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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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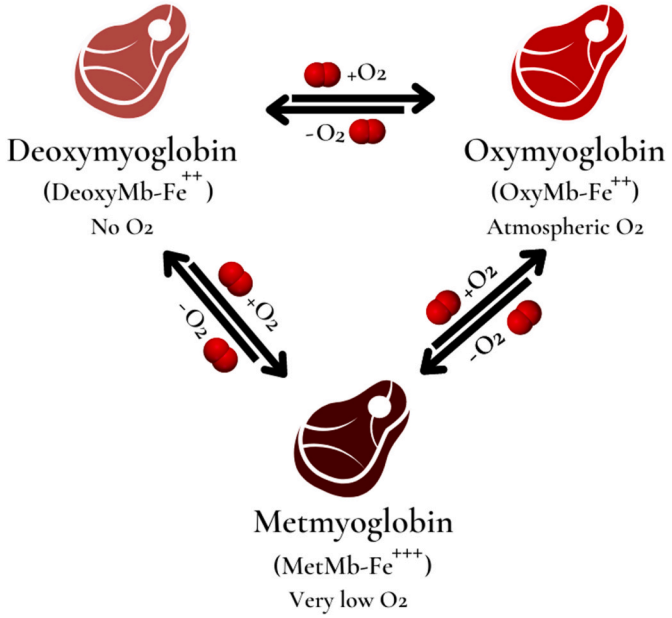

Fig. 1. Deoxymyoglobin, oxymyoglobin, and metmyoglobin: the three myoglobin states, existing in dynamic equilibrium, responsible for the fresh meat colour. Adapted from Hawthorne et al. (2020). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version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

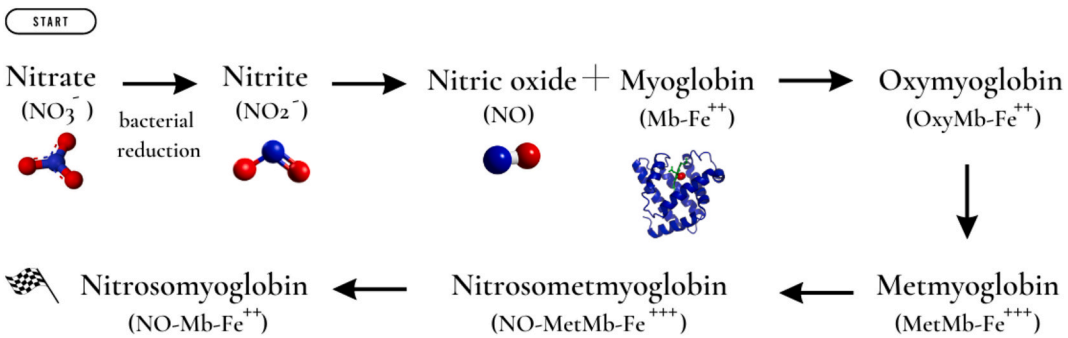

Fig. 2. Nitrosomyoglobin formation in meat products after nitrates addition. Adapted from Hammes (2012).

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.

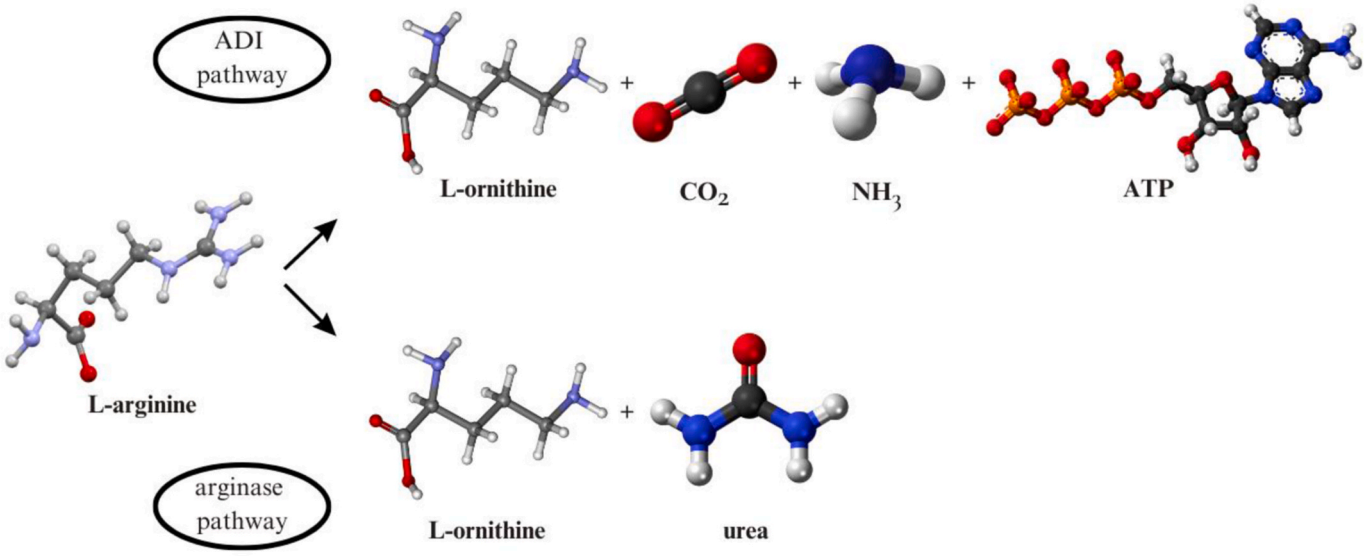

Fig. 3. Simplified pattern showing the arginase and ADI pathways involved in bacterial arginine catabolism.

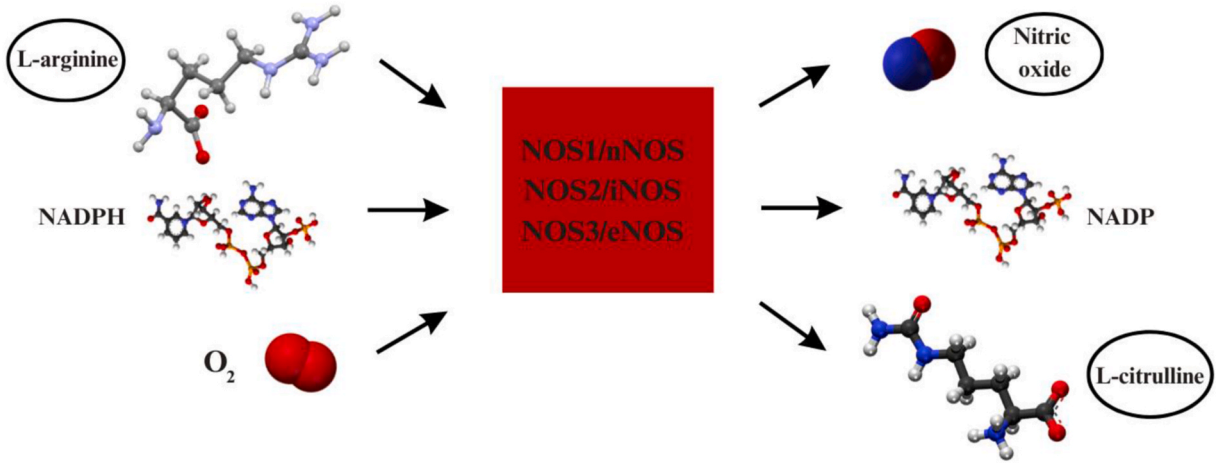

Fig. 4. Nitric oxide synthase pathway (NOS). The reaction involves the hydroxylation of L-arginine by O_2 and NADPH to form N-ω-hydroxy-L-arginine, which is then oxidised to produce NO and L-citrulline. Adapted from Abán et al. (2018).

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 $\times 10^8$			
		S. carnosus 506	4.00×10^8	4.20 $\times 10^8$			
		S. simulans 392	4.40×10^8	3.90 $\times 10^8$			
		S. saprophyticus 389	4.30×10^8	4.40 $\times 10^8$			
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 $\times 10^8$			
		S. carnosus 506	4.50×10^8	4.20 $\times 10^8$			
		S. simulans 392	3.10×10^8	3.50 $\times 10^8$			
		S. saprophyticus 389	0.90×10^8	3.40 $\times 10^8$			
$MbFe^{III}$ -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 + 160 mg/kg NO_2^-		**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)
	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 +160 mg/kg NO_3^-		**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)
	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 tetracoordinated 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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CRediT authorship contribution statement

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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