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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

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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 peroxyde (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):

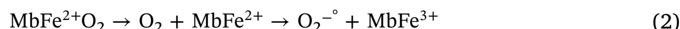
$$NO_2^- + MbFe^{2+} + H^+ \rightarrow NO^{\circ} + MbFe^{3+} + OH^- \quad (1)$$

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):


$$MbFe^{2+}O_2 \rightarrow O_2 + MbFe^{2+} \rightarrow O_2^{-\circ} + MbFe^{3+} \quad (2)$$

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.

$$ONOO^- + H^+ \leftrightarrow ONOOH \quad (3)$$

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.

$$ONOOH \rightarrow NO_3^- + H^{\cdot} \quad (4)$$

$$ONOOH \rightarrow OH^{\circ} + NO_2^{\cdot} \quad (5)$$

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

Fig. 1. Cured-meat: interactions NO_3^- vs bacteria. Bacterial stress, which is highly pH-dependent and very low partial pressure of oxygen-dependent, is enhanced by the nitrate-nitrite-peroxynitrite system which is also pH-dependent and low partial pressure of oxygen-dependent. Reactions with deoxymyoglobin condition all biochemical mechanisms related to peroxynitrite. Nitric oxide follows at least three pathways, in addition to its free transmembrane diffusion. One NO· molecule is coordinated to the Fe^{2+} center of one $MbFe^{2+}$ molecule. That forms the nitrosylmyoglobin. This reaction inhibits the prooxidant properties of $MbFe^{2+}$, but it is reversible since the nitric oxide can be displaced by oxygen in a reversible ligand-exchange reaction. NO· reacts with O_2^-· to produce a mixture of $ONOO^-$/ONOOH as a function of pH. This reaction is in competition with the reaction of superoxide dismutase (Cu, Zn-SOD) which catalyzes the dismutation of O_2^-· into H_2O_2. Catalase catalyzes the disproportionation of hydrogen peroxide to water and dioxygen. However, by binding to the heme iron, catalase binds nitric oxide to generate ferricatalase-NO, an inhibited form of the enzyme. This ferrous-nitrosyl complex is reversible by the addition of O_2^-· or H_2O_2. In competition with catalase, or if it is inhibited by NO·, H_2O_2 reacts with NO_2^- to produce a mixture of $ONOO^-$/ONOOH.

biochemistry of peroxynitrite in biological systems is highly pH-dependent (Pryor & Squadrito, 1995).

The formation of peroxynitrite occurs biologically in two different pathways. The best known is the reaction of nitric oxide with the superoxide radical anion (O_2^-·) (Denicola et al., 1998):

$$O_2^{-\cdot} + NO^{\cdot} \rightarrow ONOO^- \quad (6)$$

A less described but demonstrated way in vivo is the generation of peroxynitrite during the reaction of nitrite (NO_2^-) and hydrogen peroxyde (H_2O_2) (Kono, Shibata, Adachi, & Tanaka, 1994; Oury, Tatro, Ghio, & Piantadosi, 1995; Radi, 2013):

$$NO_2^- + H_2O_2 \rightarrow ONOO^- + H_2O \quad (7)$$

These two reactions can coexist depending to the biochemical ecosystem.

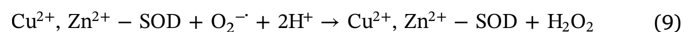
3.2. Superoxide - hydrogen peroxide - peroxynitrite

Reactions with deoxymyoglobin condition all biochemical mechanisms related to peroxynitrite. The following reactions take place in competition depending on the kinetics and the concentrations of reactants.

Nitric oxide follows at least three pathways (Fig. 1), in addition to its free transmembrane diffusion. One NO· molecule is coordinated to the Fe^{2+} center of one $MbFe^{2+}$ molecule. That forms the nitrosylmyoglobin (Andersen, Johansen, Shek, & Skibsted, 1990). This reaction inhibits the prooxidant properties of $MbFe^{2+}$, but it is reversible since the nitric oxide can be displaced by oxygen in a reversible ligand-exchange reaction (Arnold, Bohle, & Jordan, 1999), and possibly by NO_2^- (Eq. (8)). It is a substrate competition:

$$NO^{\cdot} + MbFe^{2+} \leftrightarrow MbFe^{2+}NO \quad (8)$$

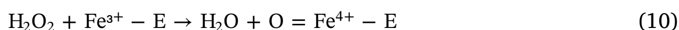
As mentioned (Eq. 4), NO· reacts with O_2^-· to produce a mixture of $ONOO^-$/ONOOH as a function of pH. This reaction is in competition with the reaction of superoxide dismutase (Cu, Zn-SOD) which catalyzes the dismutation of O_2^-· into H_2O_2. (Eq. (9)):



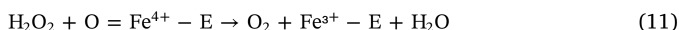
$$Cu^{2+}, Zn^{2+} - SOD + O_2^{-\cdot} + 2H^+ \rightarrow Cu^{2+}, Zn^{2+} - SOD + H_2O_2 \quad (9)$$

Catalase, a tetramer of four polypeptide chains with four porphyrin hemes (iron), catalyzes the disproportionation of hydrogen peroxide to water and dioxygen in two steps. The first step involves the ferricatalase removing and binding one oxygen atom and releasing the rest of the hydrogen peroxide molecule as water (Eq. 10). The second step is the catalase breaking down another hydrogen peroxide molecule by

releasing oxygen gas and water (Eq. (11)):



$$H_2O_2 + Fe^{3+} - E \rightarrow H_2O + O = Fe^{4+} - E \quad (10)$$



$$H_2O_2 + O = Fe^{4+} - E \rightarrow O_2 + Fe^{3+} - E + H_2O \quad (11)$$

However, by binding to the heme iron, catalase binds nitric oxide to generate ferricatalase-NO, an inhibited form of the enzyme (Brown & Borutaite, 2006). This ferrous-nitrosyl complex is reversible by the addition of $O_2^-\cdot$ or H_2O_2 (Kim & Han, 2000). In competition with catalase, or if it is inhibited by NO·, H_2O_2 reacts with NO_2^- to produce a mixture of $ONOO^-$/ONOOH (Eq. (5)). NO· does not inhibit glutathione peroxidase, the other main H_2O_2-removing enzyme, but $ONOO^-$ does inhibit the peroxidase (Padmaja, Squadrito, & Pryor, 1998).

3.3. Abiotic factors

3.3.1. Anaerobic conditions

The absence of oxygen has several impacts. First of all, the low oxygen partial pressures facilitate the formation of deoxymyoglobin. This allows the nitrite to react with $MbFe^{2+}$ to form NO· (Eq. (1)). $MbFe^{2+}$reacts with unbound O_2 yielding $MbFe^{3+}$ and superoxide radical ($O_2^-\cdot$) (Eq. (2)) (autoxidation) ((Møller & Skibsted, 2006). These two molecules, NO· and $O_2^-\cdot$, are precursors of the peroxynitrite.

Otherwise, aerobic/facultative anaerobic, and anaerobic bacteria sense environmental changes in oxygen concentration and adapt their regulatory mechanisms to ensure that the most energetically favorable process is active under a given environmental condition. The majority of denitrifying bacteria are facultative aerobic heterotrophs that switch from aerobic respiration to denitrification when oxygen runs out. This forces the bacterium to use nitrate as an available terminal electron acceptor (González, Correia, Moura, Brondino, & Moura, 2006). The low oxygen partial pressures induce the transcription of the nitrate reductase gene, and the two electron reduction of nitrate to nitrite.

Likewise, the absence of oxygen severely inhibits the expression of many TCA-cycle enzymes. Thus, under anaerobic conditions and in the absence of alternative electron acceptors, they usually convert carbohydrates to a mixture of products by fermentation to maintain redox balance (Fig. 2). The major soluble products are acetate, D-lactate, formate, and ethanol (Fig. 2), depending on the species (*Km* and *Vm* of the enzymes that are involved). These end products are exported from the bacteria. The protons of weak acids cause of the substantial acidification of the growth medium during mixed-acid fermentation.

Staphylococcus aureus may grow anaerobically either by using nitrate as a terminal acceptor (nitrate respiration) or by fermentation metabolism on carbohydrates. It produces both acetate and D-lactate. However, the D-lactate is mainly produced in bacteria grown under fermentative conditions. Moreover, in the presence of nitrate, *Staphylococcus aureus* secretes higher amounts of acetate (Fuchs, Pané-Farré, Kohler, Hecker, & Engelmann, 2007) at the expense of D-lactate.

Clostridium botulinum has a complete glycolysis pathway, but an incomplete TCA cycle. It appears to be capable of both acidogenic (acetate, D-lactate, butyrate) and solventogenic (ethanol) fermentation. In regard to *Clostridium perfringens*, the addition of nitrate causes butyrate to disappear and marks a decrease in ethanol quantities. Acetate is the main product in this case (Ishimoto, Umeyama, & Chiba, 1974). Mixed-acid fermentation is used by genera such as *Escherichia*, and *Salmonella*. It yields significant amounts of acetate, D-lactate, ethanol, and, to a lesser degree, succinate, as well as formate that is then reduced to CO_2 by periplasmic formate dehydrogenases. Acetate, D-lactate, and formate are the predominant end products and accumulate significantly during anaerobic growth on carbohydrates (Lü et al., 2012). Under anaerobic growth conditions, *Listeria monocytogenes* mainly synthetizes D-lactate, but small amounts of acetate, formate, ethanol, and carbon dioxide are also formed (Romick, Fleming, & McFeeters, 1996). It is worth mentioning once again that the addition of nitrate increases acetate production at least for *Staphylococcus aureus* and *Clostridium*. To date, no data are available for *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes*. This element is important because acetic acid is more effective in inhibiting microbial growth than lactic acid (Tangkham, Comeaux, Ferguson, & LeMieux, 2012). Their toxicity is pH dependent (see below "acidic pH").

3.3.2. Acidic pH effect

In the pH range of cured meats (5.0–6.0), the meat's acidity level is a key element. We have seen that the autoxidation of oxymyoglobin is catalyzed by the acidity (Eq. (2)). In oxymyoglobin, oxygen bonded in $MbFe^{2+}O_2$ is stabilized by hydrogen bonding from the imidazole ring (pKa ≈ 6.0) of the distal histidine (histidine 64). At pH ≈ 5.2, the dominant form of imidazole group is protonated, and the affinity for O_2 is lowered. In acidic conditions, the oxygen leaves the heme and allows the nitrite to react with $MbFe^{2+}$ to form NO· and $MbFe^{3+}$ (Eq. (1)). This reaction is carried out in competition with the autoxidation of $MbFe^{2+}O_2$ (Eq. (2)).

Moreover, the molecules that are in the protonated form (acids) readily diffuse through the membrane. This free diffusion is governed by the pKa of the acid and pH of the solution. The higher the acidity, the higher the transfer rate (Table 1). Once they have crossed the membrane into the cytoplasm, at pH values around 7.0–7.5, these molecules dissociate into a proton and an anion. The passive diffusion depends on the concentration gradient. The molecule permeates through the membrane until a balance of concentration is reached between the outside and inside the cell.

The effectiveness of nitrite and its oxidized form, peroxynitrite, is highly pH-dependent. Peroxynitrous acid (Eq. (3)) can cross bacterial membranes more easily by passive diffusion than peroxynitrite (Fig. 1). In an acidic medium, the fraction of undissociated acid is increased. At pH 6, ONOOH represents 86%, and at pH 5.0 it is around 98% (Table 1). In the cytoplasm, the $ONOO^-$ concentration is slightly higher.

This is also true for $O_2^-\cdot$ (pKa 4.88) and its pronated form, the perhydroxyl radical ($HO_2\cdot$) (Eq. (12)) (Bielski, Arudi, Cabelli, & Bors, 1984). Under the latter conditions, more superoxide was protonated and uncharged. Then the penetrance of superoxide was proportional to the concentration of this species. The permeability coefficient of $HO_2\cdot$ was determined to be 9×10^{-4} cm/s, slightly lower than that of water and far higher than the value of the anionic form ($O_2^-\cdot$, $< 10^{-7}$ cm/s) (Korshunov & Imlay, 2002). < 1% is protonated in the cytoplasm. However, in cured meats, at pH 6.0, $HO_2\cdot$ represents around 7% and at pH 5.0 around 43% (Table 1). Its solubility is similar to O_2 and it can cross membranes by passive diffusion (Guidot et al., 1995). In the cytoplasm, the concentration of $O_2^-\cdot$ is around 99%.

$$O_2^{-\cdot} + H^+ \leftrightarrow HO_2^{\cdot} \quad (12)$$

Transmembrane nitrite transfer by passive diffusion (Fig. 1) proceeds in the form of undissociated nitrous acid (HNO_2). At pH 5.0, a low fraction of HNO_2 (about 0.2%) can be easily compensated by its significantly higher membrane permeability, and its higher speed, compared with nitrite anion.

$$NO_2^- + H^+ \leftrightarrow HNO_2 \quad (13)$$

Obviously, with a pKa 11.6, the pH has no influence on H_2O_2 and it is 100% protonated.

The excreted fermentation products (Fig. 2) such as acetic acid (pKa 4.75) and lactic acid (pKa 3.85) can reenter the cells by passive diffusion at a low external pH. These undissociated fermentation acids dissociate at the higher internal pH (7.0–7.5):

$$CH_3COO^- + H^+ \leftrightarrow CH_3COOH \quad (14)$$

$$CH_3CHOHCOO^- + H^+ \leftrightarrow CH_3CHOHCOOH \quad (15)$$

A difference of about one pKa unit has significant consequences on

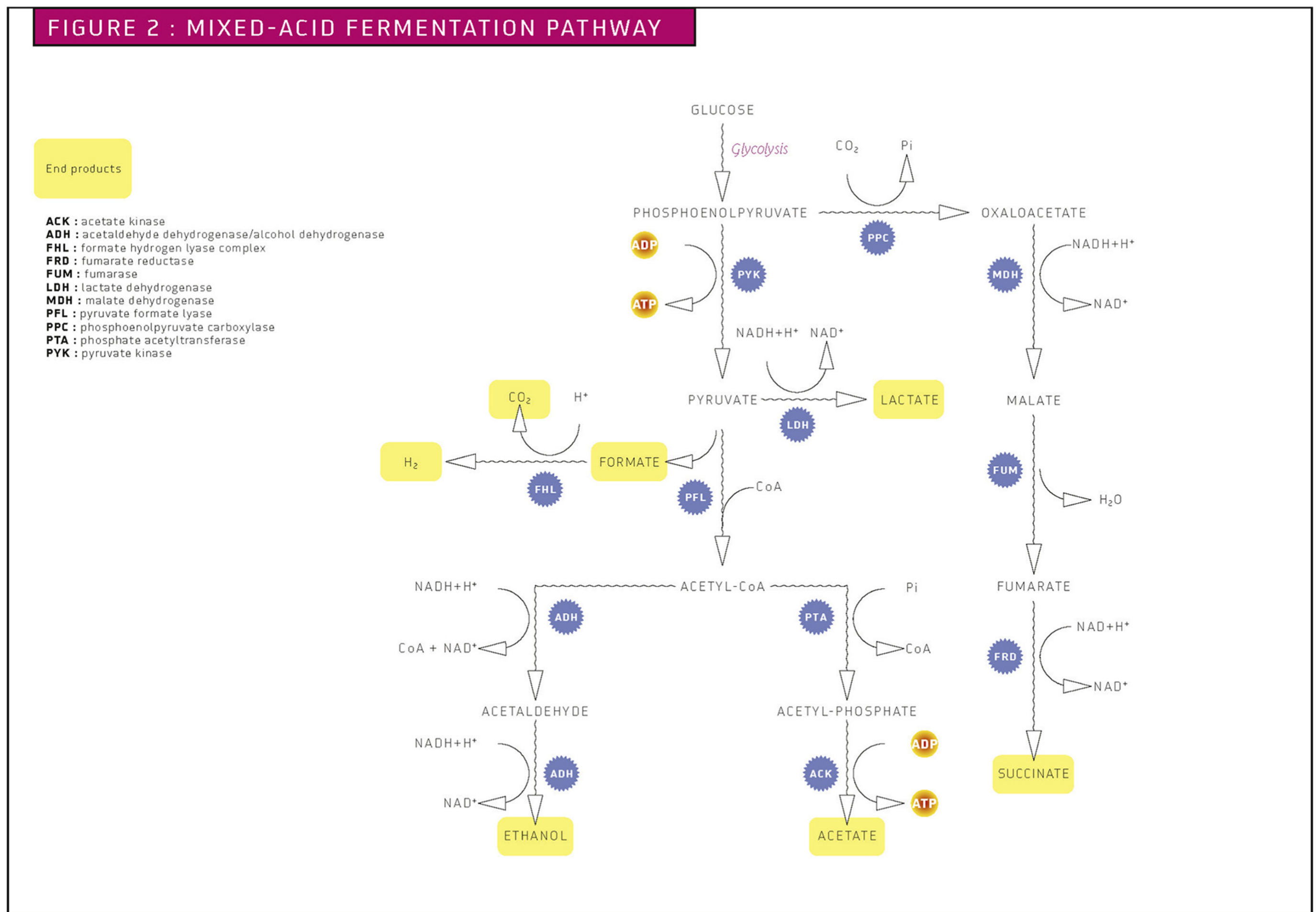


Fig. 2. Mixed-acid fermentation pathway in bacteria. Mixed acid fermentation is the biological process by which a six-carbon sugar e.g. glucose is converted into a complex and variable mixture of acids. It is an anaerobic fermentation reaction that is common in bacteria. The mixture of end products produced includes lactate, acetate, succinate, formate, ethanol and the gases H_2 and CO_2. The formation of these end products depends on the presence of certain key enzymes in the bacterium. The proportion in which they are formed varies according to bacterial species.

the two weak acids' passive diffusion rate. CH_3COOH and $CH_3CHOHCOOH$ represent around 36% and 7% at pH 6, and around 5% and 0.7% at pH 5.0, respectively (Table 1).

In a metabolic situation in a medium at pH 7, the bacteria regulate the excretion of dissociated weak acids and H^+ as well as the external pH drop in order to maintain their homeostasis down to the minimum growth pH. They regulate their own metabolism. However, in cured meats, the pH range is already acidic (5.0–6.0). This context is a constraint for bacterial metabolism that creates the risk of chronic imbalance. The bacteria must manage this situation through the products they excrete, as well as the difference in pH between the cytoplasm and the external environment. The higher the ΔpH, the greater the stress due to the H^+ concentration and the greater the concentration of protonated acids that backscatter (Table 1).

The bacteria must (i) limit the import of H^+ from the external environment via ATPase, (ii) control the electroneutral H^+ influx by undissociated weak acids, (iii) decrease the increase in cell turgor pressure, and (iv) counteract the toxic effect of fermentation anion accumulation. It is clear that effects on cytoplasmic pH, perturbation of membrane lipids and effects of anion accumulation are likely to be greatest at low pH values. At pH values close to the pKa of organic acid, inhibition is multifactorial (Russell, 1992).

It is interesting to analyze the impact of acetate for several reasons (Fig. 2): (i) it is considered the most inhibiting acid for the bacteria, (ii) its pKa (4.75) allows a higher protonated form rate, (iii) the addition of nitrate increases acetate production for certain bacteria, and (iv) accumulation of the acid anion in the cytoplasm has some significant effects on the bacteria anion balance (Roe, McLaggan, Davidson, O'Byrne, & Booth, 1998). Glycolysis oxidizes glucose to two pyruvate molecules while generating only two ATP molecules. Oxidation of glucose, however, also produces two NADH molecules, which corresponds to four reducing equivalents. Because NAD^+ serves as a substrate for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), bacteria must reoxidize NADH to maintain glycolytic flux. In the absence of a functional TCA cycle, they achieve this by placing the reducing equivalents onto partially oxidized metabolic intermediates, predominantly: (i) from pyruvate: D-lactate, formate, and ethanol, (ii) from oxaloacetate: succinate, which bacteria excrete into their environment along with acetate. Excretion of these metabolic intermediates sacrifices energy to consume reducing equivalents, whereas acetate production generates energy in the form of ATP (1 molecule of ATP per molecule of acetate) (Wolfe, 2005). Thus, cell metabolism is modulated by its redox state and energy availability (see FEDOX concept below). Pyruvate formate Lyase (PFL) catalyzes the reversible conversion of pyruvate and coenzyme A (CoA) to formate and acetyl-CoA. This acetyl-CoA with an inorganic phosphate is converted into acetyl phosphate via phosphotransacetylase (PTA). Acetate kinase (ACK) converts acetyl phosphate into acetate along with the phosphorylation of ADP. Thus, acetate production is the more energy-efficient fermentative pathway. The reactions, catalyzed by ACK and PTA, are reversible. Usually, the ACK *Km* for acetyl phosphate is significantly lower than for acetate (as with *Salmonella enterica serovar*

Typhimurium, for example) (Chittori, Savithri, & Murthy, 2012). The concentration of cytoplasmic acetate must therefore be higher to reverse the reaction. There is competition on ACK between acetyl phosphate and acetate as a function of their *Km*, *Vm* and concentration. This competition is directly related to ΔpH, to the difference (pH-pKa acetic acid), and to the balance between the acetate concentrations inside and outside the bacteria. The reverse process is particularly deleterious to the bacteria on an energetic level. Instead of producing 1 ATP, the cell consumes 1 ATP per molecule of acetate, while increasing the acetyl-CoA concentration. This imbalance has a direct impact on the homeostasis of pyruvate and the productivity of anaerobic glycolysis (Fig. 2).

Thus, anaerobiosis at a pH of 5 to 6, and high concentrations of fermentation acids (acetic acid and lactic acid in particular) can inhibit the growth of many bacteria. The ΔpH-mediated anion accumulation provides a mechanistic explanation for the effect of fermentation acids on microbial ecology and metabolism. This is reinforced by the nitrate which increases the concentration of acetic acid, at least for *Staphylococcus aureus* and *Clostridium*.

3.3.3. Ascorbate effect

Sodium ascorbate is commonly used in cured meat systems to increase the speed and extent of curing, in particular the rate of nitric oxide production and nitrosylmyoglobin formation. In the pH range of cured meats (5.0–6.0), ascorbic acid is mainly dissociated into ascorbate (pKa 4.1). Ascorbate reduces Fe^{3+} of metmyoglobin to Fe^{2+} of deoxymyogloblin and dehydroascorbate (Richards, 2013):

$$MbFe^{3+} + \text{Ascorbate} \rightarrow MbFe^{2+} + \text{DHA} \tag{16}$$

However, ascorbate can also participate in the reduction cycle of metmyoglobin reductase (MMR). This regeneration cycle of deoxymyoglobin generates NO· from nitrite binds to deoxymyoglobin. It maintains the production of NO·, and O_2^{-}·, and H_2O_2, and consequently $ONOO^{-}$/ONOOH. Ascorbate increases $ONOO^{-}$/ONOOH synthesis. It promotes its antimicrobial activity. Although it is an antioxidant, it increases oxidative stress (Fig. 1).

3.3.4. Sodium chloride effect

Microbial growth is largely dependent upon the amount of moisture in a system available as a growth medium. The typical expression of this is the water activity (a_w) of the product. Salting of meat provides a drying action, increases ionic strength and decreases water activity. These conditions play an important role in salt's antimicrobial activity in processed meats. But this does not fully explain the preservative effect. The chloride anion has a major role in this activity for at least two reasons. Cl^{-} causes the formation of nitrosyl chloride (NOCl), which is a powerful nitrosylating agent (Devine & Dikeman, 2014). It increases oxidative stress. Chloride accelerates the kinetic effect on the acid-catalyzed autoxidation of oxymyoglobin (Eq. (2)), and this autoxidation promotes the formation of peroxynitrite. This autoxidation is caused by an increased ionic strength with a rate enhancement comparable to that caused by the postmortem pH decrease. If the level is the same, this acceleration makes it possible to fight the targeted bacteria more quickly (Andersen, Bertelsen, & Skibsted, 1988).

4. Bacteriostatic and bactericidal effects chemical mechanisms of nitrite and other hurdle technologies against pathogenic bacteria

Nitrite plays an important function as a bacteriostatic and bactericidal agent. This role has varying degrees of effectiveness in either preventing or controlling the multiplication of certain bacteria. This activity is directly related to the level of oxidative stress caused by $ONOO^{-}$/ONOOH. However, this oxidative stress depends on the concentrations of NO_2^{-}, NO·, O_2^{-}· and H_2O_2. Their concentration levels and their production and reaction kinetics also depend on pH, NaCl and ascorbate rates.

To multiply, the bacteria produces energy (ATP), but it must also protect itself from oxidation related to the synthesis of ATP and other exogenous causes. This situation can be defined by the concept of FEDOX balance (Function-Energy-Defense OXidation) (Majou, 2015). There is a permanent compromise in chemical kinetics. However, depending on the level of oxidative stress, these defense systems are more or less overwhelmed. Chronic FEDOX imbalance leads to oxidative stress. The consequences are directly lethal in the short term or sublethal with a variable latency phase.

In cured meats - an anaerobic or microaerobic, acidic and highly oxidizing ecosystem - antioxidant defenses are preponderant. Some species are more resistant than others to acidic, oxidative, and nitrative stresses caused by $ONOO^{-}$/ONOOH. The toxic effect of peroxynitrite may stem from its oxidation of zinc fingers, protein thiols, membrane lipids, cysteine and arginine biosynthesis and iron-sulfur proteins (McLean, Bowman, Sanguinetti, Read, & Poole, 2010) of biological molecules. Peroxynitrite acts predominantly as an agent that promotes modifications of DNA bases, including the formation of 8-hydroxydeoxyguanosine or 8-nitroguanine (Jourd'heuil, Kang, & Grisham, 1997; Kamat, 2006). Peroxynitrite produces DNA strand breaks, activating the nuclear repair enzyme poly(ADP)ribosyltransferase (PARS), which results in ADP ribosylation, NAD^{+} and ATP consumption (Zingarelli, O'Connor, Wong, Salzman, & Szabó, 1996). Otherwise, nitric oxide inhibits the iron-sulfur enzymes due to the formation of iron-NO complexes such as catalase, ferrochelatase, aconitase, etc. (Stern, Liu, Bakken, Shapleigh, & Zhu, 2013).

Furthermore, the addition of nitrate increases acetate production at least for some bacteria (*Staphylococcus aureus* and *Clostridium*) with a deleterious effect on the bacteria ATP level at pH 5–6. The most

Table 2
Various reductases. Bacteria have a number of mechanisms (i) to switch from aerobic respiration to denitrification when oxygen as an available terminal electron acceptor runs out (reductases); (ii) to minimize the deleterious effects of oxidizing agents. These mechanisms include the repair of damage caused by oxidizing agents, in particular damage to DNA, as well as the prevention of such damage.

	Type	Oxygen	Mini growth pH[a]	Nitrate reductase	Nitrite reductase	Nitric oxide reductase	SOD	Catalase	AhpC	Regulator	DNA-binding protein	Others
Escherichia coli	Gram −	Aerobic/ anaerobic	4.4	+	+	+	+	+	+	OxyR	Dps	
Salmonella typhimurium	Gram −	Aerobic/ anaerobic	3.8	+	+	+	+	+	+	OxyR	Dps	
Clostridium botulinum	Gram +	Anaerobic	4.6–5.0	−	+	- ?	+	−	+ *Cl. Perf.*	?	− *Cl. Perf.*	
Staphylococcus aureus	Gram +	Aerobic/ anaerobic	4.0	+	+	+	+	+	+	PerR	MgrA	Carotenoid pigments
Listeria monocytogenes	Gram +	Aerobic/ anaerobic	4.0–4.3	−	- ?	- ?	+	+	+	PerR	Fri	

[a] From ANSES (France).

resistant are gram-negative aerobic/facultative anaerobic bacteria (*Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Enterobacter* spp., *Pseudomonas* spp., etc.), and the most fragile are gram-positive anaerobic bacteria (*Clostridium* spp., etc.). Table 2 presents the situation of the species that are most often present in cured meats.

4.1. Salmonella enterica serovar typhimurium, and Escherichia coli

These species are gram-negative aerobic/facultative anaerobic rods that are non-sporulating, glucose fermenting and oxidase-negative (Kumar, 2012). Usually, they use oxygen for aerobic respiration. However, successful colonization of microaerobic and anaerobic environments depends on the ability to use alternative anaerobic electron acceptors, including nitrate, nitrite, fumarate, dimethyl sulfoxide (DMSO) and trimethylamine-*N*-oxide (TMAO). The electron acceptors with greater redox potential are used preferentially. For instance, in *Escherichia coli*, nitrate is the preferred electron acceptor over fumarate (Jones et al., 2011).

They are some very effective anti-oxidant defenses located in the periplasm. First of all, they possess three different nitrate reductases, comprising the periplasmic Nap with three subunits (ABC), and the two membrane-bound isoenzymes (NarG and NarZ) whose active site is in the cytoplasm (Stewart, Lu, & Darwin, 2002). Nitrite generated by Nap and Nar can be further reduced by one of two different pathways: denitrification and nitrite reduction to ammonia. During the denitrification process, *Salmonella enterica serovar* Typhimurium and *Escherichia coli* produce NO· as an obligate intermediate, which is quickly reduced to N_2O by the anoxically functioning nitric oxide reductase (NorVW-flavorubredoxin) or HmpA (monomeric flavohemoglobin), under anaerobic conditions (Crawford & Goldberg, 1998; Gardner, Gardner, Martin, & Salzman, 1998). Hmp has also been shown to be capable of catalyzing the reduction of several alkylhydroperoxide substrates into their corresponding alcohols using NADH as an electron donor (Bonamore & Boffi, 2008). The second pathway, ammonification, where NO· is generated in low concentrations as a by-product, is conducted by two distinct nitrite reductases (cytoplasmic NirB and periplasmic NrfA), which generate ammonia in the cytoplasm and periplasm, respectively (Spiro, 2007). Nap and NrfA are co-regulated and together represent the periplasmic pathway for the reduction of nitrate, via nitrite, to ammonia. In the cytoplasm, this process is accomplished by the Nar-NirB pathway (Lundberg, Weitzberg, Cole, & Benjamin, 2004). In *Escherichia coli*, there is a differential pattern of nitrite reductase gene expression whereby the *nrfA* operon is preferentially expressed only at low nitrate concentrations. Maximal *nirB* expression occurs only at high levels of nitrate. Nitrite, the substrate for each enzyme, is shown to be a less potent regulatory signal for either operon compared to nitrate which is the more potent inducer by > 100-fold (Wang & Gunsalus, 2000).

Salmonella enterica serovar Typhimurium and *Escherichia coli* also possess other groups of periplasmic enzymes which have antioxidant effects, such as superoxide dismutases (SOD) expressing one or more isoform in the periplasm. There is one periplasmic CuZn-SOD in *Escherichia coli* (SODC) (Battistoni et al., 2000) and three SODC enzymes in *Salmonella enterica serovar* Typhimurium (SODCI, SODCII, SODCIII). SODCII is the homologous SODC in *Escherichia coli* (Sly, Guiney, & Reiner, 2002). The role of periplasmic superoxide dismutase is to block the penetration of external superoxide into the cytosol of gram-negative bacteria (Korshunov & Imlay, 2002). So, SODs protect periplasmic or inner membrane targets by diverting superoxide and limiting peroxynitrite formation (Eq. (4)) (De Groote et al., 1997; Wink et al., 1997).

The detoxification of peroxynitrite involves, among others, the oxidative stress response enzymes alkyl hydroperoxide reductases (AhpC), a bacterial peroxiredoxin, and catalases (Kat). Three catalases and two alkylhydroperoxide reductases (Ahp subunit C and TsaA) are identified in the cytoplasm of *Salmonella enterica serovar* Typhimurium. KatE and KatG are involved in H_2O_2 degradation, as well as AhpC and TsaA (Thiol-specific antioxidant) (Hébrard, Viala, Méresse, Barras, & Aussel, 2009). AhpC catalyzes the reduction of organic hydroperoxides (ROOH) and H_2O_2. TSA represents a group of enzymes that can reduce hydroperoxides in the presence of a thiol-containing electron donor. In *Escherichia coli*, three enzymes are involved in H_2O_2 scavenging: two catalases (KatE and KatG) and an AhpC (Seaver & Imlay, 2001). AhpC and KatG are identified as peroxynititrases (Mclean, Bowman, Sanguinetti, et al., 2010). Peroxynitrite causes upregulation of *katG* and a*hpC* genes (McLean, Bowman, & Poole, 2010). The expression of hydrogen peroxide-inducible proteins is controlled by the positive transcriptional regulator OxyR in *Escherichia coli* and *Salmonella enterica serovar* Typhimurium (Christman, Morgan, Jacobson, & Ames, 1985; Storz & Imlay, 1999). It is activated by a disulfide bond formation between two cysteine residues and induces the expression of *oxyS* (which encodes a small, non-translated regulatory RNA), *katG*, a*hpC*, *gorA* (which encodes glutathione reductase), *dps* (which encodes DNA-binding protein), and *grxA* (which encodes glutaredoxin 1). Glutaredoxin 1 deactivates OxyR by reducing the disulfide bond, forming an autoregulatory feedback loop (Zheng, Aslund, & Storz, 1998). So, the elimination of H_2O_2 is important for aerobic microorganisms as well as for anaerobic microorganisms.

Moreover, in *Salmonella enterica serovar* Typhimurium and *Escherichia coli*, OxyR with sigma factor RpoS (RNA polymerase, sigma S) and IHF (integration host factor) regulate the expression of *dps*, a Dps gene (DNA-binding proteins from starved cells) (Halsey, Vazquez-Torres, Gravdahl, Fang, & Libby, 2004; Marshall, Bowe, Hale, Dougan, & Dorman, 2000). Conversely, Fis and H-NS (heat-stable nucleoid-structuring) proteins can bind at the *dps* gene promoter and down-regulate its activity. Dps protein belongs to the ferritin superfamily. In response to oxidative stress, it protects DNA against oxidative damage and hyper-compacts the nucleoid into a crystalline-like structure in stationary phase. Dps levels are very low in exponentially growing cells and rise sharply as cells enter stationary phase. During the stationary phase of growth in *Escherichia coli*, the conformation of DNA changes from a relaxed state to a compacted state, a process requiring Dps. Also, Dps protein performs a pivotal function in the survival of stationary phase *Salmonella enterica serovar* Typhimurium against oxidative stress (Yoo et al., 2007).

At the concentration levels used in cured meats, nitrate alone has no bactericidal effects (Talon, Leroy, Vermassen, & Christieans, 2015). Its effect is completely inhibited by the combined action of periplasmic nitrate and nitrite reductases. Contradictory results exist on the effect of nitrite on the growth of *Salmonella enterica serovar* Typhimurium, with some studies showing inhibition (Christieans, Picgirard, Parafita, Lebert, & Gregori, 2018; Hospital et al., 2014) and others inefficiency (Bayne & Michener, 1975; Tompkin, 2005). The action is induced by the added nitrite and the resulting $ONOO^-$/ONOOH. However, $ONOO^-$/ONOOH is inhibited in whole or in part by peroxynitrases. There is a more or less significant FEDOX imbalance which has a bacteriostatic effect in the first few days. So, according to the mechanisms described above and because of their resistance to oxidative stress, the survival of these species is clearly dependent on many factors: acidic pH, ΔpH (transmembrane pH gradient), anaerobic conditions, NaCl and $NaNO_2$ concentrations, bacterial concentration, water activity (drying effect), and temperature (Gwak et al., 2016) with hurdle effects. Of course, the level of nitrite concentration has effects in some cases, but always in combination with other factors, and is probably strain-dependent. The minimum pH that enables the growth of *Salmonella enterica serovar* Typhimurium is around 3.8 (ANSES, 2011). Often a decline in population is observed in the maturation and storage phase, i.e. during a decline in water activity (Talon et al., 2015).

4.2. Clostridium botulinum - Clostridium perfringens

Clostridium botulinum is a gram-positive, strict anaerobic spore-

forming rod that is SOD-positive and catalase-negative. In foods with low acid levels, such as meat products, spores of *Clostridium botulinum* can germinate, grow and produce toxins. Although all botulinogenic clostridial strains have traditionally been classified as *Clostridium botulinum*, it is recognized that *Clostridium botulinum* contains four distinct genetic and physiological groupings (Lund, Peck, & Peck, 2000). Group I strains, whose spores are heat resistant, can germinate and proliferate at aw > 0.94 (10% NaCl) and pH > 4.6. Those in group II, whose spores are heat sensitive, can germinate and proliferate at aw > 0.97 (5% NaCl) and pH > 5.0 (Talon et al., 2015). Thus, their minimum growth pH is 4.6–5.0. Group II poses a safety hazard for modern food processing methods that consist of mild pasteurization treatments, anaerobic packaging, extended shelf lives and chilled storage.

As a gram-positive, strict anaerobic, SOD-positive (Sebaihia et al., 2007), catalase-negative bacteria, *Clostridium botulinum* is very sensitive to oxidative stress. Its optimal growth is at a potential redox of −350 mV (Smoot & Pierson, 1979). Therefore, the antioxidant defenses of certain strains are not suited to chronic oxidative stress in anaerobiosis. Nitrite has a double action on *Clostridium botulinum*. On the one hand, through exogenous $ONOO^-$/ONOOH produced in meat as for gram-negative bacteria. On the other hand, NO° blocks the catalase in the meat. There is production of H_2O_2 in meat that penetrates the *Clostridium botulinum* which is devoid of catalase. It is the weak point of this bacterium. NO_2^- penetrates the bacterium by nitrite transporter NirC (Giordani et al., 2015). The *Clostridium* genus has a nitrite reductase (Brinkac et al., 2008; Sekiguchi, Seki, & Ishimoto, 1983). There is competition on NO_2^- between H_2O_2 and nitrite reductase. One part of NO_2^- is denitrified (nitrite reductase) and another is transformed into $ONOO^-$/ONOOH by reaction with H_2O_2 (Eq. (5)). This H_2O_2 attacks DNA in particular. The higher the NO_2^- concentration, the more nitrite reductase is saturated and the more $ONOO^-$ is produced.

Nitrite is effective against *Clostridium botulinum* (Hustad, Cerveny, Trenk, Deibel, & Kautter D0a, Fazio T., Johnston R.W., Kolari O.E., 1973). The properties of curing with nitrite that make it an effective antibotulinal compound are dependent on interactions of nitrite with several other factors (Hospital, Hierro, Stringer, & Fernández, 2016). According to the mechanisms described above, those factors include acidic pH, ΔpH (transmembrane pH gradient), pO_2, NaCl rate, aw (drying effect), ascorbate rate, temperature, heat treatment, spore level, and the nature of the competing flora, with hurdle effects (Archer, 2002; Roberts & Gibson, 1986).

The case is identical for *Clostridium perfringens*, which is a gram-positive, spore-forming anaerobe. Its minimum growth pH is 5.0. Spore formation can be controlled by adding nitrite to meat or poultry products. It has been proven in multiple experiments that nitrite effectively inhibits *Clostridium perfringens* (Perigo & Roberts, 1968; Sauter, Kemp, & Langlois, 1977). It is well established that *Clostridium perfringens* possesses the *ahpC* gene that is rapidly and fully induced when the bacteria are exposed to air or to various oxidative stress under anaerobic conditions. (Jean, Briolat, & Reysset, 2004). And it lacks the *dps* gene (Takeyasu et al., 2004), which makes it more sensitive to oxidative stress. The same situation could be assumed for *Clostridium botulinum*.

4.3. Staphylococcus aureus

Staphylococcus aureus is spherical gram-positive bacteria that is immobile and forms grape-like clusters. It is aerobic/facultative anaerobic and catalase-positive. Its minimum growth pH is 3.8–4.0. *Staphylococcus aureus* grows anaerobically both in the presence of nitrate by nitrate respiration and under fermentative conditions on glucose (Burke & Lascelles, 1975). It reduces nitrate to ammonia in two steps: (i) nitrate is taken up by a nitrate transporter (NatT) and reduced by a nitrate reductase (Nar) with four GHIJ subunits, to nitrite, which is subsequently excreted by a nitrite export-import transporter, SACOL 2363, and (ii) after depletion of nitrate, the accumulated nitrite is imported and reduced by an NADH-dependent nitrite reductase, Nir with two BD subunits, to ammonia, which then accumulates in the medium. Nitrate reductase is a membrane-bound enzyme involved in energy conservation, whereas nitrite reductase is a cytosolic enzyme involved in NADH reoxidation (Fuchs et al., 2007). Cytoplasmic assimilatory, and periplasmic dissimilatory nitrate reductases are absent in staphylococci (Morozkina & Zvyagilskaya, 2007). For *Staphylococcus aureus*, the influence of nitrate on gene regulation seems to be different from that in *Escherichia coli*. The presence of nitrate and/or nitrite induces nitrate reductase and nitrite reductase activities (Neubauer & Götz, 1996). But, the nitrate/nitrite regulation system also expresses in the absence of nitrate (Fuchs et al., 2007). Like *Escherichia coli*, *Staphylococcus aureus* has an Hmp activity that is greatest during microaerobic/anaerobic growth or during nitrosative stress conditions (Gonçalves, Nobre, Vicente, Teixeira, & Saraiva, 2006). Moreover, in the presence of nitrate, *Staphylococcus aureus* secretes higher amounts of acetate (Fuchs et al., 2007) at the expense of D-lactate. This weakens *Staphylococcus aureus* as we saw above.

Most *Staphylococcus aureus* strains form yellowish-orange or golden colonies due to the presence of carotenoid pigments. The main pigment is the membrane-bound orange-red C_{30} triterpenoid staphyloxanthin. Carotenoid pigments protect the strains against desiccation and photosensitization, and are known to quench toxic singlet oxygen. On this last point, carotenoids are potent antioxidants due to their numerous conjugated double bonds, which make them an important survival factor for detoxifying ROS. In addition to pigments, most *Staphylococcus aureus* strains possess several enzymes that are used in the detoxification of reactive oxygen and nitrogen intermediates; particularly, superoxide dismutases, catalases, glutathione peroxidases, globins, and peroxiredoxins (Gaupp, Ledala, & Somerville, 2012).

Staphylococcus aureus has two SOD-encoding genes, *sodA* and *sodM*. The products of these genes combine to form three SOD activity zones; two homodimers and a heterodimer (Clements, Watson, & Foster, 1999). SODA and SODM are Mn cofactor enzymes (Valderas & Hart, 2001). During in vitro aerobic growth of *Staphylococcus aureus*, the transcription and activity of both SODs increase in the post-exponential growth phase and remain high during the stationary phase, with SODA being responsible for the majority of SOD activity (Clements et al., 1999). Transcription of *sodA* and *sodM* is most strongly induced by either internally or externally generated $O_2^-\cdot$, respectively (Karavolos, Horsburgh, Ingham, & Foster, 2003).

Staphylococcus aureus has a single monofunctional heme-containing tetrameric catalase encoded by the *katA* gene (Horsburgh, Clements, Crossley, Ingham, & Foster, 2001). In addition to catalase, *Staphylococcus aureus* has several peroxiredoxins that are induced upon treatment of *S. aureus* with H_2O_2 [i.e. Thiol peroxidase, Organic hydroperoxide resistance (Ohr), and AhpC] (Wolf et al., 2008). KatA is the major determinant in resistance to H_2O_2 (Horsburgh, Clements, et al., 2001), while AhpC confers resistance to a broader spectrum of ROS (Cosgrove et al., 2007). The *katA* gene and the *ahpCF* operon are regulated by PerR (Peroxide operon regulator) (Horsburgh, Ingham, & Foster, 2001). PerR is a member of the Fur family of regulators, and it has been identified as a peroxide-sensing protein. The PerR regulon includes many genes involved in the oxidative stress response and iron storage, including *katA*, *ahpCF*, *mrgA*, *bcp*, and *trxA* genes (Horsburgh, Clements, et al., 2001). In addition, the transcription of *katA* is positively affected by the ferric uptake regulator (Fur) (Horsburgh, Ingham, & Foster, 2001), which is a transcriptional regulator that is partially responsible for maintenance of iron homeostasis in many bacteria.

Staphylococcus aureus exclusively utilizes PerR (peroxide operon regulator) to regulate *mrga* gene expression as an oxidative stress response. MrgA protein is the DPS homolog. PerR is a peroxide-sensing protein (Morikawa et al., 2007). Unlike *Escherichia coli*, *Staphylococcus aureus* appears to lack both the genes for Fis and H-NS (Ohniwa, Ushijima, Saito, & Morikawa, 2011). Therefore, the staphylococcal *mrgA* gene cannot be induced toward the stationary phase, although it is expressed under oxidative stress. This also weakens *Staphylococcus*

aureus in its stationary growth phase by increasing its sensitivity to oxidative stress. This oxidative stress could have both origins: (i) exogenous peroxynitrite, (ii) endogenous peroxynitrite. Nitrate reduction is faster than nitrite reduction in resting bacteria (Neubauer & Götz, 1996). A high rate of nitrate reduction could lead to an internal accumulation of cytoplasmic nitrite, possibly as a result of a less efficient export. Then, there could be double competition (i) on NO_2^- between H_2O_2 and the excretion transporter, (ii) on H_2O_2 between catalase and NO_2^-, with the result being endogenous peroxynitrite (Eq. (5)). The concentration level of nitrate alone in cured meats has no bactericidal effects (Meisel, Gehlen, Fischer, & Hammes, 1989). *Staphylococcus aureus* is quite resilient to nitrosative stress, in part due to its ability to detoxify NO· via a well-characterized flavohemoprotein (Hmp), a nitric oxide reductase (Gonçalves et al., 2006). As with *Salmonella enterica* serovar Typhimurium, contradictory results exist on the growth of *Staphylococcus aureus* in the presence of nitrite, with some studies showing inhibition (Fang, Post, & Solberg, 1985; Tompkin, Ambrosino, & Stozek, 1973) and others inefficiency (Bang, Hanson, & Drake, 2008; Lechowich, Evans, & Niven, 1956). The FEDOX is also more or less imbalanced, causing bacteriostatic effect in the first few days. And we can apply the same conclusions, namely that the survival of this species is probably strain-dependent and clearly dependent on a combination of many factors: acidic pH, ΔpH, anaerobic conditions, NaCl and $NaNO_2$ concentrations, bacterial concentration, water activity (drying effect), and temperature with hurdle effects.

The effects of nitro-oxidative stresses are well documented in *Staphylococcus xylosus*, traditionally used as a major starter culture in the production of raw fermented sausages and for its contribution to color and flavor development. However, *Staphylococcus aureus* and *Staphylococcus xylosus* have very similar genotypes and phenotypes. The in situ overall gene expression profile of *Staphylococcus xylosus* in meat supplemented with nitrate and nitrite at the levels used in the meat industry reveals that *Staphylococcus xylosus* is subject to nitrosative-oxidative stresses. To overcome these stresses, *Staphylococcus xylosus* has developed several oxidative stress resistance mechanisms, such as modulation of the expression of several genes involved in iron homeostasis (siderophores) and in antioxidant defenses (three catalases, AhpC, thioredoxin, thioredoxin reductase). Most of which belong to the Fur (ferric uptake regulator) and PerR regulons, respectively. *Staphylococcus xylosus* has also counteracted these stresses by developing DNA and protein repair including ribonucleotide reductases (Vermassen, de La Foye, Loux, Talon, & Leroy, 2014).

4.4. *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, aerobic to facultatively anaerobic, non-spore-forming, rod-shaped bacterium. It is catalase-positive, oxidase-negative and nitrate-reductase negative (Glaser et al., 2001). It possesses a manganese-containing SOD that is inducible when exposed to elevated levels of O_2 (Dallmier & Martin, 1990). The typical 2 Cys peroxiredoxin gene has been identified. Members of the 2-Cys peroxiredoxin subfamily have two highly conserved cysteines, and this family includes bacterial AhpC (Dons et al., 2014). Its minimum growth pH is around 4.0–4.3. As with *Staphylococcus aureus*, *Listeria monocytogenes* exclusively utilizes PerR to regulate *fri* gene expression as an oxidative stress response (Olsen et al., 2005). *Fri* gene encodes the only ferritin-like protein of this pathogen, Fri (non-heme iron-binding ferritin), a Dps homolog protein. *Listeria* Fri is endowed with the capacity to detoxify concurrently free iron and H_2O_2. *Fri* transcription is restricted to the exponential growth phase, whereas the Fri protein has a long half-life and is also detected in significant amounts in stationary phase bacteria. The transcription of *fri* is derepressed by PerR. But its expression is downregulated by Fur, the ferric uptake regulator (Fiorini, Stefanini, Valenti, Chiancone, & De Biase, 2008).

At the concentration levels used in cured meats, nitrate alone has no bactericidal effects (Talon et al., 2015) due to its lack of nitrate reductase. Nitrite could have significant bacteriostatic activity against *Listeria monocytogenes*. But its growth kinetic is also dependent on the interaction of the same variables, particularly regarding exponential growth rates and stationary phase time (Buchanan, Stahl, & Whiting, 1989; Duffy, Vanderlinde, & Grau, 1994; Hospital et al., 2012). Thus, nitrite only has a small effect on the growth of *Listeria monocytogenes* on cooked, sliced meat products. The effect of water activity was much more marked (Stegeman, Jansen, Zegveld, Verkleij, & Stekelenburg, 2006). As with *Staphylococcus aureus*, contradictory results exist on the growth of *Listeria monocytogenes* in the presence of nitrite. As a negative point, it is necessary to take into account its greater sensitivity to oxidative stress (exogenous peroxynitrite) in stationary phase due to the lack of *fri* transcription (Dps homolog). But as positive point, *Listeria monocytogenes* is nitric oxide reductase negative and Fur activity is switched off by nitric oxide at micromolar concentration. The complexation of NO° with Fur yields a species stable in anaerobic conditions (D'Autréaux, Touati, Bersch, Latour, & Michaud-Soret, 2002). This Fur inhibition is reflected by the derepression of gene expression under Fur control such as *fri* gene.

5. Conclusion

In cured meats, anaerobic and acidic conditions are the most effective parameters at different stages. Firstly, bacteria have a fermentative metabolism that is unbalanced by external acidic pH (5–6). In this environment, the products of mixed-acid fermentation - in particular acetic acid and lactic acid - have a toxic effect. If nitrate (NO_3^-) serves as a source of nitrite (NO_2^-), this latter contributes to oxidative stress by being the precursor of peroxynitrite ($ONOO^-$), a short-lived, reactive peroxide, which is a strong oxidant. Thus, bacterial stress, which is highly pH-dependent and non-oxygen-dependent, is enhanced by the nitrate-nitrite-peroxynitrite system which is also highly pH-dependent and non-oxygen-dependent. In a medium where homeostasis is unbalanced by acidity and anaerobiosis, nitrate and nitrite have a synergistic and aggravating effect depending on their concentration. It is a hurdle technology which cause bacterial stress as other hurdles such as sodium chloride, ascorbate, and aw.

Bacteria have different enzymatic pathways which are species-dependent and strain-dependent. Nitrate reductases and nitrite reductases reduce the two nitrogen compounds to NH3 and NO·. Nitric oxide reductase reduces NO· to nitrous oxide. The antioxidant defenses are preponderant in limiting the formation and action of peroxynitrite. Detoxification of peroxynitrite involves, SOD, oxidative stress response enzymes alkyl hydroperoxide reductases (AhpC), flavohemoglobin or nitric oxide reductase (Hmp), catalases. And Dps protects DNA against oxidative damage. In this environment, certain species are more resistant than others to acidic, oxidative, and nitrative stresses caused by $ONOO^-$/ONOOH. The most resistant are gram-negative aerobic/facultative anaerobic bacteria and the most fragile are gram-positive anaerobic bacteria. The gram-positive aerobic/facultative anaerobic bacteria are also well equipped to defend themselves against chronic stress. However, these bacteria have a greater sensitivity to the oxidative stress in stationary phase due to the lack of transcription of the *dps-like* gene).

The major chemical mechanisms of nitrate and nitrite preservation described in this review should help to prioritize the effects of different hurdle technologies in cured meat processing. Thus, the use of nitrate and nitrite may be substituted by modifications of product composition, and processes.

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