC+ than those prepared with 75 mg/kg of nitrite. The negative relation between nitrite concentration and volatiles from amino acid catabolism has been reported in other studies on dry fermented sausages (Hospital et al., 2015; Perea-Sanz et al., 2018). Secondly, differences were also found among sausages prepared with 75 mg/kg of nitrite with and without bee products. Batches HP75 and HPJ75 contained higher levels of compounds from amino acid catabolism. Honeys have significant amounts of 2- and 3-methylbutanoic acids (Machado et al., 2020), which through different reactions could give rise to branched aldehydes and alcohols (Smit et al., 2009).

Finally, among compounds originating from microbial esterification only ethyl esters were detected. These compounds derive from the esterification of ethanol and organic acids by both LAB and staphylococcal esterases (Flores & Piornos, 2021; Talon et al., 1998). Ethyl acetate was the dominant compound in this group in all batches being significantly higher ($p<0.05$) in those formulated with bee products, probably due to the additional concentration of ethanol incorporated via propolis extract.

4. Conclusions

The use of mixtures of chestnut honey, propolis and royal jelly together with reduced levels of nitrate/nitrite in the production of fermented sausages provides greater antioxidant activity than the use of nitrate/nitrite alone. This effect was confirmed by significant differences in TBARs and volatile profile, but it did not affect the organoleptic properties of the products, nor the typical microbiota of sausages. Therefore, this combined strategy could be of interest to obtain nitrate/nitrite-reduced dry fermented sausages with a sensory quality comparable to a standard product, and with an increased oxidative stability during shelf life.

CRediT authorship contribution statement

Xavier F. Hospital: Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Eva Hierro:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Izaskun Martín-Cabrejas:** Writing – original draft, Methodology, Investigation. **Natalia Caballero:** Investigation. **Begoña Jiménez:** Investigation. **Vanesa Sánchez-Martín:** Investigation. **Paloma Morales:** Funding acquisition, Formal analysis. **Ana I. Haza:** Funding acquisition, Formal analysis. **Manuela Fernández:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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