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Innovative strategies to enhance the red colour of nitrite-free meat products

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Summary

Traditionally, nitrates and nitrites have been used as curing agents in meat products to achieve a stable red colour, prevent lipid oxidation, and improve quality and safety. However, the process of meat curing is currently under scrutiny due to its correlation with the formation of carcinogenic N-nitrosamines, which are associated with an increased risk of developing colorectal cancer. Furthermore, synthetic additives have been linked to other adverse health effects, including cardiovascular and heart diseases. Consequently, permitted quantities are decreasing and legislation is becoming stricter in order to protect consumers, who are paying closer attention to the ingredient lists and reducing their consumption of foods containing artificial substances. This is prompting demand for healthy and high-quality meat products made with natural components.

Therefore, this research project aims at investigating innovative strategies to replace nitrates and nitrites in meat-based foods, with a particular focus on colour enhancement. This is because colour is a key sensory attribute that influences consumer acceptance of meat products. The strategies being investigated involve the use of selected meat-associated coagulase-negative staphylococci (CNS), a porcine liver extract containing zinc protoporphyrin (ZnPP), and a commercially available polyphenol-rich ingredient (NATPRE T-10 CUR HT), applied either individually or in combination. Firstly, certain CNS strains have been selected for their ability to produce the red pigmentation characteristic of traditionally cured meats thanks to their nitric oxide synthase (NOS) activity. The NOS pathway involves the degradation of L-arginine into L-citrulline, and is linked to nitric oxide production. This process can result in the formation of nitrosylmyoglobin, the protein complex that gives meat products their distinctive pinkish-red colour. Thirty-five CNS strains were subjected to genotypic and phenotypic screening to investigate their NOS activity. This involved carrying out a series of polymerase chain reactions with specific primer pairs to ascertain the presence of NOS genes, followed by detecting arginine degradation and citrulline production in both a meat simulation medium and under real meat conditions. The investigation identified the two most promising strains:

Staphylococcus equorum L33 and *Staphylococcus saprophyticus* L49. In fact, they were found to positively affect the colour of nitrite-free fermented minced beef loin after 7 and 10 days of storage, demonstrating its similarity to traditionally nitrite-cured beef. The positive impact of the inoculation on the colour of the meat samples was also confirmed by the analytical levels of arginine and citrulline detected in the meat models. Additionally, the two selected strains were found to impart a cured red colour to nitrite-free dry-fermented pork sausages at the investigated ripening time frames (7, 14, and 21 days). Therefore, regardless of the initial meat matrix or the ripening conditions, *S. equorum* L33 and *S. saprophyticus* L49 proved to have a positive effect on colour, producing results comparable to those achieved using synthetic additives. The NOS activity of the selected bacteria was not found to be the main mechanism responsible for the significant improvement in redness, as low nitrosyl-heme values were detected in the inoculated sausages using high-performance liquid chromatography (HPLC). Other mechanisms, such as catalase activity, reducing power, oxygen consumption and proteolysis, may also contribute to colour enhancement in CNS-containing treatments. The red colour typically associated with nitrite-cured meat products was observed in dry-fermented pork sausages formulated with the ZnPP-rich extract. The intense red colour observed in these sausages suggests that ZnPP inclusion could enhance their colour without the need for nitrite. These results therefore support the use of the ZnPP-extract as a colouring ingredient in meat-based products. The polyphenol-rich ingredient was also found to improve the colour of the meat, although the resulting redness was milder than that achieved using the other two strategies. Combining NATPRE T-10 CUR HT with ZnPP extract or NOS-positive CNS strains did not enhance the colour further, confirming that the brightest redness was obtained by using ZnPP or staphylococci alone.

In addition to colour, the impact of potential alternatives to nitrites on the untargeted metabolome, lipid oxidation and olfactory profile of dry-fermented sausages was investigated to assess consumer acceptability. The analyses demonstrated that the treatments containing NOS-positive CNS not only resembled the typical colour of nitrite-cured products, but also closely replicated their metabolomic and sensory characteristics. In fact, the inoculated pork sausages had a similar composition of

metabolites to that observed in samples treated with nitrite. They also exhibited low lipid oxidation, probably associated with superoxide dismutase and catalase activity, as well as the characteristic dry-cured odour. In contrast, ZnPP-containing treatments differed significantly from traditional cured products and all other formulations, particularly with regard to the presence of biogenic amines, liver-derived off-odours and high levels of lipid oxidation. While ZnPP extract produced a colour similar to that achieved by nitrite curing, it also led to the formation of histamine and other polyamines, which are undesirable from safety and flavour perspectives. This suggests that the porcine liver extract needs to be further refined to ensure food safety and sensory acceptance. Conversely, the polyphenol-rich plant-based ingredient was found to effectively improve oxidative stability and preserve bioactive compounds, thanks to its strong antioxidant capacity. The most promising strategy appeared to be the combination of staphylococcal strains, particularly *S. equorum* L33, with NATPRE T-10 CUR HT, as this closely mimicked the nitrite-cured control in terms of both chemical and sensory attributes, while significantly limiting lipid oxidation.

These findings suggest that the innovative natural curing approaches investigated could mark a turning point in the replacement of nitrates and nitrites in meat products. In fact, while the preparation method for obtaining the ZnPP-rich extract strategy requires careful refinement and optimisation, strategies involving NOS-positive CNS and NATPRE T-10 CUR HT are very promising.

Contents

1. Introduction

1.1 The role of meat and meat products in human nutrition

Meat and meat products are nutrient- and energy-rich foods that play a significant role in human health and diet, and fulfils most of the body's requirements (Stadnik, 2024). In particular, beef and pork are among the top food sources of essential macronutrients, including high-quality and digestible proteins (the most valuable component found in meat), fats, and carbohydrates, as well as several micronutrients like bioavailable iron, zinc, potassium, magnesium, selenium, and phosphorus (McNeill, 2014). In terms of protein content, meat is rich in both essential and non-essential amino acids. Compared to other types of red meat, beef contains higher levels of the essential amino acids valine, lysine, and leucine, while pork is rich in threonine, arginine, and methionine (Geletu et al., 2021). Meat also contains essential unsaturated fatty acids, such as oleic acid, linoleic acid, linolenic acid, and arachidonic acid. These fatty acids are necessary constituents of mitochondria, cell walls, and other active metabolic structures (Wood et al., 2008). Conversely, the micronutrients are crucial for physical and cognitive development, physiological functioning, blood health, and immunity (Beal & Ortenzi, 2022). For example, iron is a key mineral in many neurodevelopmental processes and is essential for oxygen transfer in the circulatory system. Its deficiency can affect children's growth and development (McCann, Perapoch Amadó & Moore, 2020). Zinc plays a vital role in cell growth and replication, bone formation, and strengthening the immune system (Molenda & Kolmas, 2023). Zinc can also regulate anti-inflammatory and antioxidant processes. For this reason, it is used in supplementary doses to treat heart disease and cancer (Santos, Teixeira & Schoenfeld, 2020). Potassium helps metabolism, the transmission of nerve impulses, muscle growth and the maintenance of the body's acid–base balance, while magnesium is important for a number of bodily functions, including maintaining blood pressure, preventing tooth decay and ensuring bone health. In addition, phosphorus provides energy, helps to form phospholipids, and contributes to the development of bones and teeth (Ahmad, Imran & Hussain, 2018). Meat is also a good source of essential B-complex vitamins, including thiamine, riboflavin, niacin, vitamin B6 and vitamin B12. In this regard, thiamine

is involved in several chemical reactions that are necessary for the growth and maintenance of the human body. Furthermore, riboflavin is essential for releasing energy from proteins, fats and carbohydrates, and it also helps with the absorption and utilisation of iron (Suwannasom et al., 2020), while niacin is implicated in a variety of intracellular enzyme systems, including those related to energy production. Moreover, vitamin B6 plays a key role in the functioning of different enzymes that catalyse essential chemical reactions in the human body. It is also involved in synthesising neurotransmitters and heme iron (Santos et al., 2023). Additionally, vitamin B12 is only found in foods of animal origin and plays an important role in DNA synthesis and stability (Halczuk et al., 2023). Red meat contains traces of vitamin A, which is a fat-soluble vitamin necessary for maintaining healthy tissues and normal vision. Meat is also rich in pantothenic acid and biotin, and other bioactive compounds including taurine, carnitine, carnosine, ubiquinone, glutathione, and creatine (Pereira & Vicente, 2013), which are all vital for various metabolic processes, comprising those related to blood and neurological functions.

Despite their positive nutritional aspects, meat and meat products may also contain other nutrients and non-nutritive components, such as sodium, saturated fat, heterocyclic aromatic amines, and polycyclic aromatic hydrocarbons (Stadnik, 2024). Since these compounds have been linked to negative health outcomes, including carcinogenic, mutagenic, and genotoxic effects, specialists advise against frequent and excessive meat consumption. However, they suggest consuming meat in moderation as part of a healthy lifestyle, as it is considered a fundamental component of a balanced diet (Leroy et al., 2023). In fact, moderate red meat consumption in well-varied and complete diets results in positive health benefits. Restricting intake would mean obtaining these essential bioactive molecules from other foods or through fortification or supplementation. While this is theoretically possible, it may be difficult to achieve in practice due to resource limitations or a lack of nutritional awareness (Geiker et al., 2021). In particular, certain critical stages of life, such as pregnancy, breastfeeding, infancy and old age, require nutrient-dense bioavailable foods to fulfil nutritional needs (Beal et al., 2024). For instance, the consumption of red meat products is particularly beneficial for

the elderly suffering from degenerative loss of skeletal muscle mass because of the high biological value protein and bioavailable iron derived from meat (Paddon-Jones & Leidy, 2014).

1.2 The perishability of fresh red meat and the importance of processing

Fresh red meat is highly perishable due to its high nutrient and moisture content, and its neutral pH. These specific physicochemical properties enable spoilage and the growth of undesirable microorganisms (Zhou, Xu & Liu, 2010), thus causing quality degradation and significant meat losses worldwide. In addition to microbial and enzymatic activity, lipid and protein oxidation is also involved in meat deterioration. Without processing, meat deteriorates within a few days, resulting in rancidity and off-flavours, as well as nutritional losses (Nethra et al., 2023). In this context, meat processing plays a crucial role in extending shelf-life and ensuring a sustainable food chain (Djekic & Tomasevic, 2016). Indeed, it increases the availability of meat after slaughtering, preserves the nutritional value of traditional products and helps to withstand global food scarcity, thereby reducing food waste and enhancing microbial safety (Karwowska, Łaba & Szczepański, 2021). Furthermore, the different composition of ingredients, manufacturing methods, and additives used in processed meats provide a wider variety of eating experiences than fresh red meat (De Smet & Van Hecke, 2024). In fact, meat undergoes physical and chemical changes during processing. Physical changes involve modifications to tissue structures, leading to a tenderising effect mainly due to proteolysis and the release of free amino acids. This improves the sensory characteristics of the product, such as its appearance, colour, flavour, texture, aroma, and overall quality (Tangatarovich & Salamat, 2025). Conversely, the addition of food additives or prolonged storage can result in molecular interactions that lead to dehydration (Gómez et al., 2020).

Dry-curing is one of the most important food preservation techniques, involving salting, curing and ripening. Based on factors such as temperature, relative humidity, drying period and water content, it reduces water activity through drying and improves meat quality by adding salt, spices, sugar, ascorbate and curing agents (Mediani et al., 2022). Apart from the concentration of flavour

compounds due to water loss, the quality of dry-cured meat products is also affected by proteolysis and lipolysis, which enhance flavour development and palatability (Flores, 2018). These processes impart beefy, buttery, nutty, and roasted-nut flavours to meat, together with an umami taste (Hwang et al., 2019). Regarding the ingredients used in dry-curing, sodium chloride (NaCl) is one of the most common and plays a multifunctional role in preserving meat. It extends the shelf-life of meat by reducing its water activity and microbial load, while also imparting organoleptic (odour, flavour and texture) and technological (myofibrillar protein extraction, water retention capacity, gel formation and emulsification) qualities to the final product (Orsi et al., 2025). Specifically, the level of salt directly affects the solubility of myosin and actin, which are myofibrillar meat proteins. Once salt has been added, these proteins become soluble, and the viscosity of meat batters increases. This occurs partly because the proteins can interact with each other more easily to form networks, and partly because they can stabilise the interface of meat fats, creating a stable emulsion (Weiss et al., 2010). This process results in the formation of the desired texture in meat products. Additionally, spices, which the Food and Drug Administration (FDA) defines as " *aromatic vegetable substances in whole, broken or ground form that are used for flavouring rather than nutrition and from which no essential flavouring oil has been removed* ", are often used in meat processing thanks to their unique taste, odour and flavour-boosting properties, as well as their antimicrobial, antifungal and antioxidant effects (Mediani et al., 2022). Spices contain volatile oils (mainly terpenes), organic acids, phenolic compounds (such as anthocyanins, tannins, and flavonols) and alkaloids, which interact positively with meat proteins and lipids to produce desirable aromas. Different spices can release different chemical classes, including acids, aldehydes, alcohols, ketones, hydrocarbons, sulphur compounds and terpenes (Domínguez et al., 2019), thus influencing the overall sensory characteristics of the final meat product (Jung et al., 2014). Sugar, which is usually glucose, lactose or saccharose, is sometimes used in the manufacturing of processed meats to balance saltiness and facilitate microbial fermentation by acting as a substrate for starter cultures (González-Fernández et al., 2006). Sugar also plays an important role in lipolysis and lipid oxidation, particularly with regard to the levels of organic

acids, fatty acids and volatile compounds, thus improving flavour formation of dry-cured meat products (Qu et al., 2022). In addition, sodium ascorbate (E301) is commonly used as a curing accelerator, as well as for its antioxidant properties. It inhibits oxidative processes by interacting with iron ions, which are recognised catalysts for the oxidation of lipids in meat products (Karwowska et al., 2022). This delays the process of rancidity and extends the product's shelf life.

Nitrates and nitrites are the most widely used multifunctional synthetic additives in the meat industry for preserving cured meat products (Jo et al., 2020; Shakil et al., 2022). Typical food preservatives used in meat processing include sodium and potassium salts of nitrates (E251 and E252) and nitrites (E249 and E250), respectively (Zhang et al., 2023). These additives are responsible for several properties that make them indispensable for cured meat products. Firstly, they react with myoglobin in muscle tissues to give meat its distinctive pinkish-red colour. Indeed, this colour develops through several complicated reaction steps until the nitrosylmyoglobin complex is formed (Honikel, 2008). Furthermore, thanks to their strong antioxidant activity, nitrates and nitrites delay rancidity and the development of unpleasant flavours by limiting the oxidation of lipids and proteins (Aminzare et al., 2019). The mechanism responsible for both the antioxidant effect and colour development exerted by nitrite is the same, and relates to nitric oxide production. This process involves reactions with heme proteins and metal ions, the binding of free radicals by nitric oxide, and the formation of nitroso- and nitrosyl compounds, which exhibit antioxidant properties (Sindelar & Milkowski, 2011). Notably, nitric oxide can be depleted through self-oxidation, bind to the iron ion in haemoglobin to prevent its oxidation, and terminate radical chain reactions in lipid oxidative processes (Jo et al., 2020). They also inhibit the growth of spoiling and pathogenic microorganisms, particularly *Clostridium botulinum* (Bedale, Sindelar & Milkowski, 2016). Furthermore, the distinctive flavour and aroma of cured meat are influenced by nitrite-dependent factors, including the inhibition of lipid oxidation and the control of rancid flavour compounds, as well as the concurrent development of complex aroma and flavour notes (Ursachi, Perța-Crișan & Munteanu, 2020).

However, the precise chemical identity of the cured flavour is still unknown and is the subject of active research studies.

1.3 Problems related to the use of nitrates and nitrites in meat curing

The discovery of carcinogenic and genotoxic N-nitroso compounds (NOCs) in the early 1950s raised concerns about the safety of using nitrites and nitrates in meat processing, threatening the healthy development of the global meat industry (Zhang et al., 2023). These synthetic substances can act as nitrosating agents, reacting with various meat components, including amino acid residues of tryptophan, histidine, arginine, tyrosine and cysteine; proteins; sulfhydryl and phenolic compounds; secondary, tertiary and quaternary amines; amides; ascorbic acid; and myoglobin to form N-nitrosamines, which can have carcinogenic and teratogenic effects on the human body (Ayaseh et al., 2022; Shakil et al., 2022). N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR) and N-nitrosomorpholine (NMOR) are the volatile N-nitrosamines most frequently reported in meat products. Among them, NDMA and NDEA are considered the most potent in terms of carcinogenicity and genotoxicity (Flores & Toldrá, 2021).

In 2007, the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) conducted a review of sufficient epidemiological data, concluding that consuming red or processed meat likely increases the risk of developing colorectal cancer. Furthermore, researchers found that nitrite is ten times more toxic and reactive than nitrate, particularly in acidic environments such as the stomach. The fatal oral dosage for humans is 80–800 mg of nitrate per kg of body weight, compared to just 33–250 mg of nitrite (Shakil et al., 2022). Long-term consumption of increasing quantities of red meat, particularly processed meat, has been associated with an increased mortality rate, as well as an increased risk of colorectal cancer (Battaglia Richi et al., 2015). Given the cancer risk associated with these products, the International Agency for Research on Cancer (IARC) classified all types of mammalian muscle meat, including beef and pork as probably carcinogenic (Group 2A). Processed meat, which is treated using methods such as curing, salting and

fermentation to enhance flavour or extend shelf-life, has been classified as carcinogenic to humans (Group 1) (IARC, 2008). Consequently, European Union Regulation 2023/2108 introduced significantly lower permitted levels for the use of nitrites and nitrates in animal-derived products from 9 October 2025. For example, the permitted amount of nitrite in standard meat products has decreased from 150 mg/kg to 80 mg/kg of nitrite ions (equivalent to approximately 120 mg/kg of nitrite). By contrast, the maximum permitted level in traditionally cured meats varies by product, ranging from 30 to 150 mg/kg of nitrite/nitrate ions. Some specialists have even begun recommending a reduction in meat consumption. However, reducing the intake of meat and meat products alone may not significantly mitigate the carcinogenic effects. Furthermore, as previously described, this approach can have several disadvantages, including a loss of nutritional value.

Additionally, nowadays, consumers are much more interested in finding out how food products are made and which ingredients they contain. Consequently, people are paying particular attention to ingredient lists and reducing their consumption of foods containing artificial or chemically sounding substances (Fernandes et al., 2024). In fact, consumers prefer high-quality meat products made with minimal and natural components because they are perceived as healthier and more environmentally friendly (Roobab et al., 2021). Therefore, health and sustainability trends have prompted consumers to embrace the ‘clean label’ movement (Tomasevic et al., 2018), favouring food products with simple and easily understandable ingredient lists, featuring natural and recognisable components, thus minimising artificial additives (Asioli et al., 2017). This phenomenon is having a significant impact on the meat industry, triggering a renewed interest in healthier and more sustainable alternatives to traditional cured products (Aschemann-Witzel, Varela, & Peschel, 2019). Accordingly, meat manufacturers should develop new ingredients and formulations to meet this demand. While this presents challenges for meat processors, it also creates opportunities for innovation and the development of new clean-label products (Inguglia et al., 2023). In particular, the industry is seeking natural preservatives to enhance the colour of their processed products. This is because colour is the most important quality factor influencing consumers' decisions about which meat products to

purchase (Jin et al., 2018; Tomasevic et al., 2021). Currently, replacing the various functions of nitrite and nitrate in meat and meat products with other natural substances appears difficult and challenging. However, scientific research is moving in this direction, with innovative natural alternatives to synthetic additives being investigated.

1.4 Research framework aimed at improving the red colour of nitrite-free meat products

The discovery of carcinogenic and genotoxic nitrosamines originating from nitrites in processed meats had a serious negative impact on the industry. Furthermore, the 'clean label' movement has gained widespread acceptance, with fewer ingredients becoming the norm in food processing. Consequently, meat manufacturers and researchers have recently been actively exploring nitrate and nitrite replacers, primarily to improve colour and offer healthier and more natural options. In order to reduce the risk of nitrosamine formation and mitigate potential human health hazards, innovative meat curing methods are being explored.

1.4.1 Nitrate-rich plant-based extracts as an alternative to synthetic nitrite

Plant extracts and ingredients are the most extensively researched and promising alternative to synthetic additives in meat products (Ferysiuk & Wojciak, 2020). Leafy green vegetables, for example, are a key natural source of nitrates and contain lower levels of nitrites (Ranasinghe & Marapana, 2018). Celery, lettuce, and spinach have been found to contain over 2,000 mg of nitrate per kg. Therefore, plant extracts offer the greatest potential for providing processed meats with natural sources of nitrate without the need for the direct addition of synthetic sodium nitrite (Sebranek & Bacus, 2007). Once added to meat, nitrates can be converted into nitrites by nitrate-reducing bacterial cultures, providing the classic benefits of nitrites, such as colour, flavour, antioxidant and antimicrobial properties. Furthermore, plants, fruits, herbs, and spices contain various bioactive compounds, particularly phenolic compounds, which have excellent free radical scavenging properties and are beneficial to human health (Beya et al., 2021). The main phenolic compounds found in plant extracts include phenolic acids (e.g. rosmarinic acid, caffeic acid, and gallic acid),

phenolic diterpenes (e.g. carnosol and carnosic acid), flavonoids (e.g. quercetin, catechin, apigenin, naringenin, kaempferol, and hesperetin), and volatile oils (e.g. carvacrol, thymol, eugenol, and menthol) (Brewer, 2011). These substances are characterised by their ability to inhibit or prevent the growth of spoilage and pathogenic microorganisms (García-Díez et al., 2016), as well as slowing down oxidative reactions within the meat matrix. Therefore, nitrite-free meat products can be coloured using commercially available vegetable juices and powders, which also reduce carcinogenic substances and increase levels of bioactive compounds, thereby ensuring consumer welfare.

Various meat products have been made using plant extracts from different parts of plants that undergo different pre-treatments, such as powdering, juicing, and infusing, in varying proportions. Celery (*Apium graveolens*) products, such as celery juice concentrate and celery powder, are the most commonly used ingredients containing natural nitrates in studies involving cured meat foods (Usinger et al., 2016). In fact, celery juice and powder appear to be highly compatible with processed meats due to their low vegetable pigment content and mild flavour profile. Horsch et al. (2014) evaluated the effect of celery juice concentrate on the colour of cooked pork ham, comparing it with the effect of conventional nitrite treatment. Overall, similar redness values were observed for the celery concentrate and the conventional treatment at equal nitrite concentrations. Indeed, the 100 mg/kg celery juice treatment produced a comparable redness to the 100 mg/kg conventional sodium nitrite treatment throughout the storage period. Ham treated with 200 mg/kg of celery juice also exhibited similar redness to ham treated with 200 mg/kg of conventional sodium nitrite. In addition, Jin et al. (2018) investigated the effect of using celery powder as a natural curing agent on the quality of pork sausages during cold storage. They found that sausages containing 0.8% celery powder exhibited better colour coordinates than control sausages containing 0.01% sodium nitrite and did not affect the sensory characteristics of the final products negatively. The researchers supposed that the improved redness was probably due to the nitrates and other pigments (including betalains) found in celery. Moreover, Kim et al. (2017) investigated the impact of fermented spinach (*Spinacia oleracea*) extract on the colour development of cured pork. They found that the redness values of the meat increased

proportionally to the amount of extract added, concluding that nitrite sources from fermented spinach extract have significant potential for use in meat products. Furthermore, research studies have focused on beetroot (*Beta vulgaris*) because of its high nitrate content and its abundance of betaxanthins, betacyanins, and other bioactive phytochemicals, including phenolic compounds (Domínguez et al., 2020). Sucu and Turp (2018), for example, investigated the effect of replacing nitrite with 0.35% beetroot powder on the colour development of Turkish dry-fermented beef sausages over 84 days of storage at 4 °C. They observed a significant increase in colour parameter values and concluded that including beetroot enhanced redness and protected the desired red colour during storage. In contrast, Ozaki et al. (2021) examined the use of radish (*Raphanus sativus*) powder as a nitrite substitute in dry-fermented sausages, evaluating its impact on cured characteristics during ripening. The researchers developed three treatments: a control containing 150 mg/kg of sodium nitrite and sodium nitrate; a control containing neither sodium nitrite nor sodium nitrate; and a control containing either 0.5% or 1% radish powder. They found that sausages containing radish powder did not exhibit any colour differences during processing (at 14 and 35 days) compared to the control treatment containing synthetic additives. This appeared to be due to the formation of cured pigments, as well as the drying process. In addition, Hernández et al. (2021) evaluated the colour of cooked hams containing 90% meat, 1.5% salt, 0.5% phosphate, 8% water, and varying concentrations of sodium nitrite (0, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 80, 100, 130, or 150 mg/kg). The researchers also investigated the colour of hams containing NATPRE T-10 HT S, a polyphenol-rich extract, at concentrations of 5, 10, or 20 g/kg. Redness values increased with added nitrite concentrations up to approximately 80 mg/kg; values remained practically the same at higher concentrations. Conversely, the red colour remained constant for the uncured ham samples treated with NATPRE T-10 HT S, indicating that hams containing nitrite and those containing the polyphenol-rich ingredient exhibit similar red pigmentation.

However, despite the positive results of research studies, it should be noted that there is a global need for uniform regulations to facilitate the use of plant extracts as natural ingredients in meat

production. For instance, manufacturing meat products with nitrate derived from vegetable extracts has posed complex labelling issues in the USA. From a regulatory perspective, the United States Department of Agriculture (USDA) has classified plant-derived nitrates as either antimicrobial or flavouring/seasoning agents, rather than curing agents (Rivera, Bunning & Martin, 2019). Consequently, products formulated with plant extracts instead of curing agents must be labelled "uncured". In fact, the term "cured" actually refers to the process of using nitrite and/or nitrate with salt and other ingredients to improve meat preservation (Honikel, 2008). To avoid false or misleading labelling, products can also be labelled as "*No nitrates or nitrites added, except for those naturally occurring in [name of natural source of nitrite]*" (Flores & Toldrá, 2021). The European Union has also published two opinions from the Standing Committee on the Food Chain and Animal Health (SCFCAH) concluding that the use of vegetable extracts in meat products constitutes the deliberate use of a food additive. Additionally, the Standing Committee on Plants, Animals, Food and Feed (ScoPAFF) has confirmed that plant extracts used for technological purposes in food processing continue to be classified as additives. In any case, although vegetable extracts must be listed on the label, their composition does not need to be disclosed. This is of great interest to the industry, since the name of the chemical additive does not have to be specified (Bernardo et al., 2021).

1.4.2 Zinc protoporphyrin as a potential natural alternative to nitrite

Another interesting area of research into colour development in nitrite-free meat products concerns the inclusion of zinc protoporphyrin (ZnPP). ZnPP is a naturally occurring red metalloporphyrin that is structurally similar to heme, except that it incorporates zinc into the porphyrin ring instead of iron (Wakamatsu, 2022; Yoo, Bae & Jeong, 2025). ZnPP was first discovered in Parma ham, a traditional Italian dry-cured ham renowned for its distinctive red colour achieved without the addition of nitrites or nitrates (Wakamatsu et al., 2020). ZnPP is actually considered to be the primary colour-forming pigment in Parma ham rather than nitrosylheme. Therefore, it can act as a natural colouring agent, giving meat-derived foods their stable and bright red colour. Due to its ability to positively influence

colouration independently of the addition of synthetic additives, numerous researchers have tried to elucidate the complex biochemical processes involved in ZnPP formation in meat products (Wakamatsu et al., 2004). Three different ZnPP formation mechanisms have been identified. The first mechanism involves a non-enzymatic reaction in which, under anaerobic conditions, the zinc ion (Zn^{2+}) replaces the ferrous ion (Fe^{2+}) in heme. This reaction is inhibited by oxygen, nitrite/nitric oxide, or heating. The second mechanism involves enzymatic reactions initiated by an endogenous enzyme called ferrochelatase (FECH). This enzyme is located in the inner mitochondrial membrane of mammalian cells and exhibits high activity in environments characterised by low oxygen levels (Bou et al., 2020). Such conditions are often found in dry-cured meat products. Indeed, the curing process itself creates a low-oxygen environment, thereby reducing iron bioavailability and enabling zinc incorporation (De Maere et al., 2018). The third mechanism involves bacterial enzymatic reactions that degrade meat proteins and heme, followed by metal exchange (Becker et al., 2012; Yang et al., 2024). Lactic acid bacteria (LAB) are considered the main contributors to ZnPP synthesis in various meat matrices (Wakamatsu et al., 2020; Wu et al., 2023). In fact, LAB strains exhibit optimal ZnPP-forming activity in mildly acidic conditions (pH values of around 5.5–6.5) and anaerobic environments, both of which are typical of fermented meat products (Wakamatsu et al., 2020). However, ZnPP formation appears to be the result of a combination of enzymatic, non-enzymatic, and bacterial processes rather than a single mechanism, suggesting a synergistic interaction (Zhai et al., 2023). The contribution of each of these mechanisms varies depending on the meat matrix and environmental factors. Therefore, the processing conditions must be considered when studying and optimising ZnPP formation (Yoo, Bae & Jeong, 2025).

Despite the colour-forming potential of the process being so promising, to date, only a small number of studies have focused on the formation of ZnPP during the production of nitrite-free meat products. De Maere et al. (2016), for instance, investigated the colour of sausages produced without synthetic preservatives. Specifically, they evaluated the formation of the naturally occurring pigment ZnPP in nitrite-free dry-fermented sausages produced at various pH levels. They found that ZnPP

only formed in sausages at pH values above 4.9, with its concentration increasing dramatically during the latter stages of production (up to day 177), confirming that pH and time are crucial factors in its formation. Interestingly, a statistically significant correlation was also found between ZnPP generation and product redness. Other researchers have stated that pH is critical for FECH activity in ZnPP formation. In this regard, Wakamatsu et al. (2019) revealed that the optimal pH for ZnPP development varies by organ: 5.0–5.5 for the porcine heart, 4.5 for the porcine liver and 5.5–6.0 for the porcine kidney. Variations in ZnPP formation have also been observed in different types of meat. For example, the process appears to be slower in pork than in horse meat or porcine liver (De Maere et al., 2018). Consequently, producing ZnPP using porcine liver could be a significant step forward in obtaining a potential food colouring ingredient for the development of nitrite-free meat products (Llauger et al., 2023). In this regard, Llauger et al. (2024) demonstrated that ZnPP-rich pork liver homogenates can effectively preserve the colour of nitrite-free liver pâtés, even when subjected to thermal processing. This makes ZnPP an ideal solution for a variety of product applications and clean-label trends, as it aligns with consumer preference for minimally processed products. Therefore, ZnPP derived from animal by-products appears to be a promising natural alternative to synthetic additives, potentially revolutionising the colouring of meat products while eliminating the risk associated with carcinogenic N-nitrosamines (Llauger et al., 2024; Yoo, Bae & Jeong, 2025).

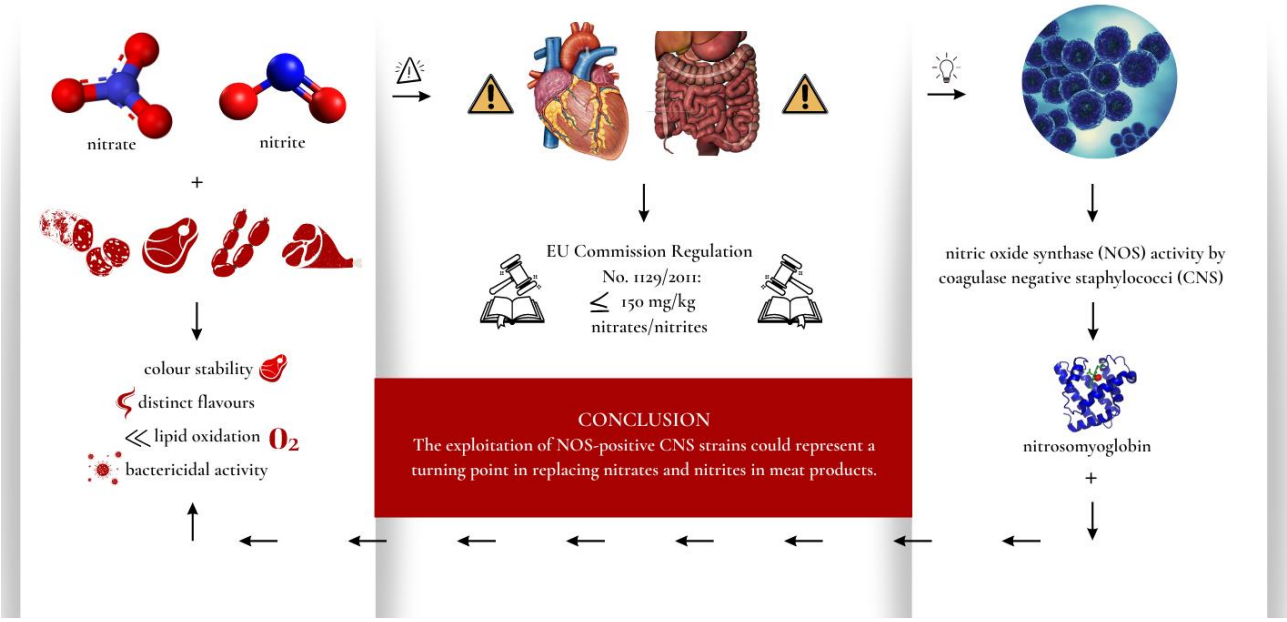
Researchers have also started investigating ways to improve the efficiency of ZnPP production technology. Particularly, ultrasound-assisted extraction methods have been shown to increase FECH activity by up to 33%, reduce processing times by 50% and rise ZnPP yields (Abril et al., 2021). Additionally, drying temperatures below 20 °C have been shown to preserve enzymatic activity, thus ensuring consistent ZnPP yields and minimising degradation during processing (Abril et al., 2022). Such technological innovations enable the large-scale production of ZnPP and stable meat colouration. However, these methods require significant investment in specialised equipment and precise environmental control, limiting their scalability and cost-effectiveness in the meat industry (Yoo, Bae & Jeong, 2025). In fact, despite valuable research on this topic, practical challenges still

persist, including optimising processing conditions, regulating pH, controlling oxygen exposure and ensuring cost-effectiveness. The industrial application and scalability of ZnPP should be explored further. Addressing these challenges is crucial for the successful industrialisation of ZnPP and the development of standardised practices, thus realising its potential as a natural meat colourant (Yoo, Bae & Jeong, 2025). However, a significant criticism of the research is the variability of ZnPP formation in different types of muscle and meat, which complicates the standardisation of meat processing. Moreover, ZnPP has not yet been approved by the FDA as a food colourant, primarily due to a lack of clear safety specifications and regulatory frameworks governing natural compounds (Simon et al., 2017). In fact, unlike synthetic colourants, natural ones are not subject to the same certification process (Simon et al., 2017). This regulatory gap poses significant concerns regarding the approval process. Furthermore, the sensory properties of ZnPP in finished products should be carefully investigated. While a few studies have been conducted on the inclusion of ZnPP in meat products, none have focused on the impact of this ingredient on the final product.

Therefore, future research should focus on understanding the ZnPP biochemical formation mechanisms, optimising cost-effective production methods, and resolving regulatory issues. The effect of ZnPP on the flavour, aroma and taste of treated meat should also be considered. Overcoming these barriers could establish ZnPP as a transformative solution for nitrite-free meat production, providing benefits for both the meat industry and consumers. While ZnPP has been shown to contribute to colour enhancement and potentially influence oxidative stability in meat products, its antioxidant effects remain unclear and require further investigation (Wakamatsu et al., 2020). Furthermore, the long-term health consequences of ZnPP have yet to be studied, necessitating targeted investigation to assess its safety and potential benefits.

1.4.3 <u>Chapter 1</u>: Replacement of nitrates and nitrites in meat-derived foods through the utilization of coagulase-negative staphylococci: A review

Graphical abstract



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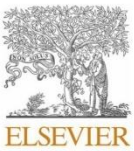

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Review Article

Replacement of nitrates and nitrites in meat-derived foods through the utilization of coagulase-negative staphylococci: A review

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ABSTRACT

Nitrates and nitrites, which are synthetic additives, are traditionally used as curing agents in meat-based products. These synthetic additives are employed in the preparation of fermented meat foods to improve quality characteristics and microbiological safety, develop distinct flavours and red-colour stability, and counteract lipid oxidation. Nitrites also display significant bacteriostatic and bactericidal action against spoilage microorganisms and foodborne pathogens (such as *Clostridium botulinum* and *Listeria monocytogenes*). However, meat curing is currently under scrutiny because of its links to cardiovascular diseases and colorectal cancer. Based on the current literature, this review provides recent scientific evidence on the potential utilisation of coagulase-negative staphylococci (CNS) as nitrate and nitrite substitutes in meat-based foods. Indeed, CNS are reported to reproduce the characteristic red pigmentation and maintain the typical high-quality traits of cured-meats, thanks to their arginine degradation pathway, thus providing the nitrite-related desirable attributes in cured meat. The alternative strategy, still based on the NOS pathway, consisting of supplementing meat with arginine to release nitric oxide (NO) and obtain a meat characterised by the desired pinkish-red colour, is also reviewed. Exploiting NOS-positive CNS strains seems particularly challenging because of CNS technological adaptation and the oxygen dependency of the NOS reaction; however, this exploitation could represent a turning point in replacing nitrates and nitrites in meat foods.

1. Introduction and background

Nitrates and nitrites are typically used as curing salts in meat-derived foods because they contribute to a tangy flavour, inhibit pathogenic microorganisms, and yield nitrosomyoglobin – the advisable and characteristic pinkish-red colour of meat and meat products (Cardinali et al., 2018; Janssens et al., 2013).

Meat colour is greatly influenced by the level of oxidation of myoglobin, an iron- and oxygen-linking protein-containing heme (Li et al., 2016). Myoglobin is a water-soluble protein composed of eight α-helices linked by short nonhelical segments. Histidine-93 is considered the most important myoglobin residue because it gets an essential character in structure and activity. Myoglobin also encloses the heme group, a tetrapyrrole prosthetic group situated within a protein's hydrophobic capsule (Mancini and Hunt, 2005). The heme group coordinates a divalent iron atom that is able to create six bonds. The tetrapyrrole nitrogen atoms act as tetradentate ligands, an additional axial ligand connects to the proximal histidine-93, while the second axial location is free for reversibly tie up other ligands. A distal histidine (His-64) imidazole group does not coordinate iron and can interact with diatomic gases. Meat colour mainly depends on the present ligand and the valence of iron (De Maere et al., 2016).

Myoglobin can exist in three different forms: deoxymyoglobin, oxymyoglobin, and metmyoglobin (Fig. 1). Deoxymyoglobin is characterised by the absence of a ligand at the sixth coordination area and a heme iron in the ferrous state (Fe^{2+}), which gives meat purplish-red or purplish-pink colour. Oxymyoglobin, the second myoglobin form, results from exposure to oxygen and induces the formation of a bright red colour. Molecular oxygen is placed in the sixth coordination zone, with iron being a divalent atom. On the other hand, metmyoglobin derives from the oxidation of ferrous myoglobin to ferric iron (Fe^{3+}) and gives meat a brown colour (Mancini and Hunt, 2005). As shown in Fig. 2, another form of myoglobin can be generated when nitric oxide (NO), a volatile compound produced by nitrate degradation, links to the heme iron contained in the myoglobin molecule (Elroy et al., 2015; Hammes, 2012) resulting in the production of the typical pinkish-red colour of

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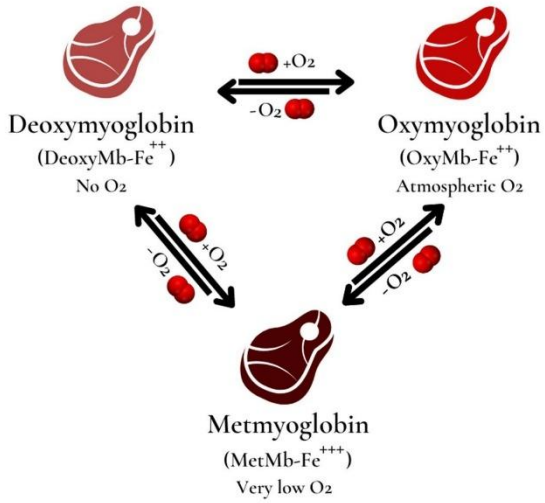

Fig. 1. Deoxymyoglobin, oxymyoglobin, and metmyoglobin: the three myoglobin states, existing in dynamic equilibrium, responsible for the fresh meat colour. Adapted from Hawthorne et al. (2020). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cured meat (Higuero et al., 2020; Ras et al., 2018b).

Common meat curing practices involve the addition of nitrates/nitrites, salt, sugars, reducing substances, spices, and phosphates. In this framework, synthetic agents bear the main responsibility for the development of the distinct flavour of cured meat foods: nitrites interact with the lipid and protein fractions, thus producing different volatile and non-volatile compounds that provide flavour enhancement (Sebranek and Bacus, 2007). Nitrites also bind to particular amino acids residues containing sulphur in meat proteins, resulting in the formation of reduced sulfhydryl residues characterised by a distinct flavour (Hammes, 2012). Moreover, nitrates may impact the activity of microorganisms and endogenous proteases and lipases, thus indirectly affecting the development of fermented meat aroma (Zhang et al., 2023).

Nitrites also exhibit an antioxidant ability, counteracting the cleavage of unsaturated fatty acids and the production of secondary oxidation flavour substances. The antioxidant activity of synthetic additives has been linked to oxygen depletion via self-oxidation to nitrogen dioxide (NO_2) in the existence of oxygen (Honikel, 2008; Jo et al., 2020). Moreover, synthetic agents can stabilise the heme-bound iron, thus inhibiting the release of free iron ions and reducing lipid oxidation (Andrée et al., 2010). Consequently, cell membranes are preserved from lipid peroxidation, resulting in the shelf-life extension due to the increased stability of fermented meat products during conservation (Shen et al., 2023).

Besides their colour, flavour, and antioxidant effects, nitrites are also added as supplements to meat products because of their antibacterial properties against pathogenic bacteria such as *Clostridium botulinum* and *Listeria monocytogenes* (Armenteros et al., 2012; Hospital et al., 2015; Majou and Christieans, 2018). Nitrite exhibit a more robust antimicrobial effect towards Gram-positive rather than Gram-negative bacteria (Pichner et al., 2006), and this inhibition depends on a spectrum of active intermediate compounds such as NO, NO_2, dinitrogen trioxide (N_2O_3), peroxynitrite ($ONOO^-$), and S-nitrosothiol (RSNO) (Gaupp et al., 2012; Rivera et al., 2019). These intermediates act via N-nitrosylation, disulfide formation, S-nitrosylation, and lipid peroxidation, altering enzyme, peptides, cell wall, and membrane structures in sensitive bacteria (Sánchez Mainar et al., 2017b; Shen et al., 2023). Heat, pH, water activity (a_w), salts, redox potential (Eh), and different curing agents can enhance the antimicrobial action of nitrites in meat-derived foods.

Despite the already mentioned great technological and non-technological benefits brought by synthetic curing agents, limiting the addition of nitrites and nitrates to meat and fermented meat foods is crucial since they appear to be harmful for human health. Moreover, the consumer demand for antioxidants and natural flavours is increasingly marked. In fact, nowadays there is a rising consciousness of artificial ingredients and an increasing attractiveness towards more natural and sustainably produced foods. The meat industry has been influenced by this new consumer trend, intended as negative attitudes and feelings associated with consuming processed meat products, mainly because of the presence of synthetic additives (Inguglia et al., 2023). Investigating new strategies and addressing clean label trends in meat processing seem to be necessary for cured-meat manufacturers.

Based on the background concerning the advantages and concerns of nitrates and nitrites usage in cured meats, this review is structured to explore potential novel natural alternatives. The review article provides a comprehensive description of the technological role of coagulase-negative staphylococci (CNS) in fermented meats in terms of colour enhancement (by acting on nitrate reductase and NOS activities), flavour generation, and bioprotection.

1.1. Literature search

A literature search was conducted on PubMed, MEDLINE, Scopus, Web of Science, Science Direct, Food Science and Technology Abstracts, Springer, Nature, Wiley, and MDPI databases. The keywords used for the literature search were 'synthetic curing agents', 'cured meat products', 'coagulase-negative staphylococci', 'nitric oxide', 'nitric oxide synthase', 'L-arginine', 'nitrate reductase', 'meat extender', and 'natural alternatives'. Articles published between 2002 and 2023 were selected for the current review.

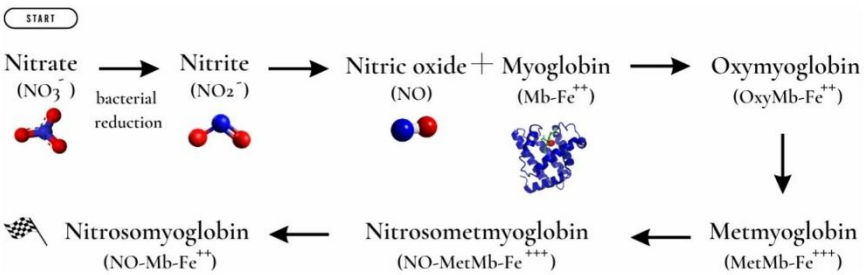

Fig. 2. Nitrosomyoglobin formation in meat products after nitrates addition. Adapted from Hammes (2012).

2. Negative health effects of nitrates and nitrites and the ensuing need to look for alternative solutions

The concern about the possible harmful role of nitrites and nitrates was raised in the 1960s. Since then, there has been an argument on the safety of meat foods. More recently, different studies have confirmed that these agents can cause acute and long-term reactions on the health of human beings; for instance, they can lead to methemoglobinemia and cancer (Majou and Christieans, 2018; Govari and Pexara, 2018). In fact, the ingestion of synthetic curing agents might increase exogenous exposure to potential carcinogenic drivers such as nitrosamines, other N-nitroso compounds (NOCs), and their precursors. As a consequence, the EU Commission (Regulation No. 1129/2011) has set a legal limit of 150 mg/kg for the utilisation of nitrates (E251 and E252) and nitrites (E249 and E250) in the manufacturing of cured meats.

Nitrates are inactive and relatively non-toxic compounds; however, their reduced forms like nitrites (which are active curing substances), NO, and NOCs can cause negative health effects (Milešević et al., 2022). Nitrates are converted to nitrites mainly through the nitrate reductase activity present in both natural meat microflora and added starter cultures (mainly staphylococci and micrococci) (Sun et al., 2019). The toxicity of nitrites is considered to be almost 10 times larger than that of nitrates. Honikel (2008) stated that the fatal oral dose for humans is approximately 80–800 mg of nitrates per kg of body weight, and 30–250 mg of nitrites per kg of body weight.

The long-term toxicity of chemically produced curing additives is related to their capability to create carcinogenic NOCs in the food source and, consequently, in the human organism (Choi et al., 2017). NOCs form when nitrosating factors derived from nitrite degradation interact with amide compounds. There are two main classes of NOCs: N-nitrosamines (NA) and nitrosoamide-like substances (i.e., N-nitrosoureas, N-nitrosoguanidines, and N-nitrosocarbamates) (Hammes, 2012). Govari and Pexara (2018) reported that NA is an etiological factor in several types of cancer in humans.

Besides the high toxicity of nitrates and nitrites recognised by authorities, the awareness that today's consumers have about food ingredients, food manufacturing, and non-communicable diseases (mainly diabetes, obesity, and cancer) should be considered (Riazi et al., 2016). This awareness is continuously leading people to eat healthy food products, thus reducing the risk of certain disorders (Asioli et al., 2017; Leroy et al., 2015). Meat and meat-related products consumption is often perceived unhealthy because of the large content of lipids, cholesterol, synthetic additives, and antimicrobials, which are potentially linked to various degenerative syndromes (Leroy et al., 2015). Nowadays, consumers pay significant attention to the term 'clean label', which appeared for the first time during the 1980s and dramatically exploded 10 years ago. The term refers to minimally processed ingredients, easy-to-understand ingredient lists, and E-number reduction.

Ensuing the collocation of nitrates- and nitrites-cured meat in the classification of Group 1 carcinogens drawn up by IARC and considering new consumer needs, several researchers have attempted to find nitrate and nitrite substitutes. Vitamins, spices, and herbal extracts have all been considered because of their antioxidant properties, mainly due to their high levels of essential oils and phenolic compounds (Efenberger-Szmechtyk et al., 2021). The antioxidant capacity of polyphenols is associated with their free-radical-scavenging activity, their role as reducing agents, their potential chelation of pro-oxidant metals, and their quenching of singlet oxygen. Betalains, carotenoids, and anthocyanins are present in herbal extracts and exert antioxidant effects (Awad et al., 2022).

Shah et al. (2014) reported that rosemary extract is characterised by a strong capacity to inhibit lipid oxidation in cooked pork meat. Besides, Šojić et al. (2018) investigated sage's antioxidant and antimicrobial effects, concluding that sage could be successfully used in the formulation of fresh pork sausages. The antioxidant and antimicrobial activity of cinnamon extract has also been confirmed (Awad et al., 2022). Moreover, Shan et al. (2009) demonstrated that clove was the most effective plant extract in retarding lipid oxidation in raw pork. In addition, Wang et al. (2021) substituted synthetic agents with rose extract in the preparation of dried cured sausages and found that the sausage quality was significantly better than that of a sausage with 150 mg/kg of nitrite. Furthermore, Ozaki et al. (2021) added oregano essential oil and radish powder to cooked pork and beef sausages and proved that this blend could enhance their colour while inhibiting the growth of mesophilic bacteria.

Phytic acid, a 'generally recognised as safe' natural and biodegradable molecule generally extracted from oilseeds, cereals, nuts, and legumes could also be employed in the food industry as a natural substitute for nitrates and nitrites. Boukhris et al. (2020) investigated the antibacterial potential of phytic acid against the proliferation of three foodborne bacterial pathogens: *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* Typhimurium. Phytate proved to be particularly effective against Gram-negative bacteria, demonstrating strongly inhibitory efficacy on the growth of *S.* Typhimurium less than 1 h after treatment. The antimicrobial effect of phytic acid is attributed to the mechanism of cell membrane damage (Bloot et al., 2022). Phytic acid sequesters polyvalent cations that command molecular interactions in the outer membranes of target microorganisms, permeating them. This permeation causes fast ion entrance, determining an increased osmotic pressure and, consequently, lysis. Furthermore, due to its iron-binding capacity originating from an entirely inactive chelate, phytic acid has a strong antioxidant function that can limit hydroxyl radical (•OH) production due to iron catalytic activity (Bloot et al., 2021). Besides its antioxidant and antimicrobial effects, phytic acid also possesses anti-nutritional properties because of its ability to bind to proteins and minerals (Feizollahi et al., 2021). This phenomenon can potentially limit the bioavailability of these dietary nutrients, thus promoting mineral and protein deficiencies. Therefore, this trait should be considered before using phytic acid as a nitrate/nitrite replacer in meat products.

In order to carter to consumers' preference for natural food additives, another industrial cured meat production strategy could be the inoculation of selected starter cultures (especially CNS) into the meat matrix to standardise the manufacturing operation and to reduce the variability of the final desired product (Alfaia et al., 2018; Jeong et al., 2016; Sánchez Mainar et al., 2017a). CNS are a group of Gram-positive cocci characterised by a lack of the virulence factor coagulase and commonly used as starters in meat food product manufacturing.

The naturally present microbiota should not be underestimated because it is important to tailor the quality and safety attributes of the resulting fermented food; however, these characteristics are not always predictable and under control: they are affected by several factors, including the muscle part used, the ingredients added, and the manufacturing conditions (Fiorentini et al., 2009). Consequently, microbial starters are used in cured-meat foods to standardise the final production. However, fermented meats can be manufactured using production technologies that cause differences in composition, acidification rate, production temperature, drying procedure, and size (Sánchez Mainar et al., 2017b). Therefore, CNS survival and proliferation in different environments depend on their competitiveness, including their ability to adapt to fluctuating oxygen levels parameters and use different energy sources (Bonomo et al., 2009; Stavropoulou et al., 2018). Fortunately, CNS staphylococci are described as biofilm-formers. In particular, *S. xylosus* and *S. equorum*, often present in cured sausages and manufacturing rooms, have a great ability to create biofilms (Leroy et al., 2009). This ability can aid them stay alive in production environments and colonise meat derivatives.

3. Importance of CNS in fermented meats

The interest toward using starter cultures and carefully selected microbial strains in cured meat producers has grown, useful including

lactic acid bacteria (Lebert et al., 2007) and catalase-positive cocci (mainly CNS) (Bonomo et al., 2009; Chen et al., 2016; Heo et al., 2020).

Species diversification can be noticeably broad among CNS. *S. xylosus, S. carnosus* and *S. simulans* species are authorized in several countries as starters in the production of fermented sausages. *S. xylosus* is often described as the most prevailing species in European traditionally fermented meat products; however, *S. saprophyticus* and *S. equorum* can also be present in some meat foods (dos Santos Cruxen et al., 2017). In addition, *S. haemolyticus*, *S. sciuri*, *S. epidermidis*, *S. succinus*, *S. vitulinus*, *S. pasteuri*, and *S. warneri* can also be present in meat-derived products (Mainar and Leroy, 2015; Zeng et al., 2021).

Generally, CNS rely on arginine and other free amino acids as major meat energy sources (Chen et al., 2016). In fact, arginine amount increases post-mortem because of the asset of endogenous proteolytic enzymes, such as aminopeptidase (Cao et al., 2022; Janssens et al., 2014). The arginine deiminase (ADI) pathway, the pathway involving the arginase enzyme, and the remarkable nitric oxide synthase (NOS) pathway are different routes for arginine degradation in CNS. Sánchez Mainar et al. (2014) ascertained that these three catabolic pathways compete for the substrate L-arginine. Indeed, the ADI pathway leads to energy production, improves survival under acidic stress situations via ammonia release, and increases the availability of intermediate carbamoyl phosphate for pyrimidine production (Rimaux et al., 2011). This pathway involves three cytoplasmic enzymes: ADI encoded by *arcA*, ornithine transcarbamoylase encoded by *arcB*, and carbamate kinase encoded by *arcC* (Leroy et al., 2017). Depending on the pH, the intermediate L-citrulline can be in part excreted and consequently turned into L-ornithine. Overall, the ADI reaction catalyses the transformation of 1 mol of L-arginine into 1 mol of L-ornithine, producing 1 mol of carbon dioxide, 2 mol of ammonia, and 1 mol of ATP. Evident ADI activity has been demonstrated by strains of *S. carnosus*, *S. epidermidis*, and *S. haemolyticus* (Janssens et al., 2014). Sánchez Mainar et al. (2017a) proved that oxygen and glucose affect the ADI-based arginine conversion of CNS strains, albeit by influencing the kinetics rather than full repression. The utilisation of arginine by CNS is greatly based on environmental state, especially the atmospheric one. In fact, aerobiosis can displace ADI activity toward arginase activeness, which is an alternative pathway that converts L-arginine into L-ornithine and urea (Sánchez Mainar et al., 2014). Consequently, the arginase reaction is generally less significant in fermented sausages characterised by poor oxygen concentrations, a situation that normally takes place at the end of fermentation and in the central part of sausages with a large diameter. The equilibrium existing between ADI and arginase pathways is shown in Fig. 3.

NOS represents the third arginine-converting pathway, where L-arginine is converted into L-citrulline and NO which can then react with myoglobin to form nitrosylmyoglobin ($MbFe^{II}NO$) – the red pigment found in cured meat (Alahakoon et al., 2015) (Fig. 4). This reaction requires oxygen and nicotinamide–adenine–dinucleotide phosphate (NADPH) as a cofactor, and may represent a significant strategy to replace nitrates and nitrites in derived-meat foods (Crane et al., 2010).

Furthermore, CNS are of particular technological relevance because they can preserve meat from oxidation through catalase activity, take part in flavour and aroma formation, and generate a desirable colour via nitrate reductase activity (Mainar and Leroy, 2015; Ravyts et al., 2012; Talon et al., 2007). In fact, CNS develop meat flavours by fermenting carbohydrates, secreting esterases, inducing lipid β-oxidation, and converting amino acids (Khusro and Aarti, 2022). They usually impact proteolytic characteristics during the fermentation stage, and their beneficial effects persist until the ripening is over (Yu et al., 2021). In addition, the CNS colour-generating mechanism appears to be influenced by parameters such as the peculiar growth attributes of the bacterial cultures employed, temperature, pH, moisture content, curing agent distribution, redox potential, and pigment concentration (Bosse et al., 2016; Casaburi et al., 2005).

4. Technological roles of CNS in meat fermentation

4.1. Colour-enhancement resulting from NOS staphylococcal activity

In meat and meat-derived products, nitrate added as curing agent is firstly converted to nitrite by the staphylococcal nitrate reductase action, leading to NO, which is responsible for colour development.

Staphylococci display two different nitrate reductase mechanisms: assimilative and dissimilative. As stated by Hammes (2012), in the dissimilative nitrate reduction process, microorganisms use nitrate as a terminal electron acceptor, thereby synthesizing adenosine triphosphate (ATP). Therefore, this process releases energy and is inhibited by oxygen; in this case, anaerobic conditions must be maintained throughout fermentation. On the other hand, in assimilative nitrate reduction, staphylococci utilise nitrate and assimilate it in their cells in the form of amine groups. Because assimilative nitrate reduction is inhibited by amino acids and ammonia, solely dissimilative staphylococcal cultures appear to be suitable in amino acid-rich meat matrixes.

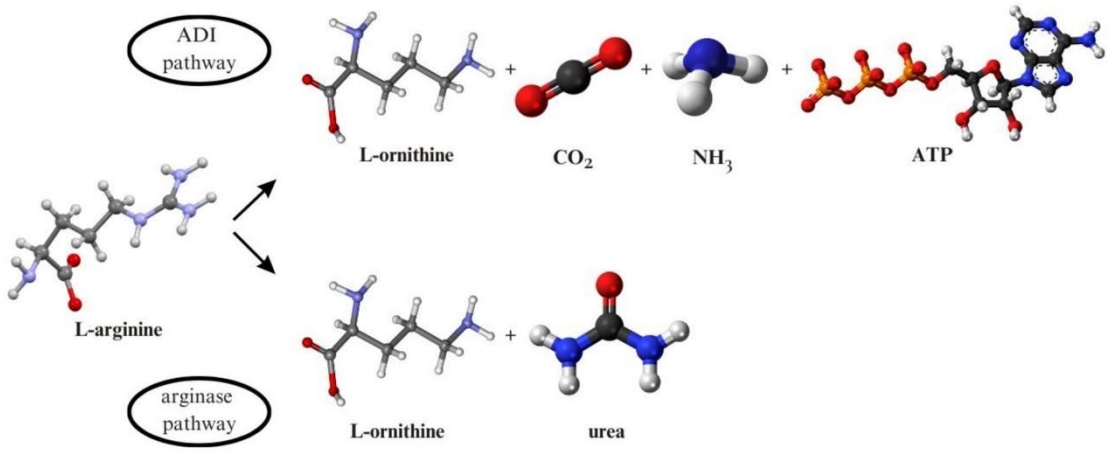

Fig. 3. Simplified pattern showing the arginase and ADI pathways involved in bacterial arginine catabolism.

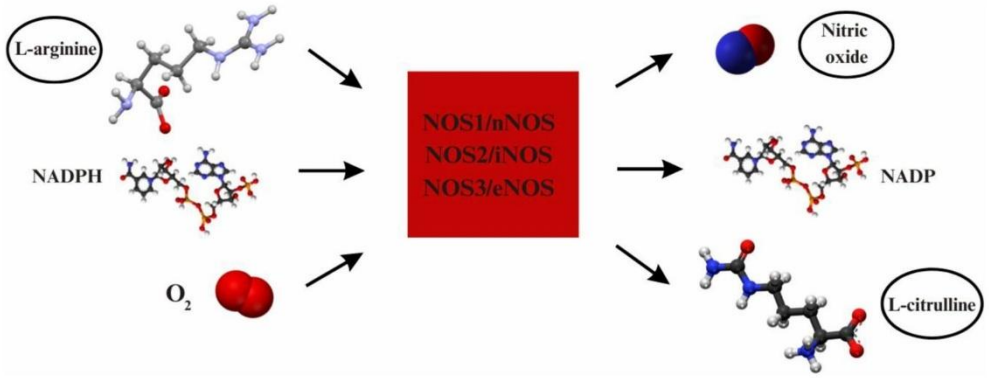

Fig. 4. Nitric oxide synthase pathway (NOS). The reaction involves the hydroxylation of L-arginine by O_2 and NADPH to form N-ω-hydroxy-L-arginine, which is then oxidised to produce NO and L-citrulline. Adapted from Abán et al. (2018).

The technologically crucial nitrate reductase activity varies appreciably between staphylococcal species, and it is generally marked in the species of *S. carnosus* and *S. xylosus* (Bosse et al., 2016; Chen et al., 2022). The nitrate reductases of these two species are particular enzymes encoded by the *nar* operon constituting of four genes (*narGHJI*) (Rosenstein et al., 2009). Nitrate reductase activity also appears important in other CNS species, such as *S. equorum* and *S. lentus*. NO synthesis by the nitrate reductase results from an anaerobic environment combined with a reduced nitrate content and is associated with nitrite buildup in a medium (Maia and Moura, 2015).

Nevertheless, to correctly develop colour using nitrate salts, it is not enough to rely only on a specific CNS strain with a confirmed nitrate reductase action because manufacturing conditions should also be contemplated. Talon et al. (2007) proved that desired activity reaches the maximum pick during exponential growth and is generated by anaerobic growth status in the presence of nitrates. The initial and the final meat pH values also play important roles because nitrate reductase activity generally diminishes below pH measurement equal to 5.2. In particular, CNS dependence on pH could be a critical parameter for colour formation when only nitrate salts are used (Vermassen et al., 2016). Mild acidification conditions in meat models have been demonstrated to support nitrate-reducing strains belonging to *S. equorum* species, while counteracting *S. saprophyticus* strains. Regarding temperature conditions, nitrate reduction can be obtained at 15 °C–20 °C although the process appears to be particularly efficient at temperatures higher than 30 °C (Sánchez Mainar et al., 2017a).

Apart from the nitrate reductase activity of CNS strains, an innovative and still under-investigation method consists of meat colour generation through the NOS staphylococcal pathway (Sánchez Mainar et al., 2014), which involves a unique L-arginine conversion mechanism (Cao et al., 2022; Ras et al., 2018a). It is assumed that the ability of CNS to exhibit NOS activity induces the release of potential colour-yielding NO resulting from L-arginine (Zając et al., 2022). In fact, the NOS pathway synthesizes NO by oxidizing the L-arginine guanidium group, depleting NADPH-H^+ as a co-factor and producing L-citrulline as a co-product (Fig. 4). The *nos* gene has been identified in the genome of *S. carnosus*, *S. equorum*, *S. saprophyticus*, *S. warneri*, and *S. xylosus* deriving from foods of animal source or processing environments (Ras et al., 2018a). Furthermore, the NOS-encoding gene has been found in a *S. haemolyticus* strain, which also displays phenotypic NOS-like activity under aerobic conditions (Sánchez Mainar et al., 2014). NOS activity can be estimated through different methods, which are generally based on measuring the amount of L-citrulline released in the investigated matrix, the amount of nitrite (intended as the final product of NO oxidation), the amount of nitrosomyoglobin produced, or the exploitation of fluorescent probes able to react with NO (Sapp et al., 2014). Vaish and Singh (2013) determined NOS effectiveness using a NOS activity assay kit and radioactive L-arginine monohydrochloride as substrate.

Several researchers have tested the ability of certain CNS species to positively affect the colour of meat products or culture media both in the reduced presence of nitrates and nitrites and in their total absence. Table 1 shows results obtained in terms of $MbFe^{II}NO$ or nitrosyl pigment formation after bacterial inoculation, while Table 2 indicates the colour change (in a*-values) of the tested product after CNS inoculation. Gøtterup et al. (2007) proved that *S. carnosus* 953 registered a particularly high $MbFe^{II}NO$ formation rate in MRS broth supplemented with metmyoglobin and nitrite, proving that the strain could be used as a starter culture to enhance colour formation. Gøtterup et al. (2008) tested the red colour intensity and the $MbFe^{II}NO$ concentration after the inoculation of sausages with *S. carnosus*, *S. simulans*, and *S. saprophyticus* first in the presence of 160 mg/kg of nitrates and thereafter in the presence of the same amount of nitrites. They reported that both parameters were significantly affected by the type of strain used in nitrate-cured but not in nitrite-cured sausages. CNS ability to produce $MbFe^{II}NO$ was also tested by Li et al. (2016), who stated that MRS broth supplemented with metmyoglobin and treated with *S. xylosus* had two absorbance peaks at almost 545 nm and 580 nm wavelengths, which are characteristic absorbance peaks of red myoglobin derivative compounds. They also compared the colour of both *S. xylosus*-supplemented MRS broth and nitrite-free MRS broth, showing that the inoculated broth exhibited a red colour, while the other maintained its brown colour. Huang et al. (2020) analysed the absorbance peaks of synthetic agents: free dry pork sausages were inoculated with *S. vitulinus*, *S. carnosus,* and *S. equorum*. The red derivative found in inoculated sausages was nitrosomyoglobin. Besides, they demonstrated that CNS could strongly improve the a*-values of investigated sausages. In particular, *S. carnosus* showed the highest colour-enhancing ability among the three considered species.

Szymański et al. (2020) studied the potential of *S. carnosus* ATCC 51365 to form nitrosyl pigments and red colour in pork meat containing 15 mg/kg of sodium nitrite. They reported that both dimensions were characterised by similar values in inoculated meat and in nitrite-cured meat (supplemented with 100 mg/kg of sodium nitrite). Moreover, Li et al. (2013) compared the red colour intensity of nitrite-free pork meat inoculated with *S. xylosus* A1 and that of meat to which was added 100 mg/kg of nitrite. They stated that inoculated meat had similar a*-values as nitrite-cured meat.

As previously mentioned, being the NOS pathway arginine-dependent, a reduced arginine availability can strongly inhibit the activity of NOS enzymes (Rath et al., 2014). Therefore, the inclusion of

Table 1
Results, intended as nitrosylmyoglobin ($MbFe^{II}NO$) or nitrosyl pigments formation, after meat products/culture media inoculation with coagulase-negative staphylococci (CNS). Where no unit of measure is indicated, the authors referred to arbitrary unit. d = day.

Meat product/ Culture medium	Aim of the work	CNS tested	Result (value)		Commented result	Instrumentation	Reference
$MbFe^{III}$ -MRS broth + 0.4 mM NO_2^-	CNS ability to produce $MbFe^{II}NO$		$MbFe^{II}NO$ formation rate		*S. carnosus 953 showed the highest* $MbFe^{II}NO$ formation rate among all CNS tested	Electron spin resonance (ESR) spectroscopy	Gøtterup et al. (2007)
		S. equorum 308	0.14 nmol/min				
		S. carnosus 953	0.72 nmol/min				
		S. xylosus 913	0.67 nmol/min				
		S. saprophyticus 389	0.41 nmol/min				
Fermented sausages +160 mg/kg NO_2^-	CNS ability to produce $MbFe^{II}NO$		[$MbFe^{II}NO$]		The type of strain imposed no significant effect on the $MbFe^{II}NO$ formation in nitrite-cured sausages	Electron spin resonance (ESR) spectroscopy at 150 K	Gøtterup et al. (2008)
			2 d	**6 d**			
		CTR	4.50×10^8	3.80×10^8			
		S. carnosus 506	4.00×10^8	4.20×10^8			
		S. simulans 392	4.40×10^8	3.90×10^8			
		S. saprophyticus 389	4.30×10^8	4.40×10^8			
Fermented sausages +160 mg/kg NO_3^-	CNS ability to produce $MbFe^{II}NO$		[$MbFe^{II}NO$]		The type of strain imposed a significant effect on the $MbFe^{II}NO$ formation in nitrate-cured sausages	Electron spin resonance (ESR) spectroscopy at 150 K	Gøtterup et al. (2008)
			2 d	**6 d**			
		CTR	1.40×10^8	2.00×10^8			
		S. carnosus 506	4.50×10^8	4.20×10^8			
		S. simulans 392	3.10×10^8	3.50×10^8			
		S. saprophyticus 389	0.90×10^8	3.40×10^8			
$MbFe^{III}$ -MRS broth (no NO_2^-/NO_3^-)	CNS ability to produce $MbFe^{II}NO$	*S. xylosus*	absorbance peaks at λ 545 nm and 580 nm		548 nm and 579 nm are the maximal absorbance peaks for $MbFe^{II}NO$	UV–Vis spectrophotometer	Li et al. (2016)
Dry pork sausages (no NO_2^-/NO_3^-)	CNS ability to form NO-Mb		NO-Mb absorbance peaks at		The red derivative found in inoculated sausages was considered NO-Mb	UV–Vis spectrophotometer	Huang et al. (2020)
		CTR	λ 415, 536 and 590 nm				
		S. vitulinus	λ 395, 476, 535 and 560 nm				
		S. carnosus, S. equorum	λ 395, 476, 535 and 576 nm				
Pork meat + NO_2^-	CNS ability to form nitrosyl pigments		[nitrosyl pigments]		The sample added with *S. carnosus* and a reduced amount of NO_2^- showed similar results as the CTR having a higher content of NO_2^-	Determined following the Hornsey method	Szymański et al. (2020)
		CTR + 15 mg/kg NO_2^-	28.6 mg/kg				
		CTR + 100 mg/kg NO_2^-	40.50 mg/kg				
		S. carnosus ATCC 51365 + 15 mg/kg NO_2^-	36.50 mg/kg				

L-arginine in the culture medium or directly into the meat batter could increase bacterial NOS activity and enhance the final desired colour. In fact, arginine has been recognised by the World Health Organisation as a safe food additive (Smith et al., 2011; Zając et al., 2022), and it is generally added to meat to improve flavour and texture, inhibit fat oxidation, and increase water-holding capacity (Zhou et al., 2014). L-arginine incorporation in the manufacture of meat products has recently received considerable interest (Ning et al., 2019). Vaish and Singh (2013) demonstrated that adding L-arginine (50 mM) considerably promoted NOS activity during bacterial cultivation. Furthermore, L-arginine addition in meat batter increased a*-values, leading to no evident difference with respect to the control sample containing nitrite. The same experiment was conducted in MRS broth, where NOS induction by L-arginine increased NO and $MbFe^{II}NO$ production (Luo et al., 2020).

4.2. Biochemistry of NOS

NOS enzymes are a family of cytochrome P_{450}-like flavohemeproteins that catalyse, in a two-step reaction, the 5-electron oxidation of L-arginine to produce L-citrulline and NO.

$$\underbrace{\text{L-arginine} + 1.5\ \text{NADPH} + 1.5\ \text{H}^+ + 2\ \text{O}_2}_{\text{First step}} \rightarrow \underbrace{1.5\ \text{NADP}^+ + \text{L-citrulline} + \text{NO} + 1.5\ \text{H}_2\text{O}}_{\text{Second step}}$$

The reaction exhausts 1.5 mol of NADPH and 2 mol of oxygen (O_2) per mol of L-citrulline formed. First, a fundamental hydroxylation of L-arginine occurs, and this leads to the production of N-ω-hydroxy-L-arginine (NOHA), which remains largely bound to the enzyme and acts as a substrate for NOS. Secondly, the intermediate is oxidised to create L-citrulline and NO (Feng, 2012). NO is a highly reactive gaseous nitrogen species containing an atom of nitrogen and another of oxygen: seven electrons from nitrogen and eight from oxygen are implicated in the creation of an uncharged molecule (N≡O) (Habib and Ali, 2011). NO is characterised by high reactivity towards biological molecules with

Table 2
Coagulase-negative staphylococci (CNS) colour formation capacity, reported in a*-values, after x number of h (hours) or d (days) of meat products/culture media inoculation with staphylococcal strains. All a*-values are reported in arbitrary unit.

Meat product/ Culture medium	CNS tested	Result (a*-value)		Commented result	Instrumentation	Reference
Fermented sausages		**16 h**	**40 h**	The red colour intensity of nitrite-cured sausages was not significantly affected by the type of strain	Gardner color guide	Gøtterup et al. (2008)
+160 mg/kg NO_2^-	*CTR*	12.80	11.90			
	S. carnosus 506	11.50	11.40			
	S. simulans 392	11.70	11.70			
	S. saprophyticus 389	10.90	11.30			
Fermented sausages		**16 h**	**40 h**	The red colour intensity of nitrate-cured products was significantly affected by the type of strain	Gardner color guide	Gøtterup et al. (2008)
+160 mg/kg NO_3^-	*CTR*	10.90	7.90			
	S. carnosus 506	10.80	12.70			
	S. simulans 392	10.40	9.80			
	S. saprophyticus 389	10.40	8.00			
Pork meat (no NO_2^-/ NO_3^-)		**12 h**		Meat added with *S. xylosus* showed similar a*-value as that of the nitrite-cured meat	ZE-6000 colorimeter	Li et al. (2013)
	CTR	9.70 ± 0.62				
	CTR + 100 mg/kg NO_2^-	13.03 ± 0.66				
	S. xylosus A1	12.79 ± 0.72				
Pork meat (no NO_2^-/ NO_3^-)		**1 d**	**3 d**	Similar a*-values were found between inoculated and uninoculated meat	Chromameter CR-400	Mainar and Leroy (2015)
	CTR	9.10 ± 1.41	6.05 ± 1.52			
	S. haemolyticus G 110	9.52 ± 1.52	5.26 ± 1.40			
	S. carnosus 1505	7.66 ± 1.41	5.96 ± 1.35			
Pork meat +200 mg/kg NO_3^-		**1 d**	**3 d**	*S. carnosus* positively affected meat colour after three days	Chromameter CR-400	Mainar and Leroy, 2015
	CTR	9.41 ± 1.29	9.86 ± 1.42			
	S. haemolyticus G 110	9.62 ± 1.42	6.17 ± 1.36			
	S. carnosus 1505	7.72 ± 1.62	9.77 ± 1.49			
Cured raw ham + NO_3^-		**10 d**	**30 d**	*S. carnosus* LTH 3838 showed a good red colour enhancing ability	Chroma Meter CR-200	Bosse et al. (2016)
	CTR	16.00	14.50			
	S. carnosus LTH 3838	14.20	15.00			
	S. carnosus LTH 7036	12.40	13.80			
$MbFe^{III}$-MRS broth (no NO_2^-/NO_3^-)		**18 h**		Inoculated MRS showed a red colour, while CTR still maintained a brown colour	ZE-6000 colorimeter (transmittance)	Li et al. (2016)
	CTR	2.10				
	S. xylosus	3.80				
Pork meat (no NO_2^-/ NO_3^-)		**18 h**		No significant differences were found between inoculated meat and nitrite-cured meat	ZE-6000 colorimeter (reflectance)	Li et al. (2016)
	CTR	9.66 ± 0.44				
	CTR + 100 mg/kg NO_2^-	13.03 ± 0.48				
	S. xylosus	12.76 ± 0.53				
Dry pork sausages (no NO_2^-/NO_3^-)		**6 d**		*S. carnosus* showed the highest colour enhancing ability among all the strains tested	WSF colorimeter	Huang et al. (2020)
	CTR	9.00				
	CTR + 90 mg/kg NO_2^-	12.90				
	S. vitulinus	11.70				
	S. carnosus	13.50				
	S. equorum	12.00				
Pork meat + NO_2^-		**28 d**	**56 d**	The sample added with *S. carnosus* and a reduced amount of NO_2^- showed similar a*- values as that of CTR having a higher content of NO_2^-	Reflection colorimeter CR-300	Szymański et al. (2020)
	CTR + 15 mg/kg NO_2^-	11.13 ± 0.22	10.55 ± 0.19			
	CTR + 100 mg/kg NO_2^-	11.30 ± 0.22	10.74 ± 0.26			
	S. carnosus ATCC 51365 + 15 mg/kg NO_2^-	11.45 ± 0.18	11.18 ± 0.23			

unpaired orbital electrons, such as free radicals or transition metal ions. The reactivity of NO depends on its physical characteristics: minuscule size, lipophilicity, and high diffusion rate (Förstermann and Sessa, 2012). NO has a short lifetime, limited by scavenging reactions performed by myoglobin.

NOS enzymes, which are heme-iron enzymes, facilitate the reaction using (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), reduced NADPH, and molecular oxygen. Electrons are transferred from NADPH through flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to the heme iron, where oxygen is bound and activated (Feng, 2012). NOS has three isoforms: NOS1 or neuronal nitric oxide synthase (nNOS), NOS2 or inducible nitric oxide synthase (iNOS), and NOS3 or endothelial nitric oxide synthase (eNOS) (Roman et al., 2002). All NOS isoforms have similar amino acid sequences and related cofactor demands. Moreover, they all require the presence of heme, oxygen, pterine cofactors, and electrons. NOS enzymes differ in structure, regulation, distribution, and synthetic capacity; however, they all catalyse the incorporation of oxygen and the release of NO from the terminal guanidino nitrogen group of L-arginine and L-citrulline as a co-product. Each isoform is the result of a recognisable and specific gene. Generally, the isoforms can be classified in two groups: constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS). While cNOS is calcium

(Ca^{2+})-dependent and is always present, iNOS is independent of Ca^{2+} and is induced by inflammatory agents such as cytokines or lipopolysaccharides. Based on this categorisation, nNOS and eNOS are integrally expressed and need high concentrations of Ca^{2+} in addition to the activation of calmodulin (CaM) to form NO for a limited time duration. In contrast, iNOS is expressed only after stimulation and locally generates elevated levels of NO for extended periods of time (Habib and Ali, 2011).

The three NOS isoforms are composed of sites of wide homology – the oxygenase and reductase domains – although each isoform displays distinctive attributes. These differences have a considerable effect on the enzymatic functions of each isoform. NOS enzymes are functional dimers, and each monomer contains two domains: the first one is a N-terminal oxygenase domain able to bind L-arginine, BH_4, and a tetra-coordinated zinc atom, and the second is a C-terminal reductase domain with an autoinhibitory region and binding areas for FAD, FMN, and NADPH. A Ca^{2+}/CaM binding site connects the two high homology regions. The oxygenase domain remains homodimeric whereas the reductase domain is monomeric, implying that the two subunits are linked through their oxygenase domains (Roman et al., 2002).

Overall, nNOS and eNOS are directly activated by high levels of intracellular Ca^{2+}, the Ca^{2+}–CaM complex, and, consequently, the CaM–NOS association, while iNOS is already constrained to CaM and is completely active. Habib and Ali (2011) stated that CaM provides a specific conformational change that improves electron flow from NADPH to flavins and enhances electron movement from FMN to heme. Consequently, CaM is a necessary compound involved in the NOS reaction.

Heme plays an essential role in the dimerization process and in its absence NOS would only exist as monomers. Heme is a unique element for which there is a full demand for the composition of active nNOS dimers. Heme is also crucial for eNOS dimerization, and it performs a similarly fundamental role in iNOS dimerization. Monomers of all the isoforms of NOS cannot bind BH_4 and, therefore, cannot catalyse L-citrulline and NO generation. Heme is bound through a cysteine thiolate ligand, and forming this bond is the most important step in the dimerization mechanism. While the heme request for dimerization is common to all the three isoforms, the presence of BH_4 is not: nNOS and eNOS can create dimers without BH_4, whereas iNOS strongly relies on the presence of BH_4 (Ishimura et al., 2004).

4.3. Flavour-generation

CNS activity is decisive for flavour improvement. CNS contribute to flavour development through four different mechanisms: (1) carbohydrate fermentation, (2) amino acid conversions, (3) lipid β-oxidation, and (4) esterase activities (Sánchez Mainar et al., 2017b; Ravyts et al., 2007). CNS convert carbohydrates into volatile compounds, giving to meats a distinctive buttery aroma, and organic acids.

In addition to carbohydrate metabolism, the amino acids conversion by CNS determines the release of volatile and non-volatile compounds with a strong aroma potential (Mora et al., 2015). For instance, staphylococci can transaminate and decarboxylate the amino acids valine, leucine, and isoleucine into the corresponding aldehydes and alcohols (Sánchez Mainar et al., 2017b). In particular, leucine-deriving compounds (i.e., 3-methyl butanal, 3-methyl butanol, and 3-methyl butanoic acid) usually develop the typical fermented sausage flavour.

On the other hand, lipolysis occurs through the enzymatic hydrolysis of the lipid portion present in meat. Lipolysis is primarily carried out by endogenous enzymes and CNS lipases (Bonomo et al., 2009). Indeed, CNS enzymes firstly oxidise the released fatty acids to enoyl-CoA, which is hydrated to hydroxyacyl-CoA and then oxidised to ketoacyl-CoA. This activity results in the production of β-ketoacids, short-chain free fatty acids, and methyl ketones. The latter contribute to cured flavour development and can be further transformed in secondary alcohols (Stahnke et al., 2006). Furthermore, aromatic ester compounds, such as ethyl esters, can be released by CNS esterase activity (Sánchez Mainar et al., 2017b).

4.4. Bioprotection

In the context of nitrate and nitrite replacement, the inhibition of pathogens and spoilage microorganisms could be achieved through bacteriocin-producing bacteria (Li et al., 2016). Bacteriocins are generally described as ribosomally synthesised peptides showing an inhibitory effect against spoilage bacteria and pathogens (mainly *C. botulinum*, *L. monocytogenes*, and *S. aureus*) (Christieans et al., 2018). The presence of certain amino acids, activity, mode of action, inhibition spectrum, mode of excretion, and thermostability are the factors generally used to classify bacteriocins.

Overall, 21 distinct bacteriocins produced by staphylococci are described in the literature (Sánchez Mainar et al., 2017b). Most bacteriocins are categorised as class I (lantibiotics) while others as class II (peptides) and class III (proteins). Among CNS, three plasmid-encoded class II bacteriocins are epidermicin NI01, aureocins A70 and aureocins A53. The first is produced by *S. epidermidis*, while the other two are produced by *S. aureus*. Instead, the plasmid-encoded lysostaphin produced by *S. simulans* ATCC 1362 belongs to class III bacteriocins, which are thermo-labile proteins larger than 10 kDa (Sánchez Mainar et al., 2017b).

Two major methods can be used for the employment of bacteriocins in the preservation of cured meat: the first one is the addition of purified bacteriocins as a food biopreservative, while the second is the utilisation of protective starter culture strains to allow in-situ production. Regarding the first option, the exploitation of nisin in processed meat products is considered safe by the two most important food safety authorities (i.e., the European Food Safety Authority and the Food and Drug Administration). In contrast, concerning the second procedure, several researchers have studied the bacteriocin production phenomenon by bioprotective cultures in fermented meat foods (Sánchez Mainar et al., 2017b). Unfortunately, the scientific literature regarding the effect of staphylococci-produced bacteriocins in meat foods is still somewhat limited. Thus, in view of the removal of synthetic curing agents, further research would be needed in order to evaluate the microbiological meat safety and the possible bioprotective role exerted by staphylococci in meat products, especially toward pathogens.

5. Conclusions and future perspectives

The safety of nitrates and nitrites as food additives is of concern; therefore, natural alternatives must be investigated and considered in the formulation of cured meat products. A promising strategy involves harnessing the ability of CNS to produce NO via the NOS pathway. CNS represents a ubiquitous group of fundamental and crucial microorganisms with important metabolic potential for starter culture innovation. In fact, CNS contribute to colour improvement through their nitrate reductase and NOS activities, flavour enhancement through their metabolism of carbohydrates, amino acids, and fatty acids, as well as antimicrobial action through their bacteriocin-producing activity. Temperature and acidification are the two conditions mainly driving the staphylococcal presence during meat fermentation, affecting the outcome of the process and the resulting cured meat product. Safety aspects are essential when selecting CNS strains, including the absence of genes encoding for the production of biogenic amines and transferable antibiotic resistance. Moreover, the ability of CNS strains to replace synthetic curing agents should be investigated, taking into account the technological adaptation of CNS and the oxygen requirement of the NOS pathway. Generally, a decrease of oxygen availability occurs moving towards the centre of a cured product, particularly in sausages with large calibres. This reduced oxygen availability is not optimal for the NOS reaction; accordingly, manufacturing tests that include the monitoring of oxygen should be performed.

In conclusion, despite the challenges arising from their use, NOS-positive CNS strains may represent a turning point in replacing nitrates and nitrites in meat-derived products.

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Lara Premi: Investigation, Visualization, Writing – original draft, Writing – review & editing. **Gabriele Rocchetti:** Writing – original draft, Writing – review & editing. **Luigi Lucini:** Supervision, Writing – review & editing. **Lorenzo Morelli:** Supervision, Writing – review & editing. **Annalisa Rebecchi:** Conceptualization, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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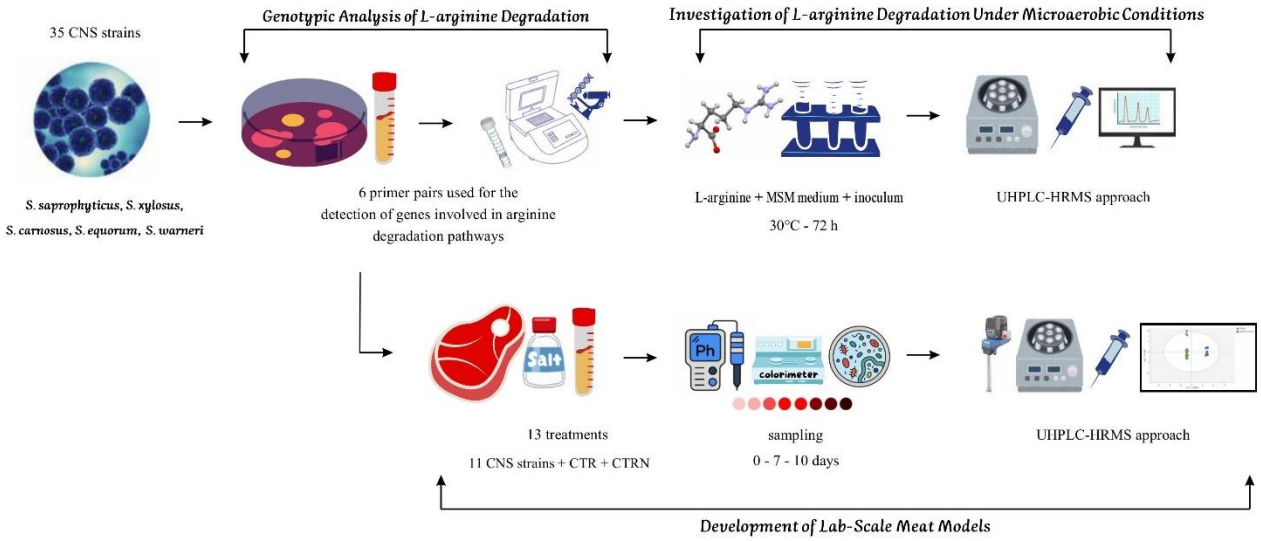
1.5 Objective of the thesis

Based on evidence from the literature, the thesis aimed to investigate various natural strategies as an alternative to the use of synthetic nitrites, primarily with the intention of improving and maintaining the red colour of meat products.

The research project first investigated whether several CNS strains could use L-arginine – the initial amino acid involved in the NOS pathway, whose degradation produces L-citrulline and nitric oxide – as an alternative energy source in meat. The study therefore emphasised the importance of the staphylococcal NOS pathway as a potential technological solution for enhancing the redness of nitrite-free meat products. In addition to evaluating the impact of staphylococcal cultures on the colour of fermented beef, a targeted metabolomics-based approach was employed to further investigate the NOS pathway. Additionally, this research work aimed to explore the potential of using the NOS-positive CNS strains that showed promise in the initial trial, a ZnPP-rich porcine liver extract, and NATPRE T-10 CUR HT (a commercially available polyphenol-rich plant ingredient) – either individually or in combination – to produce red nitrite-free dry-fermented pork sausages. Nine different model sausage treatments were prepared and ripened, and their colour attributes were investigated alongside technological microbiota counts, water activity, pH, moisture content, hemin and nitrosyl-heme amounts, ZnPP content, aqueous extract analyses, and urea-PAGE electrophoresis. Previously evaluated colour and technological quality parameters were combined with untargeted metabolomics and lipid peroxidation, and integrated with sensory odour evaluation. The aim of these analyses was to gain a deeper insight into the aroma characteristics of dry-fermented sausages and to identify the most promising formulation in terms of not only redness retention, but also chemical and sensory perspectives.

2. <u>Chapter 2</u>: Coagulase-negative staphylococci enhance the colour of fermented meat through a complex cross-talk between the arginase and nitric oxide synthase activities

Graphical abstract



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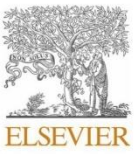

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Coagulase-negative staphylococci enhance the colour of fermented meat through a complex cross-talk between the arginase and nitric oxide synthase activities

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ABSTRACT

The nitric oxide synthase (NOS) activity was investigated in 35 staphylococcal strains from five distinct coagulase-negative staphylococci (CNS) species. NOS pathway is related with the production of nitric oxide, potentially leading to the formation of nitrosomyoglobin imparting a pinkish-red colour to fermented meats. NOS activity was investigated employing both genotypic and phenotypic methods. Among the strains studied, 16 were found to contain the gene encoding NOS, while 25 strains possessed the arginase gene. Strains from *Staphylococcus saprophyticus*, *S. equorum*, and *S. carnosus* species exhibited phenotypic NOS-like activity. This was evidenced by the production of L-citrulline and a comparably lower release of L-ornithine in meat simulation media fermented under microaerobic conditions by those staphylococcal cultures. The authentic NOS activity was further examined using lab-scale meat models. The characteristic red colouration, typically associated with sodium nitrite-cured meat products, was observed. Strains L31, L49, and V10 (*S. saprophyticus*) demonstrated a pronounced effect on meat colouration, achieving shades comparable to nitrite-enhanced meat; however, a concurring up-accumulation of putrescine and spermine (likely originating from ornithine) was also outlined. These findings suggest the potential of meat-associated CNS to promote colour development in nitrite-free meats, offering a promising area for enhancing the visual appeal of such products.

1. Introduction

Coagulase-negative staphylococci (CNS) are distinguished by their spherical morphology, and exhibit facultative anaerobic capabilities, enabling survival in both aerobic and anaerobic environments. In addition to being Gram-positive and non-motile, these organisms possess catalase activity and do not form spores, as detailed by researchers (Heo, Lee, & Jeong; 2020). The widespread occurrence of those microorganisms in food has been well-documented, especially in products derived from animals, with meat being a prominent example (Voidarou et al., 2021). In this context, *S. xylosus*, *S. saprophyticus*, *S. carnosus*, and *S. equorum* represent the species most frequently isolated from meat (Bosse, Gibis, Schmidt, & Weiss, 2016; Charmpi et al., 2020; Ravyts, De Vuyst, & Leroy, 2012; Zeng et al., 2021). Notably, *S. xylosus* is often predominant in fermented meat products, especially those prepared without the addition of starter cultures (Aquilanti, Garofalo, Osimani, & Clementi, 2016; Huang et al., 2020; Stavropoulou, De Maere, et al., 2018). Moreover, meat-related foods may also harbour other, less prevalent species such as *S. warneri*, *S. sciuri*, *S. epidermidis*, *S. succinus*, *S. vitulinus*, and *S. pasteuri* (Khusro & Aarti, 2022; Ras, Bailly, et al., 2018; Ratsimba et al., 2017).

In the field of food technology, certain CNS are widely used as functional commercial starters in meat production (Li, Zhu, Chen, Zhou, & Wu, 2022). These CNS play a significant role in providing antioxidant protection through their catalase activity (Heo, Lee, & Jeong, 2020; Stavropoulou, De Vuyst, & Leroy, 2018). This activity is crucial for achieving the distinctive pinkish-red colour in meat by facilitating nitrate reductase action (Leroy, Verluyten, & De Vuyst, 2006; Vanderhaeghen et al., 2014). In addition, they have a crucial impact on improving the mouthfeel and aroma characteristics of meat products, as

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demonstrated by various studies (Chen et al., 2021; de Almeida et al., 2018; Huang et al., 2020; Sallan, Kaban, & Kaya, 2022). Their metabolic processes produce a range of different end-products, such as alcohols, aldehydes, organic acids, free fatty acids, and ketones. In addition to their use as starter cultures, CNS are being increasingly utilised for a range of technological applications. This is due to their ability to withstand nitrosative stress, acidic conditions, and osmotic stress (Leroy, Vermassen, Ras, & Talon, 2017).

In fermented meat, carbohydrates are predominantly consumed by lactic acid bacteria (Lebert et al., 2007). As a result, CNS are compelled to utilise alternative endogenous energy sources, such as free amino acids (Sánchez Mainar, Matheuse, De Vuyst, & Leroy, 2017). Among these, L-arginine, which is abundantly present in meat, serves as a critical substrate (Cao et al., 2022; Janssens et al., 2014). The CNS can metabolise L-arginine through three distinct pathways: arginine deiminase (ADI), arginase, and nitric oxide synthase (NOS) (Premi, Rocchetti, Lucini, Morelli, & Rebecchi, 2024). Notably, the NOS pathway leads to the formation of L-citrulline and nitric oxide (NO). NO, a colourless gas, can bind to the ferrous heme-iron of myoglobin, forming nitrosylmyoglobin ($MbFe^{II}NO$), the desirable red pigment in cured meat products (Alahakoon, Jayasena, Ramachandra, & Jo, 2015; De Maere et al., 2016; Gøtterup et al., 2008; Higuero, Moreno, Lavado, Vidal-Aragón, & Cava, 2020). The three isoforms of NOS – neuronal nitric oxide synthase (nNOS or NOS1), inducible nitric oxide synthase (iNOS or NOS2), and endothelial nitric oxide synthase (eNOS or NOS3) – catalyse the hydroxylation of L-arginine to L-citrulline and NO. These enzymes are differentiated by their regulation, structure, distribution, and requirement for oxygen, heme, pterin cofactor, and electrons for functioning (Ras, Bailly, et al., 2018). Additionally, oxygen and nicotinamide-adenine-dinucleotide phosphate act as cofactors in this reaction (Zając, Zając, & Dybaś, 2022). Although the scientific literature regarding the assessment of CNS–NOS activity is somewhat limited, the NOS pathway is increasingly recognised for its potential role in developing meat products devoid of synthetic additives (Crane, Sudhamsu, & Patel, 2010). Within this context, previous research conducted by different scientists has shown that CNS cultures are capable of converting metmyoglobin to nitrosomyoglobin in meat systems without nitrite, potentially through the NOS pathway (Huang et al., 2020; Li, Kong, Chen, Zheng, & Liu, 2013; Ras, Leroy, & Talon, 2018).

Chemical additives, such as nitrates and nitrites, are frequently used in the production of cured meat products. These additives have various functions, including developing a unique tangy flavour, giving the meat its characteristic red colour, preventing lipid oxidation, and inhibiting the growth of harmful microorganisms (Huang, Luo, Li, & Xu, 2022; Premi et al., 2024; Sallan et al., 2022). Therefore, there is evidence that these artificial curative agents contribute to the formation of carcinogenic compounds (Flores & Toldra, 2021; Govari & Pexara, 2018; Majou & Christieans, 2018), e.g. by interacting with proteins found in muscle tissue, which could result in the formation of toxic compounds (i.e., nitrosamines) in the gastrointestinal tract of humans (De Mey, De Maere, Paelinck, & Fraeye, 2017; Ras, Bailly, et al., 2018).

Therefore, starting from this important background scenario, this study aimed to investigate how 35 CNS strains can use L-arginine as an alternative energy source associated with meat. Furthermore, we aimed to highlight the importance of the NOS pathway as a potential solution for the technological challenge of removing and replacing nitrates and nitrites, in order to improve the redness of the products.

Besides assessing how the staphylococcal cultures impacted the meat colour, we also used an untargeted metabolomics-based approach to provide more insights into the complex cross-talk existing between the arginase and NOS activities, being these latter able to potentially affect both aroma and flavour in the final product.

2. Materials and methods

2.1. Staphylococcal strains

For this investigation, 35 CNS strains from species such as *S. saprophyticus*, *S. xylosus*, *S. carnosus*, *S. equorum*, and *S. warneri* were utilised. These strains were isolated from fermented meat products and originated from the laboratory collection at Università Cattolica del Sacro Cuore (Cremona, Italy), except for *S. carnosus* TM300 (Department of Microbial Genetics, Universität Tübingen, Germany) and *S. saprophyticus* DSM 20229 (DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Leibniz Institute, Germany). Species-specific polymerase chain reactions (PCRs) were conducted to authenticate all staphylococcal strains at the species level. The strains were preserved at −20 °C in brain heart infusion (BHI) medium (Oxoid, Basingstoke, Hampshire, United Kingdom), supplemented with 30% (vol/vol) glycerol as a cryoprotectant.

2.2. Genotypic analysis of L-arginine degradation

The genomic DNA of all 35 CNS strains was extracted from colonies grown on mannitol salt agar (MSA) (Oxoid) medium and resuspended in 20 μl of lysis solution (microLYSIS®-Plus DNA, Microzone, Labogen, UK). These samples were then processed in a T100™ thermal cycler (Bio-Rad, Hercules, California, USA) following the manufacturer's heat treatment protocol (Microzone).

For the detection of genes involved in arginine degradation pathways, the methodology utilised six distinct primer pairs (*arcA*, *arg*, NOS-1, NOS-2, NOS-3, and NOS-4), following the PCR protocols from the study by Sánchez Mainar, Weckx, and Leroy (2014). The dimensions of the PCR products were later evaluated on a 1.5% (mass/vol) agarose gel (Promega).

2.3. Media preparation for assessing phenotypic characteristics

The experimental procedure involved the use of a meat simulation medium (MSM) enhanced with 3 g/L of L-arginine, prepared in accordance with the formula established by Sánchez Mainar et al. (2014). All components were solubilised in deionised water. Prior to sterilisation, the pH of the medium was adjusted to 5.9 to accurately reflect the typical initial acidity encountered in meat fermentation processes.

2.4. Investigation of L-arginine degradation under microaerobic conditions

The CNS strains were subcultured in BHI broth medium at 37 °C for 16 h to prepare the inocula for the screening tests. Microaerobic conditions were established to simulate the anaerobic environment characteristic of meat products. A 100-μL aliquot of the staphylococcal subculture was introduced into 10 mL of MSM, followed by incubation at 30 °C. Sampling occurred at the 72-h mark. The analysis entailed combining 200 μL from each sample with 800 μL of a 50% ethanol solution, subsequently undergoing an ultrasonic bath treatment. The samples were centrifuged at 14,000 rpm for 15 min and then filtered through a 0.45 μm syringe filter. Consequently, the concentrations of L-arginine, L-citrulline, and L-ornithine were quantified employing an ultra-high-performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) method (Section 2.5.4). The data presented in Table 2 are organised according to the concentrations of L-arginine, L-citrulline, and L-ornithine detected in the control samples (MSM culture medium only).

2.5. Development of lab-scale meat models

For the lab-scale meat models, two beef loins weighing about 2 kg each were utilised. The meat was subjected to autoclaving at a

temperature of 110 °C for 2 min to eradicate surface bacterial contamination. To ensure that no protein denaturation occurred, a 0.5-cm lightly browned surface layer of autoclaved loin was removed before being used for experimentation. After that, a total of 3500 g of beef loin, sourced from two distinct batches, were processed into vertical slices, subsequently minced using a mincer with an 8 mm mesh diameter, and thoroughly mixed. Each experimental treatment utilised 230 g of this minced meat, seasoned with 2.5% salt (NaCl). The study comprised 13 distinct treatments of meat batter, each executed in duplicate: the control (CTR), consisting solely of meat batter with added salt; the nitrite-cured batch (CTRN), prepared with 150 mg/kg of sodium nitrite ($NaNO_2$) in compliance with EU Commission Regulation No. 1129/2011 for cured meat products; and 11 additional treatments. These latter treatments were conducted by inoculating the meat batter with *nos*-positive CNS strains, including eight strains of *S. saprophyticus* (FT20E, L30, L31, L34, L43, L49, MT20D, and V10). Additionally, the experiment included *S. equorum* L33, *S. carnosus* TM300, and *S. xylosus* X100 strains. The inoculation level for each staphylococcal strain was approximately 10^6 cfu/g.

Each meat mixture portion, weighing 115 g and containing NaCl along with either $NaNO_2$ or various staphylococcal strains, was transferred into glass jars. The jars, covered with two layers of blotting paper and secured with a rubber band, were used to simulate the transpiration process characteristic of the curing phase. These jars were then stored in a controlled environment, maintained at a temperature of 22 °C and a relative humidity of 85%. Product analyses were conducted immediately after production and subsequently at 7 and 10 days of storage.

2.5.1. Evaluating pH levels in meat model samples

The pH value of the products was determined using an edge® blu pH meter HI2002-02 (Hanna Instruments, Smithfield, Rhode Island). For each treatment replication, three separate pH measurements were performed.

2.5.2. Assessment of colour attributes in meat models using the CIELab system

The colour evaluation was conducted using the CIELab system, where L^* denotes brightness (ranging from 0, representing black, to 100, indicating white), a* signifies chromaticity in the red-green spectrum (with positive values indicating red and negative values indicating green), and b* indicates chromaticity in the yellow-blue range (with positive values for yellow and negative values for blue). This analysis was performed using a ColorFlex EZ spectrophotometer (HunterLab, Virginia, USA). The colour components L*a*b* were measured both in the untreated starting meat and in the meat products following 7 and 10 days of storage at 22 °C. Prior to measurement, the blotting papers were removed from the jar tops, and the samples were exposed to air in a laboratory environment at 23 ± 1 °C.

The ΔE value was employed to contrast the colour of the inoculated products with the nitrite-containing meat. The formula $\sqrt{((\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2)}$ was used to calculate the ΔE value.

2.5.3. Microbiological analysis of lab-scale meat preparations

Each sample, weighing 10 g, was placed in a sterile plastic bag and homogenised with 90 mL of peptone saline. This mixture was then agitated in a BagMixer 400 P (Interscience, Osaka, Japan) for 4 min. Subsequent decimal dilutions were prepared, and the staphylococci count on MSA (Oxoid, Italy) was ascertained. The incubation of the plates was conducted at 37 °C for 48 h. Upon completion of the incubation, colony counts were performed, and the results were expressed as the number of colony-forming units per gram (cfu/g).

2.5.4. Targeted and untargeted UHPLC-HRMS profiling of key metabolites

Each sample, weighing 1 g, was placed into a conical test tube containing 10 mL of a 50% ethanol solution. The samples were then homogenised using a Polytron™ PT1200E homogeniser (Kinematica, Malters, Switzerland), followed by centrifugation at 5500 rpm for 15 min. Subsequently, the samples were filtered through 0.20 μm regenerated cellulose syringe filters. The concentrations of L-arginine, L-citrulline, and L-ornithine were then determined using an Orbitrap mass analyser. Specifically, a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific) coupled with a Vanquish UHPLC pump and equipped with a heated electrospray ionisation-II probe (Thermo Scientific) was employed for this analysis.

Individual standard stock solutions of each compound, obtained from Sigma-Aldrich, were prepared at a concentration of 1000 μg/L. This preparation involved dissolving each standard in 0.1% HCl water, with the solutions subsequently stored under dark conditions at 4 °C until required for further analysis. In the process of refining the UHPLC-HRMS method, mixed standard working solutions were formulated at seven concentration levels, ranging from 1 to 500 μg/L, using 0.1% formic acid in water as the solvent. The mass-to-charge (m/z) transitions for each compound were as follows: L-arginine (parent ion: 175.1190 m/z; fragment ion: 60.0568 m/z; retention time: 1.23 min); L-citrulline (parent ion: 176.1030 m/z; fragment ion: 113.0718 m/z; retention time: 1.45 min); and L-ornithine (parent ion: 133.0972 m/z; fragment ion: 90.0560 m/z; retention time: 1.31 min). All compounds were fragmented using an optimised collisional energy of 15 eV (Supplementary Material).

Chromatographic separation was conducted under isocratic conditions, employing a mixture of 50:50 v/v water:acetonitrile (both acidified with 0.1% formic acid) over a 12-min period. This was performed using an ACQUITY Premier Peptide CSH C18 Column (130 Å, 1.7 μm, 2.1 × 150 mm, 1/pk). The flow rate was set to 200 μL/min, with an injection volume of 6 μL. A full-scan mass spectrometry analysis was conducted in the 50–250 m/z range, using a positive ionisation mode and a mass resolution of 70,000 at m/z 200. The automatic gain control target and the maximum injection time settings were 1×10^6 and 200 ms, respectively. The heated electrospray ionisation parameters included a sheath gas flow of 35 arbitrary units, an auxiliary gas flow of 15 arbitrary units, a spray voltage of 3.5 kV, and a capillary temperature of 320 °C. Prior to data collection, the mass spectrometer was calibrated with Pierce™ LTQ ESI Positive Ion Calibration Solution (Thermo Fisher Scientific).

Additionally, to evaluate the untargeted profiles of low-molecular-weight metabolites related with quality and potential sensory issues, the raw mass features were elaborated using the software MS-DIAL (version 4.90) (Tsugawa et al., 2015). Automatic peak finding, LOWESS normalization, and annotation via spectral matching against the comprehensive database FooDB (https://www.foodb.ca, accessed: April 2024) were performed. The mass range 50–250 m/z was searched for features with a minimum peak height of 10,000 cps. The MS tolerance for peak centroiding and accurate mass tolerance was set to 0.05. The identification step was based on mass accuracy, isotopic pattern, and spectral matching. Gap filling using peak finder algorithm was performed to fill in missing peaks, considering 5 ppm tolerance for m/z values. Under our experimental conditions, a level 3 of confidence in annotation (Blaženović et al., 2018) was achieved.

2.6. Statistical analysis of experimental data

The results were subjected to a one-way analysis of variance (ANOVA), followed by Duncan's post hoc test ($p < 0.05$), utilising IBM SPSS Statistics software (version 28.0). The findings are presented as mean values based on three independent replicates ($n = 3$) ± standard deviation. The metabolomics dataset was elaborated through both unsupervised and supervised multivariate analyses using the software MetaboAnalyst 6.0. Two statistical approaches were used, namely hierarchical cluster analysis (HCA, Euclidean distance) and orthogonal projections to latent structures discriminant analysis (OPLS-DA). The OPLS-DA prediction model was also inspected for its prediction ability, permutation testing, and absence of outliers. Finally, the variables

importance in projection (VIP) approach was used to select discriminant metabolites, considering as a minimum threshold a VIP score >1.

3. Results and discussion

3.1. Evidence for nos gene expression

The investigation revealed a notable prevalence of the *arcA* gene in *S. carnosus* and *S. xylosus* species, whereas the arg gene was more frequently observed among strains of *S. saprophyticus*, *S. equorum*, and *S. xylosus*. Contrastingly, the *nos* gene was primarily found in *S. saprophyticus* and *S. carnosus* species. Notably, as illustrated in Table 1, the arg gene emerged as the most prevalent among the tested L-arginine degradation determinants. A total of 25 strains, encompassing 11 *S. xylosus* (84.6%), all nine *S. saprophyticus* (100%), and all five *S. equorum* strains (100%), were identified to carry this gene. In agreement with those previously reported by Sánchez Mainar et al. (2014), none of the six *S. carnosus* tested possessed the arg gene. Furthermore, the ADI-encoding *arcA* gene was detected in 14 out of 35 CNS strains investigated, including one *S. saprophyticus* strain (DSM 20229) (11.1%), eight *S. xylosus* strains (61.5%), and all *S. carnosus* strains (except 24 S) (83.3%). These findings are in line with those reported by Janssens et al. (2014), who conducted tests on six strains of *S. carnosus* and eight strains of *S. saprophyticus* to determine the presence of the *arcA* gene. Their study revealed that all the *S. carnosus* strains and only two of the *S. saprophyticus* strains contained the *arcA* gene in their genomes. Additionally, Sánchez Mainar et al. (2014) also found the *arcA* gene in all 13 *S. carnosus* strains they analysed.

In our study, the *nos* gene was detected in 16 staphylococcal strains: eight *S. saprophyticus* (88.9%), six *S. carnosus* (100%), one *S. equorum* (20%), and one *S. xylosus* (7.7%), based on the primer pairs utilised. These results were in accordance with those obtained by Sánchez Mainar et al. (2014), who found that all evaluated *S. saprophyticus* strains possessed the *nos* gene when using the NOS-4 primer, with 66.7% also testing positive with the NOS-2 primer. Our findings showed similar proportions, being 88.9% and 44.4%, respectively. Intriguingly, all *S. saprophyticus* strains isolated from salami possessed this NOS-specific genetic marker when tested with the NOS-4 primer pair. Moreover, *S. saprophyticus* strains FT20E, L30, L31, and L34 were also identified to have this genetic component using the NOS-2 primer.

All strains of *S. carnosus* examined in this study were found to carry the *nos* determinant; however, this was only evident when the PCR

Table 1
Genotypic screening for the presence of *arc*A, *arg*, and *nos* genes.

Species	Strains	Origin	Genes					
			arcA	*arg*	*nos*			
					NOS-1	NOS-2	NOS-3	NOS-4
S. saprophyticus	9		1 (11.1%)	9 (100%)	–	4 (44.4%)	–	8 (88.9%)
	DSM20229	Urine	+	+	–	–	–	–
	FT20E	Piacenza salami without starters	–	+	–	+	–	+
	L30	Argentine llama salami without starters	–	+	–	+	–	+
	L31	Argentine llama salami without starters	–	+	–	+	–	+
	L34	Argentine llama salami without starters	–	+	–	–	–	+
	L43	Argentine llama salami without starters	–	+	–	+	–	+
	L49	Argentine llama salami without starters	–	+	–	–	–	+
	MT20D	Piacenza salami without starters	–	+	–	–	–	+
	V10	Piacenza salami without starters	–	+	–	–	–	+
S. equorum	5		–	5 (100%)	1 (20%)	–	–	–
	L33	Argentine llama salami without starters	–	+	+	–	–	–
	L35	Argentine llama salami without starters	–	+	–	–	–	–
	L36	Argentine llama salami without starters	–	+	–	–	–	–
	L48	Argentine llama salami without starters	–	+	–	–	–	–
	L50	Argentine llama salami without starters	–	+	–	–	–	–
S. carnosus	6		5 (83.3%)	–	–	6 (100%)	–	–
	TM300	Fermented sausages	+	–	–	+	–	–
	09 S	Piacenza salami without starters	+	–	–	+	–	–
	15 S	Piacenza salami without starters	+	–	–	+	–	–
	19 S	Piacenza salami without starters	+	–	–	+	–	–
	24 S	Piacenza salami without starters	–	–	–	+	–	–
	28 S	Piacenza salami without starters	+	–	–	+	–	–
S. xylosus	13		8 (61.5%)	11 (84.6%)	1 (7.7%)	–	–	–
	FT2H	Piacenza salami without starters	–	+	–	–	–	–
	FT8B	Piacenza salami without starters	+	+	–	–	–	–
	FT8C	Piacenza salami without starters	–	–	–	–	–	–
	FT40C	Piacenza salami without starters	–	+	–	–	–	–
	L5	Argentine llama salami without starters	–	+	–	–	–	–
	L16	Argentine llama salami without starters	+	+	–	–	–	–
	L20	Argentine llama salami without starters	+	–	–	–	–	–
	LM2	Piacenza salami without starters	+	+	–	–	–	–
	M1S	Piacenza salami without starters	+	+	–	–	–	–
	MT40A	Piacenza salami without starters	+	+	–	–	–	–
	R6	Piacenza salami without starters	+	+	–	–	–	–
	SN21	Piacenza salami without starters	–	–	–	–	–	–
	SSP2	Piacenza salami without starters	+	+	–	–	–	–
	X100	Fermented sausages	–	+	+	–	–	–
S. warneri	1		–	–	–	–	–	–
	L39	Argentine llama salami without starters	–	–	–	–	–	–

analysis employed the NOS-2 primer pair, aligning with findings previously reported by Sánchez Mainar et al. (2014). In addition, the presence of the *nos* gene was confirmed only in one *S. equorum* strain (L33) and one *S. xylosus* strain (X100), specifically when the NOS-1 primer pair was used in the PCR analysis. Contrary to the observations by Sánchez Mainar et al. (2014), none of the strains subjected to genotypic analysis exhibited the *nos* gene when PCR was employed using the NOS-3 primer pair. Moreover, the *S. warneri* L39 strain, isolated from llama salami, was unique in that it did not exhibit any of the genes investigated in this study.

Based on the results obtained from the genotypic investigation, 11 strains (comprising eight *S. saprophyticus*, one *S. equorum*, one *S. carnosus*, and one *S. xylosus*) possessing the *nos* gene were selected for subsequent experiments. These trials were designed to determine the predominant arginine-catabolic pathway in both the meat simulation medium supplemented with L-arginine and in lab-scale meat models. Notably, among the *nos*-positive *S. carnosus* strains, only the TM300 strain was included in these subsequent trials, in line with its prior use in the research conducted by Sánchez Mainar et al. (2014).

3.2. Analysis of L-arginine degradation in microaerobic conditions

The presence of L-citrulline and L-ornithine in the medium fermented by the investigated bacterial cultures led to the assumption that all 11 strains were capable of degrading the L-arginine (3 g/L) present in the MSM medium within the 72-h examination period. In addition to replicating the analyses conducted by Sánchez Mainar et al. (2014), this study also quantified the residual L-arginine in the fermented media, facilitating further insights. Notably, significant interspecies heterogeneity was observed (Table 2), aligning with the findings of Sánchez Mainar et al. (2014). Specifically, *S. carnosus* TM300 was found to completely utilise the L-arginine in the culture medium within the designated timeframe (leaving a residual amount of 0.05 g/L), while *S. equorum*, *S. xylosus*, and all strains of *S. saprophyticus* demonstrated a slight increase in L-arginine levels. This phenomenon is possibly attributable to the comparatively mild extracellular proteolytic activity of *S. carnosus* (Wagner, Doskar, & Götz, 1998; Wang, Wang, Xia, Sun, & Kong, 2021) and the more pronounced proteolytic activity of the other staphylococcal species, which has been substantiated by numerous studies. The pronounced proteolytic potential of *S. equorum* strains isolated from Spanish dry-cured meats was particularly highlighted by Landeta, Curiel, Carrascosa, Muñoz, and de las Rivas (2013). Additionally, the proteolytic capabilities of 34 *S. saprophyticus* strains were documented by Sun, Cao, Feng, Xu, and Zhou (2019). Furthermore, Sun et al. (2023) affirmed the robust protease activity of *S. xylosus*, underscoring its role in accelerating protein proteolysis in dry sausages.

Table 2
Experimental results on the conversion of L-arginine (beginning with a concentration of 3 g/L) by the investigated strains into L-citrulline and L-ornithine in the meat simulation medium (MSM) after 72 h of microaerobic conditions, as well as the residual L-arginine concentration.

Species	Strain	Genes	L-arginine (g/L)	L-citrulline (mg/L)	L-ornithine (mg/L)
S. saprophyticus	FT20E	*arg*, NOS2-4	4.10 ± 0.01^{e}	17.01 ± 0.40^{b}	8.16 ± 0.13^{c}
S. saprophyticus	L30	*arg*, NOS2-4	3.99 ± 0.01^{cd}	19.00 ± 2.01^{b}	7.96 ± 0.72^{bc}
S. saprophyticus	L31	*arg*, NOS2-4	3.98 ± 0.01^{cd}	16.10 ± 1.01^{b}	9.28 ± 0.59^{e}
S. saprophyticus	L34	*arg*, NOS4	4.01 ± 0.01^{d}	19.01 ± 0.50^{b}	9.41 ± 0.20^{e}
S. saprophyticus	L43	*arg*, NOS2-4	4.04 ± 0.10^{de}	22.30 ± 3.15^{b}	7.04 ± 0.20^{a}
S. saprophyticus	L49	*arg*, NOS4	3.93 ± 0.04^{c}	21.00 ± 2.50^{b}	8.36 ± 1.12^{cd}
S. saprophyticus	MT20D	*arg*, NOS4	4.03 ± 0.01^{de}	15.90 ± 0.95^{b}	7.11 ± 0.39^{ab}
S. saprophyticus	V10	*arg*, NOS4	3.10 ± 0.04^{b}	18.70 ± 0.10^{b}	7.24 ± 0.13^{ab}
S. equorum	L33	*arg*, NOS1	4.30 ± 0.01^{f}	22.01 ± 2.50^{b}	9.22 ± 0.01^{de}
S. carnosus	TM300	*arcA*, NOS2	0.05 ± 0.00^{a}	23.70 ± 0.85^{c}	7.63 ± 0.39^{abc}
S. xylosus	X100	*arg*, NOS1	3.11 ± 0.03^{b}	13.05 ± 0.25^{a}	6.84 ± 0.39^{a}

The results are expressed as mean values ± standard deviation (n = 3). Different superscript letters within each column indicate significant differences determined by a one-way analysis of variance followed by Duncan's post hoc test ($p < 0.05$).

Therefore, considering the inclusion of bacteriological peptone (11 g/L) in the MSM medium used for phenotypic screening in the current research, it appears that the analysed strains initially converted peptone into free amino acids, including L-arginine. Subsequently, a portion of the excess L-arginine was utilised in the NOS pathway to produce L-citrulline, a compound integral to nitric oxide production.

Furthermore, our investigation identified *S. equorum* L33, *S. saprophyticus* L43 and L49, and *S. carnosus* TM300 as the strains exhibiting the highest L-citrulline production (22.01, 22.30, 21.00, and 23.70 mg/L, respectively). This increased L-citrulline synthesis is likely linked to the NOS pathway, considering that neither the arginase nor the ADI pathways contribute to L-citrulline release. Contrastingly, the research by Sánchez Mainar et al. (2014) observed L-citrulline presence in the medium fermented under a microaerobic environment by only three out of nine *S. saprophyticus* strains, differing from our results, where all evaluated *S. saprophyticus* strains produced L-citrulline ranging from 15.90 mg/L (MT20D strain) to 22.30 mg/L (L43 strain). Conversely, *S. xylosus* X100, despite possessing the *nos* gene similar to other tested strains, showed the lowest L-citrulline production at 13.05 mg/L. This finding aligns with the observations of Sánchez Mainar et al. (2014), who reported minimal L-citrulline release by a limited number of *S. xylosus* strains under microaerobic conditions. These results underscore the species- and strain-level variability in L-citrulline production among staphylococci.

In the context of metabolic pathways, the ADI reaction catalyses the transformation of L-arginine into L-ornithine, carbon dioxide, and ammonia (Lindgren et al., 2014), whereas the arginase pathway hydrolyses L-arginine into L-ornithine and urea (Xiong et al., 2016). Our findings elucidate the coexistence of both pathways among the staphylococci. Specifically, based on the genotypic analyses, the L-ornithine amounts produced by *S. saprophyticus*, *S. equorum*, and *S. xylosus* (ranging from 6.84 to 9.41 mg/L) appear to be attributable to the arg gene pathway. In contrast, the L-ornithine production by *S. carnosus* TM300 (7.63 mg/L) is likely due to the ADI pathway, as indicated by the presence of the *arcA* determinant.

3.3. Development and analysis of lab-scale meat models

3.3.1. pH analysis and microbiological assessment

The observed decrease in pH values detected across the samples subjected to different treatments was predominantly attributed to the activity of lactic acid bacteria, which are naturally present in meat, rather than CNS. Specifically, the CTR sample (without $NaNO_2$ and inoculum) exhibited a decline in pH from 5.55 (immediately post-production) to 5.13 and 5.14 after 7 and 10 days of storage, respectively (Table 3). Conversely, the pH of samples treated with staphylococcal strains ranged from 5.17 to 5.29 after 7 days of incubation at 22 °C and 85% relative humidity. These values were also comparable to those of the CTRN sample (containing $NaNO_2$), which registered a pH of 5.20. The findings suggest that the fermentation pattern mirrored the process typically observed in salami curing and effectively prevented over-acidification, a known causative factor for meat colour defects (Aro

Table 3
Results from experiments conducted on the pH levels and staphylococcal counts in various lab-scale meat model treatments.

Treatment	Strain	pH values			Staphylococcal count (cfu/g)		
		0 days	7 days	10 days	0 days	7 days	10 days
CTR	–	5.55 ± 0.03	5.13 ± 0.02	5.14 ± 0.00	$< 10^4$	1.80×10^6	7.40×10^5
CTRN	–	–	5.20 ± 0.01	5.21 ± 0.01	$< 10^4$	9.60×10^5	9.00×10^4
S. saprophyticus	FT20E	–	5.17 ± 0.01	5.20 ± 0.01	3.20×10^6	2.87×10^7	7.00×10^6
S. saprophyticus	L30	–	5.29 ± 0.01	5.32 ± 0.02	4.25×10^6	9.40×10^7	5.25×10^7
S. saprophyticus	L31	–	5.20 ± 0.02	5.24 ± 0.01	4.38×10^6	9.35×10^7	3.50×10^7
S. saprophyticus	L34	–	5.27 ± 0.01	5.30 ± 0.01	5.35×10^6	1.86×10^7	1.00×10^7
S. saprophyticus	L43	–	5.27 ± 0.01	5.29 ± 0.01	4.28×10^6	3.41×10^8	8.30×10^7
S. saprophyticus	L49	–	5.28 ± 0.01	5.27 ± 0.01	6.80×10^6	2.55×10^8	7.25×10^7
S. saprophyticus	MT20D	–	5.23 ± 0.02	5.27 ± 0.01	9.30×10^6	3.78×10^8	1.15×10^7
S. saprophyticus	V10	–	5.21 ± 0.01	5.24 ± 0.01	6.65×10^6	4.00×10^8	7.20×10^7
S. equorum	L33	–	5.23 ± 0.01	5.27 ± 0.01	6.10×10^6	8.90×10^7	5.10×10^7
S. carnosus	TM300	–	5.17 ± 0.02	5.19 ± 0.01	2.12×10^6	3.10×10^8	4.00×10^6
S. xylosus	X100	–	5.28 ± 0.02	5.23 ± 0.01	7.95×10^6	3.99×10^8	4.00×10^7

CTR: meat batter containing only 2.5% NaCl; CTRN: meat batter containing 2.5% NaCl and cured with 150 mg/kg $NaNO_2$.

et al., 2010).

The microbiological analysis primarily focused on monitoring the behaviour of staphylococcal cultures during the storage period of the treated meat models. As shown in Table 3, the initial staphylococcal concentration (t0) was less than 10^4 for both CTR and CTRN, while that of the inoculated strains consistently fell within the range of 10^6 cfu/g, specifically between 2.12×10^6 (*S. carnosus* TM300) and 9.30×10^6 (*S. saprophyticus* MT20D) cfu/g. Bacterial counts on MSA after 7 days revealed an approximately 1 log increase for *S. equorum* L33 and four strains of *S. saprophyticus* (FT20E, L30, L31, and L34), while a roughly 2 log rise was noted for the remaining strains. Furthermore, a slight reduction of about 1 log was observed at the 10-day for the inoculated samples, with the exception of *S. carnosus* TM300, which recorded the most significant decrease from 3.10×10^8 cfu/g (at 7 days) to 4.00×10^6 cfu/g (at 10 days). The 7-day staphylococcal concentration of CTR was equal to 1.80×10^6 cfu/g, while that of CTRN was even slightly lower (9.60×10^5 cfu/g), coherently with the antibacterial activity exerted by nitrites. During the following storage period the staphylococcal population gradually decreased, reaching values equal to 7.40×10^5 for CTR and 9.00×10^4 cfu/g for CTRN. These results are in accordance with those reported by Rocchetti, Falasconi, et al. (2021) and Rocchetti, Rebecchi, et al. (2021), which documented the concentration changes in staphylococcal cultures during the fermentation of Italian salami. Moreover, Wang et al. (2022), demonstrated that the staphylococcal concentration present in fermented sausages was lower at the twelfth day of ripening compared to the first stages of the ripening process. This could be associated with the low pH (because of the organic acids produced by the LAB naturally present in meat) and reduced water activity at the final maturation period.

3.3.2. Evaluation of colour attributes in lab-scale meat models

The CTR sample initially showed a redness value (a^*value) of 8.26, which decreased to 6.61 and 6.63 after 7 and 10 days of storage, respectively (Table 4). In contrast, the CTRN sample displayed an increase in a^* value to 12.45 and 12.61 over the same storage periods. Intriguingly, the intensity of the red colour in the resulting products was significantly influenced by the specific strain used (Fig. 1). For instance, *S. saprophyticus* FT20E, *S. carnosus* TM300, and *S. xylosus* X100 did not effectively replicate the pinkish-red hue typical of fermented meat, achieving a^*values of 8.28, 8.42, and 6.02, respectively, after 7 days, closely mirroring the CTR. On the contrary, Li, Luo, Kong, Liu, and Chen (2016) observed good red colour development in the meat batter inoculated with the strain isolated from dry sausages and belonging to *S. xylosus* species. Moreover, Huang et al. (2020) demonstrated that *S. carnosus* strain showed higher colour formation capability than *S. equorum* strain, obtaining a^* value equal to 13.50 in inoculated nitrite-free pork sausages after 6-day fermentation. This result exceeded the red colour of the control sample containing 90 mg/kg of nitrite (a^* value equal to 12.90). Contrarily, our findings are completely different: after a 7-day fermentation period, the colour of the meat batter inoculated with *S. equorum* L33 showed higher a^* values (11.41) than that added with *S. carnosus* TM300 (8.42). Coherently with our results, Sánchez Mainar and Leroy (2015) inoculated pork meat with *S. carnosus* 1505, obtaining similar a^* values between inoculated and uninoculated

Table 4
Results from an experiment examining the colour analysis of lab-scale meat models following 7- and 10-day storage.

Treatment	Strain	a*- (0 days)	a*- (7 days)	a*- (10 days)	7 days				10 days			
CTR	–	8.26 ± 1.39	6.61 ± 0.55^{a}	6.63 ± 0.87^{a}	**ΔL* CTRN**	**Δa* CTRN**	**Δb* CTRN**	**ΔE CTRN**	**ΔL* CTRN**	**Δa* CTRN**	**Δb* CTRN**	**ΔE CTRN**
CTRN	–	–	12.45 ± 0.34^{e}	12.61 ± 0.22^{f}								
S. saprophyticus	FT20E	–	8.28 ± 0.40^{ab}	7.17 ± 0.32^{ab}	2.99	17.38	0.29	4.54	13.25	29.59	0.94	6.62
S. saprophyticus	L30	–	12.16 ± 0.17^{e}	12.79 ± 0.28^{f}	0.79	0.08	1.18	1.43	1.95	0.11	0.19	1.50
S. saprophyticus	L31	–	12.40 ± 0.22^{ef}	12.01 ± 0.51^{e}	0.00	0.00	0.61	0.78	2.78	0.36	4.34	2.73
S. saprophyticus	L34	–	11.41 ± 0.76^{c}	12.02 ± 0.85^{e}	7.47	1.08	0.05	2.93	3.77	0.35	1.93	2.46
S. saprophyticus	L43	–	11.21 ± 0.83^{c}	11.67 ± 0.57^{cd}	6.48	1.53	0.88	2.98	0.44	0.89	0.06	1.18
S. saprophyticus	L49	–	13.33 ± 0.69^{f}	12.59 ± 0.34^{ef}	1.44	0.78	0.10	1.52	0.00	0.00	0.26	0.51
S. saprophyticus	MT20D	–	11.49 ± 0.74^{cd}	10.29 ± 0.67^{c}	1.39	0.92	0.05	1.53	19.73	5.38	1.78	5.19
S. saprophyticus	V10	–	13.00 ± 0.48^{ef}	12.33 ± 0.57^{e}	4.62	0.30	0.25	2.27	4.43	0.08	0.98	2.34
S. equorum	L33	–	11.41 ± 0.56^{c}	12.04 ± 0.84^{e}	0.02	1.08	0.89	1.41	1.12	0.32	2.26	1.92
S. carnosus	TM300	–	8.42 ± 0.17^{b}	8.24 ± 0.57^{b}	0.26	16.24	0.00	4.06	11.44	19.09	1.89	5.69
S. xylosus	X100	–	6.02 ± 0.96^{a}	6.81 ± 0.80^{a}	1.42	41.34	0.50	6.58	0.30	33.64	0.50	5.87

Redness (a*) values are expressed as mean values ± standard deviation (n = 3).
Significant differences within each column, as determined by one-way analysis of variance followed by Duncan's post hoc test ($p < 0.05$), are indicated with different superscript letters.
For each treated meat model, the ΔE values are also presented, which are calculated in relation to the CTRN.
CTR: meat batter containing only 2.5% NaCl; CTRN: meat batter containing 2.5% NaCl and cured with 150 mg/kg $NaNO_2$.

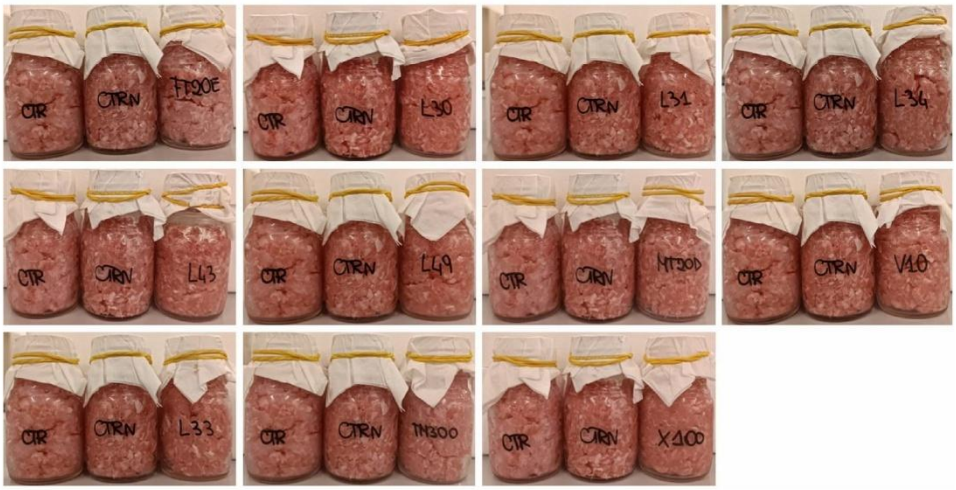

Fig. 1. Effect of meat inoculation with *S. saprophyticus* (FT20E, L30, L31, L34, L43, L49, MT20D, V10), *S. equorum* (L33), *S. carnosus* (TM300), and *S. xylosus* (X100) on the colour appearance of lab-scale meat models after 7-day fermentation. All inoculated meat samples are shown in comparison with CTR (meat batter containing only 2.5% NaCl) and CTRN (meat batter containing 2.5% NaCl and cured with 150 mg/kg $NaNO_2$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

samples (5.96 and 6.05, respectively). They stated that the red colour was achieved after a 3-day fermentation only in the meat models prepared with the inclusion of both *S. carnosus* 1505 and nitrate.

In addition, certain strains, specifically *S. saprophyticus* L31, L49, and V10, demonstrated superior capability in replicating the characteristic red colour of nitrite-cured meat, as evidenced by the highest and statistically significant a^*values. The L49 and V10 treatments, in particular, exhibited a^*values of approximately 13 and 12.5 after 7 and 10 days of storage, while L31 recorded slightly lower values. Moreover, as shown in Table 4, the ΔE values (calculated in comparison to CTRN) were 0.51, 2.34, and 2.77 for L49, V10, and L31, respectively, at the 10-day mark. The most notable results were observed in the L49 treatment, with assessed values after 10 days of incubation being $\Delta E = 0.51$, $\Delta L^* = 0.00$, $\Delta a^* = 0.00$, and $\Delta b^* = 0.26$. This suggests that the colour appearance of the L49 treatment paralleled that of the CTRN sample. The L31 and V10 treatments also achieved a red colour similar to products with added chemical additives. These encouraging findings underscore the potential for using meat-associated CNS strains in the production of low-nitrite meats due to their positive impact on meat colouration, supporting the assertions made by Szymański, Łaszkiewicz, Siekierko, and Kołożyn-Krajewska (2020).

3.3.3. Evaluation of the cross-talk between arginase and NOS activities in lab-scale meat models

Finally, the meat models underwent both a target and untargeted UHPLC-HRMS analysis to determine the presence of L-arginine, L-citrulline, and L-ornithine, together with assessing the accumulation of biogenic amines and aromatic compounds potentially affecting taste and sensory properties of the product. As indicated in Table 5, an increase in L-arginine levels after 7 days could be observed, relative to the CTR (t_0), which varied depending on the proteolytic activity of the staphylococcal strains. Notably, the L49-treated meat model exhibited the highest concentration of L-arginine (979.85 μg/kg). Concerning L-citrulline and L-ornithine, which are products of arginine degradation, a substantial increase from the initial quantities (5.65 and 19.74 μg/kg, respectively) was noted following a 7-day fermentation period. The L-citrulline concentration in the CTR sample (80.22) was lower than in the inoculated models, which ranged from 80.24 to 225.03 μg/kg. Therefore,

Table 5
Results from the experiment show the concentrations of L-arginine, L-citrulline, and L-ornithine in the meat models after 7 days of storage.

Treatment	Strain	Genes	L-arginine (μg/kg)	L-citrulline (μg/kg)	L-ornithine (μg/kg)
CTR (t0)	–	–	431.27 ± 12.37^{a}	5.65 ± 0.75^{a}	19.74 ± 5.60^{a}
CTR (t7)	–	–	896.52 ± 169.06^{d}	80.22 ± 6.34^{bc}	2384.09 ± 331.13^{c}
CTRN (t7)	–	–	984.56 ± 42.06^{d}	136.91 ± 13.76^{e}	3577.11 ± 21.61^{e}
S. saprophyticus	FT20E	*arg*, NOS2-4	578.94 ± 25.97^{abc}	106.08 ± 3.84^{cd}	3190.65 ± 151.61^{d}
S. saprophyticus	L30	*arg*, NOS2-4	890.86 ± 214.65^{d}	137.38 ± 1.38^{e}	1243.93 ± 50.40^{b}
S. saprophyticus	L31	*arg*, NOS2-4	947.81 ± 326.90^{d}	172.07 ± 12.44^{f}	3533.49 ± 196.11^{e}
S. saprophyticus	L34	*arg*, NOS4	508.67 ± 101.75^{ab}	88.49 ± 5.40^{cd}	3596.64 ± 18.33^{e}
S. saprophyticus	L43	*arg*, NOS2-4	844.56 ± 28.95^{cd}	136.24 ± 16.75^{e}	1204.43 ± 121.92^{b}
S. saprophyticus	L49	*arg*, NOS4	979.85 ± 144.61^{d}	225.03 ± 9.94^{g}	5490.11 ± 274.21^{g}
S. saprophyticus	MT20D	*arg*, NOS4	864.51 ± 177.53^{b}	80.70 ± 3.80^{bc}	1301.76 ± 36.16^{b}
S. saprophyticus	V10	*arg*, NOS4	901.99 ± 138.02^{d}	138.16 ± 4.73^{e}	4271.42 ± 18.50^{f}
S. equorum	L33	*arg*, NOS1	763.57 ± 63.71^{bcd}	108.49 ± 21.09^{d}	4337.07 ± 312.70^{f}
S. carnosus	TM300	*arcA*, NOS2	915.49 ± 160.66^{d}	139.38 ± 8.38^{e}	3376.28 ± 329.21^{de}
S. xylosus	X100	*arg*, NOS1	696.48 ± 104.92^{abcd}	80.24 ± 39.41^{bc}	3508.75 ± 0.27^{de}

The results are expressed as mean values ± standard deviation (n = 3).
Different superscript letters within each column indicate significant differences determined by a one-way analysis of variance followed by Duncan's post hoc test ($p < 0.05$).
CTR: meat batter containing only 2.5% NaCl; CTRN: meat batter containing 2.5% NaCl and cured with 150 mg/kg $NaNO_2$.

S. saprophyticus L49 emerged as the most abundant L-citrulline producer (225.03 μg/kg), being this latter an amino acid associated with nitric oxide release. This finding confirms the high $a \times$ value (13.33) and the development of a red colour after 7 days in the meat inoculated with this strain. Also, *S. saprophyticus* L31 and V10 strains revealed significant L-citrulline amounts (172.07 and 138.16 μg/kg, respectively), surpassing the levels in the CTRN sample (136.91 μg/kg), as reflected in their a^* values (12.40 and 13.00, respectively). Conversely, *S. xylosus* X100 produced the lowest amount of L-citrulline (80.24 μg/kg) after 7 days of fermentation, which was consistent with the lowest a^*values (6.02) measured with a colorimeter. The comparison of L-citrulline levels between the CTR samples and inoculated samples after 7 days suggest that the increased L-citrulline levels are related with NOS pathway. This inference is based on the understanding that the arginase pathway transforms L-arginine into L-ornithine and urea, while the ADI pathway converts it into L-ornithine, carbon dioxide, and ammonia. Given the findings from the genotypic analysis, the elevated L-ornithine concentrations observed in meats treated with strains from the *S. saprophyticus*, *S. xylosus*, and *S. equorum* species could be attributed to arginase activity. In contrast, the L-ornithine content in meat treated with *S. carnosus* TM300 is likely a consequence of the ADI pathway.

As the next step, an untargeted metabolomics-based approach was used to provide more insights into the complex cross-talk existing between the arginase and NOS activities. As a general consideration, 262 metabolites were putatively identified using their MS1 isotopic profiles against the comprehensive FooDB, with a marked enrichment of amino acids (e.g., glycine, alanine, proline, histidine, lysine, leucine, and valine), small peptides and analogues, followed by polyamines (e.g., tyramine, putrescine, norspermidine, and spermidine), carbonyl compounds (such as 1,3-propanedial), fatty acids and their derivatives. A summarizing table containing all the annotated metabolites together with their relative abundance values and spectral information is provided as supplementary material. The following unsupervised hierarchical clustering analysis (HCA) clearly discriminated CTR and CTRN samples after 7 days *vs* the other inoculated meat samples; in particular, the HCA heat map (supplementary material) showed two main subclusters according to the measured metabolites (m/z range: 50–250). On one side, we found V10, X100, L49 and MT20D strains, while on the other side the remaining inoculated samples were outlined, namely FT20E, TM300, L34, L33, L43, L30 and L31. Therefore, the small-molecular-weight-based untargeted profiling confirmed a clear impact of the inoculated strains on the chemical profile of fermented meat.

To this aim the most promising strains in terms of redness preservation, namely V10, L49 and L31, hereinafter referred to "Citrulline-Colour group", were evaluated and compared against the CTRN sample, to find potential marker compounds related with both quality and aromatic profiles of the fermented meat products. To maximize the group-discrimination, a supervised statistical approach based on OPLS-DA was carried out and the corresponding score plot is provided as Fig. 2. Overall, the model showed a high prediction ability ($Q^2 = 0.889$), clearly discriminating the Citrulline-Colour group *vs* the CTRN samples. Interestingly, 48 discriminant VIP marker compounds showed an overall up-accumulation for the Citrulline-Colour group, while 70 metabolites were found to be discriminant markers of the CTRN group (supplementary material). Among the up-accumulated metabolites in the Citrulline-Colour group (Fig. 2) we found putrescine (VIP score: 2.32; $Log_2FC = 5.21$), spermidine (VIP score: 2.46; $Log_2FC = 2.93$), tyramine (VIP score: 2.05; $Log_2FC = 2.05$), and 1,3-propanedial (VIP score: 1.98; $Log_2FC = 0.40$). Therefore, our preliminary metabolomics findings suggested a higher biogenic amines accumulation in the Citrulline-Colour group, together with a significantly higher lipid oxidation, when compared with CTRN group. Taken together, these findings suggest a higher ability of sodium nitrite to cope with lipid oxidation during 7 days of storage: in this regard, 1,3-propanedial (also known as malondialdehyde, MDA) is a beta-dicarbonyl and highly reactive compound occurring as a marker for oxidative stress, as a result of lipid peroxidation of polyunsaturated fatty acids (Domínguez et al., 2019). Therefore, the higher oxidative stress combined with the acidic

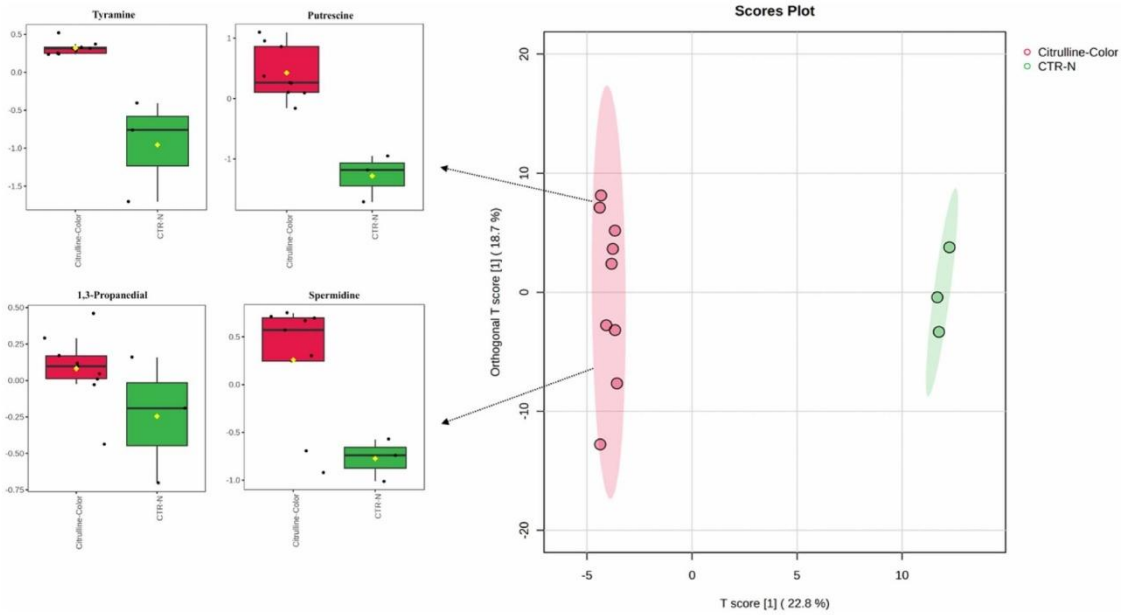

Fig. 2. OPLS-DA score plot outlining the discrimination between "Citrulline-Colour group" (i.e., V10, L49 and L31 strains) *vs* the CTRN sample (i.e., added with sodium nitrite). The up-accumulation of some key metabolites dealing with biogenic amines (i.e., tyramine, putrescine, and spermidine) and lipid peroxidation (1,3-propanedial) is also graphically provided. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

environment of the meat (pH 5–5.3) could have contributed to observe the strong up-accumulation of MDA and the highlighted biogenic amines (Fig. 2). As far as the accumulation of polyamines is concerned, their de novo synthesis begins with the formation of putrescine from the amino acid ornithine by decarboxylation. Putrescine is then converted to spermidine and spermine by other enzymatic reaction (Munoz-Esparza et al., 2019). Overall, looking at our findings, it is possible to hypothesize that CNS strains possessing both arg and *nos* genes (such as L49 and V10) promote on one side the conversion of arginine in NO (likely related with an improved redness of the product); however, on the other side, the conversion of arginine in ornithine could also lead to the accumulation of putrescine and spermidine, potentially involved in off-flavours and quality loss of the product (Fig. 2). In this regard, biogenic amines represent an important index for product stability and quality (Schirone et al., 2022); therefore, further studies are required to better evaluate the changes of sensory quality of the fermented products and/or the best combination of different strains to cope with an increased oxidative stress and biogenic amine production.

Taken together, the set of genotypic and phenotypic analyses conducted in the present study allowed us to demonstrate the correlation between high L-citrulline production and significant colour development in inoculated meat samples. However, future works are warranted to evaluate the spectrum of biogenic amines and aromatic compounds potentially contributing to spoilage conditions of the fermented meat product.

4. Conclusions

This research shows that meat colouration is positively influenced when the natural meat microflora is enriched with specific coagulase-negative staphylococci having nitric-oxide synthase activity. Specifically, the utilisation of *S. saprophyticus* L49, V10, and L31 facilitated the attainment of a* values equal to or surpassing those of the nitrite-containing sample produced with the addition of 150 mg/kg of sodium nitrite. The promising results of the colour attributes of inoculated meat samples were supported and confirmed by the analytical amount of L-arginine, L-citrulline, and L-ornithine detected in the lab-scale meat models. Consequently, this study demonstrates the potential of using staphylococcal strains, selected for their NOS activity, as viable alternatives to nitrate and nitrite in the production of derived meat products. However, further research is imperative to thoroughly evaluate the safety aspects of using staphylococci as starter cultures. Also, our preliminary findings suggest a complex cross-talk between arginase and NOS activites, leading to the concurring accumulation of biogenic amines. The absence of genes responsible for transferable antibiotic resistance must be investigated, as well as defining the impact of these cultures on the sensory profile of the fermented meat product.

5. Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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CRediT authorship contribution statement

Lara Premi: Writing – original draft, Visualization, Investigation, Formal analysis. **Gabriele Rocchetti:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Chiara Rossetti:** Methodology, Formal analysis. **Michele Dallolio:** Methodology. **Luigi Lucini:** Writing – review & editing, Supervision. **Annalisa Rebecchi:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors have declared no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116333.

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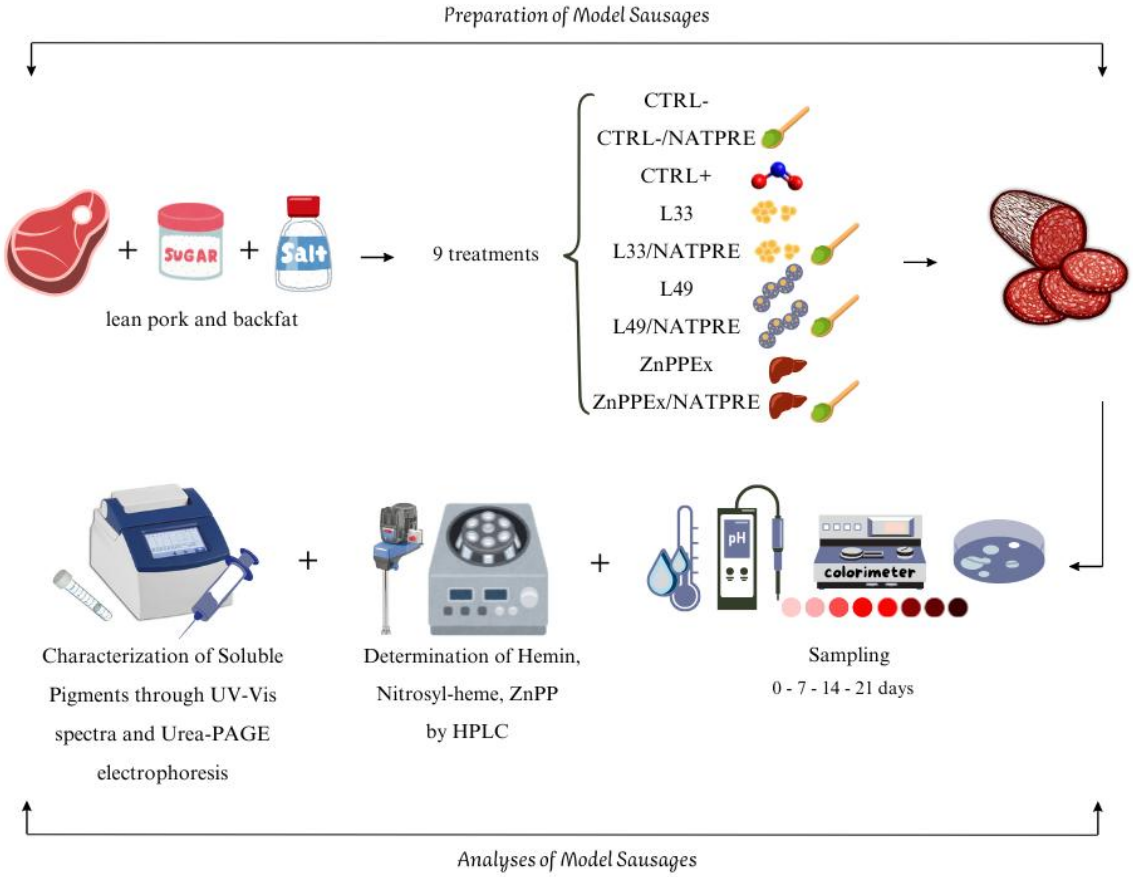
3. <u>Chapter 3</u>: Selected *Staphylococcus spp.* and ZnPP-containing liver extract are nitrite-free alternatives to enhance red colour of dry-fermented sausages

Graphical abstract



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Selected *Staphylococcus* spp. and ZnPP-containing liver extract are nitrite-free alternatives to enhance red colour of dry-fermented sausages

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ABSTRACT

As an alternative to adding pure nitrite sources to dry-fermented sausages, we examined three different strategies involving the addition of two meat-associated coagulase-negative staphylococci (CNS) showing nitric-oxide synthase (NOS) activity, a porcine liver extract rich in the pigment zinc protoporphyrin (ZnPP), and a polyphenol-rich ingredient (NATPRE T-10 CUR HT) alone or in combination with the other strategies. We studied the colour development of nine dry-fermented sausages originating from a meat batter control without nitrite (CTRL-), control with nitrite (CTRL+), inoculated with *Staphylococcus equorum* (L33), inoculated with *Staphylococcus saprophyticus* (L49), formulated with a ZnPP-rich extract (ZnPPEx), formulated with NATPRE alone (CTRL-/NATPRE) or in combination with the other strategies (L33/NATPRE, L49/NATPRE, ZnPPEx/NATPRE, respectively). The colour was affected by the treatment, ripening period, and their interaction ($P < 0.001$). After ripening, all treatments resulted in good colour when compared to the CTRL-. Nitrosyl-heme was always detected, but the CTRL+ treatment and those involving residual nitrite sources through the addition of NATPRE recorded higher values than the other samples, which produced similar results. Treatments involving the addition of the ZnPP-rich ingredient resulted in high ZnPP levels. The aqueous extracts showed the presence of soluble ZnPP forms in the ZnPPEx and ZnPPEx/NATPRE sausages, whereas the addition of nitrite and NATPRE resulted in a loss of myoglobin solubility. The mechanism of NOS-positive staphylococcal strains to maintain colour is unclear, but L49 was able to maintain reduced oxymyoglobin until the end of ripening. These findings reveal the potential of these strategies to promote colour in dry-fermented meats.

1. Introduction

Chemically pure nitrates and nitrites are synthetic additives widely used in the manufacturing of cured meat products. They primarily contribute to develop the characteristic flavour and pinkish-red colour due to the formation of nitrosomyoglobin (Huang, Luo, Li, & Xu, 2022), prevent lipid oxidation, and inhibit the growth of pathogens and undesirable microorganisms (Premi, Rocchetti, Lucini, Morelli, & Rebecchi, 2024; Skibsted, 2011). However, these meat curing agents appear to be involved in the production of N-nitroso compounds (NOCs) such as N-nitrosamines (Flores & Toldrá, 2021), genotoxic substances possibly causing human gastrointestinal cancer (Schrenk et al., 2023). Moreover, nowadays, consumers are concerned about the use of chemical additives, and they are moving towards clean-label products (Inguglia, Song, Kerry, O'Sullivan, & Hamill, 2023). In addition, the recent legislative modifications by the Commission Regulation (EU) 2023/2108 have decreased the maximum amount of permitted levels of added nitrites and nitrates in meat products and have established maximum residual levels of nitrites in order to mitigate consumer exposure to carcinogenic N-nitrosamines. Consequently, there has been an increase in the

Abbreviations: a_w, water activity; CNS,, coagulase-negative staphylococci; LAB,, lactic acid bacteria; NATPRE,, NATPRE T-10 CUR HT; NOS,, nitric oxide synthase; RBC,, red blood cell; RH,, relative humidity; ZnPP,, zinc protoporphyrin; ZnPPEx,, zinc protoporphyrin extract.

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investigation of natural substitutes of nitrite, mainly to produce the characteristic red colour of dry-cured meats.

Different nitrite-free alternatives have been reported in the literature (Delgado-Pando, Ekonomou, Stratakos, & Pintado, 2021; Shakil et al., 2022; Zhang et al., 2023). Among them, coagulase-negative staphylococci (CNS) having a nitric oxide synthase activity (NOS) gained lots of interest. In this regard, Premi et al. (2024) demonstrated that some NOS-positive CNS strains isolated from fermented meat products can develop the characteristic cured colour in nitrite-free lab-scale meat models. Besides their colour enhancement activity, CNS are also effective in producing aromatic compounds (Chen et al., 2021; Sallan, Kaban, & Kaya, 2022) and limiting oxidation processes through their catalase activity (Heo, Lee, & Jeong, 2020). Another interesting strategy involves the usage of zinc protoporphyrin (ZnPP), a red-purple complex originating from heme proteins, as a colour enhancer in meat-based products (Schivazappa et al., 2024; Wakamatsu, Kawazoe, Ohya, Hayakawa, & Kumura, 2020). In this respect, Llauger et al. (2024) proved that the ZnPP extracted from porcine liver positively affects the colour of meat products; therefore, it can be used as a food-grade colouring ingredient contributing to the development of meat products without nitrites. Other opportunities in the reformulation of meat products consist in the use of phenolics and plant extracts rich in phenolic compounds (Hernández et al., 2021; Honda, Miura, Masuda, & Masuda, 2016; Wu, Yin, Xiao, Zhang, & Richards, 2022). In this regard, a blend of fruit and spice extracts which usage has been recently investigated is NATPRE T-10 CUR HT (hereafter simply referred as NATPRE). Hernández et al. (2021) assessed the colour of nitrite-containing cooked hams with those formulated with a similar NATPRE product (NATPRE T-10 HT S at concentrations of 5, 10, and 20 g/kg), demonstrating that no significant differences in colour were found between them. These authors hypothesized that the colour of uncured hams was enhanced by the antioxidant capacity of polyphenols, which limits the formation of metmyoglobin. This reasoning was supported by the lack of detection of residual nitrite amount in the hams and by the determination of generated nitrous oxide (N_2O) by GC–MS. However, vegetables and vegetable extracts use to have elevated amounts of nitrates (Zhong et al., 2022). Accordingly, it is relevant to investigate the ingredient nitrite and nitrate levels as well as the presence of nitrosyl-heme from haemoproteins in the final product.

Starting from this background scenario, the objective of this research work was to explore the use of NOS-positive CNS strains, liver extracts rich in ZnPP, and NATPRE, alone and in combination, for creating red-coloured nitrite-free dry-fermented model sausages. To that aim, nine different treatments of model sausages were prepared and ripened, and their colour was investigated together with technological microbiota counts, a_w, pH, moisture content, the amount of hemin, nitrosyl-heme and ZnPP, the analyses of aqueous extracts and urea-PAGE electrophoresis.

2. Materials and methods

2.1. Staphylococcal strains

For this investigation, *Staphylococcus equorum* L33 and *Staphylococcus saprophyticus* L49 were utilised. These two NOS-positive CNS strains were isolated from fermented meat products, originated from the laboratory collection at Università Cattolica del Sacro Cuore (Cremona, Italy), and previously tested in the study conducted by Premi, Rocchetti, Rossetti, et al. (2024). The strains were preserved at −80 °C in brain heart infusion (BHI) broth (Scharlau, Sentmenat, Barcelona, Spain), supplemented with 20 % (vol/vol) glycerol as a cryoprotectant.

2.2. Preparation of the porcine liver extract having a high zinc protoporphyrin content

Autolyzed porcine liver homogenate (160 g), obtained as described by Llauger et al. (2023), were added to 640 mL of acetone to extract porphyrins (VWR International, Radnor, Pennsylvania, United States). This mixture was stirred for 15 mins at 4 °C in subdued light conditions. Then, the mixture was centrifuged (Avanti JXN-30 centrifuge model, Beckman Coulter, Brea, California, United States) at 30,000 ×*g* for 15 mins at 4 °C. After centrifugation, the supernatant was transferred into a rotary vacuum evaporator (Büchi R 114 model, Büchi corporation, New Castle, United States) until the evaporation of acetone was achieved. The remaining liquid containing ZnPP was then filtered using a folded qualitative filter paper n° 315 (VWR International). Then, 170 mL of phosphate buffer (50 mM; pH 7) and 630 μL of haemolyzed red blood cell (RBC) fraction was incorporated into the resulting mixture to obtain a 1:4 M ratio between ZnPP and added heme, respectively.

The RBC fraction was obtained from commercial blood from a local slaughterhouse that contained tripolyphosphate solution (0.4 %, *w/v*) as an anticoagulant. Blood was centrifuged at 2540 ×*g* for 15 mins at 5–10 °C, and the cellular fraction was diluted 1:1 with MilliQ water. After 30 mins under stirring, the RBC fraction was centrifuged at 20,900 ×*g* for 30 mins at 15–20 °C to remove erythrocytic stroma. After stirring 3 h at 4 °C, the aqueous mixture containing the RBC fraction and the extract rich in ZnPP was lyophilised using a freeze-drying equipment (Coolvacuum Technologies, Granollers, Barcelona, Spain). The heme content of the RBC fraction was calculated using the molar extinction coefficient of 7.12 measured at 523 nm (Snell & Marini, 1988).

The amount of ZnPP in the fresh autolyzed homogenate and in the lyophilized extract was ascertained by measuring its fluorescence using the Thermo Scientific Varioskan® Flash (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with excitation at 416 nm and emission at 588 nm. To do so, sample aliquots (1 g and 20 mg for autolyzed fresh homogenate and lyophilized extract, respectively) were extracted with 80 % (*v/v*) aqueous acetone solution, shaken for 30 s using a vortex mixer, and centrifuged using the 5430 R centrifuge (Eppendorf, Hamburg, Germany) at 4000 rpm for 15 mins at 4 °C.

2.3. Preparation of model sausages

A total of 10 kg of lean pork and backfat (80:20) was cut, minced, and mixed. 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 study comprised 9 distinct treatments of meat batter, each executed in triplicate: the control (CTRL-), consisting solely of meat batter added with sodium ascorbate ($C_6H_7NaO_6$, 0.05 %); the nitrite-cured meat batter (CTRL+), prepared with sodium ascorbate (0.05 %) and 150 mg/kg of sodium nitrite ($NaNO_2$); and 7 additional treatments (CTRL-/NATPRE, L33, L33/NATPRE, L49, L49/NATPRE, ZnPPEx, and ZnPPEx/NATPRE) prepared as described in Table 1. The staphylococcal strains were inoculated at a level of approximately 6 log CFU/g. On the other hand, the treatments involving the addition of ZnPP extract (referred as ZnPPEx) were formulated to contain 40 mg ZnPP/kg in the meat batter, while those marked with "NATPRE" were added with a 1 % of NATPRE T-10 CUR HT (Prosur, Murcia, Spain), a polyphenol-rich extract, which also declares to contain 10–12 % ascorbic acid, having a strong antioxidant activity. External analyses performed using the ISO method 6635:1984 showed values of 313 ± 130 mg NO_3^-/kg and 1030 ± 370 mg NO_2^-/kg.

For each treatment, meat batter portions weighing 83 g and containing all the already mentioned ingredients (Table 1) were transferred into custom-made bags made of a Tublin® 05 film (TUB-EX ApS, Tårs, Denmark), an innovative plastic material specifically used for the production of dried meat because of its permeation level (water vapour permeability of 1350 g/50 μ/m^2/24h (7 °C/50 %RH) and oxygen transmission of 744 ccm/24 h (7 °C/50 % RH) and 2156 g/m^2/24 h (23 °C/50 % RH)). Bags were sealed using an EVT-10 model vacuum packaging machine (Technotrip, Terrassa, Barcelona, Spain) and then ripened in a controlled environment, maintained at a temperature of 8 °C and 60–75 % relative humidity for 21 days. Product analyses were performed immediately after preparation and subsequently at 7, 14, and

Table 1
Formulation of model sausages expressed in kg of meat batter.

Treatment	$NaNO_2$ (150 mg/kg)	H_2O (7.7 mL/kg)	Sodium ascorbate (0.5 g/kg)	NATPRE T-10 CUR HT (10 g/kg)	ZnPP extract (40 mg/kg)	*S. saprophyticus* L49 (7.7 mL/kg)	*S. equorum* L33 (7.7 mL/kg)
CTRL-	−	+	+	−	−	−	−
CTRL-/ NATPRE	−	+	−	+	−	−	−
CTRL+	+	+	+	−	−	−	−
L33	−	−	+	−	−	−	+
L33/NATPRE	−	−	−	+	−	−	+
L49	−	−	+	−	−	+	−
L49/NATPRE	−	−	−	+	−	+	−
ZnPPEx	−	+	+	−	+	−	−
ZnPPEx/ NATPRE	−	+	−	+	+	−	−

21 days of ripening.

The described experiment was repeated two times ($n = 2$) in independent trials (replicates) using two batches of meat and ZnPP extracts. Analytical determinations were performed in triplicate.

2.3.1. Evaluating pH levels in model sausages

The pH value of the products was determined using a Crison pH 25 m equipped with a puncture electrode (5232 Xerolit type, Crison Instruments™, Alella, Barcelona, Spain). For each treatment replication, three separate pH measurements were performed after calibration with commercial standard buffer solutions (pH 4.01, pH 7.00, and pH 9.21) and temperature compensation.

2.3.2. Evaluating water activity in model sausages

The a_w of the meat samples was determined using an AQUALAB 4TE (Aqualab, Sabadell.

Barcelona, Spain). For each treatment replication, three a_w measurements with ±0.003 accuracy were conducted.

2.3.3. Evaluating moisture levels in model sausages

Moisture was determined by drying 5 g of samples to constant weight for 24 h in a VWR® DRY-Line® natural convection oven (VWR International) at 102 ± 2 °C. For each treatment replication, three determinations of the moisture level were carried out.

2.3.4. Assessment of colour attributes using the CIELab system

The colour evaluation was performed using a CM-600D spectrophotometer (Konica Minolta, Chiyoda-ku, Tokyo, Japan) through the CIELab space colour. The instrument, having a fixed 8 mm aperture, was calibrated using a white calibration plate and used with a measurement time of approximately 2 s, under an illuminant D65 and a 10° standard observer, within the wavelength range of 400–700 nm. The colour components $L^*a^*b^*$ were measured after the model sausages production, and after 7, 14, and 21 days of ripening at 8 °C. The measurements were carried out in triplicate on the surface of one-centimeter-thick slices of approximately 1.5 × 5 cm of calibre immediately after removal of the outer plastic casing.

2.3.5. Microbiological analysis of meat preparations

Each sample, weighing 10 g, was placed in a sterile plastic bag and 90 mL of peptone saline were added. This mixture was then homogenized in a SMASHER® Sample Blender (bioMerieux, Marcy-l'Étoile, France) for 60 s. Subsequent decimal dilutions were prepared, and the staphylococci count on MSA (Sigma-Aldrich, Darmstadt, Germany) and the lactic acid bacteria (LAB) count on MRS agar (ISO 15214:1998) (Sigma-Aldrich) were ascertained. The incubation of the plates was conducted at 30 °C for 48 h in aerobic conditions for MSA, and for 72 h in anaerobic conditions for MRS. Upon completion of the incubation, colony counts were performed, and the results were expressed as log CFU/g.

2.3.6. Determination of hemin, nitrosyl-heme, and ZnPP in model sausages by HPLC

Quantitative determination of two derivatives of heme (hemin, nitrosyl-heme), and ZnPP was carried out in duplicate under subdued light conditions and using an 80 % (*v*/v) aqueous acetone solution containing 0.01 % L-cysteine. In brief, 2 g of samples were weighed into amber 50-mL capacity centrifuge tubes and homogenized using an UltraTurrax T25 model disperser (IKA Werke GmbH & Co. KG, Staufen, Germany) for 30 s at 13,500 rpm with 10 mL of the solvent mixture while the tube was immersed in ice. After extraction in ice for 20 mins and centrifugation at 4000 rpm for 15 mins at 4 °C, the supernatant was filtered through a quantitative circular filter paper No.42 (Whatman, Little Chalfont, Buckinghamshire, United Kingdom) and collected into a 10 mL amber volumetric flask. The obtained product was then filtered using a 0.22 μm nylon syringe filter (Teknokroma Analítica S.A., Sant Cugat del Vallès, Barcelona, Spain) and transferred into a HPLC vial. The chromatographic separation and quantification were as described elsewhere (Bou et al., 2024). Hemin and nitrosyl-heme results were expressed as mg of hemin chloride equivalent per kg dry weight matter. ZnPP was expressed as mg per kg of dry matter.

2.3.7. Characterization of soluble pigments through UV–vis spectra and urea-PAGE electrophoresis

Two grams of sample were weighed in tubes and homogenized with 10 mL of milliQ water using the model disperser for 2 mins at 10,000 rpm while the tube was immersed in ice. The samples were then centrifuged at 38,900 ×*g* for 30 mins at 4 °C. The supernatant was filtered through 0.22 μm cellulose acetate syringe filters and the absorbance was measured from 350 to 650 nm (UV1800; Shimadzu, Kyoto, Japan). An aliquot (20 μL) was taken, and soluble proteins were separated using urea-PAGE, as previously described (Wang, Hayakawa, Kumura, & Wakamatsu, 2021) to gain information about the binding of insoluble porphyrins with proteins. Briefly, filtered supernatants were individually mixed in the following proportions: 29 % sample, 50 % sample buffer (50 mM Tris-HCl at pH 6.8 and 8 M urea), 20 % glycerol, and 1 % 2-mercaptoethanol.

The hand-cast gel was prepared using a 4.5 % stacking gel (4.5 % acrylamide, 4 M urea, 125 mM Tris HCl, pH 6.8) and a 10 % separating gel (10 % acrylamide, 4 M urea, 375 mM Tris HCl, pH 8.8). The electrophoresis was run at 10 mA for 30 mins followed by 20 mA for approximately 180 mins at 4 °C. NeoPRO 10 Prestained Protein Ladder (6.5–270 kDa, Neo-Biotech, France) was used as a molecular weight marker. After electrophoresis, the gel was irradiated with a 420 nm purple light-emitting diode light source (OSSV5111A, OptoSupply Co. Ltd., Hong Kong, China). Fluorescent images were captured using a digital camera equipped with a 600 nm bandpass filter (BPB-60, Fujifilm Corp., Tokyo, Japan). To detect protein bands, the gel was stained with 0.1 % Coomassie Brilliant Blue solution (40 % methanol, 10 % acetic acid) for 10 mins and then destained with a solution containing 10 % methanol and 7 % acetic acid overnight.

2.4. Statistical analysis of experimental data

The experiment was fully replicated two different times using two batches of model sausages. The results were expressed as mean ± standard error of the mean (SEM). All 216 samples deriving from the two experimental trials (3 samples per treatment × 9 treatments × 4 ripening periods × 2 replicates) were included in the statistical analysis. The treatment and ripening were considered as fixed variables, manufacture repetition as random effect and the interaction between the fixed variables (ripening period × treatment) was also considered. When the interaction was not significant it was excluded from the model. Microbiological counts, pH, a_w, moisture content, hemin, nitrosyl-heme, ZnPP, and the colour parameters were considered dependent variables. The results were subjected to two-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference post hoc test ($P < 0.05$), utilising JMP Pro 17 statistics software.

3. Results and discussion

3.1. Analysis of model sausages

3.1.1. Microbiological assessment

As shown in Table 2, the different treatments, ripening period, and their interaction affected staphylococci and LAB counts ($P < 0.001$).

Staphylococcal counts were initially in between 4.2 and 4.8 log CFU/g in the non-inoculated samples. On the other hand, the initial concentration of CNS-inoculated meat consistently fell within the range of 6.0 and 6.3 log CFU/g, with significant differences in staphylococcal counts between treatments ($P < 0.001$). After 7 days of ripening, values exceeding 7 log CFU/g were recorded for all the inoculated model sausages, reaching values close to 8 after 14 days and remaining constant until the end of the ripening period. In contrast, levels ranging from 6.3 to 6.6 log CFU/g were recorded after 21 days of ripening for the other treatments, except for the CTRL+ treatment, which was characterised by the lowest staphylococcal concentrations (5.3 log CFU/g).

With regards to LAB, all the treatments revealed values lower than 3 log CFU/g after meat batter preparation (Table 2). As ripening proceeded, the LAB counts increased, reaching values between 3.4 log CFU/g (for CTRL+) and 5.4 log CFU/g (for L33) after 7 days of ripening, and values between 4.9 log CFU/g (for CTRL+) and 6.5 log CFU/g (for CTRL-) after 14 days of ripening, reflecting notable differences in LAB concentrations among samples ($P < 0.001$). Levels ranging from 6.0 log CFU/g (for L49/NATPRE) to 6.3 log CFU/g (for L33) were registered at the end of ripening, except for CTRL+ sample, which was characterised by the lowest value (4.2 log CFU/g) likely due to the inhibitory action exerted by nitrite. Interestingly, the polyphenol-rich ingredient influenced the staphylococcal and lactic growth in L33/NATPRE and L49/NATPRE treatments, as the counts were consistently lower than their homologues, while it did not affect the microbiological growth in the ZnPP-containing sample, since very similar values were found between ZnPPEx and ZnPPEx/NATPRE treatments.

3.1.2. pH, moisture content, and water activity

A significant interaction between factors was found for pH values (Table 3). The initial pH-value of the meat batter used for the model sausages preparation ranged from 5.56 to 5.78. Notably, the model sausages treated with NATPRE showed slightly lower initial pH-values than the corresponding treatments without this extract. This suggests that NATPRE is able to cause a slight but rapid pH reduction, presumably because the ingredient is featured by a 4 pH-value. A low initial pH-value was also recorded for the ZnPP-containing treatments. This finding can be attributed to the residual amounts of acetic acid added during the preparation of the ZnPP homogenate (Llauger et al., 2023). The treatment also determined the final pH-value. In the CTRL- and CTRL+ treatments the final pH was equal to 5.65 and 5.67, respectively. The samples treated with staphylococcal strains were characterised by a slightly lower final value (equal to 5.60 and 5.69 for L33 and L49, respectively). In fact, as previously stated by Premi, Rocchetti, Rossetti, et al. (2024), *S. equorum* L33 and *S. saprophyticus* L49 are not able to cause a significant pH reduction, thus avoiding an unwanted excessive acidification, potentially causing meat colour defects. On the other hand, the ZnPP-containing treatments showed higher pH-values with ripening. Despite these findings help to explain the observed interaction, it is important to highlight that these differences were minimal and none of the treatments can be considered as high-acid products. This is mainly explained by the relatively low amount of glucose added to the formulation, the low levels of LAB in pork, and the low process temperature (Aro et al., 2010).

The model sausages were characterised by a moisture content ranging from 60.3 % to 61.5 % at the beginning of the ripening process. The treatment had no effect on moisture content as the same meat batter was used but, as expected, this content significantly decreased during ripening (Table 4). With regards to a_w, being the main hurdle for microbial control in low-acid dry-fermented sausages (Serra-Castelló et al., 2021; Tapia, Alzamora, & Chirife, 2020), showed a significant interaction with values comprised between 0.970 and 0.972 after preparation (Table 3). The decrease of a_w values with longer ripening periods is consistent with moisture loss (35.3–41.4 % after 21 days) as this affects a_w. Remarkably, at the end of the process all treatments were characterised by a_w values ranging from 0.886 to 0.909, values usually found in Southern Europe dry-fermented sausages (Serra-Castelló et al., 2021) and described to inhibit the growth of relevant pathogens (FSIS, 2023).

3.1.3. Determination of hemin, nitrosyl-heme, and ZnPP in model sausages by HPLC

Table 4 shows the hemin, nitrosyl-heme, and ZnPP content in the

Table 2
Results from experiments conducted on the staphylococcal and lactic acid bacteria (LAB) counts (log CFU/g) in various model sausages during ripening.[1]

Parameter	Ripening time (days)	CTRL-	CTRL−/ NATPRE	CTRL+	L33	L33/ NATPRE	L49	L49/ NATPRE	ZnPPEx	ZnPPEx/ NATPRE	SEM	T	R	TxR
Staphylococci (log CFU/g)	0	4.8^{F}	4.5^{D}	4.4^{C}	6.3^{I}	6.0^{H}	6.2^{I}	6.1^{H}	4.3^{B}	4.2^{A}	0.306	<0.001	<0.001	<0.001
	7	6.6^{L}	6.1^{H}	4.7^{E}	7.4^{O}	7.2^{MN}	7.3^{N}	7.2^{M}	6.4^{JK}	6.3^{IJ}	0.291			
	14	6.5^{L}	6.3^{IJ}	5.2^{G}	7.9^{R}	7.8^{Q}	7.9^{R}	7.6^{P}	6.5^{L}	6.4^{JK}	0.314			
	21	6.6^{L}	6.3^{I}	5.3^{G}	7.9^{R}	7.7^{Q}	7.9^{R}	7.6^{P}	6.4^{JK}	6.3^{IJ}	0.304			
	SEM	0.442	0.436	0.212	0.377	0.413	0.400	0.354	0.511	0.534				
LAB (log CFU/g)	0	$< 3^{A}$	$< 3^{A}$	$< 3^{A}$	$< 3^{A}$	$< 3^{A}$	$< 3^{A}$	$< 3^{A}$	$< 3^{A}$	$< 3^{A}$	0.000	<0.001	<0.001	<0.001
	7	5.1^{J}	3.8^{C}	3.4^{B}	5.4^{K}	4.8^{G}	5.3^{K}	4.4^{F}	5.0^{I}	4.1^{D}	0.234			
	14	6.5^{R}	6.1^{LM}	4.9^{H}	6.4^{QR}	6.1^{LMN}	6.3^{P}	6.1^{LMN}	6.2^{OP}	6.1^{NO}	0.155			
	21	6.3^{OP}	6.1^{MN}	4.2^{E}	6.3^{PQ}	6.1^{LM}	6.2^{OP}	6.0^{L}	6.1^{LMN}	6.1^{LM}	0.219			
	SEM	0.437	0.767	0.433	0.318	0.433	0.318	0.550	0.384	0.667				

[1] See Table 1 for treatments description. Different superscript letters within each panel (Staphylococci, LAB) indicate significant differences within treatment and ripening combinations. SEM, T, R and TxR stand out for the standard error of the mean, the fixed factors treatment and ripening, and the interaction between them, respectively.

Table 3
Changes in pH and water activity (a_w) in dry-fermented model sausages during ripening.[1]

Parameter	Ripening time (days)	CTRL-	CTRL−/ NATPRE	CTRL+	L33	L33/ NATPRE	L49	L49/ NATPRE	ZnPPEx	ZnPPEx/ NATPRE	SEM	T	R	TxR
pH	0	5.73^{L}	5.69^{K}	5.73^{L}	5.78^{N}	5.66^{GHI}	5.76^{MN}	5.74^{LM}	5.69^{JK}	5.56^{B}	0.022	<0.001	<0.001	<0.001
	7	5.63^{EFG}	5.52^{A}	5.66^{GHIJ}	5.61^{DE}	5.56^{B}	5.64^{EFG}	5.53^{A}	5.74^{LM}	5.88^{P}	0.038			
	14	5.56^{B}	5.66^{GHI}	5.62^{EF}	5.58^{BC}	5.68^{IJK}	5.59^{CD}	5.86^{OP}	5.75^{LM}	5.65^{FGH}	0.032			
	21	5.65^{FGH}	5.57^{B}	5.67^{HIJK}	5.60^{CD}	5.56^{B}	5.69^{K}	5.57^{B}	5.84^{O}	5.74^{LM}	0.031			
	SEM	0.035	0.039	0.023	0.046	0.032	0.036	0.077	0.031	0.068				
a_w	0	0.972^{M}	0.970^{M}	0.970^{M}	0.970^{M}	0.971^{M}	0.970^{M}	0.970^{M}	0.970^{M}	0.970^{M}	0.000	0.999	<0.001	0.591
	7	0.956^{JK}	0.953^{IJ}	0.959^{KL}	0.960^{L}	0.953^{IJ}	0.959^{KL}	0.956^{J}	0.950^{HI}	0.949^{H}	0.001			
	14	0.936^{F}	0.929^{E}	0.939^{F}	0.942^{G}	0.936^{F}	0.937^{F}	0.937^{F}	0.936^{F}	0.936^{F}	0.001			
	21	0.897^{B}	0.895^{B}	0.909^{D}	0.909^{D}	0.902^{C}	0.909^{D}	0.886^{A}	0.902^{C}	0.886^{A}	0.003			
	SEM	0.016	0.016	0.013	0.013	0.015	0.013	0.018	0.014	0.018				

[1] See Table 1 for treatments description. Different superscript letters within each panel (pH and a_w) indicate significant differences within treatment and ripening combinations. SEM, T, R and TxR stand out for the standard error of the mean, the fixed factors treatment and ripening, and the interaction between them, respectively.

Table 4
Moisture, nitrosyl-heme, hemin, and Zn-protoporphyrin (ZnPP) in fermented model sausages.[1]

	Moisture (%)	Nitrosyl-heme (mg/kg)	Hemin (mg/kg)	ZnPP (mg/kg)
Treatment				
CTRL-	60.4	2.2^{bc}	2.8^{b}	1.4^{A}
CTRL-/NATPRE	60.7	20.0^{A}	27.5^{a}	2.6^{A}
CTRL+	61.2	19.4^{A}	16.7^{ab}	1.5^{a}
L33	61.5	3.3^{bc}	8.4^{ab}	2.1^{A}
L33/NATPRE	61.3	15.5^{ab}	17.4^{ab}	2.0^{A}
L49	61.5	2.8^{bc}	5.6^{b}	2.1^{A}
L49/NATPRE	60.3	13.8^{abc}	15.6^{ab}	1.8^{A}
ZNPPEx	61.0	1.1^{c}	7.2^{b}	8.3^{b}
ZNPPEx/ NATPRE	60.5	21.3^{a}	15.1^{ab}	7.3^{b}
Ripening time (days)				
0	60.9^{a}	11.9	11.2	3.5
7	54.1^{b}	9.8	13.7	3.2
14	45.4^{c}	10.0	12.1	3.2
21	37.8^{d}	12.4	14.7	3.0
RMSE	1.1	8.2	12.2	0.94

[1] See Table 1 for treatments description. Different superscript letters indicate significant differences within columns for a certain factor. RMSE stands out for the root mean standard error.

differently prepared model sausages which were affected by the different treatments but unaffected by the ripening period. The relatively small amounts of nitrosyl-heme observed in CTRL- can be attributed to the residual nitrite levels in the meat (Iacumin et al., 2019). The same explanation is valid for L33, L49, and ZnPPEx treatments which also recorded low contents. However, these findings indicate that CNS- and ZnPP-containing treatments have little influence on the formation of nitrosyl-heme, especially when compared with those treatments containing NATPRE. In contrast, the higher levels of nitrosyl-heme in the CTRL+ treatment are explained by the deliberate addition of nitrite. The treatments involving the addition of NATPRE also resulted in similar nitrosyl-heme contents to the CTRL+. Indeed, the NATPRE ingredient was found to contain nitrites and nitrates (See section 2.3). This fact explains the increased levels of nitrosyl-heme in those treatments where NATPRE compared to their respective counterparts without the polyphenol-rich ingredient. These findings contrast with those of Hernández et al. (2021), who studied a similar ingredient (NATPRE T-10 HT S) in the elaboration of cooked ham. These authors used NO_2-generated headspace gas chromatography–mass spectrometry (HS-GC–MS) and high-performance liquid chromatography with diode array detection (HPLC–DAD) to determine residual sodium nitrite content in ham samples, but not in their NATPRE ingredient. Their results indicated that sodium nitrite levels in samples treated with this ingredient were below the limits of quantification (9 mg/kg and 2.7 mg/kg, respectively). The polyphenol-rich ingredient used in our study differs from that of Hernández et al. (2021) and may have a different chemical profile. However, the determination of residual nitrite levels in the meat product may be not indicative of the formation of nitrosyl-heme (Sindelar, Cordray, Sebranek, Love, & Ahn, 2007; Terns, Milkowski, Rankin, & Sindelar, 2011). As for the heme content, it is worth noting that Hornsey's method, a widely used method for nitrosyl-heme determination, has been reported to induce the concurrent formation of hemin due to the inherent instability of nitrosyl-heme (Bou et al., 2024). This phenomenon explains why CTRL+ also exhibited the highest hemin content. This content was higher than CTRL-, L49, and ZnPPEx whereas the same treatments but involving the addition of NATPRE resulted in intermediate hemin contents that cannot be differentiated from their counterparts nor from the CTRL+.

Table 4 also shows that ZnPPEx and ZnPPEx/NATPRE treatments displayed the highest levels which, in addition, were similar between them. The high values of ZnPP in ZnPPEx and ZnPPEx/NATPRE treatments are attributed to the addition of the extract containing the preformed chromophore. The low levels of ZnPP in the remaining treatments could be explained by the presence of nitric oxide, formed from residual nitrite sources, which is known to inhibit ZnPP formation by interfering with the activity of endogenous ferrochelatase (Wakamatsu, Hayashi, Nishimura, & Hattori, 2010). In addition to that, this enzyme requires a prolonged ripening time and relatively high temperatures to produce significant amounts of ZnPP (Bou, Llauger, Arnau, Olmos, & Fulladosa, 2022; Parolari, Aguzzoni, & Toscani, 2016; Wakamatsu, 2022) which also helps to explain why this formation was also limited in the CTRL.

3.1.4. Characterization of soluble pigments through UV–vis spectra and urea-PAGE

The UV–Vis spectra of the aqueous extracts of the different model sausages after 0 and 21 days of ripening showed the same peaks but higher absorbance values at the end of the ripening period, likely due to the water loss. For this reason, only the spectra after 21 days are presented in Fig. 1 providing information about the solubility and redox state of the porphyrins. In almost all samples the maximum peak absorbance was observed at around 410 nm, the characteristic Soret band of porphyrins. In the ZnPPEx/NATPRE sample, however, the maximum shifted to 420 nm, whereas the ZnPPEx treatment showed a maximum at 410 nm with a significant secondary peak at 420 nm. This shift at higher wavelengths indicates the presence of ZnPP (Bou et al., 2024). These findings agree with the reported ZnPP content (Table 4) and suggest that at least a part of the ZnPP is soluble, likely due to bindings or interactions with soluble proteins (Abe et al., 2024; Llauger et al., 2024).

When comparing treatments with the addition of NATPRE to their respective counterparts without this ingredient, a reduction in absorbance was detected. The same phenomenon was observed when

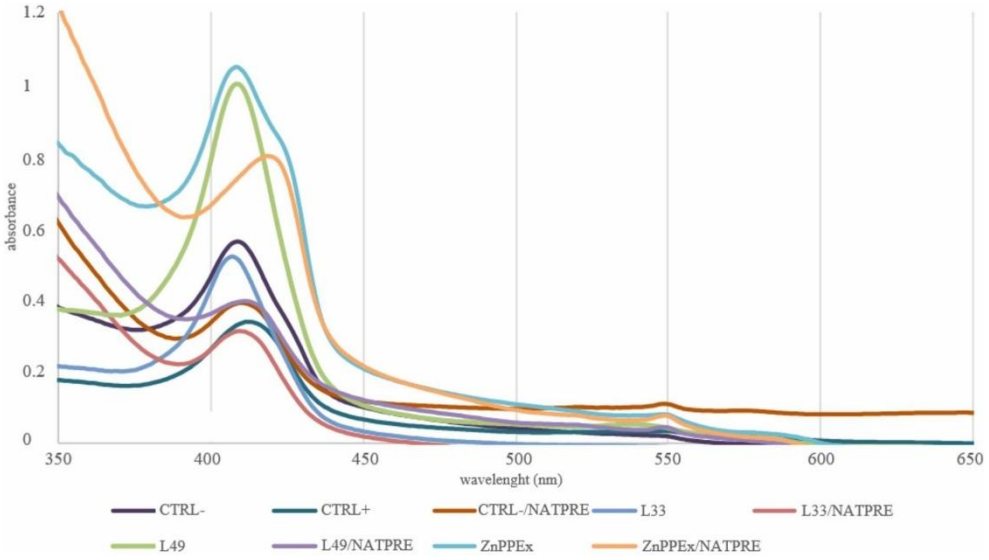

Fig. 1. Aqueous extracts spectra of the model sausages after 21 days of ripening.

comparing the CTRL+ with the CTRL-. These findings can be attributed to the formation of nitric oxide and formation of nitrosomyoglobin that further decomposes into globin and lipophilic nitrosomyochromogen as the meat pH approaches to its isoelectric point (Feiner, 2016). Moreover, the maximum absorbance intensity in the ZnPPEx treatment can be primarily attributed to the addition of the ZnPP extract, which also contains ferroporphyrins derived from its processing, and secondarily, to the relative high pH (5.84) which contributes to the stabilisation of heme proteins red colour (Richards & Hultin, 2000). Therefore, after 21 days of ripening and excluding the CTRL+ and those treatments in which NATPRE was added, pH helps to explain the greater absorbance of L49 (pH 5.68) and CTRL- (pH 5.65) compared to L33 (pH 5.60).

Notably, the L49 treatment spectra exhibited secondary distinctive maxima at 540 and 580 nm (Fig. 1), characteristic of the presence of oxygenated myoglobin and hemoglobin. In contrast, other treatments showed a secondary peak near to 545 nm, which is indicative of the presence of soluble hemichromes (Blair, Barlow, Martin, Schumaker, & McIntyre, 2020; Culbertson & Olson, 2010). The formation of hemichromes appears to be more pronounced in samples containing NATPRE or added nitrite. In these samples, insoluble hemochromes such as nitrosomyochromogen likely serve as the main red chromophore. Conversely, the presence of soluble ZnPP can also contribute to the red colour in ZnPPEx and ZnPPEx/NATPRE treatments.

The previous findings were in good agreement with the gel electrophoresis results (Fig. 2). It can be observed that two intense fluorescent bands are visible in the ZnPPEx and ZnPPEx/NATPRE treatments, confirming that at least part of the ZnPP content (Fig. 1, Table 4) was bound to soluble proteins, likely hemoglobin (Llauger et al., 2024; Wang et al., 2021). Lower-intensity fluorescent bands with the same molecular weight were also observed in the other treatments, consistent with the lower ZnPP content detected by HPLC. Among these, the L49 treatment displayed a slightly more intense fluorescent band compared to other treatments that did not include the addition of the ZnPP extract. As discussed earlier, the relatively high pH in this treatment may have contributed to a higher protein solubility, which could explain the increased fluorescence despite the similar ZnPP contents observed (Table 4).

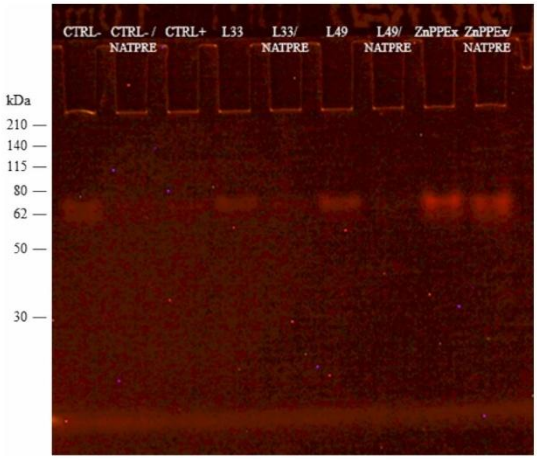

Fig. 2. Fluorescent image of Urea-PAGE electrophoresis (4 M urea, 10 % acrylamide) of dry-fermented model sausages aqueous extracts after 21 days of ripening. Emission at 420 nm and emission captured at 600 nm allows the observation of ZnPP forms.

3.1.5. Evaluation of colour attributes in model sausages

Colour is a key parameter in consumers' purchasing decisions (Purslow, Warner, Clarke, & Hughes, 2020); therefore, the colour appearance of the different model sausages was visually evaluated at different ripening times (Supplementary data S.1), and the L^* (brightness), a^* (redness), and b^* (yellowness) colour coordinates were carefully investigated (Table 5). In this regard, the statistical analysis revealed that L^*, a^*, and b^* parameters were affected by the fixed factors treatment, ripening period, and their interaction ($P < 0.001$).

Generally, brighter samples are characterised by higher L^*-values because of the lower light absorption and higher reflectance or transmittance. In the present study, the samples were featured by L^*-values

Table 5
Changes in L^*-value (lightness), a^*-value (redness), and b^*-value (yellowness) in dry-fermented model sausages during ripening.[1]

Parameter	Ripening time (days)	CTRL-	CTRL−/NATPRE	CTRL+	L33	L33/NATPRE	L49	L49/NATPRE	ZnPPEx	ZnPPEx/NATPRE	SEM	T	R	TxR
L^*	0	50.6^{J}	55.2^{NO}	52.1^{L}	55.8^{O}	45.6^{J}	52.7^{L}	54.1^{M}	50.5^{J}	54.9^{MN}	1.066	<0.001	<0.001	<0.001
	7	50.2^{K}	43.1^{FGH}	43.1^{FGH}	43.1^{GH}	41.5^{BCD}	43.2^{GH}	42.4^{DEFG}	43.1^{FGH}	42.2^{DEFG}	0.855			
	14	41.7^{BCD}	42.1^{CDEF}	43.4^{DEFG}	44.2^{J}	42.4^{DEFG}	43.3^{GH}	42.4^{DEFG}	42.9^{EFGH}	42.4^{DEFG}	0.257			
	21	40.8^{AB}	40.2^{A}	41.9^{CD}	42.9^{EFGH}	41.1^{BC}	41.5^{BCD}	40.1^{A}	42.0^{CDE}	41.5^{BCD}	0.298			
	SEM	2.649	3.404	2.347	3.113	1.020	2.536	3.164	1.973	3.222	0.486	<0.001	<0.001	<0.001
a^*	0	7.8^{E}	5.0^{A}	4.4^{A}	7.5^{E}	5.0^{A}	7.6^{E}	5.1^{A}	7.0^{D}	4.4^{A}	0.411			
	7	6.8^{CD}	6.5^{C}	9.5^{J}	9.5^{JK}	6.6^{C}	9.1^{I}	8.5^{FG}	8.6^{GH}	7.5^{E}	0.372			
	14	6.9^{CD}	7.9^{H}	9.9^{KL}	9.9^{KL}	8.4^{FG}	10.0^{L}	8.8^{HI}	9.9^{KL}	8.2^{F}	0.369			
	21	6.8^{CD}	9.0^{I}	10.0^{L}	10.5^{M}	8.9^{III}	10.1^{L}	9.1^{I}	10.1^{L}	8.9^{III}				
	SEM	0.243	0.867	1.354	0.650	0.891	0.578	0.933	0.715	1.006	0.418	<0.001	<0.001	<0.001
b^*	0	14.8^{M}	13.3^{JKL}	11.9^{EF}	14.5^{M}	13.6^{L}	16.2^{N}	13.5^{L}	15.1^{M}	14.8^{M}	0.467			
	7	13.5^{KL}	11.7^{DE}	12.8^{HIJ}	14.5^{M}	9.6^{A}	13.2^{IJKL}	12.4^{FGH}	12.4^{FGH}	13.6^{L}				
	14	11.2^{BCD}	10.8^{B}	11.9^{EFG}	13.3^{JKL}	10.9^{BC}	10.0^{A}	12.7^{HIJ}	12.5^{FGHI}	12.6^{HI}	0.365			
	21	11.5^{CDE}	9.8^{A}	11.6^{DE}	12.4^{FGH}	10.9^{BC}	12.8^{HIJK}	11.2^{BCDE}	12.7^{HIJ}	12.5^{GHI}	0.331			
	SEM	0.853	0.743	0.259	0.511	0.841	1.269	0.477	0.645	0.536				

[1] See Table 1 for treatments description. Different superscript letters within each panel (L^*, a^*, b^*) indicate significant differences within treatment and ripening combinations. SEM, T, R and TxR stand out for the standard error of the mean, the fixed factors treatment and ripening, and the interaction between them, respectively.

ranging from 45.6 (for L33/NATPRE) to 55.8 (for L33) at the beginning of the ripening, and from 40.1 (for L49/NATPRE) to 42.9 (for L33) at the end (Table 5) likely due to drying. At 21 days, L33 and ZnPPEx treatments were featured by the statistically highest L^*-values, exceeding 42, while CTRL+ showed a slightly lower value, equal to 41.9.

As for redness, the CTRL- treatment exhibited significantly lower values than those of the other eight treatments throughout the ripening period. In fact, CTRL- showed a^*-values of 7.8, 6.8, 6.9, and 6.8 after 0, 7, 14, and 21 days of ripening, respectively. Generally, a decrease in the a^*-value is the result of oxidation phenomena, which lead to the myoglobin transformation into metmyoglobin (Tomasevic, Djekic, Font-i-Furnols, Terjung, & Lorenzo, 2021). The opposite behaviour was observed for the remaining treatments. Interestingly, the CTRL-/NATPRE treatment showed redness values equal to 5.0, 6.5, 7.9, and 9.0 at the four analysed periods. NATPRE was previously used in a study conducted by Hernández et al. (2021), who stated that the colour of the nitrite-containing cooked ham was similar to the colour of the ham formulated with the polyphenol-rich extract. In the present study, model sausages treated with NATPRE showed better redness values than those of CTRL- as ripening progressed, but still lower than those characteristics of the nitrite-containing ones. In fact, the CTRL+ sample displayed an a^*-value equal to 4.4 immediately after preparation, and equal to 9.5, 9.9, and 10.0 after 7, 14, and 21 days of ripening, respectively. The similar behaviour in CTRL+ and in those samples containing NATPRE could be mainly explained by the fact that, in addition to the presence of phenolic compounds, NATPRE is also a source of nitrate and nitrite (leading to approximately 3 and 10 mg/kg meat batter, respectively). Nonetheless, other mechanisms contributing to the preservation of redness cannot be overlooked considering the relatively low ingoing amounts of nitrifying agents. Studies have shown that certain phenolics can reduce methemoglobin levels and limit heme release likely through covalent binding with the protein (Zhu et al., 2025). Notably, the colour of the CTRL-/NATPRE, L33/NATPRE, and L49/NATPRE treatments at the end of ripening did not show statistical differences, indicating that there was no synergistic effect between the staphylococcal strains and NATPRE. Therefore, the use of staphylococci specifically selected for their reducing power could play a role in the conversion process, improving the colour of the NATPRE-containing meat.

Pork meat treated with *S. equorum* L33 and *S. saprophyticus* L49 showed the characteristic red colour of nitrite-cured meat, as previously demonstrated by Premi, Rocchetti, Rossetti, et al. (2024). In fact, L33 treatment featured the highest a^*-values, equal to 7.5, 9.5, 9.9, and 10.5 after 0, 7, 14, and 21 days of ripening, respectively. Similarly, L49 showed an initial red pigmentation value of 7.6, which increased to 9.1, 10.0, and 10.1 over the same ripening periods. These findings underscore the potential for using NOS-positive meat-associated CNS strains in the production of nitrite-free meats due to their positive impact on meat colour, supporting the assertions made by Premi, Rocchetti, Rossetti, et al. (2024). Other mechanisms, such as the catalase activity, reducing power, oxygen consumption, and proteolysis (involving the generation of peptides with antioxidant properties, and the pH increase and subsequent myoglobin stability) could also be involved in the colour enhancement of the CNS-containing treatments. In fact, the redness of L33 and L49 treatments was always featured by the highest statistical significance, reaching the maximum a^*-values after 21 days of ripening. Interestingly, ZnPPEx treatment exhibited the pinkish-red hue typical of traditionally cured meat, showing a^*-values equal to 7.0, 8.6, 9.9, and 10.1 at the four analysed ripening times. The intense redness in ZnPPEx samples, as indicated by the a^*-values, revealed that the porcine liver extract having a high ZnPP content could improve the colour of dry-fermented sausages without the addition of nitrite. Therefore, these results encourage its application as a colouring ingredient in meat-based products, as previously suggested by Llauger et al. (2023). Similar to the other treatments, also the ZnPPEx/NATPRE colour was less intense than ZnPPEx, achieving a^*-values equal to 4.4, 7.5, 8.2, and 8.9 after 0, 7, 14, and 21 days of ripening, respectively.

On the other hand, b^*-coordinate indicates the yellowness likely deriving from myoglobin oxygenation and lipid oxidation (Liu, Wang, Zhang, Wang, & Kong, 2019. The initial and final b^*-values of CTRL+ were equal to 11.9 and 11.6, respectively. As shown in Table 5, in all the other cases, the b^*-value detected after 21 days of ripening was approximately three points lower than that registered at the beginning of the ripening process, indicating that lipid oxidation was limited. In fact, the initial b^*-values were in between 13.3 and 16.2, while the final values ranged from 9.8 to 12.7, reflecting significant differences in sample yellowness ($P < 0.001$).

4. Conclusions

This research demonstrates that the red colour appearance of dry-fermented sausages was improved when the meat batters with natural meat microbiota were enriched with particular CNS showing NOS activity, contained a porcine liver extract having a high ZnPP content, and/or involved the addition of the NATPRE ingredient. Specifically, the utilisation of *S. equorum* L33, *S. saprophyticus* L49, and the ZnPP extract allowed the achievement of a^*-values equal to or exceeding those of the nitrite-containing sausages prepared with the addition of 150 mg/kg of sodium nitrite. The relatively low amounts of nitrite and nitrate of NATPRE seemed to mainly improve the colour through the formation of nitrosyl-heme. However, the analyses performed on the differently prepared model sausages indicate that the usage of ZnPP extract is also a winning strategy in the replacement of synthetic additives. Furthermore, it emerges that the NOS activity performed by the selected bacteria may not be the main mechanism involved in the considerable redness improvement, because of the low nitrosyl-heme values detected in the CNS-containing sausages.

Consequently, this study proves the potential of using selected staphylococcal strains L33 and L49, and ZnPP-rich liver extract as viable alternatives for the production of dry-fermented sausages elaborated without the addition of nitrifying salts, maintaining the colour characteristics of dry-cured meat products. Further research is needed to thoroughly evaluate the impact of bacterial cultures and ZnPP-rich liver extract on the sensory profile of the final products.

Compliance with ethics requirements

This article does not contain any studies with human or animal subject.

Consent form

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2025.109919.

Data availability

Data will be made available on request.

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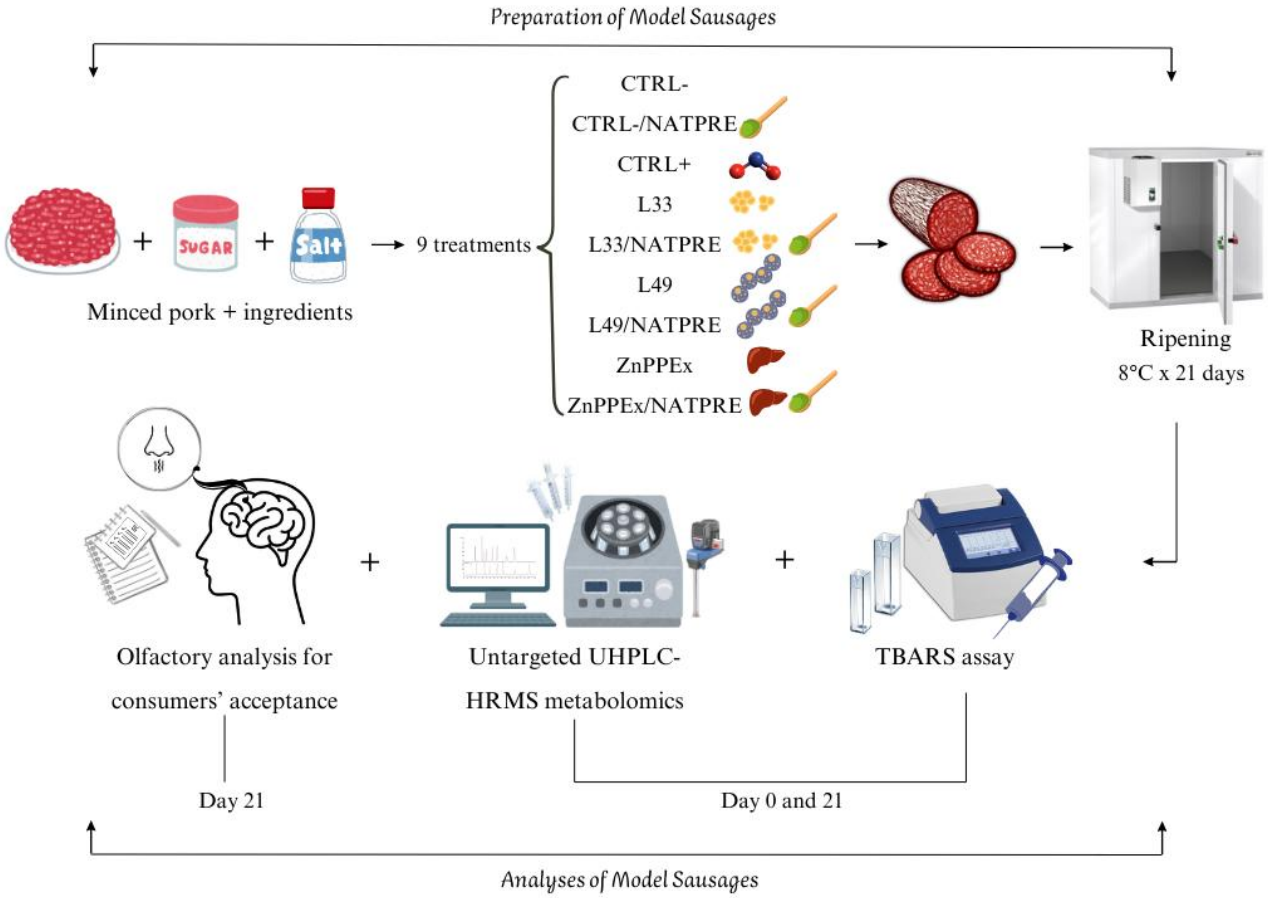
4. <u>Chapter 4</u>: Natural curing strategies for nitrite-free dry-fermented sausages: Effects of NOS-positive *Staphylococcus* spp., Zn-protoporphyrin and polyphenol-rich extracts on untargeted metabolome and sensory quality

Graphical abstract



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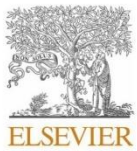

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Natural curing strategies for nitrite-free dry-fermented sausages: Effects of NOS-positive *Staphylococcus* spp., Zn-protoporphyrin and polyphenol-rich extracts on untargeted metabolome and sensory quality

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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% ± 1.1 fat (AOAC 991.36 method), 17.6% ± 1.9 protein (AOAC 928.08 method), and 60.9% ± 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 T_{21}. An additional Volcano Plot analysis was carried out for the pairwise comparisons ZnPPEx T_{21} vs T_0 and ZnPPEx/NATPRE T_{21} vs T_0. 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

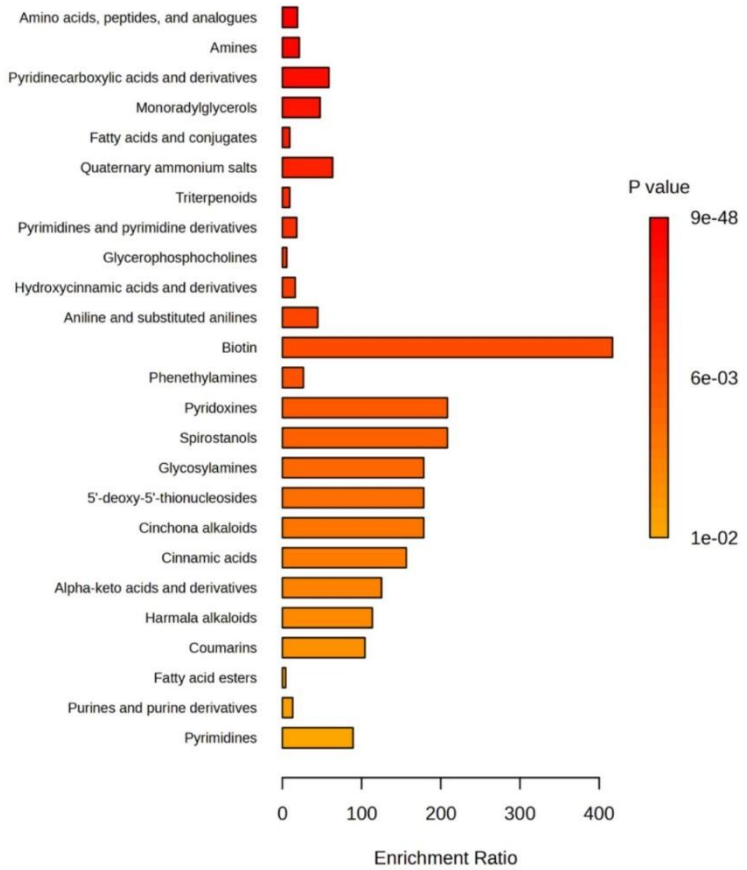

Fig. 1. Overview of enriched metabolite sets (Top 25) according to the annotated metabolites by UHPLC-HRMS. Enrichment ratio is computed by Hits / Expected, referring to the total compounds involved in a metabolite set. *Abbreviations*: Hits = observed hits; Expected = expected hits.

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 metabolic 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 metabolite 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,

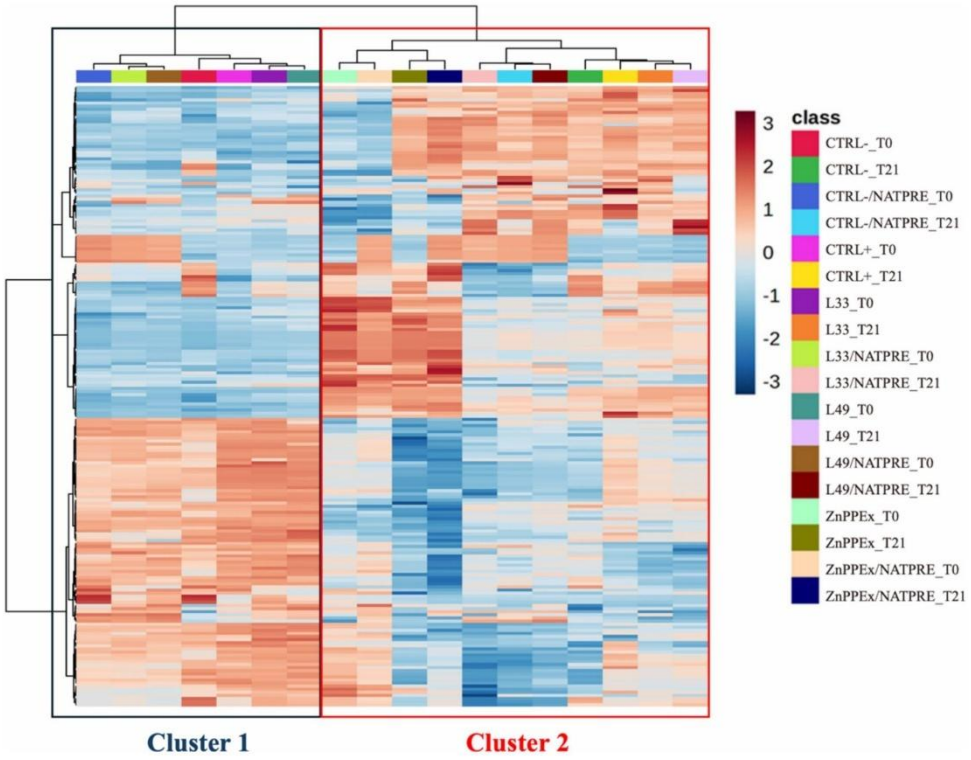

Fig. 2. Hierarchical clustering analysis (HCA) built considering the normalized relative abundance value of each annotated meat metabolite by UHPLC-HRMS of samples before (T_0) and after ripening (T_{21}). Different colours within the heat map indicate strong up- (red colour; Fold-Change = +3) and down- (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_2 FC = 8.03; $p = 2.3 \times 10^{-3}$), nervonic acid (log_2 FC = 12.33; $p = 6.9 \times 10^{-6}$), and tyramine (log_2 FC = 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_2 FC = 10.93; $p = 1.1 \times 10^{-4}$) and tyramine (log_2 FC = 8.54; $p = 6.3 \times 10^{-6}$) were confirmed, while histamine (log_2 FC = 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

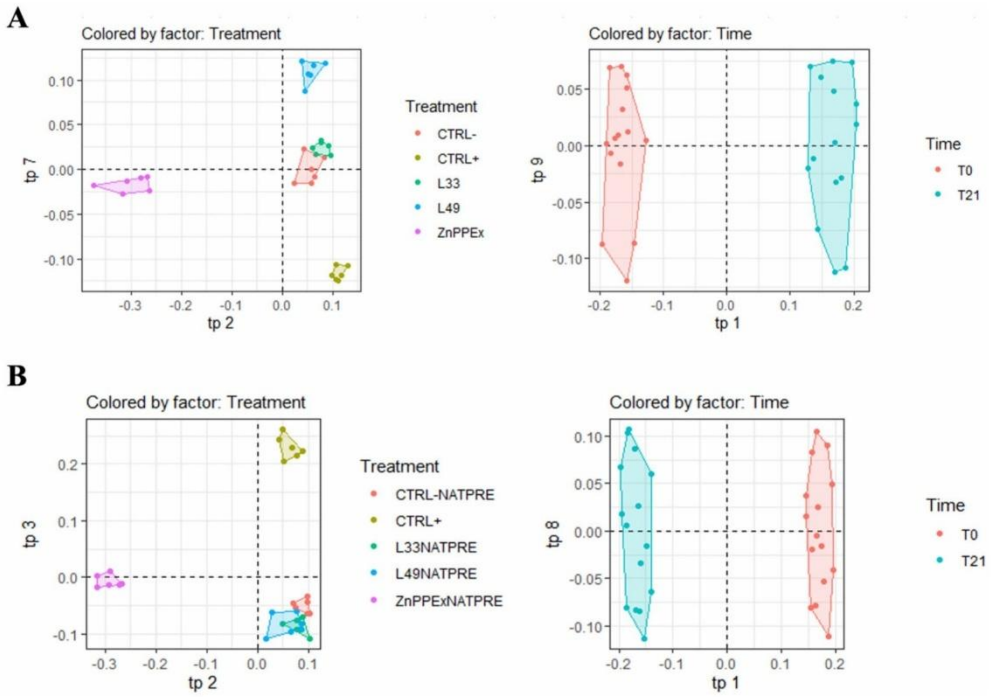

Fig. 3. Supervised AMOPLS analysis score plots built considering the metabolomic profile of the different dry-fermented sausages manufactured without (A) and with (B) NATPRE. The score plot were built considering "Treatment" and "Ripening time (T_0 vs T_{21})" as discriminant parameters. tp = contributions 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 *p*-value	RSR *p*-value	R^2Y *p*-value	Main tp		to1
no NATPRE								
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%
with NATPRE								
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 T_0. To further support these hypotheses, additional volcano plot analyses were performed by comparing the metabolomic profiles of ZnPPEx-containing samples over ripening time (from T_0 to T_{21}). These analyses revealed no significant accumulation of polyamines during ripening, while a significant ($p < 0.05$) decreasing trend was observed for spermine ($log_2FC = -0.42$) and tyramine ($log_2FC = -0.84$). 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 T_{21} as a function of the different treatments and with no NATPRE added. The log_2FC values are expressed vs the CTRL+. ns = not significant from Volcano Plot analysis.

VIP PLS-DA (no NATPRE)	Chemical class	VIP score	CTRL- (log_2FC)	L33 (log_2FC)	L49 (log_2FC)	ZnPPEx (log_2FC)
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 T_{21} (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 T_{21}, 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 T_{21}, 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 log_2FC values of lysophospholipids (such as LPC 18:1 and LPC 18:2) at T_{21} 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 T_{21} 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 ($log_2FC = 2.01$; although no significant variation was measured for this compound moving from T_0 up to T_{21}) 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 T_{21} as a function of the different treatments with NATPRE added. The log_2FC values are expressed vs the CTRL+. ns = not significant from Volcano Plot analysis.

VIP PLS-DA (NATPRE)	Chemical class	VIP score	CTRL- (log_2FC)	L33 (log_2FC)	L49 (log_2FC)	ZnPPEx (log_2FC)
(9Z)-9-octadecenoic acid	Fatty atty 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

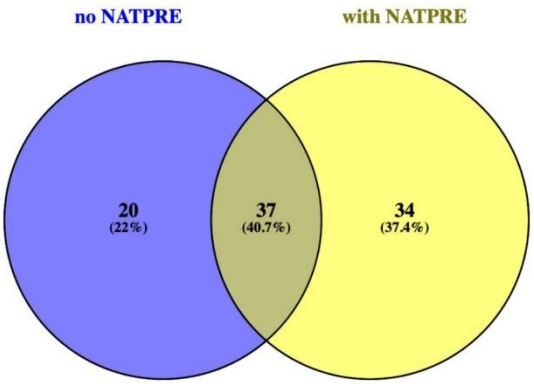

Fig. 4. Venn diagram showing the exclusive and common discriminant meat metabolites at T21, when considering samples manufactured with and without NATPRE.

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 (T_0) and after the entire ripening period (T_{21}), in order to evaluate the oxidation state of the fermented sausages (Table 4). The evaluation of lipid oxidation was intentionally focused on the initial and final stages of ripening (T_0 and T_{21}), 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 *x* treatment' significantly affected the MDA parameter, recording *p*-values < 0.001 (not shown).

Table 4
Results from experiments conducted on the TBARS (mg MDA/kg) in various model sausages during ripening. Data are expressed as mean values ± standard deviation. Superscript letters within each column (T_0 and T_{21}) 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 ± 0.06^{e}	2.43 ± 0.05^{g}
CTRL-/NATPRE	0.35 ± 0.07^{ab}	0.77 ± 0.05^{c}
CTRL+	0.32 ± 0.03^{ab}	0.53 ± 0.07^{a}
L33	1.04 ± 0.15^{d}	2.04 ± 0.08^{f}
L33/NATPRE	0.48 ± 0.08^{bc}	0.98 ± 0.00^{d}
L49	1.17 ± 0.01^{d}	1.66 ± 0.03^{e}
L49/NATPRE	0.55 ± 0.05^{c}	0.65 ± 0.01^{b}
ZnPPEx	1.79 ± 0.16^{f}	2.06 ± 0.10^{f}
ZnPPEx/NATPRE	0.28 ± 0.13^{a}	0.71 ± 0.06^{bc}

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 T_0 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 NO_3^-/kg and 1030 ± 370 mg NO_2^-/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 (T_{21}), 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 T_{21} 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 T_{21} (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 × 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.

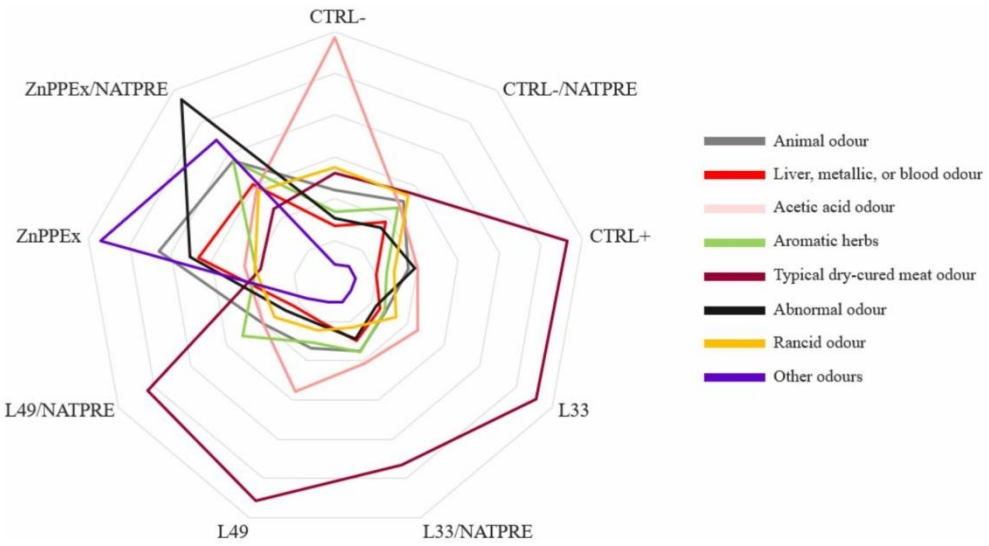

Fig. 5. Olfactory sensory evaluation of the dry-fermented model sausages at T_{21}.

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.

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)

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.

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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5. Discussion

This research project focuses on investigating potential natural alternatives to synthetic additives in meat products, especially those involved in red colour enhancement, as colour is a key factor influencing consumers' purchasing decisions. Studying potential substitutes for nitrates and nitrites is particularly important nowadays, as these substances are widely recognised as potentially carcinogenic, raising health concerns and reflecting consumer demand for healthier, more natural and sustainable foods. Although many articles in the literature have discussed the use of plant extracts as an alternative to synthetic nitrites, few scientific publications have addressed the use of ZnPP derived from animal by-products or NOS-positive staphylococcal strains. In this regard, the staphylococcal NOS pathway is definitely attracting increasing interest as it converts L-arginine to L-citrulline and nitric oxide. The latter can then react with myoglobin to form nitrosylmyoglobin, which is the pinkish-red pigment found in dry-cured meat products. Using staphylococcal strains selected for this activity as innovative starter cultures in the production of meat-based foods could therefore be an effective way of manufacturing meat products with the same visual characteristics as those containing chemical additives, while also being a 'clean' approach.

The primary step of this research was to investigate the NOS activity of several staphylococci belonging to five distinct species, using selected genotypic and phenotypic assays. Of the thirty-five meat-associated staphylococci initially analysed, sixteen were found to possess the NOS gene, as determined by the primer pairs used in the polymerase chain reactions. Based on these results, eleven strains belonging to the species *S. saprophyticus*, *S. equorum*, *S. carnosus* and *S. xylosus* were selected for subsequent experiments. These assessments involved determining the staphylococcal NOS phenotypic activity by inoculating the strains into a meat simulation medium supplemented with L-arginine. The strains were also then inoculated into real minced beef loins (i.e. minced using an 8 mm diameter mincer) to produce laboratory-scale meat models, which were stored at 22 °C with 85% relative humidity for 10 days. Targeted metabolomics techniques were employed to evaluate the concentrations of arginine and citrulline in the medium broth and the fermented meat models. The

results revealed that the meat colour enhancement achieved by inoculating certain strains was comparable to the effect of adding sodium nitrite. This study enabled us to exclude the strains that tested negative for the NOS gene or performed poorly in the meat matrix, while prioritising the most promising ones: *S. saprophyticus* L49 and *S. equorum* L33.

In addition to using staphylococci selected for their NOS activity as an alternative to pure nitrite sources, two other natural strategies were investigated in an experiment involving the production of dry-fermented pork sausages. These strategies included the use of a porcine liver extract rich in the pigment ZnPP and the incorporation of a polyphenol-rich plant ingredient (NATPRE T-10 CUR HT). Notably, no scientific research has yet examined the use of these substances in formulations for colour enhancement in dry-fermented meat products. Therefore, the main objective was to investigate the colour development of various treatments formulated with the already mentioned different natural strategies (selected NOS-positive staphylococci, ZnPP-rich porcine liver extract, and the plant extract) after 21 days of ripening at 8 °C and 60–75 % relative humidity, and to compare the results with those of nitrite-containing sausages. The colour appearance of the different treated model sausages was visually evaluated at different ripening times (7, 14, and 21 days), and the colour coordinates were carefully assessed using the CIELab system. The redness values obtained for the inoculated sausages confirmed previous findings. In fact, the dry-fermented model sausages that were inoculated with *S. equorum* L33 and *S. saprophyticus* L49 exhibited the characteristic red colour of nitrite-cured meat at all the investigated periods. This is a particularly significant outcome given that the product was made using pork instead of beef. Therefore, despite the type of meat used as the starting material being changed and the product being manufactured under different conditions (i.e., temperature, relative humidity, and ripening time), the result was still positive. These findings are crucial because they confirm those obtained in the initial trial and, for the first time, prove that NOS-positive CNS strains can develop colour independently of the meat matrix and the conditions applied. Notably, the colour remained stable throughout the ripening period, exhibiting no variation compared to the nitrite control sample. These promising outcomes suggest the potential for using NOS-positive

meat-associated staphylococci in the production of various nitrite-free prepared meats. Given the significant redness achieved in CNS-containing treatments, it has been hypothesised that the colour enhancement produced by the selected staphylococci results from several mechanisms, including catalase activity, reducing power, oxygen consumption and proteolysis, rather than just the NOS pathway. Furthermore, this study employed an innovative approach involving untargeted metabolomics to analyse the chemical impact of each tested strategy compared to the nitrite-containing meat products. Sensory analysis was also conducted to verify the olfactory attributes of the various treatments compared to the nitrite control. The results showed that the inoculated treatments most closely replicated the metabolomic profile and olfactory sensory traits of nitrite-cured sausages. In fact, these treatments exhibited low lipid oxidation, which is probably associated with staphylococcal superoxide dismutase, antioxidant peptide synthesis and catalase activity, as well as the characteristic dry-cured odour. Therefore, these selected bacterial cultures appear to be the perfect natural alternative to pure nitrite sources, positively impacting the colour of dry-fermented sausages while replicating their metabolomic profile and sensory characteristics.

Interestingly, the ZnPP-containing treatments showed the pinkish-red colour typical of traditionally cured sausages throughout the ripening process. This suggests that ZnPP could be used as a natural colour enhancer and a nitrite substitute in meat products. However, treatments formulated with the porcine liver extract were found to generate unique and undesirable metabolomic fingerprints immediately after the model sausages were prepared. This is presumably because fermentation-like compounds, such as histamine and tyramine, were introduced during the technological process of obtaining the ZnPP functional extract. Furthermore, metabolomic and sensory analyses revealed the need for further refinement, such as implementing optimised extraction protocols or using tailored starter cultures, to ensure the safety and acceptability of the final product, given the accumulation of biogenic amines and liver-derived off-odours.

Additionally, sausages treated with the polyphenol-rich ingredient exhibited better redness values compared to the control sample (CTRL-), which contained neither nitrites nor natural alternatives.

The relatively low levels of nitrite (1030 ± 370 mg NO_2^-/kg) and nitrate (313 ± 130 mg NO_3^-/kg) present in NATPRE T-10 CUR HT seemed to enhance the colour primarily through nitrosyl-heme formation. However, the redness values were lower than those of the nitrite-containing treatments. Combining NATPRE T-10 CUR HT with NOS-positive staphylococci or ZnPP produced better results than the CTRL- sample, but not as good as the single strategies, which showed the typical colour of products containing synthetic additives. Furthermore, it was found that NATPRE T-10 CUR HT effectively reduces lipid oxidation and preserves bioactive compounds. The strong antioxidant activity of this natural ingredient appears to be due to the combination of its phenolic compounds, ascorbic acid, and nitrate and nitrite content. While no synergistic effect was observed between the staphylococcal strains and the polyphenol-rich extract in terms of colour – as no improvement in results was achieved when they were used together in meat – an interaction was detected from chemical and metabolomic perspectives. The most promising strategy was actually to combine staphylococcal strains, particularly *S. equorum* L33, with NATPRE T-10 CUR HT. In fact, this combination closely mimicked the nitrite-cured control in terms of chemical and sensory attributes, while significantly limiting lipid oxidation.

These findings therefore confirm the potential of using staphylococcal strains selected for their NOS activity, either alone or in combination with NATPRE T-10 CUR HT, to replicate the colour, metabolomic and sensory effects of nitrite curing. This would provide an alternative that meets consumer demand for naturally cured meat products with a ‘cleaner label’.

6. Conclusions

This research project investigated innovative strategies for replacing nitrates and nitrites in dry-fermented meat products, with the aim of maintaining the typical colour and sensory characteristics normally achieved using synthetic additives. Specifically, dry-fermented sausages inoculated with NOS-positive staphylococci (*S. saprophyticus* L49 and *S. equorum* L33) exhibited a colour similar to that of traditional nitrite-cured products. Interestingly, these selected bacteria imparted a red colour

regardless of the initial meat source (pork or beef) or the ripening conditions (22 °C and 85% relative humidity for 10 days, or 8 °C and 60–75% relative humidity for 21 days). Furthermore, incorporating staphylococci into the sausage recipe had no negative impact on the metabolomic profile or sensory attributes of the final products; rather, it contributed to the development of the typical dry-curing odour. Therefore, these NOS-positive strains appear to be the most promising alternative to nitrites. In addition, dry-fermented pork sausages formulated with the polyphenol-rich ingredient showed comparable chemical and olfactory characteristics to nitrite-cured products, representing a suitable alternative to synthetic additives. Conversely, ZnPP-containing treatments exhibited a colour similar to that of traditionally cured products, despite their significantly different chemical, metabolomic, and olfactory profiles. Therefore, in order to improve the impact of the ZnPP-rich extract on the final product, the extraction method needs to be optimised.

In conclusion, the results of all the analyses and tests conducted during this study suggest that the proposed innovative strategies could allow meat processors to replace synthetic additives in their products without compromising quality. However, there is currently a lack of uniform regulation governing natural colourants and the use of plant extracts in meat production. Therefore, the regulatory status of these ingredients and their applications should be explored on a case-by-case basis.

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