



Natural curing strategies for nitrite-free dry-fermented sausages: Effects of NOS-positive *Staphylococcus* spp., Zn-protoporphyrin and polyphenol-rich extracts on untargeted metabolome and sensory quality

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ABSTRACT

The reduction of permitted nitrite levels in meat products by the European Union, driven by concerns related to nitrosamine formation and consumer health, has intensified the need for effective and safe nitrite replacement strategies. In this context, the present study investigated the impact of selected natural curing approaches on the untargeted metabolome, lipid oxidation, and olfactory profile of dry-fermented model sausages. Nine formulations originating from a meat batter control without nitrite; control with nitrite; inoculated with *Staphylococcus equorum* (L33); inoculated with *Staphylococcus saprophyticus* (L49); formulated with a porcine liver extract rich in zinc protoporphyrin IX (ZnPPEX); formulated with polyphenol-rich extract (NATPRE) alone (CTRL-/NATPRE); or in combination with the other strategies (L33/NATPRE, L49/NATPRE, ZnPPEX/NATPRE, respectively). Untargeted UHPLC-HRMS metabolomics, TBARS assay, and descriptive olfactory analysis were applied before and after ripening (21 days), and multivariate modelling was used to assess treatment- and time-related effects. Both treatment and ripening time shaped the sausage metabolome, whereas their interaction was not significant, indicating stable treatment-specific metabolic trajectories throughout ripening. ZnPPEX-containing formulations displayed distinct metabolic signatures after preparation, suggesting that this extract obtention process contributed to the presence of fermentation-related compounds. Among microbial strategies, L33 most closely replicated the metabolic and sensory characteristics, combining low lipid oxidation with a typical dry-cured meat odour. NATPRE effectively limited lipid oxidation and modulated the metabolomic profile, contributing to the preservation of bioactive compounds. Correlation analysis between discriminant metabolites and sensory attributes highlighted links between amino acid- and lipid-derived metabolites and both desirable and undesirable odour descriptors. Overall, the combination of starter cultures and polyphenol-based antioxidants emerged as a promising strategy for nitrite replacement, whereas ZnPPEX require further optimisation to control off-odours and biogenic amine accumulation.

1. Introduction

Chemically pure nitrates and nitrites are synthetic additives widely used in meat production due to their ability to enhance the colour and flavour of cured meat (Huang, Luo, Li, & Xu, 2022), act as antioxidants (Domínguez et al., 2019a) and inhibit the growth of undesirable microorganisms (Cardinali et al., 2018; Cui, Gabriel, & Nakano, 2010; Premi, Rocchetti, Lucini, Morelli & Rebecchi, 2024a). However, they

have been found to pose a risk to human health as they play a role in forming nitrosamines and other carcinogenic compounds (Flores & Toldrá, 2021; Schrenk et al., 2023). Consequently, European Union Regulation 2023/2108 has introduced new, lower restrictions for the use of nitrites (E249 and E250) and nitrates (E251 and E252) in animal-derived products, which will come into force in October 2025 Commission Regulation (EU), 2023. For example, the permitted amount of nitrite in fermented meat products will be reduced from 150 mg/kg of

Abbreviations: CNS, coagulase-negative staphylococci; MDA, malondialdehyde; NATPRE, NATPRE T-10 CUR HT; NOS, nitric oxide synthase; TBA, thiobarbituric acid; TBARS, Thiobarbituric Acid Reactive Substances; TCA, trichloroacetic acid; ZnPP, zinc protoporphyrin; ZnPPEX, zinc protoporphyrin extract.

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nitrite to 80 of nitrite ion (corresponding to ca. 120 mg/kg of nitrite salt). In contrast, the maximum permissible level in traditional dry cured meat products will range from 30 to 150 mg/kg expressed as nitrite/nitrate ion, depending on the product.

Therefore, several research studies are focusing on the investigation of potential effective substitutes for chemical preservatives in cured meats, primarily to recreate the distinctive pinkish-red colour of these products. Accordingly, various novel natural alternatives to nitrites have been reported in the literature (Shakil et al., 2022; Zhang et al., 2023). Specifically, coagulase-negative staphylococci (CNS) exhibiting nitric oxide synthase (NOS) activity have received particular attention. Indeed, Premi et al. (2024b) demonstrated that NOS-positive CNS strains isolated from fermented meat products could be used to replace nitrites in derived-meat products. Among these, *Staphylococcus equorum* L33 and *S. saprophyticus* L49 showed the ability to produce high levels of L-citrulline (an amino acid related to nitric oxide production and, consequently, red colour development) in microaerobic conditions. Additionally, compared to the nitrite control sample, these microorganisms exhibited the least colour variation in fermented meat models (Premi et al., 2024b) and enhanced the characteristic dry-cured colour in another study involving model dry-fermented sausages produced without nitrite sources (Premi et al., 2025). Despite their proved positive role in red colour development, the mechanisms underlying their behaviour remain unknown. In addition to their colour-enhancing properties, CNS are often used as technological functional commercial meat starters (Li, Zhu, Chen, Zhou, & Wu, 2022) thanks to their ability in producing aromatic compounds (Chen et al., 2021; Sallan, Kaban, & Kaya, 2022) and limiting oxidation processes (Heo, Lee, & Jeong, 2020; Premi et al., 2024a). Consequently, they appear a viable alternative to synthetic agents in the production of red-coloured dry-fermented meat products.

Another interesting strategy involves using zinc protoporphyrin IX (ZnPP), a natural red-purple pigment derived from heme proteins, as a colour enhancer in dry-cured meat foods (Schivazappa et al., 2024; Wakamatsu, Kawazoe, Ohya, Hayakawa, & Kumura, 2020). In fact, the ZnPP chromophore appeared to be relatively stable in the presence of light, heat, and low oxygen concentrations (Llauger et al., 2023). The ability of the ZnPP complex to impart a stable, bright red colour to meat products is well documented (Møller, Adamsen, Catharino, Skibsted, & Eberlin, 2007; Wakamatsu, Nishimura, & Hattori, 2004; Yoo, Bae, & Jeong, 2025). Indeed, Llauger et al. (2024) demonstrated that ZnPP, which was specifically extracted from porcine liver fermented homogenates (ZnPPEX), had a positive impact on the colour of meat products. Therefore, using ZnPP to colour nitrite-free meats appears to be a highly promising and innovative approach.

Other alternative solutions to traditional curing methods include the use of phenolic compounds found in plant extracts (Hernández et al., 2021; Honda, Miura, Masuda, & Masuda, 2016; Wu, Yin, Xiao, Zhang, & Richards, 2022). In this regard, NATPRE T-10 CUR HT (hereafter referred to as 'NATPRE') is a novel, natural ingredient composed of a blend of fruit and spice extracts which has recently been investigated. Particularly, Premi et al. (2025) produced dry-fermented sausages formulated with NATPRE (1%), which resulted in better redness values than untreated sausages. However, the colour enhancement was milder than that achieved using sodium nitrite. Unlike the addition of pure nitrite, the residual amounts of nitrite and nitrate found in NATPRE did not produce the characteristic cured colour. Therefore, increasing the amount of polyphenol-rich extract in the recipe could be a valuable strategy to make the sausages redder. This assumption is consistent with results obtained in cooked meat products (Hernández et al., 2021), where colour measurements of nitrite-containing cooked hams were similar to those formulated with an analogous NATPRE product (NATPRE T-10 HT S at concentrations of 5, 10 and 20 g/kg). Regardless of the presence of nitrite residual sources, the addition of plant extracts rich in phenolic compounds can prevent oxidation and affect sensory properties and microbial growth in fermented meat products

(Efenberger-Szmechtyk, Nowak, & Czyzowska, 2020).

Despite the promising results reported for each of these natural curing strategies, their application as single solutions present specific limitations. The use of NOS-positive CNS alone may not provide sufficient antioxidant protection to effectively control lipid oxidation during ripening, particularly in the absence of nitrites. Therefore, it can benefit from synergistic effects when combined with the addition of phenolic antioxidants. Similarly, although ZnPP-rich extracts are highly effective in promoting cured meat colour, their application could be associated with lipid oxidation, sensory drawbacks and the accumulation of fermentation-related compounds that may negatively affect product acceptability. Conversely, polyphenol-rich extracts such as NATPRE mainly exert antioxidant and preservative effects, but their colour-enhancing capacity remains weaker than that achieved with nitrites when used alone. These limitations highlight the need for integrated curing strategies, in which complementary technological functions are combined to achieve both visual quality and overall product stability in nitrite-free fermented meats. Starting from this background, we investigated the untargeted metabolomics and sensory profiles of dry-fermented model sausages produced with two NOS-positive CNS strains, a ZnPP-rich liver extract, and NATPRE T-10 CUR HT used both individually and in combination. A total of nine treatments were prepared and ripened for 21 days at 8 °C. Colour development and heme pigment characterisation of the same sausage formulations were comprehensively investigated in a previous companion study (Premi et al., 2025), while the present work focuses on oxidative, sensory, and metabolomic outcomes associated with these nitrite replacement strategies. Particularly, in the present work, untargeted metabolomics and the thiobarbituric acid reactive substances (TBARS) assay were integrated with the sensory evaluation of odour to gain deeper insight into the aroma characteristics of the sausages. The objective was to identify the most promising formulation, not only in terms of redness retention, but also from metabolomic and sensory perspectives.

2. Materials and methods

2.1. Preparation of model sausages

The model sausages were prepared according to the method described by Premi et al. (2025). Briefly, a total of 10 kg of lean pork loin/shoulder and backfat (80:20) from commercial pigs were obtained from a local slaughterhouse in Spain, vacuum packed, and frozen until use. Prior to preparation, the meat was stored at 4 °C overnight, then cut and minced to 6 mm using a grinder (Castellvall model AMV I-80, Riudellots de la Selva, Spain) and thoroughly mixed while maintaining the temperature at ≤ 4 °C. Each experimental treatment was prepared in the pilot plant, and utilised 1 kg of minced meat, seasoned with sodium chloride (NaCl, 2.5%) and dextrose (0.4%). The meat batter contained $15.1\% \pm 1.1$ fat (AOAC 991.36 method), $17.6\% \pm 1.9$ protein (AOAC 928.08 method), and $60.9\% \pm 0.8$ moisture (AOAC 950.46 method) (AOAC, 2016; Premi et al., 2025). The study comprised nine distinct meat batter treatments, each executed in triplicate: the control (CTRL-), consisting solely of meat batter with the addition of 0.05% sodium ascorbate; the nitrite dry-cured meat batter (CTRL+), prepared with the addition of 0.05% sodium ascorbate and 150 mg/kg of sodium nitrite; and seven additional treatments (CTRL-/NATPRE, L33, L33/NATPRE, L49, L49/NATPRE, ZnPPEX, and ZnPPEX/NATPRE), which were prepared as described in the Supplementary Table 1. Bacteria (*Staphylococcus equorum* L33 and *Staphylococcus saprophyticus* L49) previously isolated from fermented meat products were inoculated at a level of 6 log CFU/g, a value selected according to a previous dedicated study (Premi et al., 2024b). Treatments involving the addition of the porcine liver extract with a high ZnPP content (referred to as ZnPPEX) were formulated to contain 40 mg of ZnPP per kg of meat batter. The ZnPPEX came by a porcine liver fermented homogenate obtained after the addition of ascorbic acid (1000 mg/L) and acetic acid (2500 mg/L),

followed by an anaerobic incubation at pH 4.8 for 24 h at 45 °C, as described by Llauger et al. (2023). The resulting fermented homogenate was mixed with acetone (1:4, w/v) to extract porphyrins, as described by Premi et al. (2025). After stirring for 15 min at 4 °C, the mixture was centrifuged (30,000 g for 15 min at 4 °C) and the supernatant was transferred into a rotary vacuum evaporator (Büchi R 114 model, Büchi corporation, New Castle, United States) heated at 35–45 °C until the evaporation of acetone was achieved. The remaining liquid containing ZnPP was then filtered using a folded qualitative filter and quantified as described in Llauger et al. (2024). Then, a haemoglobin solution, prepared from haemolyzed porcine red blood cells, as described by Premi et al. (2025), was dissolved in phosphate buffer (50 mM; pH 7) and added to the evaporated extract containing ZnPP to achieve a ZnPP/haemoglobin 1:1 M ratio to partially solubilize ZnPP. The haemoglobin content was calculated using the molar extinction coefficient of 7.12 measured at 523 nm (Snell & Marini, 1988). The aqueous mixture containing the extract rich in ZnPP and haemoglobin was lyophilised using a freeze-drying equipment. Samples labelled 'NATPRE' contained 1% of NATPRE T-10 CUR HT (Prosur, Murcia, Spain), a polyphenol-rich extract with a strong antioxidant activity, which also claims to contain 10–12% ascorbic acid.

For each treatment, 83 g portions of meat batter, including all the aforementioned ingredients, were transferred into custom-made Tublin® 05 film bags (TUB-EX ApS, Tårs, Denmark). These were then sealed using a Technotrip EVT-10 vacuum packaging machine (Terrassa, Barcelona, Spain) and then ripened in a controlled environment at 8 °C and 60–75% relative humidity for 21 days. The experiment was repeated twice in independent trials performed on different days using two different batches of meat and ZnPP extracts. Analytical determinations were performed in triplicate ($n = 3$).

2.2. Extraction of metabolites from model sausages for untargeted metabolomic profiling

Each sample, weighing 1 g, was placed into a conical test tube containing 10 mL of an 80% methanol acidified with 0.1% formic acid (v/v) solution. The samples were then homogenised at maximum power using a Polytron™ PT1200E homogeniser (Kinematica, Malters, Switzerland) for 3 min, followed by centrifugation at 4227 xg for 15 min at 4 °C. Subsequently, the samples were filtered through 0.22 µm regenerated cellulose syringe filters.

Meat metabolites were screened using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish UHPLC pump and equipped with heated electrospray ionization-II probe (Thermo Scientific, Waltham, MA, USA) (Rocchetti et al., 2021). Chromatographic separation was achieved on ACQUITY UPLC Waters BEH C18 (2.1 × 100 mm, 1.7 µm). Each extract (3 µL) was injected at a flow rate of 200 µL/min, and the column was maintained at 40 °C. The mobile phase consisted of gradient elution based on 6–94% acetonitrile for 35 min, using 0.1% (v/v) formic acid as a phase modifier. The full scan MS analysis was performed in the mass range of 80–1200 m/z , with a positive ionization mode and a nominal mass resolution of 70,000 at 200 m/z . Moreover, data-dependent MS/MS mode was carried out only on pooled Quality Control (QC) samples in positive polarity with the selection of the most abundant ions (Top N ions = 3) by using a reduced full scan mass resolution of 17,500 at 200 m/z and working under normalized collisional energies of 23.3 eV. Raw spectral data (.RAW) were further processed using the MS-DIAL software (version 4.90). Peak finding, deconvolution, LOWESS normalization, and annotation via spectral matching against the comprehensive FooDB and in-house ESI+ MSMS libraries were performed. According to the experimental conditions, a level 2 of confidence in identification (i.e., putatively annotated compounds and structural confirmation of the most abundant mass features) was achieved (García-Pérez, Becchi, Zhang, Rocchetti, & Lucini, 2024).

2.3. TBARS assay

The thiobarbituric acid reactive substances (TBARS) assay is one of the most popular methods for determining the overall levels of oxidation in raw and cooked meat products (Abeyrathne, Nam, & Ahn, 2021). The TBARS assay was carried out immediately after the model sausages' production (T_0) and after the entire ripening period (T_{21}). Briefly, 1 g of sample was placed in a 50 mL conical test tube containing 9 mL of extraction solution (10% trichloroacetic acid, w/v) and homogenised using a Polytron™ PT1200E homogeniser (Kinematica, Malters, Switzerland) for 1 min (rotation speed 15,000 rpm). The homogenised sample was then centrifuged at 4227 xg for 15 min at 4 °C, and 250 µL of the resulting supernatant was transferred into an Eppendorf tube. Then, 750 µL of 0.5% thiobarbituric acid was added to the supernatant. The Eppendorf tubes were shaken by inverting them, incubated in boiling water for 30 min, then transferred into ice for 10 min. Spectrophotometer readings were then taken at wavelengths of 532 nm and 600 nm. Three determinations ($n = 3$) were carried out for each treatment replication.

2.4. Olfactory sensory test of model sausages

A sensory evaluation was conducted to assess the overall impact of the different treatments on the olfactory profile of dry-fermented model sausages at the end of the ripening process. Firstly, a training phase was carried out to develop descriptors that would provide maximum information about the olfactory sensory properties of the dry-fermented model sausages. The following eight descriptors were then selected: typical cured meat odour (e.g., *fuet* sausage); animal odour (e.g., skatole, musky); aromatic herbs (i.e., NATPRE ingredient); liver, metallic or blood odour; rancid odour (e.g., oxidised sunflower oil); acetic acid odour (e.g., vinegar); abnormal odour (i.e., ZnPPEx ingredient); and other odours. An additional preliminary session was carried out to unify the use of the evaluation scale among tasters.

Prior to the evaluation, the model sausages were removed from the controlled environment at 8 °C, where they had been ripening, and stored in a room at 24 ± 1 °C for 1 h. The test samples were prepared by grinding the dry-fermented sausages and placing them in closed, disposable plastic wrappers. Equal amounts and sizes of samples were provided to each of the seven trained panellists including research staff of IRTA, and a code consisting of three random numbers was used to identify the samples. Each judge received an evaluation form along with the samples. A scoring test was used to evaluate the sensory profile, with a 10-point scale chosen and definitions of the different scores provided (0 indicating the total absence of the descriptor and 10 indicating the highest intensity). The analysis was performed in individual booths, and all forms of communication were prohibited. All sensory data were collected for statistical analysis. All participants provided verbal consent after the study requirements had been fully disclosed to them. The privacy and rights of participants were also safeguarded.

2.5. Statistical and multivariate data analyses

Sensory data were analysed using a two-way analysis of variance (ANOVA) with treatment and production trial as fixed factors, including their interaction, in order to evaluate both formulation effects and batch-to-batch reproducibility. TBARS and untargeted metabolomics data were analysed by one-way ANOVA followed by Duncan's post-hoc test and multivariate approaches, respectively, using analytical replicates, as these analyses were primarily aimed at assessing treatment-driven oxidative and metabolic differences under controlled pilot-scale conditions. The software SPSS version 26.0 was used for the analysis of both TBARS and sensory dataset. The same software was used to calculate Pearson's correlation coefficients between sensory attributes and discriminant marker compounds at T_{21} from untargeted metabolomics. The multivariate statistical analysis of the omics dataset was

done using different tools, namely MetaboAnalyst 6.0 (Pang et al., 2024) and RStudio (version 4.2.3). The software MetaboAnalyst 6.0 was used for median normalization, \log_{10} transformation and Pareto scaling and to perform an unsupervised hierarchical clustering analysis (to inspect the hierarchical effect of ripening time and treatment type in an unsupervised manner), a classification of the different meat metabolites (using the enrichment tool), followed by a Volcano Plot analysis combining Fold-Change (cut-off ± 1.2) and ANOVA ($p < 0.05$) for the pairwise comparisons between the different treatments and CTRL+ at T₂₁. An additional Volcano Plot analysis was carried out for the pairwise comparisons ZnPPEx T₂₁ vs T₀ and ZnPPEx/NATPRE T₂₁ vs T₀. Thereafter, both PLS-DA and sPLS-DA models were also carried out and cross-validated using a 5-fold CV method, extrapolating the discriminant metabolites (VIP). Additionally, the “rAMOPLS” package (version 0.2) in RStudio was used to perform multifactorial ANOVA coupled with OPLS prediction modelling (AMOPLS) to better evaluate the potential interactions existing between ripening time and treatments. The model built was then inspected in terms of statistical and predictive assessments through different parameters, such as the residual sum of squares (RSS), residual structure ratio p -value based (RSR p -value) and goodness of fit (R^2Y).

3. Results and discussion

3.1. Untargeted metabolomic profiles of the dry-fermented model sausages

The untargeted metabolomics approach based on UHPLC-HRMS allowed the identification of 400 meat metabolites, according to a level 2 of confidence in annotation (i.e., putatively annotated compounds with structural confirmation based on pooled QC; Supplementary Table 2; sheet a). The compounds identified are provided with several annotation-based parameters, including average m/z , adduct type at ESI source, formula, ontology, INCHIKEY, SMILES, total identification score, S/N values, MS1 isotopic spectrum, and MSMS spectrum. Interestingly, 87.5% of the annotated compounds showed RSD values $< 30\%$, thus revealing the reliability of these compounds and supporting the robustness of the analytical workflow. As the next step, an enrichment metabolite set was used to inspect the most represented and explained classes of compounds under the experimental conditions. As reported in Fig. 1, we found an enrichment of amino acids, peptides, and analogues (49 compounds), followed by amines (including sphingosine, histamine, spermine, spermidine, cadaverine, and phytosphingosine), pyridinecarboxylic acids, lipid compounds (such as monoradylglycerols, fatty acids, and glycerophosphocholines), and other metabolites. As far

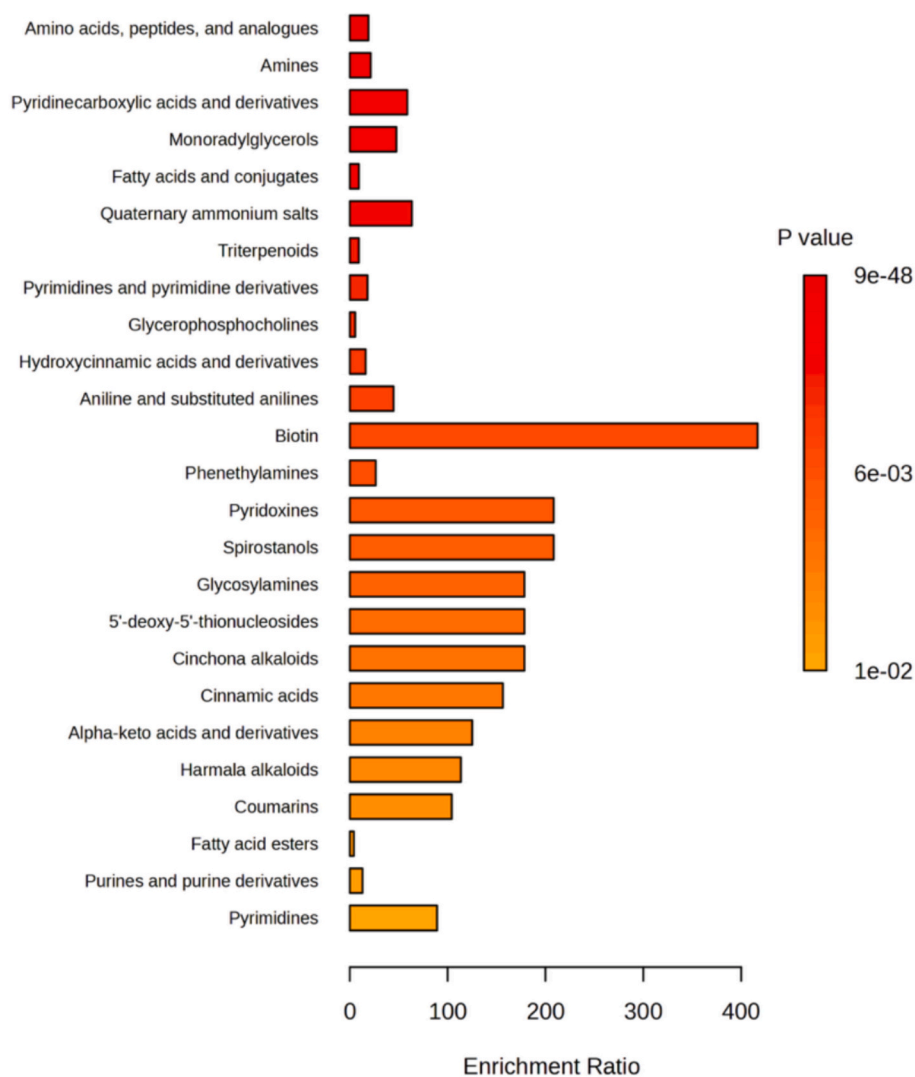


Fig. 1. Overview of enriched metabolite sets (Top 25) according to the annotated metabolites by UHPLC-HRMS. Enrichment ratio is computed by Hits / Expected, referring to the total compounds involved in a metabolite set. *Abbreviations:* Hits = observed hits; Expected = expected hits.

as amino acids are concerned, we found 14 proteinogenic amino acids, with histidine, methionine, and arginine showing the lowest RSD (%) values, namely 1.44, 1.77, and 1.93, respectively. Additionally, we measured glutathione (GSH) and its oxidised form (GSSG), two key peptides involved in redox impairment (Vašková, Kočan, Vaško, & Perjési, 2023).

The hierarchical clustering heatmap, shown in Fig. 2, clearly discriminated two major clusters among the model sausages based on their annotated metabolomic profiles. The first main cluster grouped together the T_0 samples of the CTRL- (nitrite-free control), L33, L49, and CTRL+ (nitrite-cured) treatments, as well as their respective NATPRE-supplemented variants. This indicates that at the initial stage (T_0), these formulations shared a largely similar metabolic fingerprint, regardless of the starter cultures (*S. equorum* L33 or *S. saprophyticus* L49) or the addition of the polyphenol-rich antioxidant (NATPRE). In contrast, the second major cluster separated into two distinct sub-clusters. The first sub-cluster included all samples containing the ZnPPEX, both with and without NATPRE, at both T_0 and T_{21} . The second sub-cluster grouped the T_{21} samples of the CTRL-, CTRL+, L33, and L49 treatments, along with their NATPRE variants. This pattern highlights two key insights: (i) the ripening time (21 days) has a clear impact on the metabolomic shift of the nitrite-containing and nitrite-free groups without ZnPP, and (ii) ZnPPEX addition drives a distinct metabolomic profile that is consistent over time and markedly different from all other formulations. This unique clustering of ZnPPEX treatments even at T_0

suggests that the technological preparation of the porcine liver extract, intended to enhance ZnPP levels and thus maintain a stable red colour in nitrite-free products, inherently introduces metabolites that resemble those typically formed during ripening. This can be attributed to the production process of the ZnPPEX, which involves an anaerobic incubation at 45 °C for 24 h favouring the growth of fermentative lactic acid bacteria previously described as *Lactobacillus johnsonii*, *Limosilactobacillus reuteri*, *Limosilactobacillus mucosae*, and *Lactobacillus amylovorus* (Ferrer-Bustins et al., 2023). This fact explains why the presence of NATPRE in the ZnPPEX treatments does not override this metabolic signature but might contribute to further stabilisation, as suggested by their tight clustering.

From an application perspective, the heatmap suggests that while ripening (fermentation and drying) drives a clear metabolomic evolution in traditional and starter-culture sausages, the ZnPP-based formulations already display a fermented-like metabolomic profile at T_0 , thus contributing to improved dry-cured colour without added nitrite, as observed by Premi et al. (2025). Additionally, when comparing these alternatives to the CTRL+ (nitrite dry-cured), the use of both *S. equorum* L33 and *S. saprophyticus* L49 (alone or in combination with NATPRE) appears to be the most promising alternative strategies. In this regard, by looking the heat map in Fig. 2, L33 and L49 after 21 days of ripening seem to maintain an exclusive metabolomic signature, potentially closer to ripened nitrite-cured meat (CTRL+). However, the unsupervised clustering highlighted the need for a deeper metabolomic investigation,

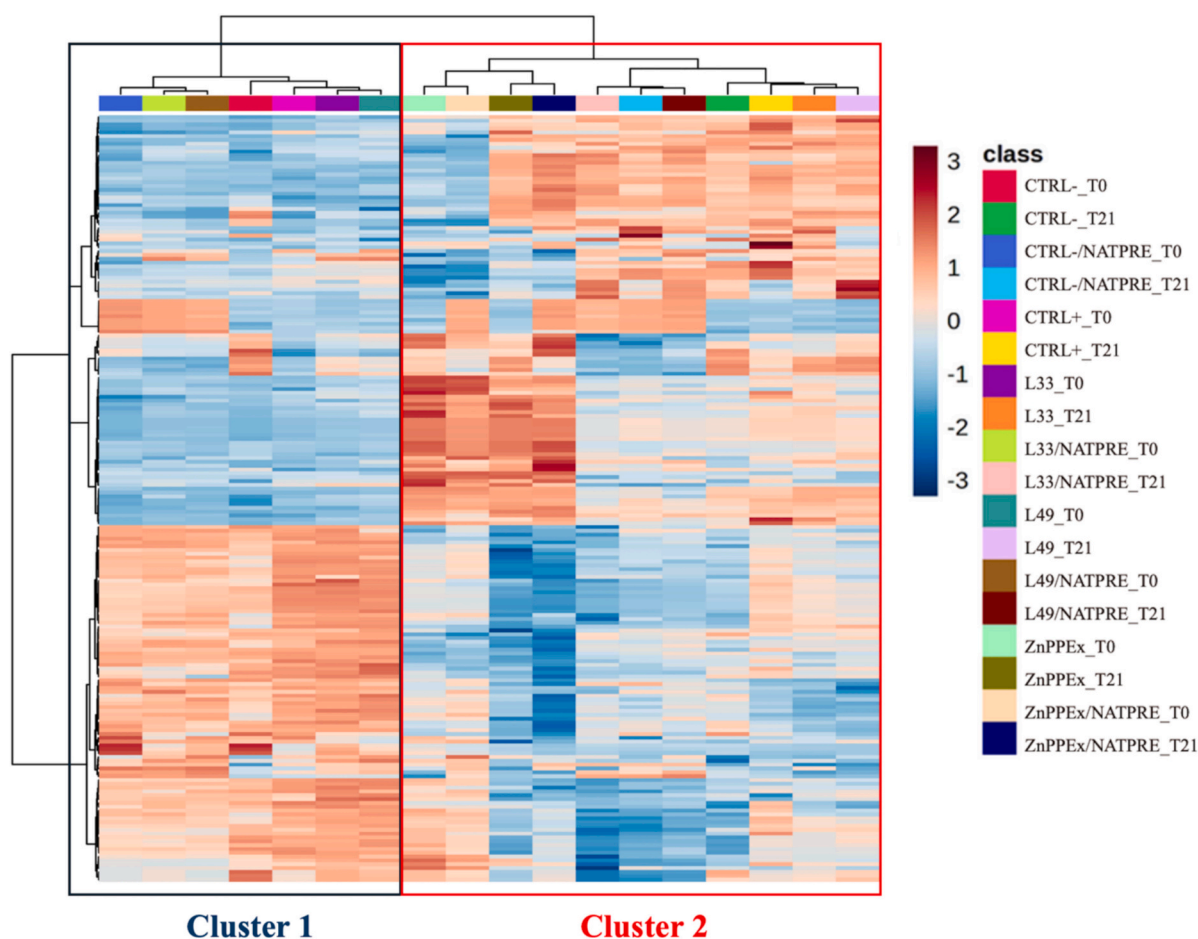


Fig. 2. Hierarchical clustering analysis (HCA) built considering the normalized relative abundance value of each annotated meat metabolite by UHPLC-HRMS of samples before (T_0) and after ripening (T_{21}). Different colours within the heat map indicate strong up- (red colour; Fold-Change = +3) and down- (blue colour; Fold-Change = -3) accumulation values, as provided by Fold-Change analysis. The following main clusters have been identified: Cluster 1: CTRL-/NATPRE_T0; L33/NATPRE_T0; L49/NATPRE_T0; CTRL-_T0; CTRL+_T0; L33_T0; L49_T0. Cluster 2: ZnPPEX_T0; ZnPPEX/NATPRE_T0; ZnPPEX_T21; ZnPPEX/NATPRE_T21; L33/NATPRE_T21; CTRL-/NATPRE_T21; L49/NATPRE_T21; CTRL-_T21; CTRL+_T21; L33_T21; L49_T21. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to better understand the behaviour of meat products manufactured with ZnPPEX and NATPRE as viable alternatives to nitrite dry-curing, particularly in relation to biochemical stability and sensory attributes. To further investigate the temporal dynamics underlying the ZnPPEX-related clustering observed in the hierarchical analysis, additional Volcano plot analyses were performed comparing T_{21} vs T_0 separately for ZnPPEX and ZnPPEX/NATPRE formulations. A total of 86 and 135 significantly modulated metabolites were detected for ZnPPEX and ZnPPEX/NATPRE, respectively. Venn diagram comparison revealed that 36.6% of the metabolites showed common temporal variations in both formulations, whereas 46.6% were exclusively modulated in ZnPPEX/NATPRE and 16.8% were specific to ZnPPEX alone, indicating a substantial modulation of the ZnPPEX-driven metabolic trajectory by NATPRE during ripening. Among the metabolites commonly affected over time, glutathione showed a significant down-accumulation in both ZnPPEX/NATPRE ($\log_2FC = -2.84$) and ZnPPEX ($\log_2FC = -4.11$), while arginine, tyrosine and serine were consistently up-accumulated in both models (supplementary table 2; sheet b). Conversely, protoporphyrin IX emerged as a ZnPPEX-specific temporal marker, showing significant up-accumulation only in the absence of NATPRE ($\log_2FC = 1.46$).

To confirm the impact of the obtention process on the metabolomic profile of ZnPPEX, we performed two supervised PLS-DA analyses at T_0 (immediately after the manufacturing of the model sausages), considering samples both without and with the NATPRE antioxidant; the results are summarised in the Supplementary Table 2 (sheet c). For the supervised model based on sausages produced without NATPRE, we found chemical similarities between CTRL+, L33, and L49 treatments, whereas CTRL- and ZnPPEX displayed clearly distinct profiles. Among the most important meat metabolites discriminating ZnPPEX from CTRL- (17 VIP compounds), we identified Heme B ($\log_2FC = 8.03$; $p = 2.3 \times 10^{-3}$), nervonic acid ($\log_2FC = 12.33$; $p = 6.9 \times 10^{-6}$), and tyramine ($\log_2FC = 8.73$; $p = 5.2 \times 10^{-5}$) as key markers. Heme B and nervonic acid are clearly related with the porcine liver used to obtain the ZnPP functional ingredient. On one side, Heme B is in fact a Fe-porphyrin IX that comes from the release of porphyrin from haemoglobin present in the liver; additionally, during the obtention process, haemoglobin was added to solubilize ZnPP. On the other side, nervonic acid is a very long-chain monounsaturated omega-9 fatty acid that plays a crucial role in the structure and function of the nervous system previously reported to be present in the pig liver (Lepine, Garleb, Reinhart, & Kresty, 1993). Regarding the PLS-DA model built at T_0 but including the effect of NATPRE, a similar pattern emerged: the ZnPPEX/NATPRE samples again showed a distinctly different metabolomic fingerprint. Among the most discriminant metabolites, nervonic acid ($\log_2FC = 10.93$; $p = 1.1 \times 10^{-4}$) and tyramine ($\log_2FC = 8.54$; $p = 6.3 \times 10^{-6}$) were confirmed, while histamine ($\log_2FC = 5.40$; $p = 1.9 \times 10^{-5}$) also emerged as relevant. These findings suggest that the technological treatment used to prepare ZnPPEX, aimed at boosting the cured colour through ZnPP formation, likely contributes to a distinct initial metabolomic signature. Interestingly, some discriminant metabolites, such as tyramine and histamine, were biogenic amines typically associated with later stages of ripening or fermentation (Leuschner, Heidel, & Hammes, 1998), but also described to be naturally present in liver (Custódio, Theodoro, & Gloria, 2016; Valero et al., 2005). Regardless of the origin of these metabolites, their presence in meat supports the hypothesis that ZnPPEX may introduce compounds that mimic advanced ripening states from the outset. This could help to explain the clear separation observed in the hierarchical clustering analysis and indicates a potential for producing nitrite-free dry-cured products with stable colour and flavour traits. Further ad-hoc targeted and quantitative metabolomic investigations will be crucial to clarify how these compounds evolve during ripening and to optimise ZnPPEX formulations in terms of both safety and sensory quality, for example, through the addition of a selected starter culture during the preparation of the porcine liver.

3.2. Focus on dry-fermented sausages manufactured with and without NATPRE

The AMOPLS analysis performed separately for sausages produced without and with NATPRE consistently showed that both the treatment and the ripening time (T_0 vs. T_{21}) had significant effects on the untargeted metabolomic profile, while their interaction was not significant (Fig. 3). Specifically, as shown in Table 1, all the factors included in the model yielded significant R^2Y values (R^2Y p -value = 0.01), but only ripening time and treatment were statistically significant based on the RSR p -value (0.01). In contrast, their interaction showed a low explanatory power (RSS = 14.1%) and a non-significant RSR p -value (0.31), highlighting the overall complexity of the model when both factors are considered simultaneously. Accordingly, under the tested conditions, treatment and ripening time independently accounted for the largest share of explained variability (RSS = 25.0% and 19.1%, respectively), while residuals captured the remaining unexplained variation (41.8%). A similar pattern was observed for the model including NATPRE (Table 1), where treatment and ripening time again showed significant effects (RSS = 28.1% and 20.0%, respectively), whereas their interaction remained non-significant (RSS = 11.8%; RSR p -value = 0.97). Residual variability was similar (RSS = 40.1%). These multivariate results clearly indicate that both technological treatments and ripening time independently shape the overall metabolomic evolution of the dry-fermented sausages, but that the specific effect of each treatment remains relatively stable throughout ripening, without notable synergistic or antagonistic interactions. The lack of a significant interaction between treatment and ripening time likely indicates that the metabolomic differences among treatments were preserved throughout the ripening process. While ripening time strongly affected the overall metabolome, the relative positioning of the treatment groups remained stable, suggesting parallel metabolic trajectories rather than treatment-specific temporal divergence. This finding points to a robust and predictable effect of the different curing strategies on sausage biochemistry, which is particularly relevant in the context of nitrite replacement.

To further elucidate these differences, two sPLS-DA models were applied (Supplementary table 2; sheet d) to maximise the separation between samples manufactured without and with NATPRE, using CTRL+ as the reference due to the application of 150 mg/kg of sodium nitrite, thus confirming the AMOPLS results showed in Fig. 3. In sausages ripened without NATPRE, 57 compounds showed a VIP score > 1 (Supplementary Table 2; sheet d), indicating high predictive value, and many displayed significant changes when comparing ZnPPEX to CTRL+. Some of these discriminant compounds, particularly relevant in meat science and according to our experimental plan, are reported in Table 2.

At T_{21} , ZnPPEX samples exhibited the highest levels (expressed as \log_2FC vs CTRL+) of biogenic amines, such as histamine ($\log_2FC = 5.39$) and tyramine ($\log_2FC = 1.94$), accompanied by a significant ($p < 0.05$) depletion of oxidised glutathione ($\log_2FC = -2.82$). Rather than indicating an effective protection against lipid oxidation, the depletion of oxidised glutathione likely reflects an increased redox turnover and utilisation of glutathione pools in response to oxidative and fermentative processes associated with ZnPPEX, possibly also influenced by the presence of ascorbic acid in the formulation. This interpretation is consistent with the elevated TBARS values observed in ZnPPEX sausages at T_{21} (2.06 mg MDA/kg), indicating that the modulation of redox-related metabolites was not sufficient to prevent lipid peroxidation. Since glutathione plays a central role in mitigating oxidative stress (Al-Temimi et al., 2023), the altered redox status observed in ZnPPEX samples highlights an active involvement of antioxidant systems rather than improved oxidative stability per se, which is technologically relevant for understanding redox dynamics during ripening (Ameer, Seleshe, & Kang, 2022). Additionally, the significantly higher abundance of histamine and tyramine may be linked to enhanced amino acid decarboxylation driven by microbial metabolism (Banicod et al., 2025) under nitrite-free dry-curing conditions or to the technological process

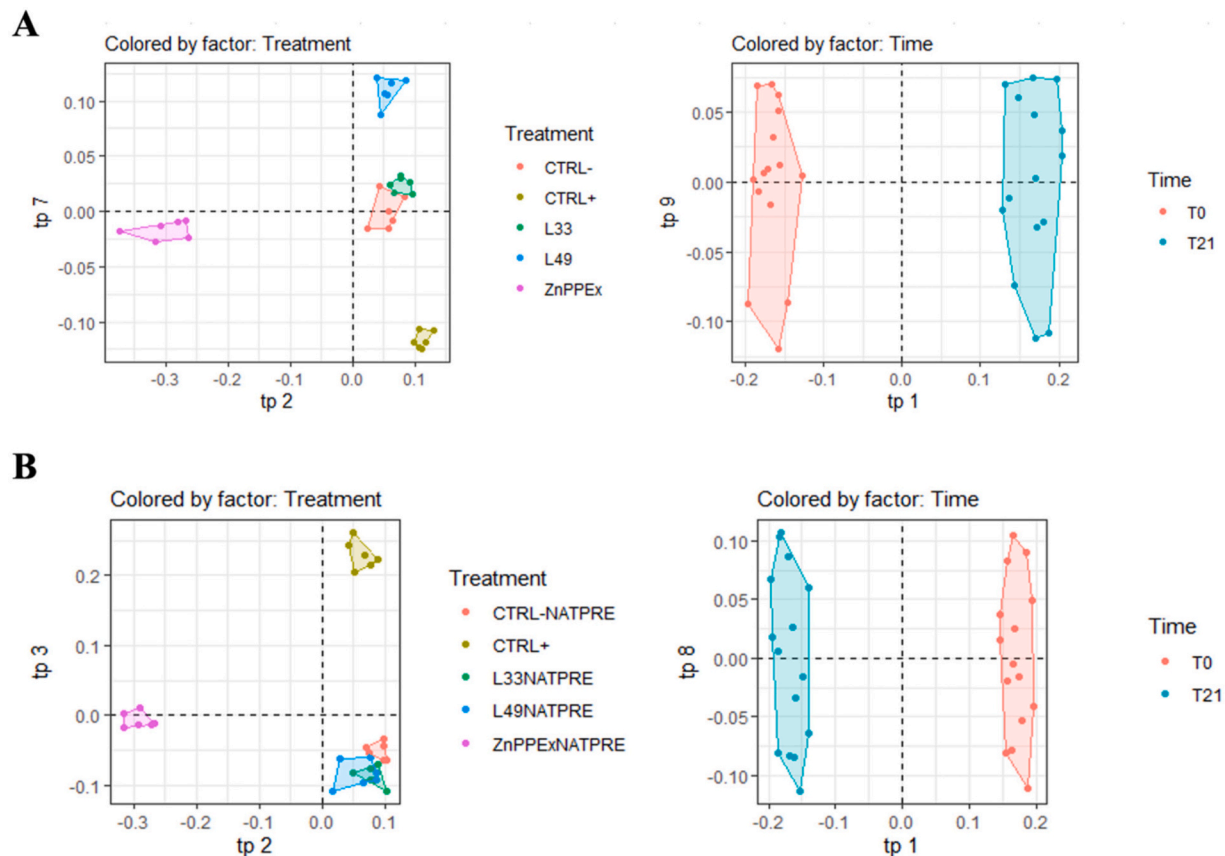


Fig. 3. Supervised AMOPLS analysis score plots built considering the metabolomic profile of the different dry-fermented sausages manufactured without (A) and with (B) NATPRE. The score plot were built considering “Treatment” and “Ripening time (T₀ vs T₂₁)” as discriminant parameters. tp = contributions of each predictive component used to build the score plot.

Table 1

Relative variability and block contributions of the AMOPLS analysis of meat metabolome considering the three factors under investigation (i.e., treatment, ripening time, and treatment x ripening time). N/A = not available; ns = not significant; Main tp = contribution of each main predictive component used to build the score plot; to1 = contribution of orthogonal component.

Effect	RSS (%)	RSR	RSS <i>p</i> -value	RSR <i>p</i> -value	R ² Y <i>p</i> -value	Main tp		to1
<i>no NATPRE</i>								
Treatment	25.0%	1.712	0.01	0.01	0.01	98.9% (tp2)	96.0% (tp7)	20.3%
Time	19.1%	1.915	0.85	0.01	0.01	98.9% (tp1)	8.5% (tp9)	18.2%
Treatment × Time	14.1%	1.300	ns	0.31	0.01	89.1% (tp3)	88.2% (tp6)	26.8%
Residuals	41.8%	1.000	N/A	N/A	N/A	16.4% (tp9)	11.9% (tp8)	34.8%
<i>with NATPRE</i>								
Treatment	28.1%	1.869	0.01	0.01	0.01	99.0% (tp2)	98.7% (tp3)	18.9%
Time	20.0%	1.992	ns	0.01	0.01	99.5% (tp1)	9.9% (tp8)	17.8%
Treatment × Time	11.8%	1.267	ns	0.97	0.01	93.6% (tp4)	74.5% (tp6)	27.9%
Residuals	40.1%	ns	N/A	N/A	N/A	19.7% (tp8)	18.5% (tp9)	35.4%

used to obtain the ZnPP functional extract, as previously suggested by inspecting the metabolomic profile of ZnPPEX at T₀. To further support these hypotheses, additional volcano plot analyses were performed by comparing the metabolomic profiles of ZnPPEX-containing samples over ripening time (from T₀ to T₂₁). These analyses revealed no significant accumulation of polyamines during ripening, while a significant ($p < 0.05$) decreasing trend was observed for spermine ($\log_2\text{FC} = -0.42$) and tyramine ($\log_2\text{FC} = -0.84$). Notably, no significant accumulation was detected for histamine over time, thus confirming our initial hypothesis that this biogenic amine represents a specific biomarker of the fermentative process used to obtain ZnPPEX. Overall, these findings indicate that while ZnPPEX effectively promotes a cured-like red colour resembling that achieved through nitrite curing, if produced under non-

controlled conditions it may also represent a source of histamine, which is undesirable from both food safety and off-flavour perspectives (Jaguey-Hernández et al., 2021). These observations support the need for further studies focusing on the quantitative evaluation of histamine and other polyamines in ZnPP extracts obtained through different processing methods. Several free amino acids, including tyrosine, proline, phenylalanine, arginine, isoleucine, and alanine, were also identified as discriminant markers (Table 2). In this regard, Lopez-Pedrouso et al. (2023) reported that during the process for obtaining the ZnPPEX various amino acids (e.g., proline, phenylalanine) and peptides were formed. The consistent down-accumulation of tyrosine vs CTRL+ at T₂₁, observed in L33, L49, and ZnPPEX treatments, aligns well with the simultaneous up-accumulation of tyramine, its decarboxylation product,

Table 2

Discriminant VIP compounds at T₂₁ as a function of the different treatments and with no NATPRE added. The log₂FC values are expressed vs the CTRL+. ns = not significant from Volcano Plot analysis.

VIP PLS-DA (no NATPRE)	Chemical class	VIP score	CTRL- (log ₂ FC)	L33 (log ₂ FC)	L49 (log ₂ FC)	ZnPPEX (log ₂ FC)
Histamine	2-arylethylamines	2.949	ns	ns	ns	5.39
Alanine	Amino acids	1.021	ns	ns	-4.00	ns
2,3-dihydroxypropyl 3-hydroxy-13-methyltetradecanoate	Fatty alcohols	3.171	ns	ns	3.18	ns
Isoleucine	Amino acids	1.479	ns	ns	ns	0.94
Homocysteic acid	Amino acids	1.252	ns	ns	1.23	1.34
Arginine	Amino acids	1.064	ns	-0.59	-0.50	-0.66
(9Z)-9-octadecenoic acid	Fatty acids	3.733	ns	ns	1.51	4.28
Oxidised Glutathione	Peptides	1.321	-1.06	ns	ns	-2.82
Tyramine	Phenethylamines	2.555	ns	1.31	1.19	1.94
Nicotinic acid	Pyridinecarboxylic acids	1.046	-1.29	ns	ns	ns
Phenylalanine	Amino acids	1.641	ns	ns	ns	0.97
Proline	Amino acids	1.760	ns	ns	ns	1.09
Tyrosine	Amino acids	1.331	ns	-0.39	-0.39	-0.82

further supporting the role of these cultures or treatments in modulating proteolysis and amino acid catabolism. Interestingly, only 11 metabolites were significantly altered in L33 vs. CTRL+ at T₂₁ (Supplementary Table 2; sheet d), confirming that L33 induced a metabolomic profile closely resembling that of the traditional nitrite dry-cured product without the addition of starter cultures. However, the observed increase in tyramine in L33 highlights the need for targeted quantitative studies to better monitor its accumulation relative to sensory acceptability and regulatory limits. In contrast, L49 triggered 30 significant metabolite changes compared to CTRL+ (Supplementary Table 2; sheet d). Among these, the up-accumulation of (9Z)-9-octadecenoic acid suggests enhanced lipolysis, while the presence of L-homocysteic acid points to oxidative degradation pathways involving sulphur amino acids like methionine and cysteine. The detection of 2,3-dihydroxypropyl 3-hydroxy-13-methyltetradecanoate, exclusively associated with L49 at T₂₁, indicates possible unique lipid metabolic routes that could contribute to specific flavour notes or oxidative stability.

In sausages produced with NATPRE, 71 compounds showed a VIP score > 1, and 63 exhibited significant changes in ZnPPEX/NATPRE vs. CTRL+ (Supplementary Table 2; sheet d). Similar discriminant metabolites emerged at T₂₁, such as histamine and oxidised glutathione (Table 3), reinforcing the notion that ZnPPEX strongly influences the metabolomic signature regardless of the antioxidant addition. Interestingly, ZnPPEX/NATPRE samples showed the highest log₂FC values of lysophospholipids (such as LPC 18:1 and LPC 18:2) at T₂₁ compared to

both the nitrite dry-cured control and the other treatments (Table 3). This could be attributed to residual endogenous phospholipase activity, potentially promoting phospholipid hydrolysis during ripening. While NATPRE provides strong antioxidant capacity, its protective effect may be less effective against enzymatic phospholipid degradation, especially in the absence of nitrite, which normally stabilises membrane structures and limits lipolytic activity (Shakil et al., 2022). These findings suggest that further optimisation of ZnPPEX production and stabilisation is needed to control excessive lysophospholipid formation, which can impact flavour, texture and oxidative stability. Notably, several bioactive compounds, such as carnosol (a diterpene lactone), retinoic acid (a retinoid), and rosmic acid (a terpene lactone), were detected at T₂₁ in CTRL-, L33, L49, and ZnPPEX. Their presence confirms the stability and functional contribution of the NATPRE extract in enhancing oxidative stability and possibly flavour, in line with prior findings (Premi et al., 2025). Interestingly, tyramine was higher in the ZnPPEX/NATPRE group (log₂FC = 2.01; although no significant variation was measured for this compound moving from T₀ up to T₂₁) while the other treatments significantly reduced tyramine levels when combined with NATPRE (Table 3). Conversely, CTRL+ was effective in preserving the levels of 3-phenyllactic acid (PLA); PLA is an organic acid produced by lactic acid bacteria with antimicrobial properties, known for inhibiting a wide range of bacteria and fungi (Rajanikar et al., 2021). Again, these findings showed that polyamines were specific biomarkers of the fermentative process to obtain ZnPPEX. Finally, the Venn diagram analysis (Fig. 4)

Table 3

Discriminant VIP compounds at T₂₁ as a function of the different treatments with NATPRE added. The log₂FC values are expressed vs the CTRL+. ns = not significant from Volcano Plot analysis.

VIP PLS-DA (NATPRE)	Chemical class	VIP score	CTRL- (log ₂ FC)	L33 (log ₂ FC)	L49 (log ₂ FC)	ZnPPEX (log ₂ FC)
(9Z)-9-octadecenoic acid	Fatty acids	3.564	-0.49	ns	ns	5.08
2,3-dihydroxypropyl 3-hydroxy-13-methyltetradecanoate	Fatty alcohols	3.003	ns	ns	1.95	3.37
3-phenyllactic acid	Phenylpropanoic acids	1.717	-0.47	-0.54	-0.47	-1.83
Carnosol	Diterpene lactones	2.400	9.64	9.06	10.09	10.01
Histamine	2-arylethylamines	3.272	ns	ns	ns	5.58
Isoleucine	Amino acids	1.064	ns	ns	ns	0.83
Homocysteic acid	Amino acids	1.103	ns	ns	1.29	ns
Proline	Amino acids	1.271	-0.61	-0.66	-0.49	0.68
Tryptophan	Amino acids	1.012	ns	-1.12	ns	ns
LPC 18:1	Lysophospholipids	2.309	ns	ns	ns	2.33
LPC 18:2	Lysophospholipids	1.096	ns	ns	ns	0.82
LysoPC(0:0/18:0)	Lysophospholipids	1.996	ns	-0.66	ns	1.64
Nicotinic acid	Pyridinecarboxylic acids	1.188	ns	ns	-0.85	-1.32
Oxidised Glutathione	Peptides	1.569	ns	ns	ns	-1.34
Phenylalanine	Amino acids	1.170	ns	ns	-0.40	0.96
Retinoic acid	Retinoids	2.224	7.72	7.21	8.33	8.10
Rosmic acid	Terpene lactones	2.090	5.01	4.16	5.58	5.63
Tyramine	Phenethylamines	1.348	-1.77	-2.49	-3.39	2.01
Tyrosine	Amino acids	1.204	ns	ns	ns	-0.85

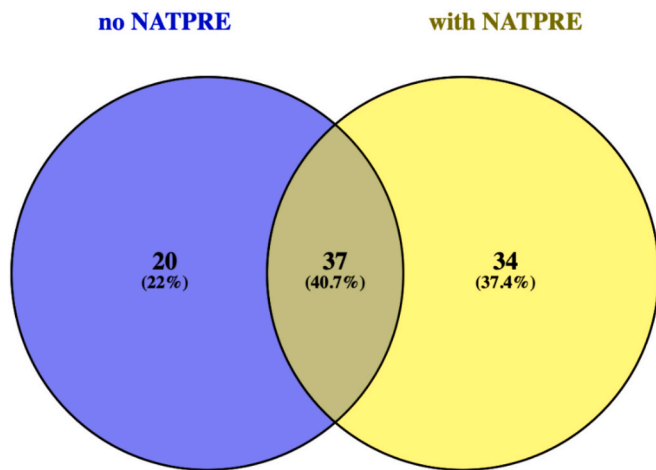


Fig. 4. Venn diagram showing the exclusive and common discriminant meat metabolites at T₂₁, when considering samples manufactured with and without NATPRE.

showed that 40.7% of discriminant metabolites (37 compounds) were shared, while 20 and 34 metabolites were exclusively associated with sausages produced without and with NATPRE, respectively. Taken together, these results highlight the potential of L33 and L49, alone or in combination with NATPRE, to partially replicate the metabolic effects of nitrite curing, while providing a cleaner-label alternative aligned with consumer demand for naturally cured meat products.

3.3. Impact of the different treatments on the lipid oxidation of dry-fermented sausages

Although certain compounds derived from lipid oxidation play a key role in developing the characteristic aroma of dry-cured products (Domínguez et al., 2019b), oxidation reactions in meat foods primarily lead to sensory and nutritional degradation. These complex reactions can occur via different pathways, resulting in a variety of volatile compounds that cause rancid odour and flavour, as well as irreversible changes to the product's colour, texture, and appearance (Purriños, Bermúdez, Franco, Carballo, & Lorenzo, 2011; Sangaré, Bony, Chèné, & Karoui, 2025). This reduces the product's shelf life and influences consumers' acceptance. Malondialdehyde (MDA) is considered to be the main marker of lipid oxidation as it produces rancid aromas even at low amounts. Various studies have determined that the accepted limit for the absence of rancidity in meat and meat products is 2.5 mg MDA/kg (Domínguez et al., 2019a; Zhang et al., 2019). In this study, we therefore measured the MDA content per kilogram of meat by means of the TBARS assay immediately after the model sausages' production (T₀) and after the entire ripening period (T₂₁), in order to evaluate the oxidation state

Table 4

Results from experiments conducted on the TBARS (mg MDA/kg) in various model sausages during ripening. Data are expressed as mean values \pm standard deviation. Superscript letters within each column (T₀ and T₂₁) indicate significant differences as resulting from one-way ANOVA followed by Duncan's post-hoc test (p -value < 0.05).

Treatment	mg MDA /kg T ₀	mg MDA/kg T ₂₁
CTRL-	1.46 \pm 0.06 ^e	2.43 \pm 0.05 ^g
CTRL-/NATPRE	0.35 \pm 0.07 ^{ab}	0.77 \pm 0.05 ^c
CTRL+	0.32 \pm 0.03 ^{ab}	0.53 \pm 0.07 ^a
L33	1.04 \pm 0.15 ^d	2.04 \pm 0.08 ^f
L33/NATPRE	0.48 \pm 0.08 ^{bc}	0.98 \pm 0.00 ^d
L49	1.17 \pm 0.01 ^d	1.66 \pm 0.03 ^e
L49/NATPRE	0.55 \pm 0.05 ^c	0.65 \pm 0.01 ^b
ZnPPEX	1.79 \pm 0.16 ^f	2.06 \pm 0.10 ^f
ZnPPEX/NATPRE	0.28 \pm 0.13 ^a	0.71 \pm 0.06 ^{bc}

of the fermented sausages (Table 4). The evaluation of lipid oxidation was intentionally focused on the initial and final stages of ripening (T₀ and T₂₁), corresponding to the starting material and the fully ripened product. While this approach captures the technologically and sensorially relevant endpoints, future studies specifically designed to investigate oxidation kinetics could benefit from the inclusion of intermediate ripening time points. Interestingly, we found that the combination of 'storage time \times treatment' significantly affected the MDA parameter, recording p -values < 0.001 (not shown).

As a general consideration, the TBARS values of all treatments were relatively higher than those reported in previous studies, but consistent with the values reported by Zhang, Peng, Li, Wu, and Guo (2017). This may be due to the high fat content (15.1%) of the meat batter used to prepare the model sausages. Moreover, it is important to note that the meat used to prepare the model sausages was formulated with 2.5% sodium chloride, typically acting as a pro-oxidant substance accelerating lipid oxidation mainly by disrupting cell membranes, releasing iron ions from biomolecules, and inhibiting the antioxidant enzymes (Decker & Xu, 1998; Mariutti & Bragagnolo, 2017).

As reported in Table 4, the highest initial MDA values were recorded in treatments that did not contain NATPRE or sodium nitrite, i.e. substances with a high antioxidant potential. In fact, the initial MDA contents for CTRL-, L33, L49, and ZnPPEX were 1.46, 1.04, 1.17, and 1.80 mg/kg, respectively. On the other hand, the MDA value at T₀ was equal to 0.32 mg/kg in the CTRL+ treatment, while ranged from 0.28 to 0.55 mg/kg in the samples containing NATPRE. As previously stated by Premi et al. (2025), external analyses performed on NATPRE revealed values of 313 ± 130 mg NO₃⁻/kg and 1030 ± 370 mg NO₂⁻/kg. Therefore, the strong antioxidant effect of this natural ingredient appeared to be due to the combination of phytochemicals (i.e., phenolic compounds), ascorbic acid, and nitrate and nitrite contents. Specifically, phenolics and terpenes, exhibit strong antioxidant capacity as they can act as hydrogen donors and effectively scavenge free radicals (Melios, Grasso, Bolton, & Crofton, 2024), while ascorbic acid acts as a reducing agent, which mainly inhibits myoglobin oxidation. Notably, the MDA values of all treatments increased during the 21-day ripening period. In this regard, the CTRL- treatment achieved the highest final value (equal to 2.43 mg MDA/kg), while the CTRL+ treatment achieved the lowest (equal to 0.53 mg MDA/kg). In fact, nitrite and nitrate indirectly inhibit lipid oxidation by competitively depleting oxygen and binding to the iron ion in haemoglobin, thereby destroying the radical chain reactions caused by nitrite-derived nitric oxide (Zhang et al., 2023). At the end of ripening (T₂₁), sausages fermented with *S. equorum* L33 showed significantly lower TBARS values (2.04 mg MDA/kg) than the untreated control (CTRL-, 2.43 mg MDA/kg), although lipid oxidation remained markedly higher than in the nitrite-cured control (0.53 mg MDA/kg). ZnPPEX-based formulations exhibited similarly elevated TBARS levels (2.06 mg MDA/kg), confirming limited control of lipid oxidation when the extract was used alone. In contrast, the addition of NATPRE consistently reduced lipid oxidation across all formulations, leading to TBARS values below 1 mg MDA/kg at T₂₁ in all NATPRE-containing treatments, including L33/NATPRE (0.98 mg MDA/kg) and ZnPPEX/NATPRE (0.71 mg MDA/kg). Other plant extracts rich in phenolic compounds such as pomegranate extract, green tea, olive, grape and other seed extracts have also been reported to limit the formation of TBARS in dry-fermented sausages (Sirvins, Goupy, Promeyrat, Ginies, & Dufour, 2025). Their industrial application is not straightforward and requires extract standardization and studies evaluating their impact on sensory properties of the commercial product.

Interestingly, sausages fermented with *S. saprophyticus* L49 showed lower lipid oxidation at T₂₁ (1.66 mg MDA/kg) compared to the negative control (2.43 mg MDA/kg). While no enzymatic activities were directly measured in the present study, this behaviour may be tentatively associated with antioxidant-related mechanisms previously reported for CNS strains, such as superoxide dismutase, catalase activity, or the production of antioxidant peptides (Chen et al., 2025).

Particularly, superoxide dismutase activity can eliminate the peroxide radicals involved in rancidity development, producing hydrogen peroxide which is then destroyed by catalase activity (Landeta, Curiel, Carrascosa, Muñoz, & de las Rivas, B., 2013). Notably, catalase is one of the most important antioxidant enzymes as plays an important role in the detoxification of pro-oxidant molecules, thereby limiting the oxidation of lipids and proteins, and preventing rancidity in meat products (Rebecchi, Miragoli, Lopez, Bassi, & Fontana, 2020). Catalase activity is a defining feature of staphylococci: most species and strains have one catalase enzyme, while some have two or three. Strains of *S. saprophyticus* and *S. equorum* have been reported to have two catalases (Mainar, Stavropoulou, & Leroy, F., 2017). Similarly, Chen et al. (2022) isolated 107 Gram-positive CNS strains, primarily belonging to the *S. saprophyticus* species, from Chinese fermented meat products. They found that 58 of these strains exhibited high catalase activity.

3.4. Olfactory analysis for consumers' acceptance and Pearson's correlations

A quantitative descriptive olfactory analysis was conducted on the dry-fermented model sausages at T_{21} , as this method is commonly used in research studies and appeared to be the most suitable for providing easily interpretable information when developing a new meat product (Ruiz-Capillas, Herrero, Pintado, & Delgado-Pando, 2021). The aim of this sensory analysis was to investigate the impact of NOS-positive CNS, ZnPPEx, and NATPRE on olfactory perception. Fig. 5 shows the evaluation results for the dry-fermented sausages with different formulations. Specifically, the olfactory analysis revealed that the L33, L49, and CTRL+ treatments produced very similar results for all eight attributes, with the same level of statistical significance. This suggests that the sensory profiles of the CNS-inoculated treatments resemble those of traditionally cured, dry-fermented sausages. Notably, the panellists did not identify any significant differences in the aromatic profile between nitrite-containing dry-fermented model sausages and sausages produced using selected NOS-positive CNS as innovative starter cultures (Supplementary Table 3). In particular, the typical cured note was perceived as being at the same level for the L33, L49, and CTRL+ treatments. In fact, the mean score for this attribute was 5.57 for the two NOS-positive CNS-inoculated treatments, compared to 5.50 for the CTRL+ treatment

(Supplementary Table 3). Thomas, Mercier, Tournayre, Martin, and Berdagué (2014) stated that the effect of nitrite on cured aroma is primarily a balance between aldehyde and ester compounds, which produce green, fatty, and fruity odour notes, and sulphur and nitrogen compounds, which produce meaty, roasted, and nutty odours. On the other hand, the volatile aroma compounds in the inoculated products are most likely enhanced by microbial metabolism. This includes proteolytic and lipolytic activity, as well as carbohydrate fermentation. Microbial degradation of amino acids is also a source of aroma compounds, such as thiols, straight-chain sulphur compounds, pyrroles and pyrazines (Hospital et al., 2015). The overall values for treatments containing NOS-positive CNS and NATPRE (L33/NATPRE and L49/NATPRE) were also comparable with those for the CTRL+ treatment. Only a slightly higher odour of aromatic herbs was registered, particularly in the L49/NATPRE treatment. Notably, the scores for typical cured meat odour were statistically similar to those of CTRL+. Conversely, the values obtained for the ZnPPEx-containing treatments differed significantly from those of the CTRL+ treatment, particularly with regard to the animal odour, as well as liver/metallic/blood odour (Fig. 5 and Supplementary Table 3). This was probably because the extract was derived from porcine liver, a particularly odorous viscera which clearly impacted the dry-fermented sausages. In these cases, values three times higher than CTRL+ were recorded. High levels of rancidity were also assessed, particularly for ZnPPEx/NATPRE. ZnPPEx-containing treatments also exhibited extremely high off-odour values, primarily in the 'abnormal odours' and 'other odours' categories. Furthermore, the desirable cured odour was significantly weaker than that of the nitrite-containing treatment. In addition, CTRL- was found to have a dry-cured meat odour similar to CTRL+, but with a stronger acetic acid smell than any of the other treatments. Finally, the level of rancid odour in CTRL- and CTRL-/NATPRE was twice that of traditionally cured sausage. Two-way ANOVA performed on sensory data revealed a significant effect of formulation for all evaluated descriptors, whereas no significant Treatment \times Trial interactions were detected, indicating that the sensory differences among treatments were consistent across independent production trials. A significant ($p < 0.05$) effect of trial was observed only for rancid odour, likely reflecting a uniform shift in intensity perception between production days rather than a treatment-dependent effect, as confirmed by the absence of interaction terms.

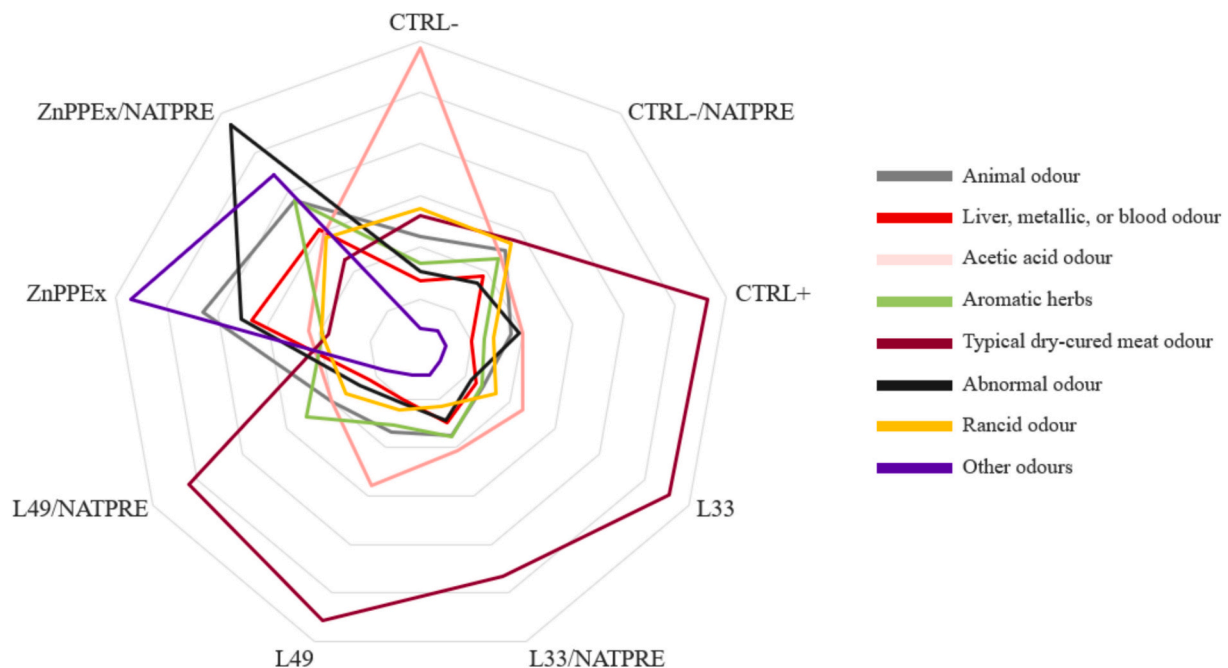


Fig. 5. Olfactory sensory evaluation of the dry-fermented model sausages at T_{21} .

From a mechanistic perspective, the better sensory profile observed in formulations combining NOS-positive CNS with NATPRE likely reflects a complementary interaction between microbial nitric oxide production and antioxidant protection. While NOS activity contributes to the formation and stabilisation of cured-like attributes through NO-mediated reactions (Premi et al., 2024a), the polyphenol-rich extract could limit lipid oxidation and preserves key aroma-active metabolites, thereby preventing the development of rancid and off-odours that could otherwise mask desirable cured notes. This functional complementarity may explain why CNS/NATPRE combinations most closely resembled the nitrite-cured control in terms of olfactory perception, despite the absence of added nitrite.

To further explore the biochemical basis of the observed olfactory perceptions, a Pearson's correlation analysis was performed between sensory attributes and the discriminant metabolites identified by supervised sPLS-DA at T21 (Table 5). Overall, several sensory descriptors showed significant associations with specific metabolites, supporting a clear link between chemical composition and odour perception in dry-fermented sausages. Animal odour and liver/metallic/blood odour were mainly associated with metabolites related to amino acid metabolism and fermentation-derived compounds. In particular, animal odour showed a strong negative correlation with tyrosine ($r = -0.650$; $p < 0.01$), while liver, metallic and blood odour were negatively correlated with phenyllactic acid ($r = -0.630$; $p < 0.01$), a microbial-derived organic acid commonly linked to fermentation processes and antimicrobial activity. These negative correlations suggest that higher levels of these metabolites may contribute to masking or reducing undesirable animal-derived notes. The aromatic herbs descriptor was strongly and positively correlated with rosmic acid ($r = 0.750$; $p < 0.01$), a terpene-derived compound originating from the NATPRE extract, confirming the sensory impact of polyphenol-rich ingredients on herbaceous and aromatic perceptions. Similarly, typical dry-cured meat odour was positively associated with phenyllactic acid ($r = 0.603$; $p < 0.01$), supporting the role of microbial metabolism in shaping desirable cured aroma traits in nitrite-free formulations. Abnormal odour showed the highest number of significant correlations and was strongly associated with (9Z)-9-octadecenoic acid ($r = 0.785$; $p < 0.01$), a lipid-derived compound linked to lipolysis and oxidative processes, in agreement with the higher off-odour perception observed in ZnPPEx-containing treatments. Likewise, the "other odours" descriptor was highly correlated with phenylalanine ($r = 0.885$; $p < 0.01$), suggesting that alterations in amino acid pools and their downstream catabolism may contribute to non-typical or poorly defined odour notes. Interestingly, the increased levels previously mentioned of LPC 18:1 and LPC 18:2 in ZnPPEx-treated samples were positively correlated ($p < 0.01$; supplementary table 2; sheet e) with the sensory perception of abnormal odours, suggesting that

enhanced phospholipid hydrolysis and membrane-derived lipid turnover may contribute to the development of undesirable aroma traits. Overall, these correlations reinforce the sensory results and highlight how untargeted metabolomics can provide mechanistic insight into aroma perception, linking specific metabolic signatures to both desirable and undesirable olfactory attributes in dry-fermented sausages. However, although the present correlation analysis provides valuable insight into the metabolic determinants of odour perception, further studies specifically designed to profile volatile compounds, for instance using headspace GC-MS, are warranted to more directly associate aroma-active molecules with individual sensory descriptors in nitrite-free dry-fermented sausages. Finally, despite its effectiveness in promoting colour development (Premi et al., 2025), the use of ZnPPEx alone resulted in elevated TBARS values (Table 4) and was associated with increased liver-derived and other odours, together with higher levels of selected biogenic amines. Overall, these findings indicate that ZnPPEx-based formulations require further optimisation, particularly in terms of extraction conditions and/or dosage, to minimise oxidative and sensory drawbacks.

4. Conclusions

This work demonstrates that innovative natural curing approaches, including NOS-positive CNS, ZnPP-rich porcine liver extracts, and polyphenol-based antioxidants, can significantly shape the metabolome and oxidative stability of dry-fermented sausages. Multivariate analyses confirmed that both the technological treatment and the ripening time strongly influenced the metabolic profile, whereas their interaction was negligible, highlighting that these factors act independently. Notably, ZnPPEx treatments generated unique metabolomic fingerprints already after preparation, supporting the hypothesis that the obtention process introduces fermentation-like metabolites. However, the presence of biogenic amines and liver-derived off-odours suggests that further refinement, such as optimised extraction protocols and/or dosage adjustment, as well as the inclusion of tailored starter cultures, is needed to ensure food safety and sensory acceptance. Conversely, the combination of *S. equorum* L33 and L49 with NATPRE emerged as very promising strategies, closely mimicking the nitrite-cured control in both chemical and sensory attributes while significantly limiting lipid oxidation. From a practical perspective, this approach aligns with current regulatory trends toward reduced nitrite usage in meat products and responds to consumer demand for clean-label and naturally cured foods, offering a technologically feasible solution for the production of nitrite-free dry-fermented sausages. These findings confirm the potential of starter cultures with NOS activity, coupled with natural antioxidants, as viable alternatives to conventional nitrite curing in dry-fermented meat products. Although the present study relied on an untargeted UHPLC-HRMS approach with level 2 compound annotation, this strategy was deliberately adopted to capture global metabolic shifts associated with alternative curing technologies. Targeted and quantitative LC-MS/MS validation of selected safety- and quality-relevant metabolites, such as biogenic amines and key lipid-derived compounds, represents a logical next step to further refine the technological and safety assessment of nitrite-free formulations.

CRediT authorship contribution statement

Lara Premi: Writing – original draft, Visualization, Investigation, Formal analysis. **Gabriele Rocchetti:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Annalisa Rebecchi:** Supervision. **Ricard Bou:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Anna Jofré:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Table 5

Significant sensory attributes mostly correlated with untargeted metabolomics profiling as resulting from Pearson's correlation analysis (p -value < 0.05). The most significant compound for each sensory attribute is also provided.

Sensory attribute	Significant correlations	Most significant correlation
Animal odour	32	Tyrosine (-0.650 ; $p < 0.01$; VIP score = 1.352)
Liver, metallic, blood odour	20	Phenyllactic acid (-0.630 ; $p < 0.01$; VIP score = 1.290)
Acetic acid odour	1	Sphingomyelin (0.393; $p < 0.05$; VIP score = 2.170)
Aromatic herbs	34	Rosmic acid (0.750; $p < 0.01$; VIP score = 1.787)
Typical dry-cured meat odour	14	Phenyllactic acid (0.603; $p < 0.01$; VIP score = 1.290)
Abnormal odour	57	(9Z)-9-octadecenoic acid (0.785; $p < 0.01$; VIP score = 4.313)
Other odours	28	Phenylalanine (0.885; $p < 0.01$; VIP score = 1.707)

Ethical statement

All sensory judges voluntarily participated in the experiment after demonstrating a comprehensive understanding of the experimental requirements and potential risks. Formal written informed consent was procured from each participant. The study also obtained formal ethical approval (approval number: CCSC 19/2024).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2026.118436>.

Data availability

Data will be made available on request.

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