Review

Mechanisms of the bactericidal effects of nitrate and nitrite in cured meats

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ABSTRACT

For cured meat products, nitrite is recognized for its antimicrobial effects against pathogenic bacteria, even though the specific inhibitory mechanisms are not well known. Nitrite contributes to oxidative stress by being the precursor of peroxynitrite ($ONOO^-$), which is the major strong oxidant. Thus, bacterial stress (highly pH-very low partial pressure of oxygen-dependent) is enhanced by the nitrate-nitrite-peroxynitrite system which is also highly pH- and low partial pressure of oxygen-dependent. Nitrite is a hurdle technology which effectiveness depends on several other hurdle technologies including sodium chloride (accelerating the autoxidation of oxymyoglobin and promote peroxynitrite formation), ascorbate (increasing $ONOO^-$ synthesis), and Aw. In this environment, certain species are more resistant than others to acidic, oxidative, and nitrative stresses. The most resistant are gram-negative aerobic/facultative anaerobic bacteria (*Escherichia coli*, *Salmonella*), and the most fragile are gram-positive anaerobic bacteria (*Clostridium botulinum*). This position review highlights the major chemical mechanisms involved, the active molecules and their actions on bacterial metabolisms in the meat ecosystem.

1. Introduction

The origins of the use of nitrate to cure meat are lost in the mists of time. Niter, was collected in ancient China and India long before the Christian era. Niter was used to refer specifically to nitrated salts known as various types of saltpeter such as calcium nitrate anhydrous [Ca $(NO_3)_2$] or potassium nitrate (KNO_3). The highest levels of dietary nitrate are found in vegetables (celery, beet, arugula, and spinach) depending on growing conditions. These salts were used to cure meat (Binkerd & Kolari, 1975). Nitrate and/or nitrite play a decisive role in cured meat products, providing specific sensory properties (flavor), stability (red color) and product safety. Nitrite is recognized for its bacteriostatic and bactericidal effects against pathogenic bacteria such as *Salmonella enterica serovar Typhimurium*, *Listeria* spp., and *Clostridium botulinum* (Hospital, Hierro, & Fernandez, 2012; Hospital, Hierro, & Fernandez, 2014; Keto-Timonen, Lindström, Puolanne, Niemistö, & Korkeala, 2012). However, the specific inhibitory mechanisms of nitrite are not well known. On the one hand, this inhibition is effective only on certain bacterial species (Tompkin, 2005). On the other hand, its effectiveness depends on several factors including pH, temperature, nitrate or nitrite concentrations, curing accelerators such as sodium chloride or ascorbate and erythorbate, the inhibitory effect of iron concentration, and the initial spoilage bacterial load.

However, due to media coverage on the relationship between additives and some diseases, consumers now want more natural, fresh and minimally processed foods with fewer artificial additives, including preservatives. This consumer demand for high nutritional quality is a strong long-term trend. Thus, the use of nitrite in meat as curing agent raises public concern because nitrite can be a precursor of nitrosamines, many of which are known to be carcinogenic (Pegg & Shahidi, 2000). In order to meet both consumer expectations and manufacturing constraints, it is essential to understand the chemical mechanisms of nitrate and nitrite additives effect in order to reduce their concentrations or potentially eliminate them. The aim of this review is to highlight the mechanisms involved, the active molecules and their actions on certain bacteria genus in the meat ecosystem.

2. Chemistry of nitrate and nitrite in cured meats

Nitrate (NO_3^-) is specifically used in certain curing conditions and products where nitrite must be generated in the product over long periods of time. Its role is to serve as a source of nitrite for curing reactions. While nitrate has the same functionality as nitrite, it acts much more slowly and is therefore used less frequently. An additional step of the conversion of nitrate to reactive nitrite is necessary. This step is performed by the bacterial reduction of nitrate to nitrite. This can be

accomplished by bacterial flora naturally found in meat or by the addition of microorganisms such as starter cultures, with nitrate-reducing properties (Heaselgrave, Andrew, & Kilvington, 2009). These strains express nitrate reductases (Nared) under anaerobic conditions including *Lactobacillus sakei*, *Lactobacillus plantarum*, Leuconostoc strains (Hammes, 2012), *Staphylococcus carnosum*, *Staphylococcus xylosus* (Bonomo, Ricciardi, Zotta, Parente, & Salzano, 2008), *Staphylococcus aureus* (Talon, Walter, Chartier, Barrière, & Montel, 1999), *Bacillus subtilis* (Burke & Lascelles, 1975), etc. In bacteria, nitrate is used as a substrate for anaerobic respiration.

The pKa of nitric acid (HNO_3) is -1.6, meaning when nitrate is dissolved in water, it all exists as nitrate anion. Nitrate transport across bacterial membranes is facilitated by protein transporters that are members of the major facilitator superfamily. The most recent studies show that two types of protein transporters are probably involved. For example, NarK1 seems to be a nitrate/proton symporter and Nark2 a nitrate/nitrite antiporter in *Paracoccus denitrificans* (Goddard et al., 2017), or NarK seems to be a nitrate/nitrite antiporter and NarU a cation symport in *Escherichia coli* (Fukuda et al., 2015). This mechanism involves the excretion of nitrite out of the cytoplasm. Nitrite is excreted until nitrate depletes. Such nitrite export was argued to be followed by nitrite import by a putative transporter called NirC with subsequent cytoplasmic reduction (Jia, Tovell, Clegg, Trimmer, & Cole, 2009). Indeed, the pKa of nitrous acid (HNO_2) is relatively low: 3.42 at 25 °C. In meat curing conditions, with a pH of 5.0–6.0, between 97.5% and 99.8% of HNO_2 is dissociated (NO_2^-) (Table 1).

Nitrite is reduced to nitric oxide (NO·) by two pathways (Fig. 1): (i) bacterial nitrite reductases (Nired) which are periplasmic (gram negative bacteria) or cytoplasmic; (ii) deoxymyoglobin of meat which has a nitrite reductase activity (Eq. (1)) (Gladwin & Kim-Shapiro, 2008; Koizumi & Brown, 1971). Nitrite is also oxidized to peroxynitrite ($ONOO^-$) by hydrogen peroxide (Eq. (7)).

In meat, at least three forms of myoglobin (Mb) exist (Fig. 1): an oxygenated one of ferrous myoglobin called oxymyoglobin ($MbFe^{2+}O_2$), an oxidized one called metmyoglobin ($MbFe^{3+}$) and the reduced form, called deoxymyoglobin ($MbFe^{2+}$). The partial pressure of oxygen (pO_2) is a major factor in the existence of these three molecules (Møller & Skibsted, 2006). In anaerobic and acidic conditions, the oxygen leaves the heme and allows the nitrite to react with $MbFe^{2+}$ to form NO· and $MbFe^{3+}$ (Eq. (1)) (Shiva et al., 2007):

$$NO_2^- + MbFe^{2+} + H^+ \rightarrow NO^° + MbFe^{3+} + OH^- \tag{1}$$
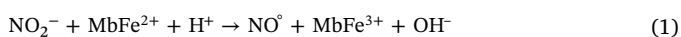
This reaction is carried out in competition with the autoxidation of $MbFe^{2+}O_2$ which is the result of acid catalysis at the meat's pH. It is the spontaneous conversion of $MbFe^{2+}O_2$ or $MbFe^{2+}$ to $MbFe^{3+}$.

Table 1
Undissociated forms according to pH. In water, weak acids have both a dissociated state and undissociated state that coexist. The acid dissociation constant is Ka (pKa = $-$log Ka). The acid that is in the undissociated form readily diffuses through the bacterial membrane. This free diffusion is governed by the pKa of the acid and pH of the solution.

	pKa (25 °C)	pH 5 undissociated	pH 6 undissociated	pH 7 undissociated
Peroxynitrous acid	6.80	98.5%	86.4%	38.6%
Perhydroxyl radical	4.88	43,1%	7%	0,7%
Nitric acid	-1.60	0%	0%	0%
Nitrous acid	3.42	2.5%	0.2%	0.02%
Hydrogen peroxide	11.6	100%	100%	100%
Acetic acid	4.75	35.9%	5.3%	0.5%
Lactic acid	3.85	6.6%	0.7%	0.07%
Formic acid	3.70	4.7%	0.4%	0.04%
Butyric acid	4.82	39.6%	6.1%	0.6%

$MbFe^{2+}$ reacts with unbound O_2 by an outer-sphere electron-transfer-yielding $MbFe^{3+}$ and superoxide radical ($O_2^-·$) (Eq. (2)). The logarithmic transformed rate constant for autoxidation of $MbFe^{2+}O_2$ depends linearly on the pH under acidic conditions (Møller & Skibsted, 2006):

$$MbFe^{2+}O_2 \rightarrow O_2 + MbFe^{2+} \rightarrow O_2^{-°} + MbFe^{3+} \tag{2}$$
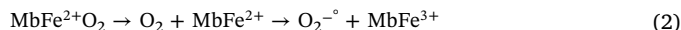
The enzyme system metmyoglobin reductase (MMR) reduces $MbFe^{3+}$ to $MbFe^{2+}$ (Mikkelsen, Juncher, & Skibsted, 1999). This allows the NO· and $O_2^-·$ cycle to continue until MMR is exhausted.

3. Mechanisms of bactericidal effects

In cured meats, peroxynitrite's mechanisms of action depend on the kinetics of its formation, decay, and diffusion through the membranes of all the molecules involved, including peroxynitrite itself. The set of reactions described depends on dissociation constants, concentrations, diffusion rates, reaction kinetics, and lifetimes of the most reactive molecules. Some molecules compete with one another. Some reactions are reversible, and several reactions can happen at the same time. The mechanisms are regulated among themselves. It is not the law of "all or nothing". Thus, we will try to present the major pathways of bactericidal effects in a reactionary dynamic (Fig. 1).

3.1. Peroxynitrite, a cytotoxic biological agent

The antimicrobial action of nitrite is attributed to reactions associated with the generation of nitric oxide (NO·). Nitric oxide reacts with neutrophil-derived superoxide inside the phagosome to yield the potent oxidant peroxynitrite ($ONOO^-$), which has an antimicrobial effect. This molecule is a key effector in the control of infections by macrophages. Its central role has been proven in a large number of models (Prolo, Álvarez, & Radi, 2014). Also, NO· aids in the killing mechanism of macrophages.

Peroxynitrite - a reactive but short-lived peroxide - is a strong oxidant and nitrating agent capable of causing the oxidation and nitration of proteins, DNA, and lipids through direct oxidative reactions or indirect, radical-mediated mechanisms. It is both an oxidant and a nucleophile, and these two chemical properties dictate much of its biochemical actions in vivo, making it the cause of a variety of cell injuries (Ferrer-Sueta & Radi, 2009). At 5–37 °C, $ONOO^-$ has an apparent acidity constant, pKa, app. of 6.8 (Pryor & Squadrito, 1995). The protonated molecule is conjugated acid peroxynitrous acid (ONOOH), which is also a strong oxidizing species in vivo (Eq. (3)), which is able to permeate biological membranes.

$$ONOO^- + H^+ \leftrightarrow ONOOH \tag{3}$$

The stability, reactivity, and capacity to permeate membranes of $ONOO^-$ and ONOOH are different.

Peroxynitrite is relatively stable. Below pH 7, > 90% of free peroxynitrous acid isomerizes to nitrate (Eq. (4)). Metmyoglobin catalyzes this isomerization (Herold & Shivashankar, 2003). The rest is cleaved by homolysis to hydroxyl radical (OH·) and nitrogen dioxide ($NO_2·$) (Eq. (5)) (Koppenol, Bounds, Nauser, Kissner, & Rüegger, 2012), which are also strongly oxidant.

$$ONOOH \rightarrow NO_3^- + H^· \tag{4}$$

$$ONOOH \rightarrow OH^° + NO_2^· \tag{5}$$

Peroxynitrite can cross cell membranes through either anion channels or passive diffusion of the anionic and protonated molecules, respectively (Denicola, Souza, & Radi, 1998). The calculated permeability coefficient for ONOOH is 8.0×10^{-4} cm/s, which is comparable to that of H_2O and is approximately 400 times greater than that of superoxide. This high permeability means that ONOOH is a highly transportable and highly reactive oxidant (Su & Groves, 2010). Therefore, the