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Unsolicited Perspective

Antimicrobials offered from nature: Peroxidase-catalyzed systems and their mimics

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ABSTRACT

The control of antimicrobial resistance requires the development of novel antimicrobial alternatives and naturally occurring peroxidase-catalyzed systems may be of great value in this era of emerging antimicrobial resistance. In the peroxidase system, a peroxidase enzyme catalyzes the oxidation of a halide/pseudohalide, at the expense of hydrogen peroxide, to generate reactive products with broad antimicrobial properties. The appropriate use of peroxidase systems needs a better understanding of the identities and properties of the generated antimicrobial oxidants, specific targets in bacterial cells, their mode of action and the factors favoring or limiting their activity. Here, the ABCs (antibacterial activity, bacterial "backtalk" and cytotoxicity) of these systems and their mimics are discussed. Particular attention is paid to the concomitant use of thiocyanate and iodide dual substrates in peroxidase/peroxidase-free systems with implications on their antimicrobial activity. This review also provides a summary of actual applications of peroxidase systems as bio-preservatives in oral healthcare, milk industry, food/feed specialties and related products, mastitis and wound treatment; lastly, this review points to opportunities for further research and potential applications.

1. Introduction

Back in 1946, Alexander Fleming warned: "*There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring 'fastness'* (resistance)". Later, history has shown that the introduction of any novel antibiotic has been rapidly followed by the emergence and spread of resistance (Fig. 1). In fact, resistance has emerged towards every antibiotic class introduced to date. The antibiotic resistance crisis spurred global efforts to develop antibacterial alternatives. Naturally occurring peroxidase-catalyzed systems are among the potential candidates. In mammals, phagocytic cells, such as neutrophils, monocytes and eosinophils, as well as exocrine secretions, such as saliva and milk, contain peroxidase-catalyzed systems that comprise part of the host innate defense system. In the peroxidase system, a particular peroxidase enzyme catalyzes the oxidation of a halide/pseudohalide, at the expense of hydrogen peroxide (H_2O_2), to generate reactive products with a wide range of antimicrobial activities [1].

Here, we review the naturally occurring peroxidase-catalyzed systems that play a role in immune defense against invading microbes, their mimics possessing antimicrobial activity, the importance of different antimicrobial oxidants, including their production, identities, mode of action, reported bacterial resistance mechanisms and mammalian cytotoxicity, as well as we highlight their use as alternative antimicrobials. This review intends to discuss key features of the peroxidase and peroxidase-mimicking antimicrobial systems to the best of the

Abbreviations: ALB, albumin; Arg, arginine; C, cytosine; CDC, Centers for Disease Control and Prevention; Cys, cysteine; DTT, dithiothreitol; EPO, eosinophil peroxidase; G, glucose; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GOD, glucose oxidase; GSC, glutathione; His, histidine; ITC, iodo-thiocyanate complex; LPO, lactoperoxidase; Lys, lysine; ME, mercaptoethanol; Met, methionine; MPO, myeloperoxidase; MRSA, multidrug-resistant *S. aureus*; NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); NMNH, reduced nicotinamide mononucleotide; NMR, nuclear magnetic resonance; Sec, selenocysteine; SeMet, selenomethionine; SPO, salivary peroxidase; TNB, 5-thio-2-nitrobenzoic acid; Trp, tryptophan; Tyr, tyrosine; U, uracil; WPI, whey protein isolate.

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knowledge available on relevant literature.

2. Peroxidase-mediated antimicrobial systems of myeloid cells

Myeloid cells of human blood contain peroxidases in their cytoplasmic granules – myeloperoxidase (MPO) in neutrophils and monocytes, and eosinophil peroxidase (EPO) in eosinophils.

2.1. Myeloperoxidase system of neutrophils

Neutrophils are the first responders of host defense towards invading bacteria and fungi, and MPO is the key component of neutrophils' antimicrobial armory [3]. Neutrophils are rich in MPO, which constitutes approximately 5% of the total neutrophil protein and occurring in the cytoplasmic granules at very high concentrations, making up about 25% of the granule proteins [4].

Circulating neutrophils are passive, but they can be quickly activated by components of opsonized bacteria, which bind to the receptors of neutrophils and trigger phagocytic machinery. The binding increases the oxygen uptake called respiratory burst and triggers the activation of membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which reduces molecular oxygen to superoxide radical ($O_2^{\bullet-}$). $O_2^{\bullet-}$ is further converted to H_2O_2 spontaneously or catalyzed by the superoxide dismutase [5–7]. Although H_2O_2 can be directly toxic to microorganisms, its reactivity is increased many orders of magnitude by other mechanisms. Neutrophils employ MPO and H_2O_2 ($E^0_{H_2O_2/H_2O} = 1.77$ V) generated during the respiratory burst to oxidize preferentially chloride (Cl^-) to hypochlorous acid (HOCl) as the initial product. HOCl/OCl^- (hypochlorite ion) equilibrium reacts with excess Cl^- to generate chlorine (Cl_2) ($E^0_{Cl_2/Cl^-} = 1.36$ V) [6]. The process of MPO-mediated bacterial killing of the neutrophil is illustrated in Fig. 2. Bacterial targets of these powerful bleaching agents include thiol groups (-SH), iron-sulfur centers, sulfur-ether groups, heme groups and unsaturated fatty acids, resulting in a loss of microbial membrane transport, an interruption of the membrane electron transport chain, a dissipation of energy reserves and a suppression of DNA synthesis because of the disruption of the interaction between cell membrane and the chromosomal origin of replication [8].

However, the relevance of the MPO system in the antimicrobial armory of neutrophils has been questioned because individuals with genetic defects in the MPO gene do not seem to be particularly susceptible to life-threatening infections. This controversy was resolved by Klebanoff and co-authors [9] with a proposition that the MPO system will substantially contribute to innate host defense only when other host defense mechanisms are overwhelmed by the exposure to pathogens.

In general, MPO also oxidizes iodide (I^-) ($E^0_{I_2/I^-} = 0.54$ V) and bromide (Br^-) ($E^0_{Br_2/Br^-} = 1.07$ V) halides, but not fluoride (F^-) ($E^0_{F_2/F^-} = 2.87$ V) and the pseudohalide thiocyanate (SCN^-) ($E^0_{SCN_2/SCN^-} = 0.77$ V). Cl^- is considered as the main physiological substrate for MPO because of its occurrence at high concentrations in plasma (100 – 140 mM), whereas, SCN^- (20 – 120 μM), Br^- (20 – 100 μM) and I^- (< 1 μM) are present in relatively low concentrations [10–12].

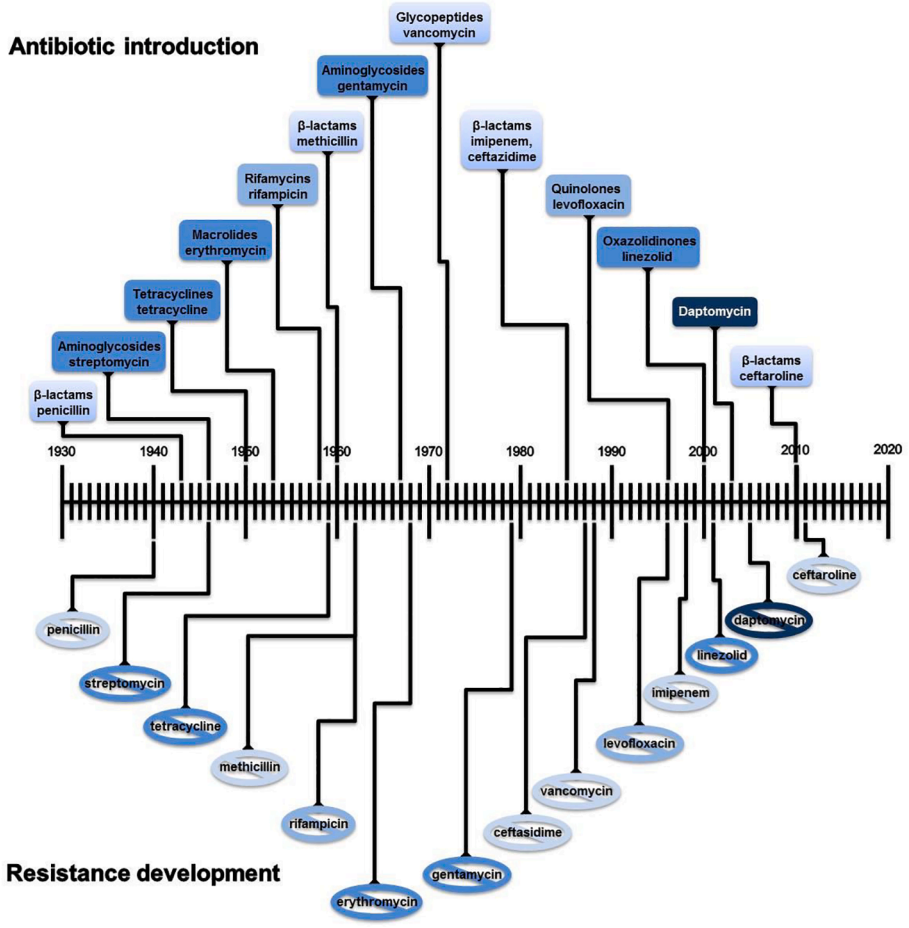

Fig. 1. Antibiotic introduction and first reported resistance. Inhibitors of: cell wall synthesis ( ), nucleic acid synthesis ( ), protein synthesis ( ) and cell membrane integrity ( ). Data taken from Centers for Disease Control and Prevention [2].

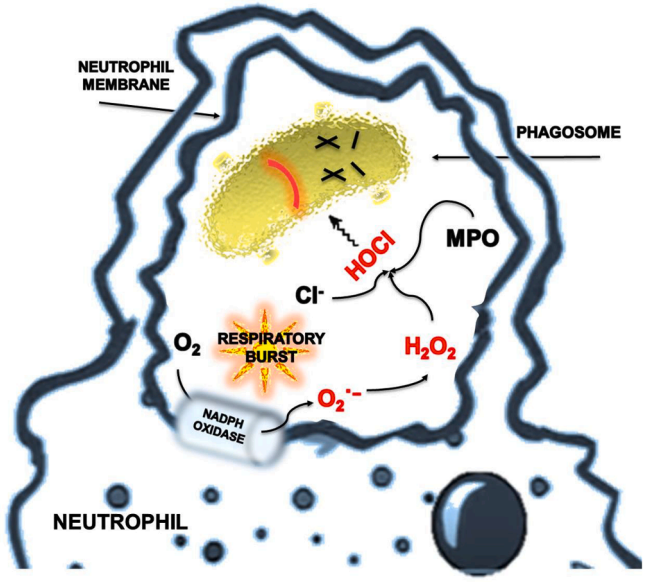

Fig. 2. Neutrophil killing of bacteria mediated by the myeloperoxidase (MPO) system. The bacterium is opsonized and thus recognized by a neutrophil. The pathogen is then engulfed (phagocytosis), triggering the respiratory burst, and killed within the phagosome by the bleaching agents formed *via* the "toxic triad" (MPO/H_2O_2/Cl^-).

2.2. Myeloperoxidase system of monocytes

Monocytes share the phagocytic function of neutrophils, but they also play a central role in the coordination of innate and adaptive immunity, presenting the antigens to T lymphocytes. Monocytes eventually leave the bloodstream and become tissue macrophages attacking microorganisms. They function also as scavengers removing dead cell debris. Monocytes contain considerably less peroxidase than neutrophils (one-third of the peroxidase in neutrophils), and their enzyme is structurally and functionally related to MPO [6,13].

Monocytes, like neutrophils, respond to stimulation by the respiratory burst, forming H_2O_2. MPO and H_2O_2 oxidize I^- to form iodinating species (a more detailed discussion on iodinating species follows in section 5.2.1.), and oxidize Cl^- to chlorinating agents, although in general, not at the same level as in neutrophils [6]. When monocytes transform into macrophages they lose their granule MPO and do not utilize MPO/H_2O_2-dependent antimicrobial mechanism [3,6].

2.3. Eosinophil peroxidase system of eosinophils

Eosinophils are important in allergic reactions and host defense against parasites [3]. Eosinophils, while activated, release EPO, and similar to MPO, use NADPH oxidase-derived H_2O_2 to oxidize preferentially Br^- and generate a brominating agent hypobromous acid (HOBr) [14]. EPO can also oxidize I^- and SCN^-, as well as Cl^- at acidic pH [15].

3. Peroxidase-mediated antimicrobial systems of saliva

Human saliva contains two peroxidases, salivary peroxidase (SPO) and MPO. SPO is synthesized and secreted by the major salivary glands, whereas MPO is derived from neutrophils that enter the oral cavity during normal extravasation [16,17]. In stimulated whole saliva of healthy adults, the overall concentration of SPO and MPO ranges from 2 to 13 μg/ml [16], the major proportion (75%) responsible for peroxidase-catalyzed reactions often being MPO [18].

The major source of H_2O_2 in the oral environment is a class of catalase-negative lactic acid bacteria, primarily Streptococci, which are the predominant microorganisms in the oral environment [10,19]. This participant of the peroxidase-catalyzed system may be generated also endogenously, i.e. by neutrophils in the process of phagocytosis and activation of oxygen metabolism [1,10]. H_2O_2 can also be supplied exogenously, by the addition of H_2O_2-producing systems, such as glucose/glucose oxidase (G/GOD), lactose oxidase and xanthine oxidase, which may produce a more effective antimicrobial system than in the case of directly added H_2O_2 [1].

Human SPO catalyzes the oxidation of halides I^-, Br^-, and pseudohalide SCN^- to respective hypo(pseudo)halites in the presence of H_2O_2. In addition, Cl^- is oxidized by MPO, but not by SPO. Based on the relative concentrations of halides present, it was considered that Cl^- was the leading substrate for MPO in all MPO-related antimicrobial systems *in vivo* because of its major role as a substrate for MPO in the oxidative killing of microbes in neutrophils [16]. However, antimicrobial studies showed that SCN^- is more easily oxidized by MPO in saliva-like conditions. Thomas and Fishman [10] investigated the oxidants produced by neutrophils in the presence of Cl^- and SCN^-. They concluded that under conditions similar to those of saliva, where SCN^- is present at 0.1–3 mM and Cl^- at 20 mM, hypothiocyanite ($OSCN^-$) was the dominant oxidant formed.

4. Peroxidase-mediated antimicrobial systems of milk

Lactoperoxidase (LPO) is an enzyme that has been found in milk from many mammalian species, and also in many types of secretions e.g. in tears, nasal fluid, airway surface fluid, uterine luminal fluid and vaginal secretions [20]. Human milk contains two peroxidase enzymes – LPO secreted from the mammary gland and MPO originated from milk leukocytes. The relative amounts vary widely from sample to sample and depend on the stage of lactation [21]. However, our knowledge of LPO in human milk is limited. The properties of human LPO are similar to bovine milk LPO, thus, surrogate bovine LPO has been generally used to study peroxidase-derived antimicrobial properties of milk. In bovine milk, LPO is, parallel to xanthine oxidase, the most abundant enzyme. Its concentration is approximately 30 mg/l, constituting about 0.5% of the whey proteins [1].

The primary role of the LPO system is to protect the lactating mammary gland and the intestinal tract of the newborn infants against invading bacteria, fungi and viruses [22]. A recent review provides a detailed, up-to-date summary of studies exploring the antibacterial, antifungal, antiviral and antiparasitic properties of LPO systems [23].

LPO preferentially catalyzes the oxidation of SCN^-, but also I^- and Br^- [24]. Another component of the LPO system, hydrogen peroxide, is not normally detected in raw milk. Many milk bacteria, Lactococci, Lactobacilli and Streptococci can produce sufficient H_2O_2 under aerobic conditions. The source of H_2O_2, as in the oral environment, can be endogenous by leukocytes in the process of phagocytosis, as well as, exogenous by the addition of H_2O_2-generating systems, such as G/GOD, sodium percarbonate, etc. [15].

The thiocyanate anion is another major component of the LPO system. It is secreted from mammary, salivary, lacrimal and gastric glands. Its concentration is partly dependent on the eating and smoking habits of a human. In human milk, it has been reported that average values range from 0.1 to 4 μM [25]. The major source of SCN^- is the detoxification reaction of cyanide (CN^-) by the enzyme thiosulfate cyanide sulfurtransferase, which primarily occurs in the liver. Also, certain vegetables from *Brassica cruciferae* (cauliflower, cabbage, kale, etc.) contain a notable amount of thiocyanate precursors, such as the glucosinolates, which upon hydrolysis produce SCN^-, and/or isothiocyanate and nitriles [1,21].

5. Mechanism of halide oxidation

The layout of reactions generating enzyme intermediates is illustrated in Fig. 3 [1,3,12,26–28]. Halide oxidation starts with the reaction of peroxidase with H_2O_2. The active site of the native peroxidase enzyme

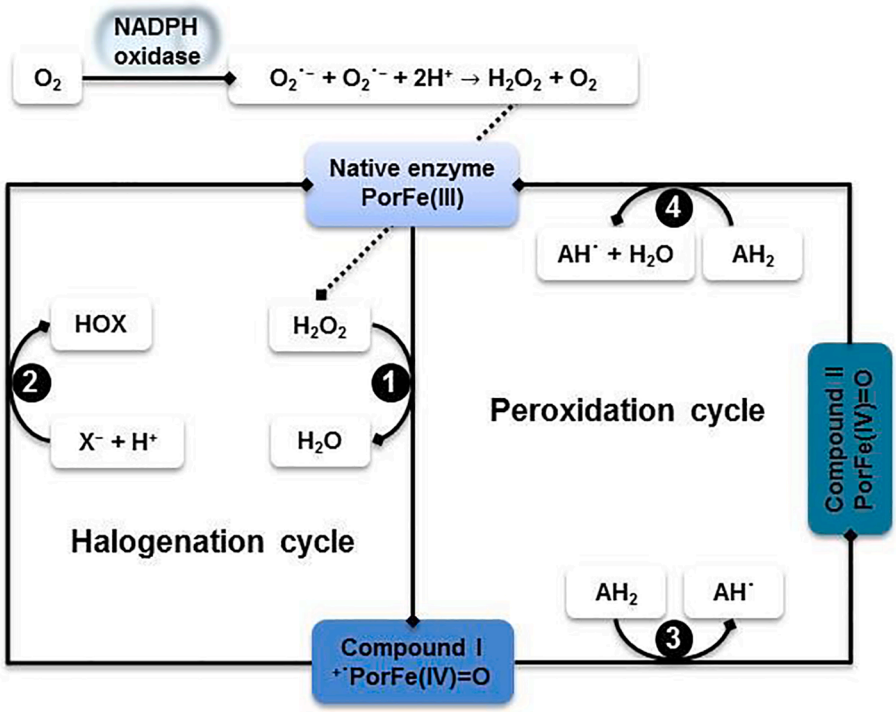

Fig. 3. Generation of catalytic intermediates of the peroxidase enzyme [1,3,12,26–28]. PorFe denotes the porphyrin-iron complex; $X^- = Cl^-$, Br^-, I^-, SCN^-; HOX = HOCl, HOBr, HOI, HOSCN; AH_2 = reducing agents. The H_2O_2 participant may be generated in neutrophils during respiratory burst by the membrane-bound NADPH oxidase. Further descriptions are given in the text.

contains a ferric heme, which is oxidized with H_2O_2 to form compound I (Reaction (1)). Compound I is an oxoiron(IV) intermediate [Fe(IV) = O] containing a porphyrin π-cation radical ($^+$Por) [29]. Compound I is converted back to the native enzyme through direct two-electron reduction of a (pseudo)halide (X^-) generating (pseudo)hypohalous acid (HOX) (halogenation cycle; Reaction (2)). Alternatively, compound I can oxidize a range of reducing substrates (AH_2) by a mechanism involving two sequential single-electron steps. The first step leads to the generation of a second enzyme intermediate, compound II, which is subsequently reduced to the native enzyme by a second molecule of reducing AH_2 substrate, releasing free radicals ($AH^{\cdot}$) (peroxidase cycle; Reactions (3), (4)). Phenolic acids, aromatic phenols, indoles, amines and sulfonates are typical reducing substrates [30]. It is considered that compound II is inactive in (pseudo)halide oxidation [12], thus the agent that oxidizes the (pseudo)halide is compound I.

Although the catalytic mechanisms of different peroxidases are similar, they are different in their ability, or inability, to oxidize various (pseudo)halides. Compound I oxidizes halides at different rates ($I^- > Br^- > Cl^-$); however, compound I of different peroxidases may have different redox potential. Thus, although all peroxidases will catalyze the oxidation of I^-, at neutral pH, only MPO will oxidize Cl^-, while EPO will oxidize it only at acidic pH and SPO and LPO will not, and none of these enzymes is capable of oxidizing F^-. The pseudohalide SCN^- will be oxidized also by LPO, MPO, EPO and SPO [31,32].

5.1. *Oxidants generated by the oxidation of thiocyanate with MPO, EPO and LPO, their antibacterial mechanism of action, bacterial resistance and mammalian cytotoxicity (summarized in Table 1)*

5.1.1. *Peroxidase oxidation of SCN^-*

MPO, EPO, SPO and LPO are all able to convert SCN^- into products with antibacterial activities. In particular, the chemistry of SCN^- oxidation by LPO has been widely studied. The chemical species which are responsible for the antimicrobial activity of the LPO/H_2O_2/SCN^- system are assumed to be several thiocyanate oxidation products. It should be noted that quantitative and qualitative measurements of these oxidation products in biological tissues are often not available, mainly because adequate methods are lacking.

The proposed mechanism for the peroxidase-catalyzed oxidation of SCN^- is depicted in Eqs. (1)–(3) [33–36]. Oxidation can go in two different ways, resulting in intermediate oxidation products, which are responsible for the antimicrobial activity. The first pathway is oxidation of SCN^- yielding thiocyanogen $(SCN)_2$ (Eq. (1)), which is unstable in aqueous solution and rapidly hydrolyzes to hypothiocyanous acid (HOSCN) in equilibrium with its conjugate base $OSCN^-$ (Eqs. (2) and (3)). The pK_a of HOSCN is 5.3, indicating that $OSCN^-$ predominates in most physiological fluids [25]. Alternatively, the second pathway is the direct production of $OSCN^-$ (Eq. (4)). Nevertheless, $OSCN^-$ is the major product observed over SCN^- oxidation at neutral pH [35]. For ease of reading, within this review the terms $OSCN^-$ or HOSCN will be used interchangeably to represent the physiological mixture composed of $OSCN^-$/HOSCN.

However, the reaction profile is complex and depending upon reaction conditions, other short-lived intermediates may be formed in varying amounts. Pruitt et al. [34] reported that, at neutral pH, addition of excess H_2O_2 or LPO/H_2O_2 to an $OSCN^-$ generated from the LPO system results in the formation of other highly reactive, short-lived antimicrobial products in addition to $OSCN^-$, which represent higher oxidothiocyano derivatives, such as cyanosulfite (O_2SCN^-) and cyanosulfate (O_3SCN^-) (Eq. (5) and (6)). The authors assumed that these higher oxidothiocyano derivatives would have better oxidizing properties and, accordingly, would be more effective microbial inhibitors than $OSCN^-$.

$$H_2O_2 + 2SCN^- + H^+ \xrightarrow{peroxidase} (SCN)_2 + 2H_2O \quad (1)$$

$$(SCN)_2 + H_2O \rightarrow HOSCN + H^+ + SCN^- \quad (2)$$

$$HOSCN \rightleftharpoons H^+ + OSCN^- \quad (3)$$

$$H_2O_2 + SCN^- \xrightarrow{peroxidase} OSCN^- + H_2O \quad (4)$$

$$OSCN^- + H_2O_2 \rightarrow O_2SCN^- + H_2O \quad (5)$$

$$O_2SCN^- + H_2O_2 \rightarrow O_3SCN^- + H_2O \quad (6)$$

Chung and Wood [37] proposed that the species responsible for the antibacterial activity of $LPO/H_2O_2/SCN^-$ system may be the CN^-. Subsequently, Modi and co-authors [38] reported that CN^- can be formed at a ratio of $[H_2O_2]/[SCN^-] > 2$, which was confirmed by ^{15}N nuclear magnetic resonance (NMR) spectroscopy and by changes in the optical spectrum of LPO. The authors showed that the activity of the system was at its maximum when $[H_2O_2]/[SCN^-]$ ratio was 1 at a pH of 6. The formation of $OSCN^-$ was also observed to be the greatest when the ratio of $[H_2O_2]/[SCN^-]$ was 1 at pH < 6. They concluded that the potential bactericidal or bacteriostatic activity of the $LPO/H_2O_2/SCN^-$ system may be related to the formation of $HOSCN/OSCN^-$ species rather than CN^- and $(SCN)_2$, since these species were not present in the

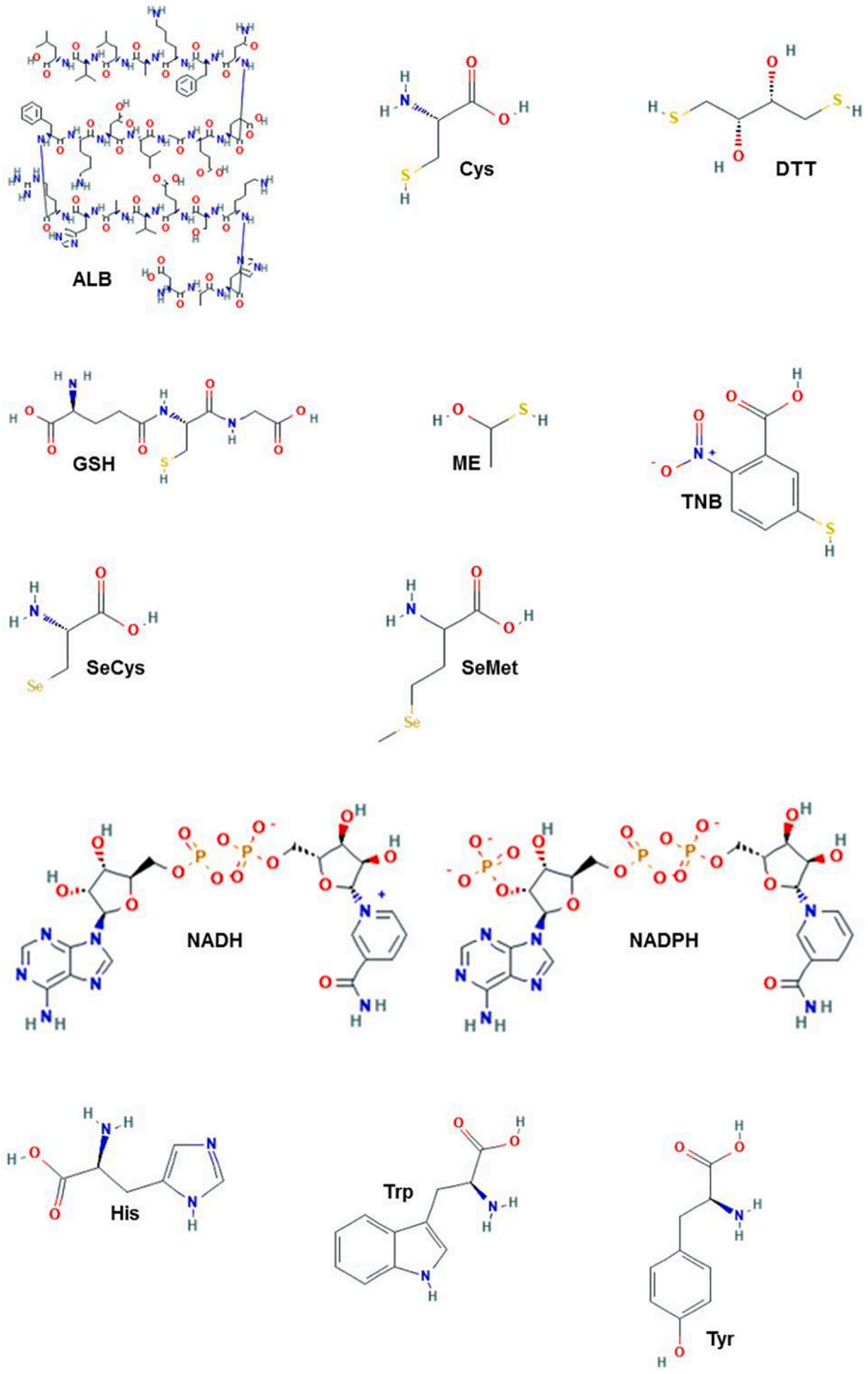

Fig. 4. Chemical structures of compounds sensitive to SCN^- oxidation products. ALB = albumin; Cys = cysteine; DTT = dithiothreitol; GSC = glutathione; His = histidine; ME = mercaptoethanol; NAD(P)H = reduced nicotinamide adenine dinucleotide (phosphate); SeCys = selenocysteine; SeMet = selenomethionine; TNB = 5-thio-2-nitrobenzoic acid; Trp = tryptophan; Tyr = tyrosine.

solution when the bactericidal activity of the system was maximum (i.e. when the $[H_2O_2]/[SCN^-]$ was 1) [38].

5.1.2. Mechanism of action of peroxidase-oxidized SCN^-

The key to the antimicrobial action of the peroxidase/H_2O_2/SCN^- system is the selective oxidation of sulfhydryl groups of microbial proteins and other low molecular weight species of the cytoplasmic thiol pool. The reaction products $OSCN^-$, HOSCN and $(SCN)_2$ react rapidly with sulfhydryl groups yielding sulfenyl thiocyanate derivatives (R-S-SCN) (Eqs. (7)–(9)) [36]. The R-S-SCN can react with another thiol group to form a disulfide bond (Eq. (10)); though steric constraints may prevent this reaction in proteins) or can undergo further modification, such as reversible hydrolysis to yield sulfenic acid (Eq. (11)).

$$OSCN^- + R-S-H \rightarrow R-S-SCN + OH^- \quad (7)$$

$$HOSCN + R-S-H \rightarrow R-S-SCN + H_2O \quad (8)$$

$$(SCN)_2 + R-S-H \rightarrow R-S-SCN + SCN^- + H^+ \quad (9)$$

$$R-S-SCN + R-S-H \rightarrow R-S-S-R + SCN^- + H^+ \quad (10)$$

$$R-S-SCN + H_2O \rightleftharpoons R-S-OH + SCN^- + H^+ \quad (11)$$

However, not all sulfhydryls are sensitive to SCN^- oxidation products in a same level. Albumin (ALB), cysteine (Cys), dithiothreitol (DTT), glutathione (GSH), mercaptoethanol (ME) and 5-thio-2-nitrobenzoic acid (TNB) are all readily oxidized, but β-lactoglobulin is poorly oxidized. HOSCN, in addition to sulfur-containing species, can oxidize selenol-containing species (–SeH), thus, selenocysteine (SeCys), selenomethionine (SeMet) residues in proteins are also targets. Under some conditions, such as the presence of LPO, sufficient H_2O_2 and SCN^-, and when all the sulfhydryls are oxidized, modification of other targets like aromatic amino acid residues [tyrosine (Tyr), tryptophan (Trp) and histidine (His)] can also occur. While amines ($-NH_2$) are not believed to be major direct targets for HOSCN, there is still evidence supporting it (reviewed in [39,40]). The cellular targets for HOSCN interaction are also reduced nicotinamide dinucleotides: NADH and NADPH can be oxidized to NAD^+ and $NADP^+$ in reversible reactions [22]. The structures of compounds oxidized by SCN^- oxidation products are depicted in Fig. 4.

When microbial cytoplasmic membranes are damaged by the oxidation of SH groups, it leads to the leakage of potassium ions, amino acids and polypeptides. Similarly, uptake of amino acids, purines and pyrimidines by the cell, and, thus, the synthesis of proteins, DNA and RNA are also impeded [41,42]. However, unlike oxidants like H_2O_2 and superoxide, $OSCN^-$ has not been reported to cause DNA damage [43]. The alteration of bacterial cell membranes and transporters also hinder glucose and oxygen uptake, thus inhibiting the glucose transport and respiration. The antimicrobial species generated by LPO-catalyzed oxidation of SCN^- can also inhibit critical Cys in several glycolytic enzymes, such as hexokinase, glyceraldehyde-3-phosphate dehydrogenase (GADPH), aldolase and glucose-6-phosphate dehydrogenase [22]. Indeed, it has been hypothesized that HOSCN's effect on bacterial growth is mainly glycolysis-mediated [25].

5.1.3. Bacterial resistance to peroxidase-oxidized SCN^-

Despite the importance of the peroxidase/SCN^- systems in host defense against pathogens, the bacterial stress response and resistance mechanisms to this specific form of oxidative stress have not been exhaustively characterized [43].

Susceptibility of bacteria to antimicrobial activity of peroxidase systems is dependent on the state of their metabolic growth. Generally, growing cells are less susceptible to inhibition or killing than resting cells. Bacteria growing aerobically are less susceptible to the peroxidase systems than are those growing anaerobically [22].

The cell wall of Gram-positive bacteria could somewhat limit the accessibility but do not completely block the entrance of the peroxidase-oxidized SCN^- products into the cell interior [22,41]. In fact, many studies showed that the Gram-positive and Gram-negative bacteria were similarly affected (summarized in [15,16]; Table 2). There is a lack of specific knowledge on the potential for scavenging of peroxidase-oxidized SCN^- products in the cell wall, but in theory, low molecular weight species (such as GSH) and repair mechanisms of oxidized free Cys residues of certain proteins recently identified in the bacterial cell envelope (such as soluble periplasmic protein DsbG) [44] may be involved in the defense against these oxidants.

The availability of H_2O_2 is an important factor for peroxidase-mediated oxidant generation, thus, bacteria which produce H_2O_2-consuming enzymes (e.g. catalase, GSH peroxidase) show low susceptibility to peroxidase/SCN^--mediated inhibition [3].

However, bacterial resistance to LPO/SCN^--mediated killing mainly occurs *via* enzymes and substrates that inactivate and reduce the generated oxidizing agents, as well as by reversing the oxidation of SH groups [65]. Peroxidase/SCN^- systems can cause both reversible and irreversible effects, displaying bacteriostatic and bactericidal activity. Several factors influence their reversible versus irreversible character. Irreversible inhibition is associated with long-term incubation, high $OSCN^-$ concentrations and particular bacterial species. Increased concentrations of reducing agents, such as GSH and Cys, can reverse the inhibition by buffering $OSCN^-$ and can ease the oxidative damage by reversing thiol modifications. It was shown that in *E. coli cysJ* was activated under the stress induced by LPO system [43]. CysJ together with CysI form the NADPH-dependent sulfite reductase, which catalyzes a key step in the production of SH compounds such as Cys, GSH and coenzyme A. The authors suggested that this operon was induced to boost the *E. coli's* own sulfhydryl production and that the activation of the sulfite reduction pathway was involved in the protective response against the LPO-mediated challenge [43]. Earlier, Mickelson and Anderson [66] reported that a NADPH-dependent cystine reductase may account for increased resistance against the LPO system in *Streptococcus agalactiae*. Another resistance mechanism was used by *S. cremoris*, which recruited $NADH_2$ oxidase enzyme to catalyze the oxidation of $HADH_2$ with $OSCN^-$ by this lowering the inhibitory effect. Another bacterial strain, *S. sanguinis* had developed high expression level of NADH-OSCN oxidoreductase that reduced $OSCN^-$ back to SCN^- [22].

5.1.4. Mammalian cell cytotoxicity of peroxidase-oxidized SCN^-

There are numerous reports that a variety of dissimilar cells are subject to peroxidase-mediated toxicity. A peroxidase together with H_2O_2 and a halide (dubbed as "cytotoxic triad") can exert toxic effects *in vitro* on bacteria, fungi, viruses, tumor cells, erythrocytes, sperm cells and many other mammalian cells. This non-specific toxicity of peroxidase systems is not surprising, given their activity is expressed by hypohalous acid/hypohalite, which are small, inorganic, highly reactive molecules and do not have a preference for reacting with one cell type over the other [67]. However, long-held debate exists as to the mammalian cytotoxicity of peroxidase/SCN^- system. This system is often regarded as a mild alternative to other peroxidase products, particularly HOCl and HOBr, based on the following characteristics: (i) conversion of SCN^- to HOSCN by a peroxidase is viewed as a detoxification mechanism to remove potentially more damaging oxidants, such as H_2O_2 and HOCl; (ii) its component SCN^- under physiological conditions is a scavenger, in particular for HOCl and HOBr; (iii) its product HOSCN reacts preferentially with free SH groups (e.g. free Cys) [68].

The literature indicates that the peroxidase/SCN^- system is specifically safe to mammalian cells, which are associated with the oral cavity and the airway. The oxidants generated from this system inhibit bacteria, fungi and viruses in the oral cavity, while at the same time the conversion of SCN^- to HOSCN limits the production of potentially tissue damaging oxidizing species, such as H_2O_2 and HOCl. Similarly, in airways this system facilitates the bacterial clearance and scavenges deleterious oxidative species [40].

Table 1
Summary of the characteristics of peroxidase-catalyzed and peroxidase-mimicking antimicrobial systems. The components of the antimicrobial complexes (involving thiocyanate and/or iodide substrates), oxidants with reported antimicrobial activities, their cellular targets in bacteria, altered functions in cellular systems, defense/resistance mechanisms in bacterial cells and toxicity against mammalian cell types are presented (corresponding references are cited in accompanying text).

System components	Antimicrobial species	Cellular targets	Cellular functions	Bacterial defense/resistance	Mammalian cytotoxicity
Peroxidase/ H_2O_2/SCN^-; H_2O_2/SCN^-	$HOSCN/OSCN^-$ $(SCN)_2$ O_2SCN^-/O_3SCN^- CN^-	Thiols (-SH; e.g. ALB, Cys, DTT, GSC, ME) Selenothiols (-SeH; e.g. Sec, SeMet) Amines (–NH_2; e.g. Lys, Arg, His, α-amino group, N-terminus) Aromatic amino acid residues (e.g. Tyr, Trp, His) NADH, NADPH	Alteration of cell membranes and transporters Inhibition of protein, DNA and RNA synthesis Inhibition of glycolysis Inhibition of glucose transport Inhibition of respiration	State of metabolic growth (e.g. growing cells, aerobic growth) Cell wall of Gram-positive bacteria (less susceptible) H_2O_2-consuming enzymes (e.g. catalase, GSH peroxidase) Reducing agents (e.g. GSC, Cys) Reducing enzymes (e.g. sulfite reductase, cystine reductase, $NADH_2$ oxidase, NADH-OSCN oxidoreductase)	Human erythrocytes Murine macrophages Human coronary artery endothelial cells
Peroxidase/ H_2O_2/I^-; H_2O_2/I^-	I_2 HOI/OI^- I_3^- I_5^- I_6^{2-} H_2OI^+ HI_2O^-	All thiols (-SH) Thioether moieties (R-S-R; e.g. Met) Amines (–NH_2) Phenolic groups (–OH; e.g. His, Tyr) Imidazole groups (NH···N; e.g. His, Tyr) Aromatic amino acid residues (e.g. Tyr, Trp, His) Carbon-carbon bonds (C = C) in unsaturated fatty acids NMNH NADH, NADPH C and U pyrimidine nucleobases	Alteration of cell membranes and transporters Destabilization of membranes Inhibition of protein, DNA and RNA synthesis Inhibition of glycolysis Inhibition of glucose transport Inhibition of respiration	Not reported	Human erythrocytes Human lymphoid cells Mouse ascetic lymphoma cells Murine plasmacytoma tumor cells
Peroxidase/ $H_2O_2/I^-/SCN^-$; $H_2O_2/I^-/SCN^-$	I_2 OI^- $OSCN^-$ ICN I_2SCN^- OH^-	Not reported	Partitioning of riboplasm Fragmentation of DNA	No resistance was observed in *E. coli*, *P. aeruginosa*, *S. aureus*, multidrug-resistant *S. aureus* (MRSA)	Human mouth epithelial cells Human cervical epithelial adenocarcinoma cells (HeLa) Horse erythrocytes

ALB = albumin; Arg = arginine; C = cytosine; Cys = cysteine; DTT = dithiothreitol; GSC = glutathione; His = histidine; Lys = lysine; ME = mercaptoethanol; Met = methionine; NAD(P)H = reduced nicotinamide adenine dinucleotide (phosphate); NMNH = reduced nicotinamide mononucleotide; Sec = selenocysteine; SeMet = selenomethionine; TNB = 5-thio-2-nitrobenzoic acid; Trp = tryptophan; Tyr = tyrosine; U = uracil.

However, other evidence shows that peroxidase/SCN^--derived oxidants are cytotoxic to other mammalian cells like erythrocytes, macrophages and endothelial cells. For instance, Grisham and Ryan [17] found that peroxidase-generated HOSCN lysed human erythrocytes at a pH of 6, oxidized the hemoglobin to methemoglobin, and together with $OCSN^-$ oxidized also the GSH. As with bacterial cells, HOSCN can target critical thiol proteins with high specificity also in mammalian cells. In another study [69], red blood cells exposed to increasing concentrations of EPO/H_2O_2/SCN^- oxidation products were first depleted of GSH, after which GSH S-transferase, GADPH, and ATPases underwent SH reductant-reversible inactivation, building up the hemolysis. The oxidants inactivated red blood cell membrane ATPases 10 – 1000 times more potently than either HOCl, HOBr and H_2O_2 did [69]. Similarly, Lloyd et al. [70] showed that HOSCN induced apoptosis and necrosis of macrophages (J774A.1) with greater efficacy and at lower concentrations than HOCl or HOBr, due to selective targeting of critical thiol residues on mitochondrial membrane proteins. Love et al. [71] reported that the cellular targets of HOSCN in J774A.1 macrophages were multiple thiol-containing proteins involved in metabolism and glycolysis (fructose bisphosphate aldolase, triosephosphate isomerase, GAPDH and creatine kinase), together with several chaperones, antioxidants and structural proteins. They concluded that the ability of HOSCN to inhibit glycolysis and perturb energy production contributed to the cell death. HOSCN also induced apoptosis of human coronary artery endothelial cells by the increase of mitochondrial membrane permeability, the release of cytochrome *c* from the mitochondria and externalization of phosphatidylserine [68].

5.2. Oxidants generated by the oxidation of iodide with/without MPO, EPO and LPO, their antibacterial mechanism of action, bacterial resistance and mammalian cytotoxicity (summarized in Table 1)

5.2.1. Oxidation of I^-

Iodide ion too can be oxidized by MPO, SPO and LPO. I^- is the most readily oxidizable of all halides *in vitro* (lower redox potential, $E^0_{I_2/I^-}$ = 0.54 V), and the peroxidase-catalyzed oxidation of I^- yields in molecular iodine (I_2) and, depending on I^- concentration and pH, hypoiodous acid (HOI), its conjugate base hypoiodite (OI^-) or other iodine species [1]. Few studies have suggested that I_2 is the major agent, which is able to damage the cells [54,55,72], but HOI/OI^- has also been identified as possessing antimicrobial properties [46–48,52]. However, the active agent responsible for peroxidase/I^--mediated bacterial killing is believed to be a mixture of iodine species that are not fully detailed due to the complex iodine chemistry [39].

Chemists earlier suggested that the reaction between I^- and H_2O_2 (without any peroxidase) takes place through a series of short-lived intermediates (such as HOI, OI^-) and observable intermediates (I_2, I_3^-) [73]. Oxidation of I^- by H_2O_2 involves two reactions: the first one is slower and produces HOI (Eq. (12)) and the second one faster forms free iodine by the reaction of the hypoiodous acid with more iodide ion (Eq. (13)). The slowness of the first reaction controls the overall rate [74]. Triiodide anions (I_3^-) also can be present in equilibrium with I^- and I_2 (Eq. (14)).

$$H_2O_2 + I^- + H^+ \rightarrow HOI + H_2O \quad (12)$$

Table 2
Antimicrobial spectrum of peroxidase-catalyzed and peroxidase-mimicking systems.

Organism	Antimicrobial system	Reference
Gram-positive bacteria		
Bacillus cereus	LPO/H_2O_2/I^-	[45]
Fusobacterium nucleatum	HRPO/H_2O_2/I^-	[46]
Micrococcus flavus	LPO/I^-/SCN^-/G/GOD	[47]
Propionibacterium acnes	LPO/I^-/SCN^-/G/GOD	[47]
Staphylococcus aureus	LPO/I^-/SCN^-/G/GOD	[47]
Staphylococcus aureus	LPO/H_2O_2/I^-	[45]
Staphylococcus aureus	LPO/SCN^-/G/GOD, LPO/I^-/SCN^-/G/GOD	[48]
Staphylococcus aureus	H_2O_2/I^-/SCN^-	[49]
Methicillin-resistant *S. aureus*	H_2O_2/I^-/SCN^-	[49]
Streptococcus dysglactiae	LPO/I^-/GOD, LPO/SCN^-/GOD	[50]
Streptococcus faecalis	LPO/I^-/SCN^-/G/GOD	[47]
Streptococcus mutans	LPO/H_2O_2/I^-	[51]
Streptococcus mutans	LPO/I^-/SCN^-/G/GOD	[47]
Streptococcus salivarius	LPO/I^-/SCN^-/G/GOD	[47]
Streptococcus uberis	H_2O_2/I^-/SCN^-	[49]
Streptococcus uberis	LPO/I^-/GOD, LPO/SCN^-/GOD	[50]
Gram-negative bacteria		
Actinobacillus actinomycetemcomitans	MPO/H_2O_2/I^-, LPO/H_2O_2/I^-, LPO/H_2O_2/SCN^-	[52]
Burkholderia cepaciae	LPO/I^-/GOD, LPO/SCN^-/GOD	[50]
Enterobacter cloacae	LPO/I^-/SCN^-/G/GOD	[47]
Escherichia coli	LPO/H_2O_2/SCN^-	[36]
Escherichia coli	LPO/H_2O_2/I^-, LPO/H_2O_2/SCN^-, LPO/H_2O_2/I^-/SCN^-	[53]
Escherichia coli	Peroxidase/H_2O_2/I^-	[54,55]
Escherichia coli	MPO/H_2O_2/I^-, LPO/H_2O_2/I^-, MPO/I^-/G/GOD	[56]
Escherichia coli	H_2O_2/I^-/SCN^-	[49,57]
Escherichia coli	LPO/I^-/SCN^-/G/GOD	[47]
Escherichia coli	LPO/SCN^-/G/GOD, LPO/I^-/SCN^-/G/GOD	[48]
Escherichia coli	LPO/I^-/GOD, LPO/SCN^-/GOD	[50]
Flexibacter psychrophilus	LPO/I^-/SCN^-/G/GOD	[58]
Fusobacterium nucleatum	MPO/H_2O_2/I^-	[51]
Klebsiella aerogenes	LPO/I^-/SCN^-/G/GOD	[47]
Porphyromonas gingivalis	MPO/H_2O_2/I^-	[51]
Proteus vulgaris	LPO/I^-/SCN^-/G/GOD	[47]
Pseudomonas aeruginosa	LPO/H_2O_2/I^-	[45]
Pseudomonas aeruginosa	H_2O_2/I^-/SCN^-	[49]
Pseudomonas aeruginosa	LPO/SCN^-/G/GOD, LPO/I^-/SCN^-/G/GOD	[48]
Pseudomonas aeruginosa	LPO/I^-/SCN^-/G/GOD	[47]
Psuedomonas aeruginosa	LPO/I^-/GOD, LPO/SCN^-/GOD	[50]
Pseudomonas fluorescens	LPO/I^-/SCN^-/G/GOD	[47]
Salmonella typhimurium	LPO/I^-/SCN^-/G/GOD	[47]
Serratia marcescens	LPO/I^-/SCN^-/G/GOD	[47]
Fungi		
Aspergillus niger	LPO/I^-/SCN^-/G/GOD	[47]
Botrytis cinerea	LPO/H_2O_2/I^-/SCN^-	[59,60]
Candida albicans	LPO/H_2O_2/I^-/SCN^-	[61]
Candida albicans	LPO/I^-/SCN^-/G/GOD	[48]
Candida albicans	LPO/I^-/G/GOD	[62]
Candida albicans	LPO/I^-/SCN^-/G/GOD	[47]
Cladosporium herbarum	LPO/I^-/SCN^-/G/GOD	[47]
Myrothecium verrucaria	LPO/I^-/SCN^-/G/GOD	[47]
Penicillium commune	LPO/H_2O_2/I^-/SCN^-/G/GOD	[63]
Penicillium digitatum	LPO/H_2O_2/I^-/SCN^-	[60]
Penicillium expansum	LPO/H_2O_2/I^-/SCN^-	[59,60,64]
Penicillium funiculosum	LPO/I^-/SCN^-/G/GOD	[47]
Penicillium italicum	LPO/H_2O_2/I^-/SCN^-	[60]
Phytophthora infestans	LPO/H_2O_2/I^-/SCN^-	[60]
Pityrosporum ovale	LPO/I^-/SCN^-/G/GOD	[47]
Saccharomyces cerevisiae	LPO/I^-/SCN^-/G/GOD	[47]
Stachybotrys atra	LPO/I^-/SCN^-/G/GOD	[47]
Trichoderma viride	LPO/I^-/SCN^-/G/GOD	[47]
Trichophyton interdigitale	LPO/I^-/SCN^-/G/GOD	[47]
Trichophyton mentagrophytes	LPO/I^-/SCN^-/G/GOD	[47]
Trichophyton rubrum	LPO/I^-/SCN^-/G/GOD	[47]

G = glucose; GOD = glucose oxidase; LPO = lactoperoxidase; MPO = myeloperoxidase.

$$HOI + I^- + H^+ \rightleftharpoons I_2 + H_2O \quad (13)$$

$$I_2 + I^- \rightleftharpoons I^{3-} \quad (14)$$

Gottardi [75], on the other hand, specified ten iodine species (I_2, I_3^-, I_5^-, I_6^{2-}, HOI, OI^-, HI_2O^-, I_2O^-, H_2OI^+ and IO_3^-) for the inorganic iodine-water system. He emphasized that the equilibria in the system are governed by H^+ and I^-, which imply that pH value and the additional iodide influence on species concentrations. In the most common case, with iodine in the presence of additional iodide at pH ≤ 6, only I_2 and I_3^- possess the main oxidation power. In the absence of additional iodide, at pH 8 – 9, HOI accounts for the major oxidation capacity. At high iodide concentration (e.g. Lugol's solution), I_5^- and I_6^{2-} species become important·H_2OI^+, OI^- and HI_2O^- are prominent only at pH > 10. IO_3^- has no oxidative activity in neutral and basic pH conditions, but can act as an oxidant in a form of HIO_3 at pH < 4 [75,76].

5.2.2. Mechanism of action of oxidized I^-

Thomas and Aune [72] have proposed that the antimicrobial activity of the peroxidase/H_2O_2/I^- system is due to the oxidation of I^- to I_2, followed by rapid iodination of cell components, namely, protein sulfhydryls. Oxidation of cell components yields in the reduction of I_2 back to I^-, so that as a result I^- is not consumed. Released I^- can be re-oxidized and take part again in the oxidation of other protein sulfhydryls. Therefore, one iodide ion can oxidize many cell components [54]. In such a way, I^- acts as a cofactor in the transfer of oxidizing equivalents from H_2O_2 to cell components (Fig. 5) [55].

Oxidation of 1 mol of R-S-H per mole of H_2O_2 or I_2 to sulfenyl iodide (R-S-I) corresponds to the Eq. (15) and (16). The release of I^- from R-S-I may occur through hydrolysis to yield a sulfenic acid derivative (Eq. (17)) or through disulfide formation (Eq. (18)).

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{\text{peroxidase}} I_2 + 2H_2O \quad (15)$$

$$R-S-H + I_2 \rightarrow R-S-I + I^- + H^+ \quad (16)$$

$$R-S-I + H_2O \rightleftharpoons R-S-OH + I^- + H^+ \quad (17)$$

$$R-S-I + R-S-H \rightarrow R-S-S-R + I^- + H^+ \quad (18)$$

Iodination and oxidation produce apparent denaturation of the proteins and enzymes, which are crucial for bacterial viability. The mode of action of oxidized iodide resembles that of thiocyanate, but differs in the following aspects: (i) all SH groups are oxidized by I^- oxidation products; (ii) oxidized I^- species are directed against a broader range of molecules, such as thioether (R-S-R) and NH_2 moieties of proteins, NADH, NADPH and reduced nicotinamide mononucleotide (NMNH); (iii) due to the cofactor role of I^-, a greater extent of cell components are oxidized; (iv) cells do not recover after removal of the oxidized iodide. These indicate that peroxidase/I^- systems have an irreversible bactericidal effect.

Moreover, Gottardi [76] has indicated that, although the exact details of microbial killing by I_2 or the reaction products occurring in inorganic aqueous solutions are not known, these oxidants have the following consequences: (i) oxidation of SH group of Cys amino acid results in failure to connect protein chains by disulfide bonds, impeding

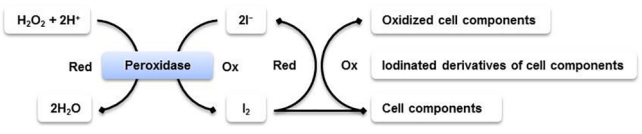

Fig. 5. The role of I^- turnover in the antimicrobial action of peroxidase/H_2O_2/I^- system [54,55,72]. Red and Ox denote the reduction and oxidation reactions.

protein folding and synthesis; (ii) iodination of phenolic (–OH) and imidazole (NH···N) groups of Tyr and His amino acids, and iodination of cytosine (C) and uracil (U) pyrimidine nucleobases can increase the bulk of molecules, leading to a form of steric hindrance in hydrogen bonds; (iii) iodine can react with the carbon–carbon double bond (C = C) of unsaturated fatty acids, leading to a change in the physical properties of the lipids and cause membrane destabilization.

5.2.3. Bacterial resistance to oxidized I^-

Our extensive review of the literature suggests that evidence of resistance in bacteria to peroxidase-mediated iodide oxidants is lacking. Moreover, to date, there are also no reports of resistance development against iodine-based disinfectants. Most researchers interpret this phenomenon as being due to the strong bactericidal activity, expressed by multiple modes of action, that include the disruption of multiple microbial metabolic pathways and destabilization of cell membrane components, causing irreversible damage to the pathogen [77,78].

5.2.4. Mammalian cell cytotoxicity of peroxidase-oxidized I^-

There are several reports on the cytotoxicity of peroxidase/I^- systems towards normal, as well as tumor cells. It was reported that LPO, in the presence of H_2O_2 and I^-, was cytotoxic for human and mouse lymphoid cells and human erythrocytes. This effect was rather rapid and highly efficient leading to 85–90% cell death within 90 min [79]. MPO or LPO when combined with G/GOD and I^- hemolysed human erythrocytes [80]. Hemolysis of this I^--dependent system was associated with the iodination of erythrocyte cell components (membrane proteins, hemoglobin). Clark et al. [81] demonstrated the cytotoxicity of I^--dependent system on mouse ascetic lymphoma cells using 4 cytotoxicity tests (^{51}Cr release, trypan blue exclusion, inhibition of glucose C-1 oxidation and loss of oncogenicity for mice). Stanislavski and co-workers [82] used antibody/glucose oxidase/lactoperoxidase conjugate to target murine plasmacytoma tumor cells. Cytotoxicity was generated when antibody/GOD/LPO targeted cells were incubated in a medium supplemented with glucose and sodium iodide.

5.3. Dual (pseudo)halides in peroxidase systems and their contribution to the antimicrobial action

5.3.1. Combination of Cl^- and Br^-

The ultimate activity of species generated by peroxidase/H_2O_2/(pseudo)halide systems may be affected because of the reaction of the initial products with other (pseudo)halides. As an example, these set of reactions can generate *trans*-halogen species. More than a century ago, inorganic chemists proposed the existence of inter-halogens, which are combinations of different halogens. The general formula of most inter-halogen compounds is XX_n^-, where n = 1, 3, 5 or 7, and X is the less electronegative of the two halogens. Electronegativity increases moving upward on the halogen group: I is less electronegative than Br, followed by Cl and F. Both binary (BrCl, IBr, and ICl) and ternary (ICl_3) inter-halogens have been since characterized. Anions of inter-halogens and poly-halides are also known; they include Cl_3^-, Br_3^-, I_3^-, Br_2Cl^- and $BrCl_2^-$. One pathway for their formation requires hypohalous acid (HOX) and halide ion (X^-), Eq. (19) [83].

$$HOX + nX^- + H^+ \rightarrow XXn^- + H_2O \quad (19)$$

Employing this mechanism, HOCl can react with Br^- to yield molecular bromine chloride (BrCl). Henderson et al. [83] showed that MPO at acidic pH *via* reaction of HOCl and Br^- generated reactive BrCl inter-halogen gas that oxidized nucleobases. Whereas, Spalteholz et al. [84] reported a direct formation of BrCl in halide oxidation by compound I of MPO, but not a formation *via* hypohalous acids.

5.3.2. Combination of Cl^- or Br^- with SCN^-

Chemically, inter-halogens are extremely corrosive species that attack a wide range of compounds and can be implicated in mutagenesis, so it is critical that scavengers govern their reactive properties. One such potential scavenger is SCN^-, an endogenous inorganic anion in human physiological fluids, with concentrations ranging from μM in plasma to mM in saliva. Ashby et al. [85] have suggested that SCN^- might limit host tissue damage by restricting the lifetime of the more detrimental oxidant HOCl. They have hypothesized that SCN^- acts as a redox buffer *via* the mechanism of non-enzymatic transfer of oxidizing equivalents from HOCl to SCN^- and, thus, the oxidizing equivalents of HOCl are preserved in $OSCN^-$, which is considered less lethal to mammalian cells (Eq. (20)).

$$HOCl + SCN^- \rightarrow OSCN^- + Cl^- + H^+ \quad (20)$$

Likewise, Nagy et al. [86] showed that HOBr reacted rapidly with SCN^- to yield HOSCN, and have proposed that SCN^- is a highly efficient scavenger of HOBr, which limits the ability of HOBr to cause biological damage to the mammalian cells. Whether this is true remains to be established, as the formed HOSCN/$OSCN^-$ may be as, or more, damaging than HOBr or HOCl. As discussed earlier, SCN^- oxidation species can exert considerable biological damage because they have greater specificity, particularly for thiols.

5.3.3. Combination of SCN^- and I^-

In chemical literature, the reaction between I_2 and SCN^- in aqueous solution under normal conditions has been known for a while, which is characterized with production of inter-(pseudo)halogen cyanogen iodide (ICN):

$$4I_2 + SCN^- + 4H_2O \rightarrow 7I^- + 8H^+ + ICN + SO_4^{2-} \quad (21)$$

On the other hand, H_2O_2 oxidation of iodide ion or molecular iodine in solutions containing SCN^- was shown to generate a complex having the probable formula $I(SCN)_2^-$ [87]. The authors suggested the reasonable equations for those reactions as:

$$H_2O_2 + I^- + SCN^- + 2H^+ \rightarrow I(SCN)_2^- + 2H_2O \quad (22)$$

$$H_2O_2 + I_2 + 4SCN^- + 2H^+ \rightarrow 2I(SCN)_2^- + 2H_2O \quad (23)$$

Schoneshbfer and Henglein [88] studied the transient complexes which could have been formed between the thiocyanate and halogen ions. Pulse radiolysis of aqueous solutions containing SCN^- and I^- under oxidizing conditions led to the formation of $ISCN^-$. During the disappearance of the various complexes in addition I_2SCN^- was formed. I_2SCN^- could be oxidized by H_2O_2 in the presence of excess SCN^- and form $I(SCN)_2^-$ [89]. Like the I_2SCN^- ion, $I(SCN)_2^-$ is unstable in aqueous solution, but its stability is enhanced at low pH, high ionic strength and low temperature [89]. However, in the recent study, the ^{13}C NMR spectroscopy performed on $H_2O_2/I^-/SCN^-$ solution showed the absence of inter-(pseudo)halogen molecules, such as ICN, $I(SCN)_2^-$ and I_2SCN^-, as well as $OSCN^-$ and I_2 [59].

The discussed literature describes the suggested reactions and generated species without the involvement of a peroxidase enzyme. Recently, Schlorke and co-workers investigated the species formed by the LPO/$H_2O_2/I^-/SCN^-$ system utilizing ^{13}C NMR spectroscopy and gas chromatography-mass spectrometry (GC–MS) [90]. They identified ICN as a yet unknown LPO-mediated oxidation product when SCN^- and an excess of I^- (in comparison to SCN^-) were applied together. This product was also formed in MPO or an enzyme-free system. Furthermore, a more recent study detected I_2SCN^- by ^{13}C NMR in LPO/$H_2O_2/I^-/SCN^-$ system [64]. In any case, it remains unknown as to what extent ICN, I_2SCN^- or other pointed species contribute to the antimicrobial activity of peroxidase-mediated systems.

5.3.4. Mimics of naturally occurring peroxidase-catalyzed systems – Simultaneous incorporation of SCN^-/I^- dual substrates in peroxidase-catalyzed and/or non-catalyzed antimicrobial systems

In general, the use of multiple drugs with different mechanisms of

action may affect multiple targets and multiple organisms simultaneously. Antibiotic combinations have been widely used to treat multidrug-resistant bacteria. The reasons why combination therapy is practiced are the following: (•) broadening antibacterial spectrum – ensures that at least one agent will cover the infecting pathogen; (•) polymicrobial infections – require more than one antibiotic to cover all bacterial pathogens; (•) synergy – increase the efficacy of the therapeutic effect, decrease the dosage while increasing/maintaining the efficacy and minimizing toxicity; (•) emergence of resistance – chances of emergence of resistance against two agents are lower as compared with a single agent [91]. Thus, incorporating two substrates (SCN^- and I^-) simultaneously into the peroxidase and/or peroxidase-free, H_2O_2 and/or G/GOD systems, hypothetically, may generate multiple oxidants from both substrates, with multiple mechanisms of action, directed against multiple cellular targets and multiple bacterial species, with little possibility of resistance development.

The SCN^-/I^- substrate couple was investigated by several researcher groups who reported contradictory results about the role this coupling played in the antimicrobial action of peroxidase-catalyzed (and/or enzyme-free) systems; some support antagonistic, the others synergistic interactions.

(i) Antagonism of the concomitant presence of SCN^- and I^- in peroxidase systems. Klebanoff [56] observed that SCN^- was inhibitory to the MPO/H_2O_2/I^- system. Although he found this paradoxical, considering SCN^- when combined with MPO and H_2O_2 exerted an antibacterial effect.

Ihalin et al. [52] investigated the effect of both LPO and MPO systems on *Actinobacillus actinomycetemcomitans* with different (pseudo) halide substrates, SCN^-, I^-, Cl^- and their combinations. They demonstrated that the oxidation of I^- had the highest antimicrobial ability followed by Cl^- and SCN^-. However, the addition of SCN^- into either MPO/H_2O_2/Cl^- or MPO (or LPO)/H_2O_2/I^- system suppressed the bactericidal action of the oxidized halide. Cl^-, on the other hand, did not affect the bactericidal effects of the MPO/H_2O_2/I^- system, but when all three (pseudo)halide substrates were present, no antimicrobial effect was recorded.

Subsequently, Ihalin and co-workers [51] studied the effects of I^-, Cl^- and SCN^-, and their combinations, with LPO and MPO on the viability of *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Streptococcus mutans* and *Streptococcus rattus*. The oxidation products of I^- were again found to be the most potent, followed by the oxidation products of Cl^- (with MPO) and SCN^- (with MPO and LPO) against all the bacteria tested. The effects were much weaker on the Streptococcus species. They reported that physiological concentrations of SCN^- suppressed the effects of LPO/H_2O_2/I^- or MPO/H_2O_2/I^- or MPO/H_2O_2/Cl^- systems, whereas, Cl^- had no effect on MPO/H_2O_2/I^- system.

Fweja et al. [45] showed that the addition of SCN^- had a negative effect on LPO/H_2O_2/I^- system when tested against *Staphylococcus aureus* in a liquid medium and mixed cultures of *S. aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* inoculated in whole milk.

Ahariz and Courtois [62] studied the susceptibility of *Candida albicans* to LPO/G/GOD system when both SCN^- and I^- substrates were simultaneously present. They observed that LPO/I^-/G/GOD reduced the colony-forming unit count to zero, but the addition of SCN^- (0.25, 2, 3 and 4 mM) progressively decreased this antifungal effect. Thus, their results also demonstrated the competition between SCN^- and I^- for peroxidase, or the scavenging effect of the SCN^-.

(ii) Synergism of the concomitant presence of SCN^- and I^- in peroxidase and/or enzyme-free systems. The incorporation of I^- as an additional substrate for the commercial LPO/SCN^-/G/GOD preservative system was carried out to broaden the antimicrobial activity of the system, considering OI^- is effective against yeast and mold, while $OSCN^-$ against bacteria [47]. The authors proposed to use this antimicrobial system as a preservative or as an antimicrobial agent in oral hygiene, deodorant and antidandruff products.

Bosch et al. [48] investigated the change in antimicrobial activity during storage of the peroxidase-catalyzed antimicrobial system, which was similar to the commercially available enzyme system described above [47] in that both SCN^- and I^- were utilized as substrates for LPO, and the system relied on G/GOD to generate the H_2O_2. The effect of I^- addition to LPO/SCN^-/G/GOD system, the chemical stability and the change in antimicrobial effectiveness during storage were studied. The addition of I^- with SCN^- increased the fungicidal and bactericidal effect against *C. albicans*, *E. coli* and *S. aureus* confirming the synergistic action between I^- and SCN^-, with the I^-/SCN^- ratio of 60:10. Whereas, the inhibition of *P. aeruginosa* growth was at the same level when the system contained or lacked the I^-. The antimicrobial stability of the LPO system was examined over the 18-month period. In general, the aged samples showed activity that was comparable to the freshly prepared solutions, although, with some organisms, longer contact time was needed for the aged system to exert the antimicrobial effect. Thus, the authors suggested the use of this antimicrobial complex as a preservative in foods and pharmaceuticals [48].

In another study, Ihalin et al. [46] targeted the antimicrobial effect of the horseradish peroxidase (HRPO)/H_2O_2/I^- system on *F. nucleatum*. They showed that in saliva (implying the presence of SCN^-) HRPO/H_2O_2/I^- combination reduced the number of viable bacteria to 37%, compared to 87% live bacteria in the saliva/HRPO/H_2O_2 system. These results suggested that saliva (SCN^- ions) did not inhibit the antimicrobial activity of the HRPO/H_2O_2/I^- system.

Schlorke and co-workers [53] investigated the killing efficiency of the inter-(pseudo)halogen ICN oxidant generated in the LPO/H_2O_2/I^-/SCN^- system using the bioluminescent *E. coli* K12 strain that allows time-resolved determination of cell viability. They concluded that the co-presence of I^- and SCN^- greatly enhanced the killing activity of the LPO system in comparison to the sole application of I^- or SCN^- in the system.

Bafort and co-authors showed that certain mixtures of LPO/H_2O_2/I^-/SCN^- had a very good antimicrobial potential against plant pathogens. They tested the efficacy of the system in *in vitro* study against *Phytophthora infestans*, *Penicillium digitatum*, *Penicillium italicum*, *Penicillium expansum* and *Botrytis cinerea* [60]. It was shown that the mixture containing an I^-/SCN^- ratio of 4.5 and diluted 3-fold inhibited pathogen growth by 63 – 100%. In another study, Bafort et al. [64] described that under precise experimental conditions (i.e. high ionic strength, an I^-/SCN^- ratio of 4.5 and acid pH of *in vitro* enzymatic reaction medium) an iodine-thiocyanate complex (I_2SCN^-) was produced which inhibited the growth of *P. expansum in vitro*, a fungus responsible for various fruit pathologies. Later, the same group suggested using again an optimal I^-/SCN^- ratio of 4.5 to favor antimicrobial efficiency and a strongly mineralized medium if high doses of substrates are used. They also recorded that although the generated oxidants were relatively stable, the long-term storage of the mixture was improved at 4 °C [59]. The authors believe that this iodine-thiocyanate complex is a "green" biochemical method for controlling plant pathogens and is as efficient as conventional chemical pesticides under controlled conditions.

Meantime, Sebaa et al. [61] used the same system implemented in [59,60,64] studies to verify the efficacy of iodine-thiocyanate complexes for Candida-colonized denture decontamination. In this study [61], the LPO/H_2O_2/I^-/SCN^- system was tested *in vitro* on the reference strain *C. albicans* and on clinical strains isolated by swabbing resin dentures. The dual substrate system inhibited the growth of Candida in the liquid medium and limited its presence in biofilms. Subsequently, an *ex vivo* clinical trial was performed to confirm the data obtained *in vitro* which showed that the mixture affected Candida carriage in 60% of the tested dentures after only 5 min incubation. Delightfully, the system was less toxic to the epithelial cells of the mouth than disinfectant chlorhexidine. The authors suggested that this antimicrobial system is a promising new strategy for the decontamination of dentures.

The research carried out by our team was also focused on the combined oxidation of I^- and SCN^- by H_2O_2, but in the absence of a peroxidase enzyme ($H_2O_2/I^-/SCN^-$ antimicrobial mixture named iodo-

thiocyanate complex or ITC). We showed that ITC was bactericidal causing rapid death of not only *E. coli*, *P. aeruginosa*, *S. aureus* and multidrug-resistant *S. aureus* (MRSA) strains in planktonic and mono-species biofilm forms, but it also could eradicate dual-species biofilms of *Streptococcus uberis* and *S. aureus* within short 30 sec exposure time [49]. We observed that the bactericidal action of ITC was derived from the combinational effect of various antimicrobial species, such as OI^-/$OSCN^-$, hydroxyl radical (OH) and I_2, the latter being the dominant antimicrobial component. This bactericidal effect was supposedly due to the simultaneous partitioning of riboplasm and fragmentation of DNA without visible cell rupture or lysis. In addition, our group addressed the emergence of bacterial resistance towards ITC and the cytotoxicity towards mammalian cells. We demonstrated that the prolonged serial passage of *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA in the presence of sub-inhibitory concentrations of ITC failed to select for resistance [49]; the continuous culture of *E. coli* under the gradually increasing selective pressure of ITC did not result in the development of resistance [92] and the ITC usage posed a low risk for resistance development *via* increased mutation rate or increased efflux [93]. To reveal the possible mechanism of resistance to ITC, we carried out whole-genome sequencing of the continuous culture of *E. coli* exposed to increasing ITC [92]. As no resistance arose despite the long-term selective pressure, we could not identify any determinants which could have contributed to ITC resistance. From the mutation list, of interest were the mutations in DNA mismatch repair endonuclease MutH that, theoretically, can lead to the development of a mutator phenotype, thus, contributing to antimicrobial resistance and cross-resistance. However, another study from our group showed that *E. coli* did not develop an elevated mutation rate when exposed to the near-lethal concentration of ITC [93].

Although we observed dose-dependent cytotoxic and hemolytic activity against human cervical epithelial adenocarcinoma HeLa cells and horse erythrocytes, overall the *in vitro* comparative cytotoxicity study of ITC showed that the beneficial antimicrobial properties of ITC outweighed its non-selective cytotoxicity [57]. Therefore, we suggest that the use of this antimicrobial may provide an effective and efficient method for killing pathogens, as well as for disinfecting and removing biofilm contamination without triggering resistance emergence in bacteria.

6. Application of peroxidase-catalyzed systems

As was discussed, the antimicrobial spectrum of the peroxidase-catalyzed and mimicking systems covers a broad range of microorganisms listed in Table 2 and described in several reviews [15,16,22,94–96]. However, the peroxidase systems had limited application as antimicrobial agents mostly because the enzyme purification from human leukocytes (MPO), human milk (LPO) or human saliva (SPO) is more expensive than traditional preservatives, the purification procedures are time-consuming, and thus, it is difficult to obtain large quantities of enzymes. Human MPO, LPO or SPO are purified but just for research objectives. Instead, LPO purified from bovine milk has been largely used for research and commercial reasons, as it is readily available and is structurally and catalytically close to human SPO and LPO. Additionally, recombinant peroxidases generated in wide panel of cell factories such as bacteria, yeast, fungus and plant have gained widespread applications [97].

LPO has found many applications thanks to its broad antimicrobial activity and presence in different body fluids. LPO systems are widely used as natural bio-preservatives in oral healthcare, milk industry, food/feed specialties and cosmetics (reviewed extensively elsewhere [1,15,20,95,96,98]). Here, we will briefly discuss some of the various examples.

6.1. Enhancement of antimicrobial activity of saliva by peroxidase system

Commercially available oral health care products, including Biotene, BioXtra, Zendium Saliva, Orabarrier and Oralbalance, used LPO with or without its substrate SCN^- to boost or restore saliva's intrinsic antimicrobial ability [96]. The H_2O_2 component of the system was generally formed *in situ* in the mouth by a G/GOD system. Many of those commercial products are available in the form of toothpaste but also chewing gum, mouth-rinse, moisturizing gel, lozengs, foams, etc. The question is, whether these products are functioning *in vivo*. In their recent review, Magacz et al. [95] compared the results of clinical trials of dentifrices enriched with LPO and *in vitro* tests of the LPO system alone or in combination with lactoferrin, lysozyme or immunoglobulins. The clinical trials involved participants from various age groups with various clinical conditions, e.g. malodor, xerostomia, caries and chronic periodontitis, as well as healthy subjects. This review presents in details the results of a set of studies proving the effectiveness of the LPO system in the prevention and alleviation of the symptoms of mentioned oral diseases, as well as the involvement of LPO system in inhibition of planktonic and biofilm bacteria associated with these diseases in *in vitro* tests. Thus, we will not discuss here individual studies demonstrating the significance of LPO system in oral health and its efficacy in oral hygiene products.

6.2. Enhancement of antimicrobial activity of milk by peroxidase system

LPO system has an antimicrobial effect against a diversity of milk-borne pathogenic and spoilage bacteria. A summary of the studies, which targeted the influence of the LPO system on various milk-borne pathogens, is demonstrated in the report of the FAO/WHO technical meeting [94]. The foremost recommended industrial application of the LPO system is in the dairy industry for the preservation of raw milk during storage and transportation (reviewed in [15,22]). The LPO system also has wide application in cheese production, as it can eliminate pathogens in the starter cultures for cheese-making and extend the shelf life of fresh cheese [15]. Another beneficial application of the LPO system is the rearing of calves. Usually, dairy calves are fed with milk substitutes and are prone to infections, such as enterotoxigenic *E. coli*. The addition of a preparation based on LPO system together with lactoferrin in the milk replacements have been shown to decrease the severity and duration of enteric colibacillosis in calves, even when calves were already infected [99].

6.3. Application in mastitis treatment

The LPO system has a potential to be applied as a treatment for bovine mastitis. Mastitis is one of the most common diseases amongst dairy cows and therefore the costliest problem to the dairy industry. It is an inflammatory condition in the udder of mammals and occurs when immune cells are released into the mammary gland in response to invading bacteria. As a result, milk from cows with mastitis has a higher somatic cell count, which is reducing the yield and quality of the milk. Treatment regimens rely solely on antibiotic usage, but the milk treated with the antibiotic is not marketable. The LPO system has been suggested as a potential solution to overcome this problem.

The consensus in the literature is that the LPO system has mainly a bacteriostatic effect against the common udder pathogens *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *E. coli*, *P. aeruginosa* and *S. aureus* [94]. Moreover, even if the peroxidase system is bactericidal to mastitis pathogens in synthetic medium, the addition of the milk to the reaction mixture inhibits the bactericidal properties of the system, suggesting that milk proteins interfere with the bactericidal agents and form adducts with them [100]. It was reported that the LPO system in mastitic milk was less effective compared to the healthy milk because of a higher concentration of catalase enzyme and reducing agents [94]. However, a WO/2012/140272 patent application [50] described a biocidal antimicrobial composition for use in the treatment of mastitis, which comprised of a peroxidase, a glycoside hydrolase (to break down disaccharide sugars into monosaccharides), an oxidoreductase enzyme (to react with the

monosaccharide sugars and release H_2O_2) and a substrate for peroxidase (dependent on which peroxidase will be used). In any case, further studies are required before introducing the LPO system as a suitable mean for *in vivo* treatment of bovine mastitis.

6.4. Application in the food industry

A large amount of the published studies have focused on the natural environment of LPO system (saliva, milk, etc.) and respective pathogens, while the antimicrobial activity of the system can also target various food pathogens. This led to the investigations on the application of LPO system for the preservation of foodstuffs, including fish, meat, fruits, vegetables, etc.

Van Hooijdonk et al. [58] described a trial investigating the potential of the peroxidase system for use in fish farming. Their peroxidase system was comprised of LPO, both SCN^- and I^- as substrates, and G/GOD as the source of H_2O_2. They used LPO system to feed the rainbow trout fry and monitor the mortality during the weaning period, which is normally high, mainly because of infections caused by *Flexibacter psychrophilus* and *Octomis salmonis*. There was 30% decrease in accumulated mortality in the test LPO group. Elotmani and Assobhei [101] studied the combined antimicrobial effects of LPO system (LPO/SCN^-/G/GOD) and nisin against the bacterial strains isolated from sardines. They reported that nisin inhibited only Gram-positive bacteria, whereas LPO system inhibited all strains studied, and even more, the combined effect was significantly higher. The authors suggested that the combination of these two could be a possible bio-preservative for fish and fish products.

The LPO system with both SCN^- and I^- substrates was successfully incorporated into edible films. Min and Krochta [63] showed that the incorporation of LPO/H_2O_2/SCN^-/I^-/G/GOD antimicrobial system into edible whey protein isolate (WPI) films inhibited the growth of *Penicillium commune* and have suggested that the LPO system with WPI films have the potential to be used in complex food systems. A similar study was carried out to develop antimicrobial edible films, by combining the defatted soybean meal, the LPO system (LPO/H_2O_2/SCN^-/I^-/G/GOD) and heat pressing [102]. The authors proposed that the antimicrobial edible films and coatings can be applied to ready-to-eat products to minimize or prevent the growth of pathogenic microorganisms, including Salmonella, during storage.

As discussed in section 5.3.4, Bafort and co-workers demonstrated that the dual I^-/SCN^- substrate LPO system was as efficient as a conventional synthetic chemical method under well-defined conditions to control pests in pre and postharvest crops. They proposed that this alternative environment-friendly and consumer-oriented biochemical method can respond more adequately to the expectations of sustainable agriculture [59,60,64].

6.5. Application in wound treatment

Wound infection can be a challenging problem, especially in the context of growing resistance to antibiotics. Topical application of peroxidase-catalyzed systems is a promising tool for wound treatment. Several patents were filed describing the use of peroxidase systems for wound healing. US 4,576,817 patent [103] proposed an organic absorbent material for body contact, such as a bandage and a pad, incorporating dry enzymes (oxidoreductase and, optionally, peroxidase) which will be activated upon contact with serum. As an oxidoreductase can be used GOD, generating H_2O_2 using the glucose from serum, and as for peroxidase can serve LPO, which will interact with produced H_2O_2 and an oxygen-accepting anion (SCN^-, Cl^- and I^-) in serum to produce oxidized species and inhibit bacteria. Whereas, US 7,731,954 B2 patent [104] proposed a wound dressing, comprised of an oxidoreductase enzyme and, optionally, peroxidase, wherein the enzyme(s) are present in hydrated condition, e.g. being present in one or more hydrogels. The third similar US 7,927,588 B2 patent [105] described skin dressings comprised of two dressings: a first dressing incorporating dry oxidoreductase enzyme; and a second dressing carrying a source of water, such that when the both are in contact water migrates from the second dressing towards the first and hydrates the enzyme. The first dressing is placed on top of the second one, and the dressings are kept separately before use. Alternatively, the embodiment includes also a peroxidase enzyme, preferably present in the hydrated condition. WO/2012/140272 patent [50], mentioned in the mastitis section, also suggested using their antimicrobial composition for wound treatment. In addition, the research conducted by our team anticipated that the ITC (H_2O_2/I^-/SCN^- complex) might in future find use as an antiseptic and disinfectant, to treat infections and/or to decontaminate surfaces and in particular to limit the spread and risk posed by antibiotic resistant bacteria [49,57,92,93].

7. Conclusions and areas for future work

The antibiotic doomsday scenario is on the horizon. If we are to keep pace with the rise of drug resistance, we need to refill the antimicrobial pipeline. But there aren't a lot of new antimicrobial drugs waiting in the wings to join the battle against resistant pathogens. Nature is offering a possible solution for this problem in a form of peroxidase-catalyzed systems. Peroxidase-catalyzed systems are widespread throughout nature as part of mammalian innate defenses against invading microorganisms. A peroxidase enzyme catalyzes the oxidation of a (psedo)halide substrate by hydrogen peroxide to generate highly reactive products with a wide range of antimicrobial properties. Over and above, the peroxidase-catalyzed systems are inspiring for the development of alternative antimicrobial therapeutics. One creative example of such mimics based on (pseudo)halide oxidation is the peroxidase-free – dual-substrate – hydrogen peroxide system which possesses even broader set of oxidants, targets more than one type of bacteria, has more than one target in a bacterial cell, thus, is not prone to trigger the emergence of facile resistance.

In any case, the actual products with antimicrobial properties and the chemistry of their reactions depend upon the specific peroxidase/peroxidase-free – substrate/substrates – oxidizer inter-relationships. A more detailed understanding of the oxidants that contribute most to lethality of peroxidase or peroxidase-like systems, modifications of microbial components and metabolism, response of microbes and detrimental effect towards mammalian cells remains an area for further study. Thus, we propose the following opportunities for future studies:

- Expand the antimicrobial screening of peroxidase/peroxidase-like systems on an extended list of microorganisms, including multidrug-resistant clinical isolates, fungi and viruses. The general rule regarding antibacterial activity spectrum has been "broader is better". This is true when treating severe infections, as clinicians do not always know the causative pathogen, and thus usage of broad-spectrum antimicrobial will save the precious time.
- Scrutinize the identities of all reactive species occurring in the mixtures of peroxidase/peroxidase-like systems; elaborate the mechanism of action studies to detect multiple targets of those multiple species in various types of microorganisms; link the chemical identities to their targets and antimicrobial actions. To have a fully characterized profile of an antimicrobial mixture one wants to know "who is there and what are they doing there?"
- Seek to reduce the mammalian cytotoxicity of peroxidase/peroxidase-like systems. It is well-documented that peroxidase systems (and likely peroxidase-mimicking systems) have a dual role, behaving as both a friend and a foe. The highlight is that as they have non-specific, broad-spectrum target mechanism, aside from mediating bacterial cell killing, destroying invading parasites, combating fungal infections and inactivating viruses, they can attack a variety of mammalian cells, including tumor cells.
- Investigate the therapeutic relevance of peroxidase/peroxidase-like systems on animal, *ex vivo* and *in vivo* models, such as wound,

lung, urinary tract, digestive, device-related infection models, dental plaque and many more. These antimicrobials have a future, but will they work in real life?

- Conduct *in vitro* resistance studies with numerous parallel evolving cultures to test the dynamics and determinants of resistance towards peroxidase/peroxidase-like systems in drug-sensitive and/or multidrug-resistant pathogens; follow the cross-resistance and collateral sensitivity on large sets of antibiotics. If one would replay the tape of adaptation to these antimicrobial systems, would the evolution result the same outcome?

Though the application potential of these antimicrobial systems is wide and is currently being explored worldwide, here as well, further research is needed. To our knowledge no peroxidase/peroxidase-like system has reached the approval as an antibacterial therapeutic so far, thus preclinical and clinical trials can be developed to explore their potential as effective pharmaceuticals, such as antimicrobial therapeutics or prophylactics. Peroxidase/peroxidase-like systems may be used in combination therapy with bactericidal antibiotics, theoretically to re-sensitize the resistant bacteria towards the antibiotic and prevent the mutagenesis in bacteria towards antibiotics by inhibiting reactive oxygen species [106]. Also, considering the peroxidase-catalyzed systems are the part of the innate host immunity which coexists peacefully with microbiota during lifetime and no resistance emerges, these systems may be used to help the host to restore natural microbiome by clearing the pathogens. Additionally, linking peroxidase/peroxidase-like systems with intriguing bioactivities other than antimicrobial effects will give more possibilities for applications and it is very likely that research in this ground will result in the introduction of novel mode of action and biological effects. For these very reasons, undoubtedly, peroxidase systems and their mimics are emerging as one of the promising nature-inspired antimicrobial strategies to tackle antimicrobial resistance.

CRediT authorship contribution statement

Lilit Tonoyan: Conceptualization, Writing - original draft, Writing - review & editing. **Diego Montagner:** Visualization, Writing - review & editing. **Ruairi Friel:** Writing - review & editing. **Vincent O'Flaherty:** Supervision, Visualization, Writing - review & editing.

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Conflict of interest

Dr. RF is an employee of Westway Health and owns shares in the company. Prof. VOF also owns shares in the company. Dr. LT and Dr. DM declare no conflict of interest.

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