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Reactive Oxygen Species (ROS)-Mediated Antibacterial Oxidative Therapies: Available Methods to Generate ROS and a Novel Option Proposal

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Review

Reactive Oxygen Species (ROS)-Mediated Antibacterial Oxidative Therapies: Available Methods to Generate ROS and a Novel Option Proposal

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Abstract: The increasing emergence of multidrug resistant (MDR) pathogens causes difficult-to-treat infections, with long-term hospitalizations and high incidence of death, thus representing a global public health problem. To manage MDR bacteria bugs, new antimicrobial strategies are necessary, and their introduction in practice is a daily challenge for scientists in the field. An extensively studied approach to treat MDR infections consists in inducing high levels of reactive oxygen species (ROS) by several methods. Although further clinical investigations are mandatory on the possible toxic effects of ROS to mammalian cells, clinical evaluations are extremely promising, and their topical use to treat infected wounds and ulcers, also in presence of biofilm, is already clinically approved. Biochar (BC) is a carbonaceous material obtained by pyrolysis of different vegetable and animal biomass feedstocks, at 200−1000 °C in limited presence of O_2. Recently, it has been demonstrated that BC capability of removing organic and inorganic xenobiotics is mainly due to the presence of persistent free radicals (PFRs), which can activate oxygen, H_2O_2 or persulfate in the presence or not of transition metals by electron transfer thus generating ROS, which in turn degrade pollutants by advanced oxidation processes (AOPs). In this context, the antibacterial effects of BC containing PFRs have been demonstrated by some authors against *Escherichia coli* and *Staphylococcus aureus*, thus giving birth to our idea of the possible use of BC-derived PFRs as a novel method capable of inducing ROS generation for antimicrobial oxidative therapy. Here, the general aspects concerning ROS physiological and pathological production and regulation, and the mechanism by which they could exert antimicrobial effects have been reviewed. The methods currently adopted to induce ROS production for antimicrobial oxidative therapy have been discussed. Finally, for the first time, BC-related PFRs have been proposed as a new source of ROS for antimicrobial therapy via AOPs.

Keywords: multidrug resistant pathogens; antimicrobial oxidative therapy; reactive oxygen species (ROS); biochar (BC); biochar-derived permanent free radicals (PFRs); advanced oxidation processes (AOPs)

1. Introduction

The incessant and rapid increase of multi drug resistant (MDR) pathogens causes the emergence of difficult-to-treat infections, with long-term hospitalizations, high-costs, and a frightening incidence of death. In the United States, more than 2.8 million antibiotic-resistant infections occur each year, resulting in 35,000 deaths [1]. Resistant bacteria are becoming an uncontrollable worldwide hazard to both humans and animals [2,3]. Management of the antimicrobial resistance through the available

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antibiotic is a global public health problem and represents a daily challenge for experts in the field. To limit the global antibiotics resistance crisis, the main solution would be to reduce the volume of the unscrupulous use of antibiotics both in medicine, agriculture and in the environment [4], as well as to perform an efficient infection control strategy to prevent the spread out of contagions. Anyway, the development of novel antimicrobial drugs remains urgent and mandatory [1]. Although several new agents based on existing classes of antibiotics are being developed, there has been little advancement on the exclusive discovery of novel agents [4].

Moreover, the capability of several bacterial and fungal species to form biofilms is an added alarming mechanism through which pathogens develop a very complex form of resistance. Biofilm producing pathogens represent a significant problem in many clinical settings, since biofilm further increases their tolerance towards conventionally prescribed antimicrobials [5,6]. The high-dosage use of antibiotics in biofilm conditions to treat chronic wounds, burns, chronic respiratory diseases, cystic fibrosis and recurrent cystitis leads to intense selective pressure, which paradoxically drives to further antibacterial resistance [4].

On this alarming scenario, the need for the development of optional per se effective therapeutic approaches, or of alternative treatments which can improve the antimicrobial efficacy of existing drugs also towards biofilms, is imperative [7]. This second strategy would allow to reduce the amounts of antibiotics to be used thus limiting the emergence of resistance in pathogens. Entirely novel antimicrobial instruments characterized by a unique mechanism of action are represented by reactive oxygen species (ROS) induced by different methods [4].

ROS have demonstrated in vitro and in vivo a significant antimicrobial action against a wide spectrum of Gram-positive and Gram-negative organisms, including MDR isolates and biofilm-producing pathogens [8]. The use of ROS could represent a new therapeutic approach for topical use on skin, mucosal membranes, or internal tissue that may be colonized with microbial inhabitants and biofilms [9].

Treatments involving ROS as antimicrobial agents are already available for topical application and are clinically approved to treat infected wounds and are being developed for clinical use also in other settings [10].

As mentioned above, ROS is the well-known acronym used in several sectors including medicine, to indicate reactive oxygen species, including radical and not radical oxygen-containing atoms and molecules, such as superoxide anion (O_2^-), singlet oxygen ($^1O_2$), peroxide (O_2^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), and hydroxyl anions (OH^-), that are constantly being formed as byproducts of the physiologic aerobic metabolism of cells [11]. Additionally, ROS can react with NO produced by cells from intracellular *L*-arginine via action of epithelial nitrogen oxide synthetase (NOS), neuronal NOS, and inducible NOS, forming other reactive species such as NO• and $ONOO^-$, while NO• in combination with O_2, provides ONOO•. These molecules are referred to as reactive nitrogen species (RNS) [11].

In normal conditions, ROS and RNS generation is kept under control by the antioxidant defenses and repair systems of cell [12]. On the contrary, when overproduced, the detoxification systems of cell fail to maintain ROS and RNS physiological levels, which accumulate, thus causing the onset of oxidative stress (OS) and inflammation. Irreversible damage to DNA, lipids, and proteins occurs, thus promoting aging, age-related diseases, and several degenerative human disorders [13].

Collectively, OS is a cascade of events that frequently triggers and accompanies molecular/cellular pathogenic events. It is responsible for several human disorders, including carcinogenesis [14,15], atherosclerosis, cardiovascular, and neurodegenerative diseases [16,17].

As occurring in humans, also in pathogens, OS builds up when prooxidants overpower antioxidants. Therefore, ROS get accumulated in the microorganism's cell, thus exceeding the cell's capacity to readily detoxify them [18].

As examples, the host immune response, as well as several antimicrobials counteract infections by inducing ROS accumulation. While the interaction between the host and pathogens causes exogenous OS in bacteria, the intracellular redox reactions, antibiotics, and not controlled aerobic respiration contribute to endogenous OS [19]. ROS cause multiple damages to the bacterial cells,

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including double stranded breaks in DNA by oxidizing dCTP and dGTP pools, which results in the misincorporation of bases into DNA. Additionally, ROS induce lipids peroxidation, and proteins carbonatization [20], thus exerting a very rapid bactericidal activity. It has been reported that ROS were able to cause 3 log CFU reduction in 30 min and total eradication in 2 hours when used against *Staphylococcus aureus* [21]. Several conventional and alternative antibiotics including metal nanoparticles and natural molecules exert their antimicrobial properties by inducing ROS hyperaccumulation in pathogens [22]. Other methods to exert ROS-mediated antimicrobial effects, include photodynamic therapy (PDT), honey reactive oxygen (HRO) therapy, and hyperbaric oxygen treatment (HBOT).

On these considerations, ROS could really represent an effective option for eradicating MDR pathogens. While procedures to induce ROS in microbial cells, such as HBOT and PDT are traditional, the use of nanomaterials and engineered medical honey are rather novel promising methods. Nevertheless, nanotoxicology is a field still not clearly defined, which lacks sufficient and unequivocable epidemiologic data, information, and regulation. Furthermore, such methods may induce ROS formation also in host cells. To make possible an enlargement of the clinical use of ROS to counteract MDR pathogens and related biofilm, the development of other delivery strategies for increasing the selectivity of ROS for microbial pathogens over the host tissue is necessary. In this regard, based on our recent studies on biochar (BC) [23,24], we profit of this review to propose a possible innovative method to be studied to produce ROS by a natural low-cost source. Biochar (BC) is a carbonaceous material obtained by pyrolysis of different vegetable and animal biomass feedstocks and waste, at 200−1000 °C in the limited presence of absence of oxygen. Due to its strong adsorption capacity, BC can exert a plethora of beneficial effect, including the removal of environmental pollutants and xenobiotics, thus preventing their uptake in plants, animals, and humans [25–28]. In microbiology, BC has demonstrated to be helpful in limiting the antimicrobial resistance by degrading/removing residual antibiotics in soil and waters [24]. The so called, environmentally persistent free radicals (EPFRs) are known to exist in significant concentration in atmospheric particulate matter (PM) and are primarily emitted from combustion and thermal processing of organic materials. While their existence in combustion has been known for over half-a-century, only recently their presence in environmental media and their healthy and/or hazardous effects have been researched [29]. Nowadays, it has been demonstrated that also BC can contain persistent free radicals (PFRs) as well, bound to the external or internal surfaces of its solid particles [29]. PFRs are reactive species due to unpaired electrons, that can persist up to several months, in contrast to traditional transient radicals [24]. Studies reported that PFRs are the main responsible for the BC capacity to degrade organic pollutants through the generation of active oxygen species (ROS) and sulfate radicals [23]. It was reported that the generated ROS, including radical (•OH, $•O_2^-$, $•O_2H$, $SO_4•^-$) and non-radical species ($^1O_2$) successfully degrade several organic pollutants, hormones and eDNA, by advanced oxidation processes (AOPs). Interesting, PFRs-mediated ROS showed antibacterial effects against *Escherichia coli* and *S. aureus* [30–32], thus supporting the idea of the possible use of BC-derived PFRs as a novel method to induce ROS generation for antimicrobial oxidative therapy. In the following sections, all that was introduced in this section will be more in deep reviewed and discussed.

2. Reactive Oxygen Species (ROS) and Oxidative Stress (OS)

2.1. Physiological and Pathological Origin of ROS

Figure 1 schematizes the main processes by which ROS can form in cells and the detrimental effects they can determine to health [11].

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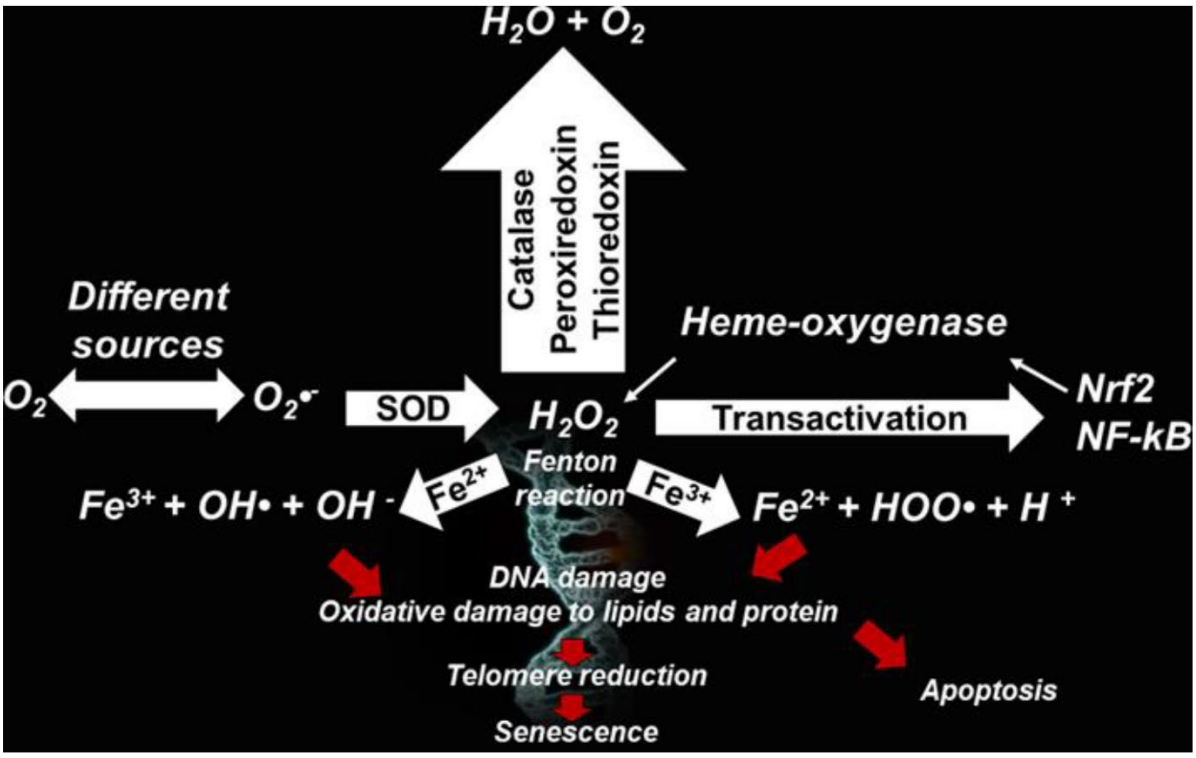

Figure 1. Schematic pathways of reactive oxygen species (ROS) production and their main effects on biological systems. Nrf2 = erythroid nuclear transcription factor-2; NF-kB = transcription factor involved in cellular responses to stimuli such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized low density lipoproteins (LDL) etc. Reproduced from our article [11].

Table 1 reports the possible enzymatic and not enzymatic endogenous sources of ROS, as well as those exogenous. The main reactive species of both oxygen and nitrogen which can be consequently produced have been also included.

Table 1. Endogenous and exogenous sources of ROS and the main reactive species of both oxygen and nitrogen (RNS) which can be consequently produced.

Endogenous Sources		Exogenous Sources	Reactive Species
Enzymatic	**Non-Enzymatic**		
NOX MPO Cytochrome P450 Lipoxygenase Angiotensin II Xanthene oxidase Cyclooxygenase FpH•	Mitochondria Respiratory chain Glucose auto-oxidation NAD• Semiquinone radicals Radical pyridinium Hemoproteins	Air Water pollution Tobacco Alcohol Heavy/transition metals Drugs Industrial solvents Cooking Radiation EPFRs BC-PFRs	$O_2^{\bullet-}$ H_2O_2 •OH •OOH ONOO• $NO_2^{\bullet}$ NO• $ONOOCO_2^-$ NO^{2+} ONOOH N_2O_3 $ONOO^-$ $ONOOCO_2^-$ $CO_3^{\bullet-}$

NADPH = nicotinamide adenine dinucleotide phosphate; MPO = myeloperoxidase; NOX = NADPH oxidase; NAD = nicotinammide adenina dinucleotide; Fp = flavoprotein enzymes; EPFRs = environmental persistent free radicals; BC-PFRs = biochar-related persistent free radicals.

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Based on the recently acquired knowledge and literature reports [33–36], respect to the Table reported by us in 2020 [11], environmental persistent free radicals (EPFRs) and biochar-related persistent free radicals (BC-PFRs) have been included among the exogenous sources of ROS in Table 1. Upon their formation according to reported processes and mechanisms the can induce ROS formation by reacting with atmospheric or water dissolved O_2, as well as with H_2O_2 and/or persulfate [24]. Briefly, phagocytic cells (neutrophils, monocytes, or macrophages) use NOX for one-electron reduction of molecular oxygen to the radical superoxide anion ($O_2^{\bullet-}$), during cellular respiration. $O_2^{\bullet-}$ is considered the primary ROS, and when reacts with other molecules through enzymatic or non-enzymatic metals-catalyzed processes, generates secondary ROS.

$O_2^{\bullet-}$ is mainly transformed by superoxide dismutase (SOD) into the hydrogen peroxide (H_2O_2), from which the highly reactive ROS hydroxyl ion ($^{\bullet}OH$) and radical $HOO^{\bullet}$, are formed through the Fenton or Haber–Weiss reactions in presence of transition metals. $O_2^{\bullet-}$ is also produced from the irradiation of molecular oxygen with UV rays, photolysis of water, and by exposure of O_2 to organic radicals formed in aerobic cells such as $NAD^{\bullet}$, $FpH^{\bullet}$, semiquinone radicals, cation radical pyridinium, or by hemoproteins.

While the radical $O_2^{\bullet-}$ does not react directly with lipids, polypeptides, sugars, or nucleic acids, $^{\bullet}OH$ and $HOO^{\bullet}$ react especially with phospholipids in cell membranes and proteins, thus causing oxidative damage, DNA damage, telomere reduction, ageing and apoptosis [11].

Furthermore, H_2O_2 can be converted by MPO to hypochlorous acid, particularly hazardous for cellular proteins [37].

Additionally, ROS can react with NO produced from intracellular *L*-arginine by cells as a defense mechanism, using three different kinds of NOS, such as epithelial NOS, neuronal NOS, and inducible NOS, thus forming reactive nitrogen species (RNS) such as $NO^{\bullet}$ and $ONOO^{-}$. Finally, $NO^{\bullet}$ in combination with O_2, can provide $ONOO^{\bullet}$, which induces lipid peroxidation in lipoproteins [11,38–40].

Table 2 summarizes the specific source of the most representative radical and not radical reactive species produced in biological aerobic systems and their physiological function.

Table 2. The most representative radicals and not radical reactive species produced in biological aerobic systems with sources and functions.

Reactive Specie	Source	Function
$O_2^{\bullet-}$	Enzymatic process Autoxidation reactions Non-enzymatic electron transfer reactions	Reduces iron complexes such as cytochrome C Oxidizes ascorbic acid and α-tocopherol
$HOO^{\bullet}$	Protonation of $O_2^{\bullet-}$	Initiates fatty acid peroxidation
$HO^{\bullet}$	By H_2O_2 via Fenton reaction and HWR	Reacts with organic and inorganic molecules *
$NO^{\bullet}$	*L*-arginine (substrate) NADPH (electron source) Nitric oxide-synthase	Intracellular second messenger Stimulates GC and PK Causes smooth muscle relaxation in blood vessels
$NO_2^{\bullet}$	Protonation of $ONOO^{-}$ Homolytic fragmentation of $ONOOCO_2^{-}$	Acts on the antioxidant mechanism ↓ Ascorbate and α-tocopherol in plasma

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$ONOO^{\bullet}$	Reaction of O_2 with $NO^{\bullet}$	Oxidizes and nitrates methionine and *L*-tyrosine Oxidizes DNA to form nitroguanine
$CO_3^{\bullet-}$	(SOD)-Cu^{2+} By reaction of $^{\bullet}OH$ reacts with HCO_3^-	Oxidizes proteins and nucleic acids
$ONOOCO_2^-$	Reaction of $ONOO^-$ with CO_2	Promotes nitration of oxyhaemoglobin's tyrosine of the via free radicals

HWR = Haber Weiss recombination; * DNA, proteins, lipids, carbohydrates; GC = guanylate cyclase; PK = protein kinases.

Whatever their origin, ROS and RNS cause indifferently detrimental oxidative modifications of cellular macromolecules such as carbohydrates, lipids, proteins, DNA, and RNA, thus determining the production of molecules, which are considered markers of OS (Table 3).

Table 3. Oxidative modification of cellular macromolecules: reactions involved and produced markers of OS.

Cellular Macromolecules	Reactions	OS biomarkers	Ref
Proteins	RNS with *L*-tyrosine	NT	[41]
	Fenton reaction of ROS with *L*-lysine, *L*-arginine *L*-proline, *L*-threonine	PC	[42]
Proteins/lipids	Michael-addition of aldehydic lipid oxidation products to *L*-lysine, *L*-cysteine, *L*-histidine	PC	[42]
Proteins/lipids	Complex oxidative process	Ox-LDL	[43]
Proteins/carbohydrates	Glyco-oxidation between *L*-lysine amino groups and *L*-arginine carbonyl groups linked to carbohydrates	AGEs	[44]
Lipids	$^{\bullet}OH$ and $HOO^{\bullet}$ mediated lipid peroxidation of poly-unsaturated fatty acids *	4-HNE, MDA, F2-IsoPs	[41]
DNA	Mutagenic oxidation	2-Hydroxy adenine 8-Oxoadenine 5-Hydroxycytosine Cytosine glycol Thymine Glycol 8-OHGua 8-OHdG	[45]

AGEs = advanced glycation end products (*N*-ε-carboxymethyl-lysine pentosidine glucosepane); 4-HNE = 4-hydroxynonenal; MDA = malondialdehyde; F2-IsoP = F2-Isoprostanes; Ox-LDL = oxidized low-density lipoprotein; PC = protein carbonyl; 8-OHGua; 8-hydroxyguanine; 8OHdG—8-hydroxy-2′-deoxyguanosine; * linoleic, arachidonic acids; NT = nitro tyrosine.

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To counteract the detrimental effects reported in Table 3, cells have developed several repair systems able to restore or eliminate lipids, proteins, and DNA damaged by the action of ROS and RNS. Particularly, cytosolic and mitochondrial enzymes which include polymerases, glycosylases, and nucleases, repair the damaged DNA, while proteinases, proteases, and peptidases make part of the proteolytic enzymes, remove damaged proteins. In addition, biological systems have developed both physiological and biochemical mechanisms to limit free radicals' production and reactive species toxicity. At physiological level, the microvascular system exerts the function of maintaining the levels of O_2 in the tissues, while at biochemical level a protective activity is exerted both by endogenous (enzymatic and non-enzymatic) and exogenous molecules, as reported in Table 4.

Table 4. Endogenous and exogenous molecules which can counteract free radicals and reactive species toxicity. ↑ Means increase; ↓ means decrease/reduction.

Process	Endogenous Molecules	Actions	Exogenous Molecules	Effect
NE	Vitamin E Vitamin C Carotenes Ferritin Ceruloplasmin Selenium GSH Manganese Ubiquinone Zinc Flavonoids Coenzyme Q Melatonin Bilirubin Taurine Cysteine Albumin Uric acid	Interact with ROS and RNS and terminate the free radical chain reactions	Vitamin C	↓$O_2^{\bullet}$ ↓$^{\bullet}OH$
			Vitamin E	↓Lipid peroxidation
			Resveratrol Phenolic acids Flavonoids	↓$O_2^{\bullet}$ ↓$^{\bullet}OH$ ↓Lipid peroxidation
			Oil Lecithin	↓$O_2^{\bullet}$ ↓$^{\bullet}OH$ ↓Lipid peroxidation
			Selenium Zinc	Antioxidant
			Acetylcysteine	Antioxidant

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E	SOD	Converts O_2 to H_2O_2 ↓Hydroxyl radical production
	CAT	Decomposes H_2O_2 to H_2O+O_2 ↓Hydroxyl radical production
	GSH-Px	Converts peroxides and hydroxyl radicals into nontoxic forms by the oxidation of GSH into GSSG
	GR	Converts glutathione disulphide to GSH
	GSTs	Catalyses the conjugation of GSH to xenobiotic substrate
	G6PD	Catalyses the dehydrogenation of G6P to 6-phosphoglucono-Δ-lactone
	Nrf2	Regulates the expression of antioxidant proteins
	ARE	Encodes for detoxification enzymes and cytoprotective proteins
	NQO1	Catalyses the reduction of quinones and quinonoids to hydroquinone molecules
	MSR	Carries out the enzymatic reduction of the oxidized form of methionine to methionine

NE = not enzymatic; E = enzymatic; CAT = catalase; GSH-Px = Glutathione peroxidase; GR = glutathione reductase; GSH = reduced glutathione; Nrf2 = erythroid nuclear transcription factor-2; ARE = antioxidant response element; NQO1 = NAD (P)H quinone oxidoreductase 1; GSTs = glutathione S-transferases; G6P = glucosio-6-fosfato; G6PD = glucosio-6-fosfato dehydrogenase; GSSG = glutathione disulfide; MSR = methionine sulfoxide reductase.GSH-Px, GR, and MSR are the main intermediaries in the processes for repairing oxidative damage.

2.2. Pathogens Responses to OS

As reported previously in the introduction, ROS cause multiple damages to the bacterial cells [22]. Anyway, bacteria can evade OS by several defenses including detoxifying methods using enzymes like catalase, alkyl hydroperoxide reductase, thioredoxin, and superoxide dismutase (SOD). Additionally, they use pigments like carotenoids; metal homeostasis, and repairing devices including DNA restoration, general stress response, and SOS response [18,46]. All these mechanisms are regulated by gene networks [46]. *E. coli* reacts to OS mainly producing SOD and catalase, which convert $O_2^{\bullet-}$ to H_2O_2 (SOD) and in turn H_2O_2 in H_2O and O_2 (catalase). While mammalian cells possess two types of SOD, *E. coli* owns three isoforms of SOD characterized by different metal *cores*. Particularly, sodA contains Mn, sodB includes Fe, and sodC comprises both Cu and Zn. *E. coli* has also two types of catalases, namely hydro-peroxidase I and hydro-peroxidase II [47]. Also, *E. coli* has several major regulators activated during OS such as OxyR, SoxRS, OhrR,and RpoS. OxyR and SoxR control the catalases and SODs transcription in relation to the $O_2^{\bullet-}$ and H_2O_2 concentration. They undergo conformation changes when oxidized in the presence of hydrogen peroxide and superoxide radicals, respectively, and subsequently control the expression of cognate genes [48]. In contrast, the RpoS regulon is induced by an increase in RpoS levels. It is a specialized sigma factor which governs the expressions of gene that lead to general stress resistance of cells [49]. These genes may be involved in eliminating oxidative agents, in repairing systems of affected biomolecules and in maintaining normal cellular physiologic circumstances. Despite the enormous genomic diversity of bacteria, OS response regulators present in *E. coli* are functionally conserved in a wide range of bacterial groups. Bacteria have developed complex, adapted gene regulatory responses to OS, probably due to the high

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level of ROS produced endogenously through their basic metabolism. Additionally, several bacterial pathogens prevent the increase of ROS by directly inhibiting the synthesis of NADPH oxidase [50]. Iron homeostasis and remodeling of metabolism are two other methods by which bacteria lessen the damage caused by ROS. Bacteria can remodel their metabolism via upregulation of the glycoxylate shunt, thus reducing endogenous ROS formation, or by redirecting the metabolism toward the pentose phosphate pathway and augmenting the production of NADH which refills the level of antioxidants. Ketoacids such as pyruvate and α-ketoglutarate can decarboxylate in the presence of ROS, thus originating toxic molecules and diminishing damage caused by ROS [51]. Iron, which is involved also in ROS generation by Fenton reactions, is crucial for the growth and survival of bacteria and paradoxically iron acquisition by siderophore action is pivotal to counteract OS [52]. Siderophores are compounds that bacteria produce when intracellular iron concentration is low to facilitate its uptake [53]. Two siderophores namely Staphyloferrin A and B have found to enhance the resistance to OS in *S. aureus*, while *E. coli* produces an enterobactin siderophore to alleviate damage by OS [51,52]. It was demonstrated that OS in turn regulates the bacterial siderophore production [53]. When *E. coli* was exposed to H_2O_2 and paraquat, the expression of enterobactin increased also in presence of high concentration of iron, which reduced the sensitivity of the isolate to both H_2O_2 and paraquat [53]. Similarly, when methicillin resistant *S. aureus* (MRSA) was exposed to the antimicrobial surface coating AGXX®, siderophore biosynthesis genes were highly upregulated. Some bacterial species produce biofilm as a highly specialized and organized form of resistance in which bacteria cooperate and stay protected by a self-produced biomass [54]. Persisters are bacterial cells in a dormant state with low metabolic activity existing in biofilm that showcase high antibiotic tolerance, can recolonize post-therapy [55], are less sensitive to ROS and demonstrated increased expression of efflux pumps. Efflux pumps major expression is another mechanism to react to OS which allows bacteria to pump out the ROS-damaged proteins [56].

3. Antimicrobial Oxidative Therapies: Available Methods to Induce ROS Formation

As previously reported in the Introduction, the induction of high levels of reactive oxygen species (ROS) by several procedures thus causing OS detrimental for bacteria cells, has been extensively studied for inhibit several species of Gram-positive and Gram-negative bacteria, viruses, and fungi. The use of ROS represents a new therapeutic approach for topical use on skin, mucosal membranes, or internal tissue that may be colonized with microbial inhabitants and biofilms [57]. It was found that the antibacterial effect of several conventional and alternative antibiotics, metal nanoparticles and natural molecules is based also on their capability of inducing ROS hyperaccumulation in pathogens [22]. Therefore, other methods have been developed and are clinically applied or in clinical trials to produce ROS finalized to antimicrobial oxidative therapy. They include the photodynamic therapy (PDT), the honey reactive oxygen (HRO) therapy, and hyperbaric oxygen treatments (HBOT) [57].

3.1. ROS Formation Induced by Conventional, Alternative and Natural Antimicrobials

Table 5 reports information concerning conventional and alternative antimicrobials including some antibiotics, nanoparticles, and natural compounds, which exert their effects by generating ROS.

Table 5. Conventional and alternative antimicrobials including some antibiotics, nanoparticles, and natural compounds, which exert their effects by generating ROS.

Type	Name	Mechanism of Action	Clinical Application	Target	Ref.
Antibiotics	Nitrofurantoin	Autooxidation of nitroaromatic anion radicals * in presence of O_2 providing O_2^- and then	UTI	*E. coli*	[18]

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		ROS thus causing OS and toxicity to bacteria			
	Polymyxin B	Accumulation of OH•	Untreatable infections	*Acinetobacter baumannii* *Pseudomonas aeruginosa* *Enterobacteriaceae* **	[58]
	Nalidixic acid Norfloxacin	Mutagenesis by oxygen free radical generation	Gastroenteritis Enteric fever Bacteremia	*Salmonella typhimurium*	[59]
	Norfloxacin Ampicillin Kanamycin Fluoroquinolones *β*-Lactams Aminoglycosides	↑ Superoxide levels ↑ H_2O_2 ↑ Lethality by accumulation of OH•	UTI	*E. coli*	[60]
	Nalidixic acid Trimethoprim Ampicillin Aminoglyoside	Post-stress ROS-mediated toxicity			[61,62]
Alternative antimicrobials	OSECs	Ebselen ***	By inhibiting TrxR in bacteria laching glutathione thus triggering OS.	Skin infections Bacteremia Endocarditis Food poisoning Pneumonia TSS	*S. aureus*	[63]
				Tuberculosis	*M. tuberculosis*	[64]
	NPs	MSNP-maleamic	↑ ROS (40% *E. coli*, 50% *S. aureus*)	UTI	*E. coli*	[65]
		MSNPs-maleamic-Cu	↑ ROS (40% *E. coli*, 30% *S. aureus*)	Skin infections Bacteremia Endocarditis Food poisoning Pneumonia TSS	*S. aureus*	

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	Metal oxide NPs	↑ ROS ↑ RNS	Skin infections Bacteremia Endocarditis Food poisoning Pneumonia TSS UTI	*E. coli* *S. aureus* *S. epiderdimis* *Photobacterium hosphoreum*	[64]
NZs	AgPd0.38	By ROS produced on Ag and Pd bimetallic alloy	Severe infections	*S. aureus* *Bacillus subtilis* *E. coli* *P. aeruginosa*	[66]
AgRuSCs	AGXX®	By ROS catalytical production	UTI	*Enterococcus faecalis*	[54,67–69]
			Skin infections Bacteremia Endocarditis Food poisoning Pneumonia TSS	MRSA	
Natural Compounds	Allicin	OS by ↑ ROS via ↓ of low MW thiols	Skin infections Bacteremia Endocarditis Food poisoning Pneumonia TSS	*S. aureus*	[70]

UTI = urinary tract infections; * by a NADH-dependent reduction; ** carbapenemase-producing; ↑ Means increase; ↓ means decrease/reduction; TSS = toxic shock syndrome; NPs = nanoparticles; *** used also in combination with ROS-producing antimicrobials such as silver nanoparticles (AgNPs}; NZs = nanozimes; OSECs = organo-selenium compounds; AgRuSCs = silver and ruthenium-based surface coatings; MRSA = methicillin-resistant *S. aureus*; MW = molecular weight.3.1.1. ROS Formation Induced by Antibiotics.

Clinically approved antibiotics such as erythromycin, by protein synthesis inhibition, and rifampicin, by inhibiting RNA synthesis, are effective on *Rhodococcus equi*, while vancomycin is antibacterial against *R. equi, Mycobacterium tuberculosis* and *S. aureus*, by inhibiting cell wall synthesis inhibition. Moreover norfloxacin, by inhibiting DNA gyrase, is effective against *R. equi, S. aureus* and *E. coli*. Clofazimine, by DNA replication inhibition, ethambutol and isoniazid, by cell wall synthesis inhibition, are antibacterial against *M. tuberculosis.* Finally, quinones, by different cellular targets, are active on *Enterococcus* sp., *Streptococcus* sp., *Staphylococcus* sp. and *Moraxela catarrhalis* [64]. Anyway, studies observed that antibiotics functioning with a primary mode of action not correlated with OS, interfering with some bacterial cell targets, as above reported, were found to cause bacterial damage also generating ROS [71,72]. As shown in Figure 2, the interaction of antibiotics with bacteria cell targets can cause both ROS hyperproduction and cell damage which in turn cause additional ROS hyperproduction, while the improved concentration of ROS cause OS in bacteria lethally amplifying cellular damage.

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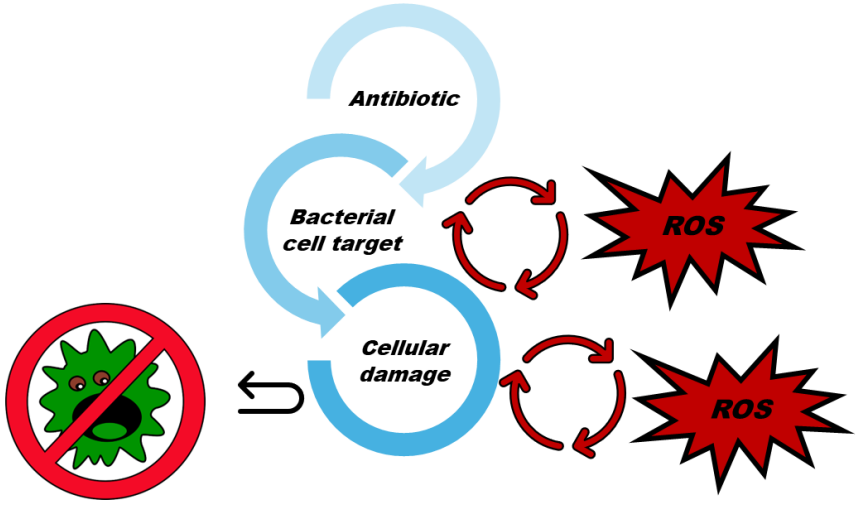

Figure 2. ROS induction by antibiotics.

Particularly, some antibiotics generate ROS through overstimulation of electrons via the tricarboxylic acid cycle and the release of iron from the iron–sulfur clusters, thus activating the Fenton chemistry. Nitrofurantoin and Polymyxin B are two commonly used ROS-mediated antibiotics [22]. Nitrofurantoin, used to treat urinary tract infections by *E. coli* [18], acts by a NADH-dependent reduction, producing nitroaromatic anion radicals. The autooxidation of these anion radicals in presence of O_2, produces O_2^-, by which ROS generate, thus causing OS and toxicity on bacteria [18]. Polymyxin B (PMB) makes part of the family of antimicrobial peptides and is active mainly on Gram-negative bacteria such as *A. baumannii, P. aeruginosa*, and carbapenemase-producing *Enterobacteriaceae* [18,73]. Due to its neurotoxic and nephrotoxic properties, PMB is advised to be used only as a last resort antibiotic [18]. Sampson et al. demonstrated that PMB, in addition to be a membrane disruptor [74], induced cell death in Gram-negative bacteria by the accumulation of OH•[58]. Anyway, Arriaga-Alba and co-workers were the first authors to report oxidative stress induction as a part of the mechanism of action of nalidixic acid and norfloxacin in *Salmonella typhimurium*. [59]. Antibiotics were shown to upregulate many oxidative stress genes in *P. aeruginosa*[22]. Wang and Zhao found out that norfloxacin was more lethal in *E. coli* deficient in catalase gene katG, than in its isogenic mutants, thus confirming a potential pathway linking hydroxyl radicals to the antibiotic lethality [60]. Also, ampicillin and kanamycin showed increased lethality to an alkyl hydroperoxide reductase ahpC *E. coli* mutant, lacking the defense system to contrast hydroxyl radicals. These studies evidenced increased superoxide levels in the bacterium, which were the source of H_2O_2 which in turn generated the highly toxic hydroxyl radical, responsible for the improved lethality of antibiotics[60]. Hong et al demonstrated that *E. coli* exposed to lethal oxidative stressors caused by antibiotics including nalidixic acid, trimethoprim, ampicillin, and aminoglycosides, did not die only during the actual treatment but also post-treatment, after the removal of the initial stressor, due to post-stress ROS-mediated toxicity[61,62]. Anyway, the connection between antibiotics action and ROS are not still clearly demonstrated. Since it was demonstrated that antibiotics work also under anoxic conditions, some reports oppose that the generation of ROS contributes to their lethality, which is instead influenced by the bacterial metabolism, iron homeostasis and iron-sulfur proteins [75–77]. Although contradictory studies exist concerning the possibly ROS influence on antibiotic lethality, it was established that numerous alternative antimicrobials work by inducing ROS-mediated OS in bacteria. Unfortunately, some ROS-producing antibiotics, such as aminoglycosides, fluoroquinolones, and β-lactam antibiotics may induce host cellular damage in specific tissues such as the renal cortex or tendons by generating OS, anyway manageable by specific antioxidant molecules [64].

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3.1.2. ROS Formation Induced by Alternative Antimicrobials

Several novel alternative antimicrobials are under development whose primary mode of action seems to be through the generation of ROS causing OS in bacteria. Generally, these antimicrobials often target the redox defenses, such as the thiol-dependent enzyme thioredoxin reductase (TrxR) in bacteria[63]. Examples of ROS-mediated antimicrobials include Ebselen, nanoparticles, nanozymes, and AGXX®.

Ebselen

Ebselen is an organo-selenium-based antioxidant drug endowed with anti-inflammatory, antioxidant, and cryoprotective effects. It acts by inhibiting TrxR in bacteria lacking glutathione, thus triggering OS, thus being lethal to these pathogens. Ebselen was recently shown to efficiently inhibit *in vitro* the growth of MDR *S. aureus,* to improve wound healing in rats, and to reduce the bacterial load in *S. aureus* skin lesions in rats [63]. Ebselen has been reported to inhibit *M. tuberculosis*. Important, Ebselen could also be combined with other ROS-stimulating compounds that block the antioxidant defenses of bacteria such as silver nanoparticles (NPs) [64].

Nanoparticles (NPs)

Mainly due to their small size (<100 nm) nanoparticles (NPs) can cause hyperproduction of ROS which can cause carbonylation of proteins, peroxidation of lipids, DNA/RNA breakage, and membrane structure destruction, thus damaging cells [78]. Among NPs, those made of silver, such as silver oxide NPs (AgNPs), titanium dioxide, silicon, copper oxide, zinc oxide, gold, calcium oxide, and magnesium oxide NPs have been ported to have antibacterial effects against both Gram-positive and Gram-negative pathogens [57]. Mesoporous silica NPs (MSNPs) containing a maleamato ligand (MSNPs-maleamic) and others containing also copper (III) coordinator ions (MSNPs-maleamic-Cu) synthesized by Diaz-Garcia et al, demonstrated antibacterial activity towards *E. coli* and *S. aureus* by OS induction [65]. The minimum inhibitory concentration values (MICs) of MSNPs-maleamic and MSNPs-maleamic-Cu established that both preparations performed better against *E. coli* than on *S. aureus* and that MSNPs-maleamic (MIC = 62.5 μg/mL) was more effective than MSNPs-maleamic-Cu (MIC = 125 μg/mL) against *E. coli* [65]. Both preparations caused a significant increase of ROS in both species (30-50% more than in control) and NPs that caused the major increase displayed lower MICs (MSNPs-maleamic, 50% in *S. aureus*, and 40% in *E. coli*), thus confirming that ROS and OS generation contribute to the antibacterial mechanism of action of MSN-maleamic and MSN-maleamic-Cu [65].

Nanozymes (NZs)

The major drawback of ROS-based antibacterial therapy consists in the low selectivity of ROS, that are detrimental also for human cells. Recently, nanomaterials possessing enzyme-like characteristics and referred to as nanozymes (NZs), have been reported to produce surface-bound ROS which were selective in killing bacterial cells over mammalian ones [66]. Particularly, ROS bound on silver and palladium bimetallic alloy (AgPd0.38) efficiently killed antibiotic resistant bacteria including *S. aureus, Bacillus subtilis, E. coli* and *P. aeruginosa* (MBC = 4-16 μg/mL) without developing drug resistance and inhibited biofilm formation [66].

AGXX®

AGXX® is a silver and ruthenium-based antimicrobial surface coating, that can be coated or deposited on various carriers such as cellulose, plastics, ceramics, or metals. AGXX® demonstrated low levels of toxicity to mammalian cells [79], and no tendency to develop resistance, due to its multiple mode of action [67]. ROS are produced catalytically by AGXX® which demonstrated to inhibit the growth of *Enterococcus faecalis*, and MRSA, as well as to prevent biofilm formation in MRSA [54,68]. In both species, AGXX® affected oxidative stress defenses such as superoxide dismutase (SodA), catalase (KatA), alkyl hydroperoxide reductase (AhpCF), thioredoxin/thioredoxin reductase (Trx/TrxR), disulfide reductase (MerA), and oxidized bacillithiol (BSH) [69]. AGXX® induced OS,

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general stress, heat shock (expression of Clp proteases), and copper stress [54,68]. In MRSA, it affected the iron homeostasis and upregulated several siderophore biosynthesis (sbn) genes [54,69], while in *S. aureus*, increased protein-thiol-oxidations, protein aggregations, and a BSH redox state were observed[67,69]. The mechanism of action of AGXX® was assessed in *S. aureus* observing two inter-connected redox cycles by which AGXX® simultaneously exerts ROS-mediated (superoxide anion, hydrogen peroxide, and highly toxic HO•) antimicrobial effect and achieves self-renewal.

3.1.3. ROS Formation Induced by Natural Compounds

In addition to synthetic compounds, also natural compounds can exert ROS-mediated antimicrobial effects. It is the case of allicin and honey, being the latter the main ingredient of a clinically approved gel formulation for topical administration. Honey is used in the honey antimicrobial therapy, that has been here discussed in a dedicated section. There are many other secondary metabolites produced by plants that may elicit oxidative stress in bacteria, such as catechins, ferulic acid, and their derivatives [64]. The combination of other ROS- and RNS-generating antimicrobials with these compounds may lead to the development of promising therapeutic strategies against different intracellular bacterial pathogens [64]. Particularly, allicin is a constituent of garlic, which works as a thiol-reactive compound; decreasing the levels of low molecular weight thiols, which should function as a defense against ROS. Their increasing causes OS to bacterial cells, thus inhibiting their growth [70].

3.2. ROS Formation Induced by Antibacterial Photodynamic Therapy

By a study on the toxicity of small concentrations of acridine red on *Paramecium* spp. it was recognized that the observed toxicity was dependent on the time of the day and the amount of daylight [80]. Later, von Tappeiner with Albert Jesionek applied clinically this approach, to treat skin carcinomas and coined the term "photodynamic phenomenon" [81–83] .Thus, anticancer photodynamic therapy was born. In those years, the successful photodynamic inactivation of bacteria was also described [80]. Anyway, while anti-cancer PDT is clinically applied from 25 years, at least in the treatment of actinic keratosis or basal cell carcinoma, its application as antimicrobial option has only more recently been rediscovered, to manage the emergence of the first drug-resistant infections in the healthcare sector during the early 1990s [84,85].

3.2.1. Basic Principles of Photodynamic Therapy

PDT is based on the combination of three elements including a not toxic compound referred to as photosensitizer (PS), light in a spectral range appropriate for exciting the PS (typically from the visible to near infrared (NIR) spectrum) and molecular oxygen [85]. The mechanism of PDT is described by the Jablonski diagram reproduced in Figure 3.

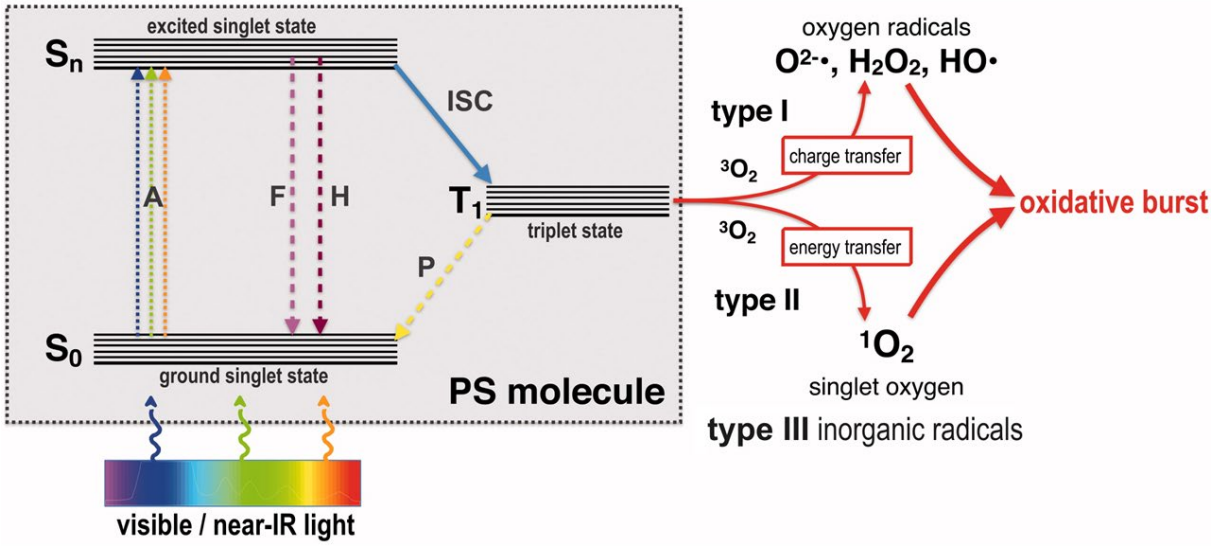

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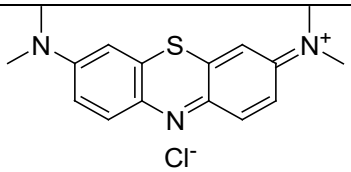
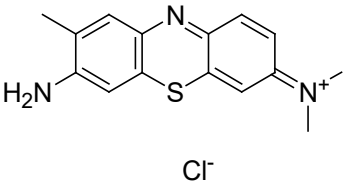
Figure 3. Jablonski diagram showing the photochemical and photophysical mechanisms of antimicrobial photodynamic therapy (PDT). S_0: ground singlet state of the PS molecule; Sn: excited singlet state of the PS molecule; T_1: triplet excited state of the PS molecule; A: absorption of light; F: fluorescence emission; H: heat generation (internal conversion); ISC: inter-system crossing; P: phosphorescence emission; $^3O_2$: ground state oxygen; $^1O_2$: singlet oxygen; $O^{2-}\bullet$: superoxide anion; HO•: hydroxyl radical; H_2O_2: hydrogen peroxide. The image is an adaptation from an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/Licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium[80].

Briefly, upon absorption of a photon (A), the PS moves from its ground singlet state (S_0) to an excited singlet state (Sn). In this status, PS can lose energy, thus returning to S_0 by emitting fluorescence (F) or heat (H) via internal conversion. On the contrary, it can pass to a longer-living excited triplet state PS (T_1), through an inter-system crossing (ISC) process. Following, it can either return to S_0 state by phosphorescence emission (P) or generating reactive oxygen species (ROS), by two mechanisms [86]. When a type I mechanism is followed, electrons are transferred to surrounding substrates thus forming of superoxide radical anions ($O^{2-}\bullet$), that undergo dismutation into hydrogen peroxide (H_2O_2) from which the highly reactive hydroxyl radical (HO•) derives via Fenton-like reactions. Differently, in type II mechanism, energy and not charge is transferred directly to the ground state molecular oxygen ($^3O_2$), leading to emergence of singlet oxygen ($^1O_2$), which is nothing else than energized molecular oxygen [87]. At this point, PS is returned in its S_0 status, ready to begin another cycle with production of additional ROS. Interesting, one PS molecule can generate thousands of molecules of $^1O_2$ before being destroyed. The singlet oxygen quantum yield describes the amount of type II mechanism [87]. During antibacterial PDT, type I and type II reactions can occur simultaneously, and the ratio between these processes depends on the type of PS utilized, its chemical structure and the specific microenvironment in which the PDT is implemented. The interplay between type I and type II mechanisms is a critical factor to consider for an optimized treatment and understanding its underlying photochemical processes [88–90]. As already reported, ROS are detrimental for bacteria by targeting several vital microbial molecules, such as proteins, lipids, and nucleic acids, thus determining bacterial death. An additional type III reaction has been recently incorporated into the familiar categories of type I and II mechanisms. In this novel process, free radicals of inorganic compounds are generated, regardless of the presence of oxygen, which would participate in the photoinactivation of microorganisms [91].

3.2.2. Photosensitizers Used in Clinical Trials

The main photosensitizers used in clinical trials are listed in Table 6 [92,93].

Table 6. Most common photosensitizers used in clinical trials.

Class	Compound	Description	Target	Abs $_{max}$ (nm)	Impact in the field	Refs.
Phenotiazinium derivatives	 Methylene Blue	3-ring p-system Auxochromic side groups Single positive charge SOQY < 0.5 Type I reactions	Dental plaque	632	1st clinically approved PS (dentistry)	[94]
			*Streptococcus mutans*	410	Standard PS *in vitro*	[95]

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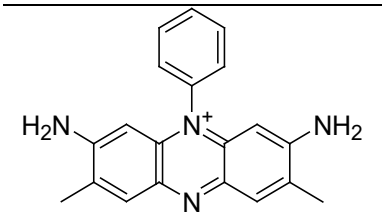
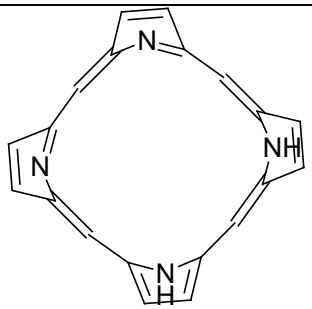
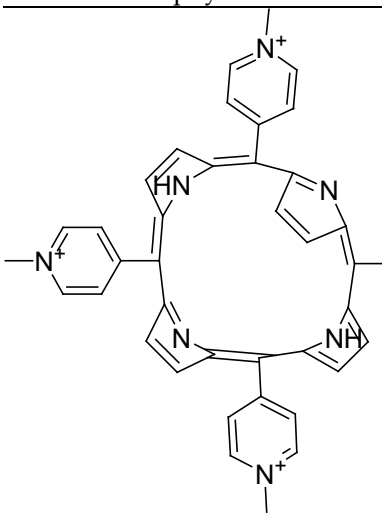
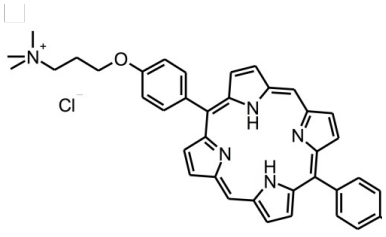
	Toluidine Blue		*E. coli*			[96]
	 Safranine O		*F. nucleatum* *P. gingivalis*	520		[97]
	 Porphyrin		*S. aureus* *P. aeruginosa* *E. faecalis*	446	Widely used as standard PS *in vitro*	[98–100]
Porphyrin derivatives	 TMPyP *	Four pyrrole cycles Up to eight positive charges SOQY = 0.5-0.8 Occurring in nature Type II reactions	MRSA ESBL *K. pneumoniae*	421	N.R.	[101]
	 XF-73 *		Staphylococci Enterococci Streptococci *S. aureus* biofilm	380-480		[102]
Chlorin derivativ		Like heterocyclic-macrocyclic compounds	*S. aureus* *E. coli*	660 (neutral)		[103]

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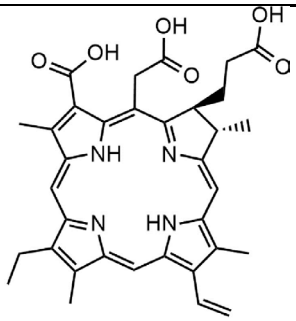
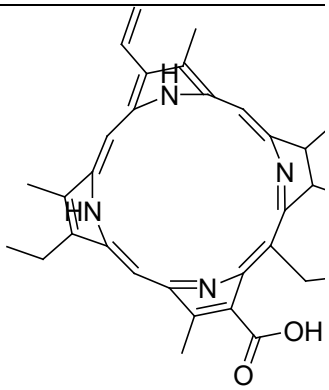
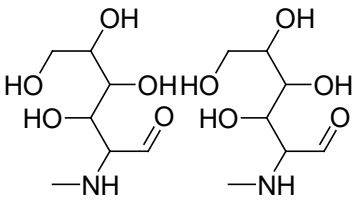
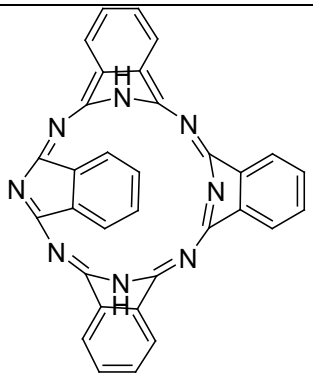
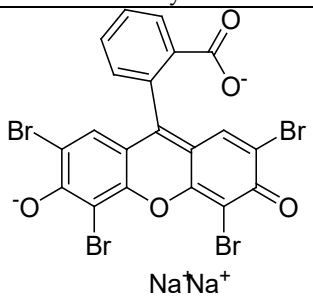
	 Chlorin e6	Neutral or up to eight positive charges SOQY = 0.5-0.8 3 pyrrole and 1 pyrroline subunits Type II reactions	*E. coli*	532 (cationic)		[104]
	  Photodithazine®		MRSA MSSA	660		[105]
Phthalocyanin derivatives	 Phthalocyanine	4 pyrrole cycles Hydrophobic and uncharged Type II reactions	*A. hidrophila*	670		[106]
Xanthene derivatives	 Eosin Y	Anionic xanthene dyes Fluorescein derivatives SOQY = 0.5-0.6 Type II reactions	MRSA MRSA biofilm *S. aureus*	N.R.	Sparse studies in recent years	[107]

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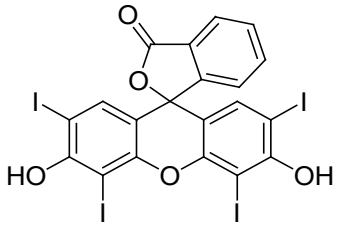
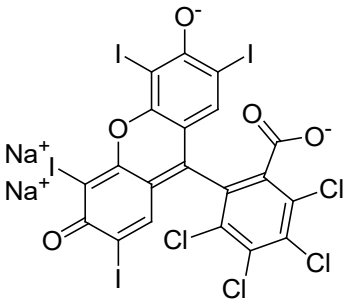
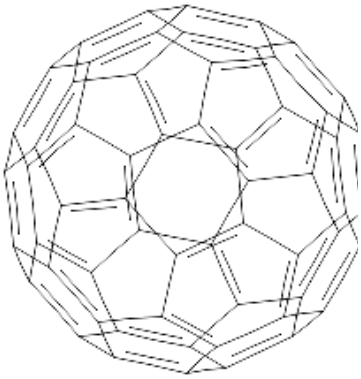
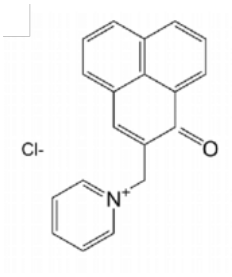
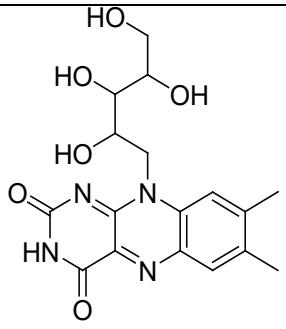
	 Erythrosine		*S. mutans* *Lactobacillus casei* *Candida albicans*	470		[108]
	 Rose Bengal		*E. faecalis* *P. aeruginosa*	532		[109]
Nanomaterials	 Fullerene C60 ***	Soccer-ball shaped cage molecules Made exclusively from carbon atoms Neutral Extended p-conjugated system Type I and II reactions	*S. aureus* *E. coli*	532	Unique class of PS Sparse studies on effect on biofilms	[110] [111]
Phenalenones	 SAPYR	Biosynthesized by plants to defend against pathogens using sun to generate singlet oxygen SOQY > 0.9 Positively charged pyridinium-methyl moiety Type II reactions	*E. faecalis* *Actinomyces naeslundii*	360-420	First water-soluble exclusive type-II PS	[112]
Riboflavin derivatives	 Vitamin B2	In PDT cationic derivatives with up to eight positive charges SOQY = 0.7-0.8 Type II reactions	MRSA EHEC	450 (cationic)	N.R.°	[113]

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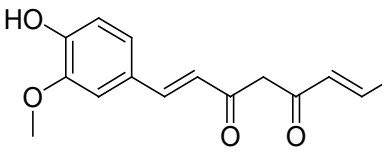
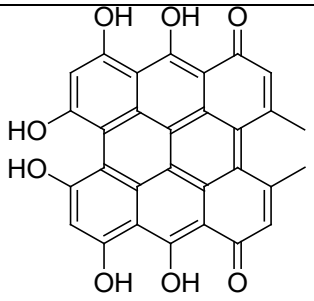
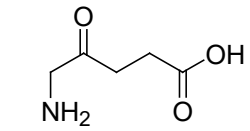
Curcumins	 Curcumin (neutral)	Naturally occurring yellow dye from the rootstocks of *Curcuma longa* Approved as food additive (E100) For use in PDT positive charges have been included in derivatives structures Type I reactions	*S. mutans* *L. acidophilus*	547	Novel positively charged derivatives with enhanced water solubility	[114] [115]
Natural compounds	 Hypericin (neutral)	Naturally occurring For use in PDT positive charges have been included in derivatives structures Type II reactions	*S. aureus* *E. coli*	593	N.R.	[116] [117]
5-ALA	 5-amminolevulinic acid	δ-amino acid in which the hydrogens at the γ position are replaced by an oxo group Metabolized to protoporphyrin IX	MRSA	410	Optical imaging agent	[118]

SOQY = Singlet oxygen quantum yield * derivatives of porphyrin; ** derivative of chlorine e6; S = Abs max, Soret band; Q = Q band; *** functionalized in multiple ways by adding positively charged moieties; ESBL = extended spectra β-lactamases; MRSA = methicillin resistant *S. aureus*; MSSA = methicillin sensitive *S. aureus;* MRSE = methicillin resistant *S. epidermidis;* EHEC = Enterohemorrhagic *E. coli*; 5-ALA = 5-aminolevulinic acid.

The most common PSs clinically used in anticancer PDT have been extensively studied also for their antibacterial properties and effectiveness in the treatment of various bacterial infections. Many published studies have determined that phenothiazinium PSs such as MB and TB are effective on planktonic bacteria. Furthermore, some studies also tested the efficacy of phenothiazinium against biofilm structures [92]. Recently, new derivatives such as dimethyl methylene blue (derived from MB) and EtNBS (N-ethylpropylsulfonamido) have been studied. These dyes possess a high cationic charge that makes them more effective against bacterial cells [92]. Rose Bengal is an anionic synthetic xanthene dye that has shown promise in several clinical trials for PDT, particularly in the treatment of localized bacterial infections. Other synthetic anionic xanthene dyes derived from Fluorescein are Eosin Y and Erythrosine (ERY). All these dyes have an absorption peak in the green wavelength range (480–550 nm). The attachment and uptake of anionic PSs by the bacterial cells are lower than cationic ones [92]. Amino-levulinic acid (ALA) is a prodrug that can be converted to protoporphyrin IX (PpIX), clinically used (in the form of the hydrochloride salt) in combination with blue light illumination for the treatment of minimally to moderately thick actinic keratosis of the face or scalp. It has demonstrated to be effective also in the treatment of bacterial infections, including periodontal infections. Additionally, a variety of evidence proved that 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT) is clinically effective in the management of *Acne vulgaris* and is recommended as an alternative treatment modality for severe acne [119]. Concerning chlorins, mainly cationic derivatives of chlorin-e6 are used for PDT. Photodithazine® is a commercially available chlorin-e6 derivative with two positive charges. Chlorine e6 (Ce6) and various phthalocyanines have strong antibacterial properties and have been used in preclinical studies and

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in some early clinical trials for antibacterial PDT. Curcumin is a natural compound found in *Curcuma longa*, and its cationic derivatives have also been investigated as possible PSs. Collectively, the use of specific PSs in clinical trials strongly vary depending on the target bacterial infection and the research objectives of each study [92,120,121].

3.2.3. Antibacterial PDT vs. Antibiotics

Unfortunately, as reported in the following Table 7, even if significant are the advantages of using antibacterial PDT (APDT) compared to antibiotics, APDT has certain limitations that need careful consideration.

Table 8. Advantages and limitations of APDT compared to conventional antibiotics.

Advantages	Limitations	Discussion on limitations
↑ BS action than antibiotics including bacteria, protozoa, fungi	Light limited penetration capabilities	Possible problems in reaching deep seated infections by scattering phenomena [122] Bacterial colonies located beneath the skin's surface or within organs could be difficult to reach * [123]
↓ Adverse effects and damage to the host tissue	Potential lack of target specificity	↑ Selectivity and efficiency of PSs toward bacteria and ↓ toxicity on mammalian cells can be achieved using proper vectors or by co-administration, conjugation or incorporation with polycationic materials, bacterial-targeting peptides, polymers, antibiotics, or antibodies [124–127]
Bactericidal effects independent of antibiotic resistance pattern	Risk of antibiotic inactivation	When in combination with certain antibiotics APDT can inactivate the antibiotics [123]
No resistance following multiple sessions of therapy	Potential side effects	Emergence of skin sensitivity, redness, and pain at the treatment site [128]

* In this case the correct choice of the wavelength and PS is pivotal; PS = Photosensitizer; BS = broad spectrum; ↑ means increase, more; ↓ means decrease/reduction, less, minor.

Despite promising results have been observed in some diseases, the clinical translation of PDT for bacterial infections has progressed slower than for cancer treatment and leisurelier than anticipated. Therefore, its widespread adoption in medical practice remains limited. Among limitations, the modest capability of light to penetrate skin, tissues and organs hampers the application of APDT for systemic infection and reduce its effectiveness in the topical treatments. The penetration of light depends on optical properties of the tissue and the wavelength of used light. There exists a heterogeneity between tissues and even within a tissue. These inhomogeneity sites (e.g. nuclei, membranes, etc.) cause light scattering, reflecting, transmitting or absorption [122]. Light within the 620–850 nm spectrum range achieves the optimum tissue penetration and PDT applications [122]. Mainly concerning potential side effects, understanding, and effectively managing them is critical for optimizing patient outcomes. To fully harness the potential of antibacterial PDT as a valuable therapeutic strategy in combating bacterial infections, it is imperative to address these drawbacks through ongoing research efforts and comprehensive approaches [120,129]. Successful outcome of antibacterial PDT depends on the selected PS. To enhance PS selectivity over host tissue, while maintaining nonselective activity against microbial species represents a pivotal challenge. The optimal PS should target both Gram-positive and Gram-negative species, accounting for their

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differential treatment responses. Several very recent studies have described the latest advancements in the field over the past years, emphasizing new PS systems relevant for antibacterial PDT's methodologies [80,130–132], which have recently reviewed by Sébastien Clément & Jean-Yves Winum [123].

3.2.4. Light Sources

The main light sources employed to activate PSs have been included in Table 7.

Table 7. Main light sources employed to activate PSs.

				Refs.
Light source	LASER	Argon	Monochromatic, coherent, and collimated light High irradiance Couplable into optical fiber bundles Expansive, cumbersome	[133]
		Diode		
		Neodymium doped Yttrium Aluminum Garnet lasers		
	Light-emitting diodes (LEDs)		Deliver a slightly wider emission spectrum than LASER Low costs No monochromatic, no coherent	[133]
	Gas-discharge lamps	Quartz-tungsten-halogen lamps Xenon-discharge lamps Sodium lamps	They can be spectrally filtered to match any PS No efficiently couplable into optical fiber bundles Cause more heating as compared to LASERs and LEDs	[134]
	Daylight		Broad-spectral range from UV to IR region Free of cost Can illuminate a very large area with high uniformity Variable in irradiance, radiant exposure is poorly controlled	[133,135]

LASER = Light Amplification by Stimulated Emission of Radiation.

In addition to the light sources reported in Table 7, "non-coherent" or "non-thermal" light sources without giving details of the actual light source used have been reported. Additionally, there are also occasional reports of other light sources such as endoscopy systems, photopolymerisers and supra-luminous diodes (SLD) [133]. All in all, for the irradiation of a given PS, parameters such as radiant exposure, light irradiance, power output, spectral emission, intensity of the respective light source, as well as the mode of light-delivery (via optical fiber or directly) are more important than the type of the light source itself.

3.2.5. Clinical Trials

Due to the multi-faceted nature of the antimicrobial photodynamic process and its diverse targets, the emergence of resistance in microbes becomes highly improbable. Consequently, the anti-bacterial PDT should be considered promising as a potent and innovative approach in combating bacterial infections [136]. In the last years, the volume of research focused on PDT as therapeutic device to inhibit a wide range of microorganisms, including bacteria is remarkably increased. Anyway, the antibacterial PDT has yet to attain the capability to tackle systemic infections. On the contrary, it has demonstrated high potential for addressing localized infections sustained by MDR bacteria including hospital-acquired pathogens like *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa*, and *Enterobacter* spp., which together constitute the group ESKAPE [137]. Additionally, in vitro and in vivo studies have demonstrated the capability of PDT of eradicating or significantly reducing biofilms, thus finding applications in dental diseases, skin infections and orthopedic implants treatment [138,139]. The ongoing advancement of antibacterial PDT systems is also marked

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by the continuous refinement of the strategies, prominently through synergistic combinations of diverse chemicals. Antibacterial PDT has been applied in about 40 clinical trials for treatment of dermatological disorders and oral infections. Clinical trials on antibacterial PDT have focused on evaluating its safety and efficacy in treating specific bacterial infections, especially those that are challenging to manage with traditional antibiotics [85]. Some of the key findings from these trials include those obtained in dermatological disorders such as acne vulgaris, and bacterial skin conditions where antibacterial PDT studies have shown positive outcomes in reducing the severity of acne lesions [140–143]. Some studies have also explored applications in treating infected wounds, especially those associated with MDR bacteria. The results suggest that antibacterial PDT can be beneficial in promoting wound healing and controlling bacterial growth [144,145]. In the field of periodontal diseases, clinical trials have shown promising results for treating chronic periodontitis, a common bacterial infection that affects the gums and supporting structures of the teeth [146–149]. Other clinical trials studies have also examined the use of antibacterial PDT in managing bacterial infections related to medical implants, such as catheters and prosthetic devices [150,151].

3.3. ROS Formation Induced by Antibacterial Honey Therapy

Honey was used in traditional medicine mainly to treat wounds due to its antimicrobial effects and healing properties. The bactericidal efficacy of honey was reported more than a century ago by Van Ketel [152], whose findings prompted extensive research on honey over the next decades. With the advent of modern medicine, the interest in honey and its medical use decreased [152]. Nowadays, honey is undergoing a revival in its consideration for antimicrobial and wound healing applications, due to the rising global antibiotic resistance, which makes the development of novel alternative therapies to combat infections necessary. Several factors can contribute to its effective antimicrobial activity, which can strongly vary depending on different microbial strains, including the geographical and botanical source, its harvesting, processing, and storage conditions. It has been demonstrated that a range of both Gram-positive and Gram-negative bacteria, including MDR strains and biofilms, fungi and viruses can be inhibited by honey. Furthermore, susceptibility to antibiotics can be restored when used synergistically with honey. Table 8 reports the antibacterial effects of honeys from different geographical sources and the related target bacteria.

Table 8. Antimicrobial effect of honey from different geographical locations and target pathogens.

Country of Origin	Honey Sample	Organisms	Ref.
Australia			
New Zealand	Manuka	*S. aureus, P. aeruginosa*	[153]
New Zealand	Manuka	*S. aureus*, MRSA, MSSA Coagulase-negative *S. epidermidis* *K. pneumonia*, ESBL *E. coli*	[154]
Australia	*Leptospermum* based honey	*S. aureus*	[155]
North America			
Canada	Canadian honey	*E. coli, Bacillus subtilis*	[156]
Cuba	Christmas vine, Morning glory Black mangrove Linen vine, Singing bean	*S. aureus, P. aeruginosa* *E. coli*, *B. subtilis*	[157]

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South America			
Chile	Ulmo honey	MRSA, *E. coli, P. aeruginosa*	[158]
Argentina	Algarrobo, citrus and multifloral honey	*S. aureus, E. faecalis, E. coli Morganella morganii P. aeruginosa*	[159]
Europe			
Scotland	Blossom, heather, Highland, Portobello Orchard	*Acinetobactor calcoaceticus S. aureus, P. aeruginosa, E. coli*	[160]
Northwest Spain	Rubus honey	*S. aureus, S. epidermidis Micrococcus luteus, E. faecalis B. cereus, Proteus mirabilis, E. coli P. aeruginosa Salmonella typhimurium*	[161]
Denmark	Heather, raspberry, rapeseed, hawthorn White clover	*S. aureus, P. aeruginosa, E. coli*	[162]
Slovakia	Honeydew honey	*P. aeruginosa, S. aureus*	[163]
Asia			
China	Buckwheat honey	*S. aureus, P. aeruginosa*	[153]
Saudi Arabia	Sider honey	*S. aureus, Streptococcus pyogenes Corynebacteria pseudotuberculosis K. pneumonia, P. aeruginosa E. coli*	[164]
Africa			
Algeria	Astragalus, wall-rocket, eucalyptus Legume, peach, juniper, buckthorn multifloral	*Clostridium perfringens, S. aureus, E. coli, B. subtili.*	[165]
Nigeria	Wildflower and bitter leaf honey	*S. typhimurium Shigella dysenteries, E. coli B. cereus, S. aureus*	[166]
Egypt	Cotton, blackseed, orange, eucalyptus Sider, clover honey	*E. coli, S. aureus Streptococcus mutans, P. mirabilis P. aeruginosa K. pneumoniae*	[167]
Egypt	Acacia, citrus, clover, coriander, cotto palm honey	*S. aureus, S. pyogenes C. pseudotuberculosis K. pneumonia, P. aeruginosa, E. coli*	[164]

ESBL =extended spectrum β-lactamase.

3.3.1. Medical Application of Honey

Although the knowledge of the antibacterial compounds involved in the antibacterial effects of honey remains still incomplete, the information on honey has remarkably expanded in the recent years. Otherwise, despite the variability of the antibacterial activity of honey limits its extensive applicability in medicine, several honeys have been approved for clinical application [168]. Currently, honey is mainly used as a topical antibacterial agent in applications on wounds. Its high viscosity

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grants an effective hydrated and protective barrier between the wound site and external environment. Honey has been used to treat wounds such as burns, trauma, and chronic wounds, where the complex wound healing process could be interrupted by infection or specific disease states (e.g., diabetes), thus limiting the development of irreversible chronic wounds, recurrent infections, amputation/limb salvage, and life-threatening conditions [152]. For mild to moderate superficial and partial thickness burns, honey was more effective than conventional treatment for reduction of microbial colonization and improved wound healing [169,170]. In a study, the application of honey on tunneled cuffed hemodialysis catheters, resulted in a comparable bacteremia-free period compared with that obtained with mupirocin treatment [171]. Honey has also been widely explored as a tissue-regenerative agent. In this case, it has been applied directly on wound or in combination with traditional wound dressings, which allow honey remaining in direct contact with the wound bed, thus providing a persistent and long-term release of antimicrobial agents to contribute to all stages of wound healing. Furthermore, the presence of reactive oxygen species (ROS) such as H_2O_2 has been shown to promote wound healing by promoting cellular repair processes and tissue regeneration [10,172]. Anyway, some limitations exist, such as being absorbed by the dressing, poor penetration into the wound site, and short-term antimicrobial action. To address these issues, tissue engineering approaches have been developed, such as its formulation in electrospun fibres and hydrogels [173–177]. Collectively, the use of honey, honey-derived, and honey-inspired products in tissue engineering applications combined with other biomaterials, may enable its use in a variety of other clinical situations outside wound care, where the combination of antimicrobial properties and tissue regeneration is desirable.

The Case of Surgihoney Reactive Oxygen (SHRO)

The first therapeutic agent based on the oxidative activity of ROS was a pharmaceutical honey gel for treating wounds, referred to as Surgihoney Reactive Oxygen (SHRO). Particularly, SHRO is an engineered sterilized honey created to act as a preventive antimicrobial agent for soft tissue infections. SHRO deriving from natural organic honey from different origins, is capable to provide a constant level of ROS over a prolonged period of time, when topically applied to a wound. Subsequently, ROS induce OS due to •OH production and inhibit the essential metabolic procedure for bacterial growth [178]. As more in detail discussed in the subsequent sections the antimicrobial activities of SHRO are mainly due to the generation of H_2O_2[179]. Other formulated honey prototypes (PT1 and PT2) were designed to furthers increase the generation of H_2O_2. Subsequently, honey ROS-based antibiotic agents differently formulated, such as sprays, nebulizers and infusions, that employ this mechanism are being developed and may be particularly useful for delivery ROS to other clinical sites. Nowadays, there are many types of therapeutic honey in the market (e.g., Paterson's curse, Rosemary, Manuka, Thyme, Revamil, Rewa Rewa, heather honey, Khadi, Kraft honey, Multifloral, and Medihoney) [180,181]. Table 9 summarizes a clinical register using SHRO in various complex and severe infections [4].

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Table 9. Summary of a clinical register of the use of SHRO in various complex infections.

Clinical site	Dosing regimen	Clinical details	Outcome	Adverse reports
Respiratory tract	Daily nebulized SHRO in respiratory nebulizer	Bronchiectasis, Several patients One patient—recurrent exacerbations with secondary infection with *Mycobacterium avium*	Reduction in bacterial load and temporary eradication of *M. avium* (1 patient)	None
Scalp	Daily topical application for 6 weeks	Fungal kerion, *T. tonsurans* Patient intolerant to oral antifungals	Complete resolution	None
Intraperitoneal	50–100 g daily via abdominal drain	Severe four-quadrant peritonitis following intraabdominal infection and corrective surgery Patients also on systemic antibiotics Often polymicrobial infections with MDR strains and *Candida* spp.	Variable, but general peritonitis Control	N.R. to SHRO use
Abdominal wall Deep soft tissue	SHRO into open cavity with each dressing	Used both prophylactically and therapeutically in around 20 patients	Prevention of infection and effective therapy in infected cavities	None
Prosthetic joints	Single dose around prosthetic joint at surgery Numerous patients	Mixed microbiology including *S. ludenensis* Given in conjunction with systemic antibiotics	Good adjunct to existing management	None
Prepatellar bursitis	Single application at debridement	On immunosuppression for psoriasis *M. malmoense* isolated from prepatellar pus Put on clarithromycin, rifampicin and ethambutol + SHRO topically	Complete healing and no further isolation of *M. malmoense*	None
Bladder	Twice-weekly instillation via suprapubic catheter	Several patients with long-term urethral or suprapubic catheters	Reduction in urosepsis	None
External auditory canal	Daily with wick or cotton wool	*Pseudomonas* otitis externa	Resolved	None
Oral infections	Daily oral application of 10 g SHRO	Recurrent aphthous ulcer, gingivitis, geographic tongue. No microbiology	Reported reduction in duration of symptoms	None
Helicobacter gastritis	Once daily 10 g SHRO for 10 days	Confirmed Helicobacter pylori gastritis MDR strain and no response to antibiotic eradication regimens	Continuation of symptoms Therapeutic failure	None

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N.R. = Not related.

With the rising age of the population in many countries, and the global epidemic of obesity and type 2 diabetes [4], the disease of chronic soft tissue lesions is becoming enormous. Most chronic breaks in the skin often become colonized with bacteria [182], that regardless their pathogenicity play an essential role in slowing tissue healing, establishing biofilm and resulting in wound slough and an unpleasant odor [182]. As reported in Table 9, in these cases where often available antibiotics are no longer functioning, the effects of SHRO on bioburden and biofilm [178] can be of great help. In fact, the early use of ROS in such lesions can control bioburden and biofilm, thus sparing conventional antibiotic use, and supporting infection control [178,183,184]. In clinical studies, ROS therapy via SHRO has demonstrated satisfactory safety and tolerability, and clinical- and cost effective in practice [183,185]. Most outstandingly, SHRO has demonstrated impressive capacity to clean up bacterial bioburden and biofilm in chronic wounds, being also active on MDR bacteria such as *P. aeruginosa*, MRSA and vancomycin-resistant enterococci (VRE) present in an ischemic ulcer [4].

Medical Grade Honey for Clinical Applications

Medical-grade honey (MGH) intended for clinical application must be sterilized by gamma irradiation to destroy potentially present bacterial spores, including those of *Bacillus* spp. and *Clostridium botulinum*, which could cause wound botulism or gangrene [186]. Several types of honey, including MGH, have recently been re-introduced into modern medicine. There is no clear definition of MGH, but according to a study by Hermann et al., MGH should satisfy the criteria reported in Figure 4 [187].

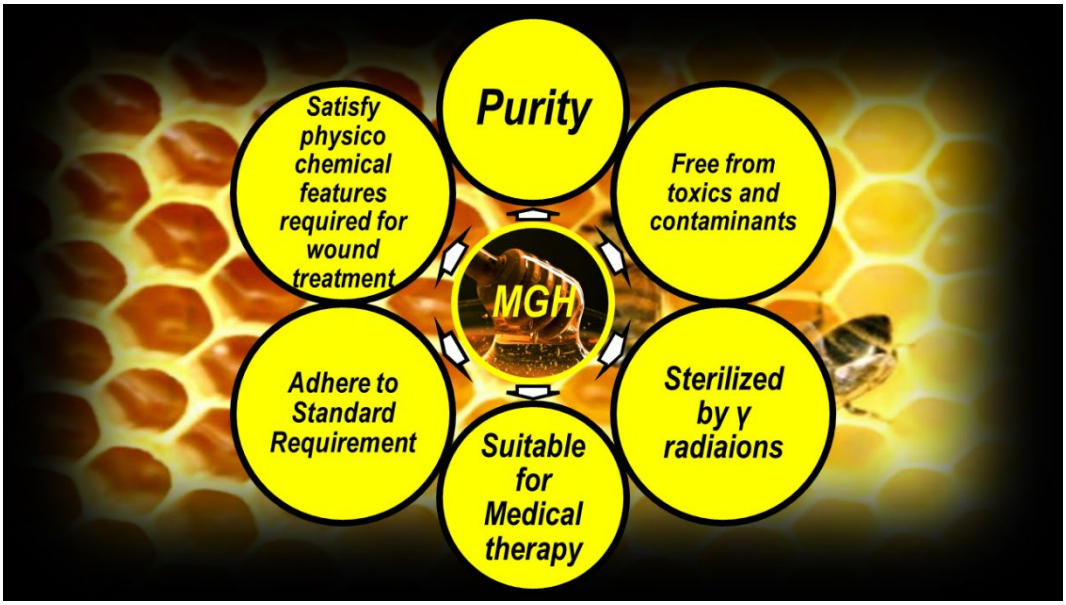

Figure 4. Characteristic and criteria that a medical grade honey (MGH) should fulfil according to Hermann et al [187].

Manuka and Revamil® are the major medical-grade honeys currently approved for clinical application. Manuka honey is produced from the manuka bush (*Leptospermum scoparium*) native of New Zealand and Australia. The raw honey used as a source for Revamil®, is instead produced by a standardized process in greenhouses. Since Revamil® honey is registered as a medical device for applications in wound healing and not as an antimicrobial agent, the antimicrobial activity is not specified for individual batches of this honey. However, in a quantitative liquid bactericidal assay, both Revamil® and manuka honeys demonstrated potent bactericidal activity [188,189], being manuka honey the most performant against *S. aureus*, *B. subtilis*, and *P. aeruginosa*. On the contrary, these honeys had identical bactericidal activity against *E. coli*. The following Table 10 reports the approved and already commercially available wound healing products based on honey.

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Table 10. Commercially available honey-based wound healing products.

Product	Description	Indications	Mechanism of Action	Ref.	Clinical Evidence
Activon® Manuka Honey Tube AM	100% MGMH	Sloughy, necrotic wounds # Malodorous wounds #	Debrides necrotic tissue Can be used in dressings or directly into cavities	[190]	Blistering and cellulitis on a type 2 diabetic patient Pediatric burn Foot ulceration Grade 5 sacral wound [190]
Activon® Tulle AM	Knitted viscose mesh dressing with 100% MH	Granulating or shallow wounds Debriding or de-sloughing small areas of necrotic or sloughy tissue	Creates a moist healing environment Eliminates wound odor Antibacterial action	[190]	Over-granulated grade 3 and 4 pressure ulcers Extensive leg cellulitis Venous ulcer, chronic wound Infections, necrotic foot [190]
Algivon® Plus AM	Reinforced alginate dressing with 100% MH	Cavities, sinuses, pressure, leg, diabetic ulcers Surgical, infected wounds Burns, graft sites Ideal for wetter wounds	Absorbs exudate Debrides, removes slough Reduces bacterial load	[190]	Chronic wounds [191] Burn wound management [192]
Algivon® Plus Ribbon AM				[190]	Autoamputation of fingertip necrosis [193]
Aurum® ostomy bags WM	MGMH added to hydrocolloids	Stoma care	Kills bacteria Suppresses inflammation Promote healthy skin around the stoma	[194]	Pyoderma gangrenosum around ileostomy [195]
L-Mesitran® Border AME	Hydrogel + honey (30%) pad on a fixation layer	Chronic wounds $	Exudate absorption Re-hydration of dry tissue Antibacterial properties	[196]	Pediatric minor burns and scalds [197]
L-Mesitran® Hydro AME	Sterile, semi-permeable hydrogel dressing ##	Chronic wounds * Superficial and acute wounds ** Superficial and partial-thickness burns *** Fungating wounds, donor sites Surgical wounds, cuts, abrasions	Donates moisture to rehydrate dry tissue Antibacterial properties	[196]	Pediatric minor burns and scalds [197] Fungating wounds [198]
L-Mesitran® Ointment AME	Ointment $$		Aids debridement and reduce bacterial colonization	[196]	Skin tears; irritation and inflammation [198]

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Manuka Dress IG MPI	Wound dressing made with 100% *Leptospermum scoparium* @	Leg and pressure ulcers First- and second-degree burns Diabetic foot ulcers, surgical and trauma wounds	Osmotic activity that promotes autolytic debridement and helps maintain a moist wound environment	[152]	Burn management [152] Difficult-to-debride wounds [199] Necrotic pressure ulcer Recurrent venous leg ulceration [200]
Medihoney® Antibacterial Honey DSC	100% sterilized MGMH	Deep, sinus, necrotic, infected surgical and malodorous wounds	Creates an antibacterial environment Debridement on sloughy and necrotic tissue, removes malodor Provides a moist environment	[201]	Wound healing [202] Prevention of catheter-associated infections in hemodialyzed patients [203]
Medihoney® Apinate Dressing DSC	Calcium alginate dressing with 100% MGMH	Diabetic foot, leg, pressure ulcers First- and second-degree partial-thickness burns Donor sites, traumatic, surgical wounds.	Provides a moist environment Osmotic potential Draws fluid through the wound to the surface Low pH of 3.5–4.5.	[204]	Venous leg ulcers [205]
Medihoney® Barrier Cream DSC	Barrier cream with 30% MGMH	Protects skin damaged by irradiation treatment or in wet areas Prevents damage caused by shear and friction	Maintains skin moisture and pH.	[206]	Treatment for intertrigo in large skin folds [207]
Medihoney® Antibacterial Wound Gel™ DSC	Antibacterial wound gel &	Burns, cuts, grazes, and eczema wounds	Creates a moist, low-pH environment Cleans the wound by osmotic effect Reduces the risk of infection	[208]	Reduction in incidence of wound infection after microvascular free tissue reconstruction [209]

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Surgihoney RO TM MH	Antimicrobial wound gel &&	Infected, chronic wounds	Antimicrobial activity by controlled release of H_2O_2 Promotes debridement and new tissue growth	[210]	Prevention of caesarean wound infection Prevention/eradication of bacterial colonies in dressing oncology long vascular lines; ulcers, surgical wounds and trauma wounds [183,185,211] In vitro activity against biofilm-producing clinical bacterial isolates [178]

MGMH = Medical grade manuka honey; AM Advances Medical manufacturer; WM Welland Medicals Ltd manufacturer; AME Aspen Medical Europe Ltd manufacturer; MPI Medicareplus International manufacturer; DSC Derma Science-Comvita manufacturer; MH Matoke Holdings Ltd manufacturer; # pressure ulcers, leg ulcers, diabetic ulcers, surgical wounds, burns, graft sites, infected wounds, cavity wounds and sinuses; $ pressure ulcers; superficial and partial-thickness burns; venous, arterial, and diabetic ulcers; ## contains 30% honey with vitamin C and E, as well as an acrylic polymer gel and water, with a polyurethane film backing; * pressure ulcers, venous and diabetic ulcers; ** cuts, abrasions and donor sites; ***first- and second-degree; $$ made of 48% medical-grade honey, medical-grade hypoallergenic lanolin, oils, and vitamins; @sterile honey from New Zealand. Non-adherent impregnated gauze; & 80% medical-grade manuka honey with natural waxes and oils; && utilizes bioengineered honey to deliver Reactive Oxygen® (RO™).3.3.2. Antibacterial Mechanisms of Honey.

The antimicrobial activity of the majority of honeys is mainly due to its capability to generate high levels of hydrogen peroxide (H_2O_2) [10,21,179,212–215] by the oxidative action the enzyme glucose oxidase (GOx) on glucose. Secreted into the nectar by bees during the preparation of honey, GOx oxidizes glucose to gluconic acid thus producing H_2O_2 [10,215–219]. The enzyme presents no activity in raw honey, due to a lack of free water. Therefore, to initiate the peroxide-dependent antimicrobial mechanism, the honey needs to be diluted. Other important antimicrobial features responsible for the non-peroxide activity of honey include low water content (osmotic effect), low pH (acidic environment), phenolic compounds, bee defensin-1 (Def-1), and methylglyoxal (MGO) (in *Leptospermum*-derived manuka honey). Table 11 summarizes the factors that confer honey its antibacterial effects.

Table 11. Components that confer honey its antibacterial effects and would healing properties.

Antibacterial factors	Sources	Mechanism of formation	Effects on Bacteria	Ref.
H_2O_2	Glu	Oxidation of Glu deriving by sucrose captured by bee from flowers	OS DNA damage	[152]
Bee Def-1	Bee's hypopharyngeal gland	Innate immune response	Create pores in membrane Interferes with bacterial adhesion Alters the production of EPSs	[215]
Acidic pH (3.4-6.1)	GluLac GluA	By enzymatic oxidation of Glu	Prevents bacterial growth	[152]
MGO *	Dihydroxyacetone	Heating	Alters bacteria fimbria and flagella	[220]
Osmotic pressure	Super concentration of sugars	N.A.	↓ Availability of free water molecules ↓ Bacterial growth	[221]

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Polyphenols	Flowers as secondary metabolites	Flowers metabolism	Pro-oxidative properties Accelerate HO• formation Oxidative DNA breakage Non-enzymatic ↑ H_2O_2	[222]

Glu = glucose; GluLac = gluconolattone; GluA = gluconic acid; EPSs = extracellular polymeric substances; Bee Def 1 = Bee defensin 1; MGO = methyl glyoxal; * exclusively in Leptospermum honeys (e.g. manuka); ↑ means increase, more; ↓ means decrease/reduction, less, minor.

Briefly, sucrose captured by bee from flowers is broken down via diastase and invertase enzymes into glucose and fructose. Glucose oxidase (Gluox) secreted by the bee's hypopharyngeal glands, in presence of O_2 and sufficient H_2O oxidizes glucose forming gluconolactone/gluconic acid, that make honey acidic and H_2O_2 [223]. H_2O_2 is the most responsible of honey's antimicrobial activity, by killing pathogens through DNA damage, and being destructive towards several cellular targets [223]. Interesting, the antimicrobial effect of hydrogen peroxide in honey increases upon dilution, enabling the glucose oxidase enzyme to bind to glucose more readily, thus resulting in a continuous production of hydrogen peroxide [215]. Moreover, honey, predominantly due to gluconolactone/gluconic acid formation, is acidic with an average pH of 3.91 (ranges between 3.4 to 6.1), which makes it powerful against microbial strains, preventing their growth. Bee Def-1 is an antibacterial peptide originating in the bee's hypopharyngeal gland and identified in bee hemolymph (the bee blood system) [224]. Within the bee it acts as an innate immune response, exhibiting activity against fungi, yeast, protozoa and both Gram-positive and Gram-negative bacteria [189]. Importantly, bee Def-1 is mainly effective against Gram-positive bacteria, most notably *B. subtilis, S. aureus* and *Paenibacillus larvae*, while it has limited effectiveness against MDR organisms [168]. Although the full mechanism of action for bee Def-1 has not been elucidated, defensin proteins from other species have been shown to create pores within the bacterial cell membrane, resulting in cell death [225]. Bee Def-1 demonstrated to play an important role in wound healing, through stimulation of MMP-9 secretions from keratinocytes [226]. It interferes with bacterial adhesion to surfaces, or in the early biofilm stage by inhibiting the growth of attached cells, and by altering the production of extracellular polymeric substances (EPS). MGO is generated in honey during storage by the non-enzymatic conversion of dihydroxyacetone, a saccharide found in high concentrations in the nectar of *Leptospermum* flowers [220]. The antimicrobial activity of MGO is attributed to alterations in bacterial fimbriae and flagella, which prohibit the bacterium's adherence and motility. Honey is a super-saturated solution of sugars. The strong interaction between these sugars with water molecules prevents the abundance of free water molecules (low water activity) available for microbes to grow [221]. Finally, the combination of different phenols acts as an enhancer of honey's antimicrobial efficacy. Produced as plant secondary metabolites, these bioactive compounds are transferred from the plant to the honey by bee and have been identified as the major responsible for the health-promoting properties of honey [222]. In alkaline conditions (pH 7.0–8.0), polyphenols can display pro-oxidative properties, inhibiting microbial growth by accelerating hydroxyl radical formation and oxidative strand breakage in DNA. They could also support the production of considerable amounts of H_2O_2 via a non-enzymatic pathway.

3.4. ROS Formation Induced by Antibacterial Hyperbaric Oxygen Therapy

Differently from antibacterial honey therapy, which is a topical treatment, antibacterial hyperbaric oxygen therapy (HBOT), making part of hyperbaric medicine, is a systemic method to treat soft tissue infections [57]. Particularly, in a typical HBOT treatment, the patient (mono-place) or more than one patient (multi-place) inhale 100% O_2 for a specified time in a pressurized chamber [227]. From the lung, the inhaled oxygen is delivered to the whole body, where induces the formation of ROS, which overwhelm the antioxidant defenses of the facultative anaerobic bacteria and aerobic bacteria causing lipids peroxidation and membranes disruption, DNA injury, protein disfunction and death. A pressure greater than 1.4 atmosphere absolute (ATA) is necessary to have an effective antibacterial effect against some facultative anaerobic bacteria and aerobic bacteria [227]. In addition

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to be directly bactericidal via ROS formation, it has been reported that HBOT enhances the antimicrobial effects of the immune system, such as those of leukocytes in hypoxic wounds (Figure 5) [228].

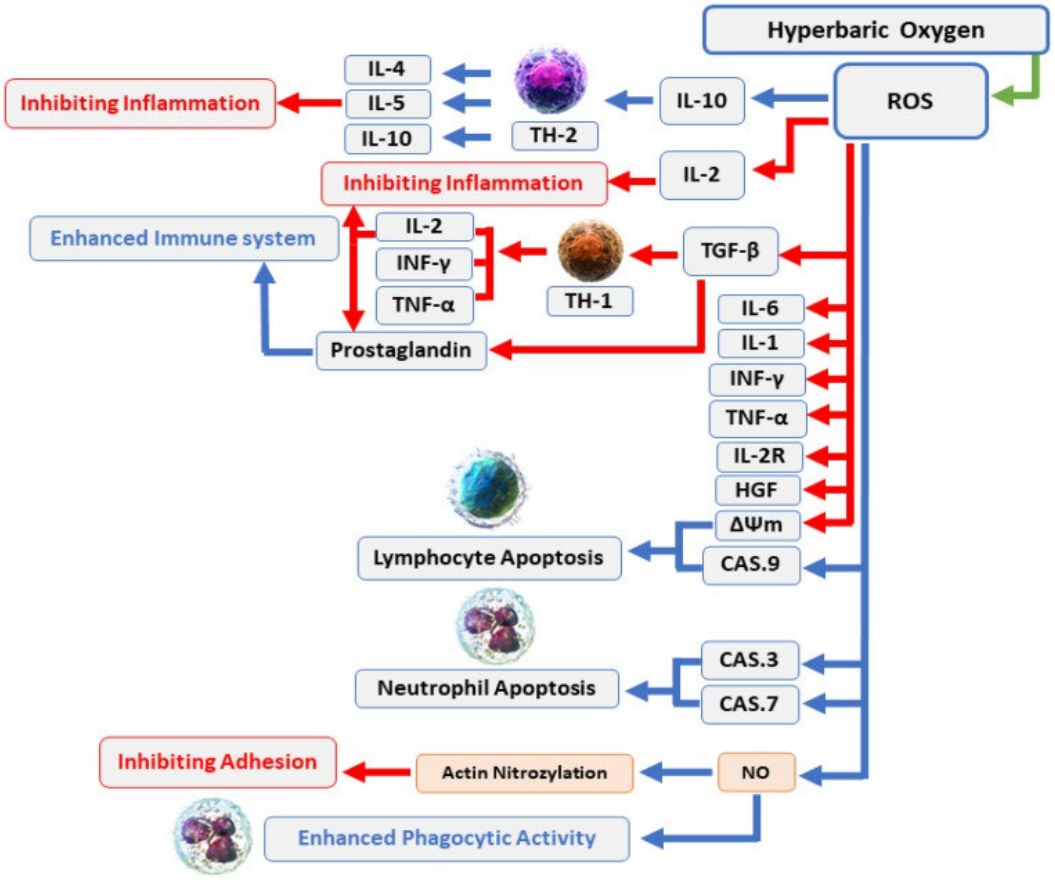

Figure 5. HBOT enhances the immune system's antimicrobial effects: Increased O_2 levels during HBOT have a variety of biological effects, including suppression of proinflammatory mediators, transitory reduction in the CD4:CD8 T cell ratio, and stimulation of lymphocyte and neutrophil death through caspase-3-, caspase-7-, and caspase-9-dependent mechanisms. In general, these effects can boost the antibacterial processes of the immune system and infection recovery. Abbreviations: ROS, reactive oxygen species; IL, interleukin; INF, interferon; TNF, tumor necrosis factor; CAS, caspase; NO, nitric oxide. [229].

Moreover, HBOT potentiates the antibacterial effects of some antibiotics such as imipenem and tobramycin (Figure 6).

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Figure 6. Events caused by hyperbaric oxygen therapy by which its antibacterial effects derive.

Hyperoxia (98% O_2 at 2.8 absolute ATA—approximately 284.6 kPa) has been shown to enhance the effect of nitrofurantoin, sulfamethoxazole, trimethoprim, gentamicin, and tobramycin in *E. coli* strains (serotype 018 and ATCC 25922) [230]. Two works by Kolpen et al report that the combination therapy of ciprofloxacin and HBOT may be potentially beneficial for the eradication of infections caused by the biofilm forming *P. aeruginosa* [231,232]. Anyway, HBOT did not show any additive or synergistic effect with other antimicrobial agents, such as distamycin and rifampicin [57]. Very recently, it has been reported that wild strains of *Pseudomonads, Burkholderias*, and *Stenotrophomonads* stopped growing under hyperbaric conditions at a pressure of 2.8 ATA of 100% oxygen [230]. HBOT is used as a primary or alternative method for the treatment of infections such as diabetic foot infection, surgical site infections, gas gangrenes, osteomyelitis, and necrotizing compartments [229]. In addition to the bactericidal effects, HBOT suppresses the production of clostridia alpha toxin in gas gangrene diseases. HBOT has demonstrated also anti-inflammatory effects, that may play a significant role in decreasing tissue damage and infection expansion. While generally safe, HBOT may have side effects such as ear discomfort, sinus pain, and temporary nearsightedness. Other contraindications include untreated pneumothorax (collapsed lung), certain medications, and claustrophobia. Although patients treated by HBOT need carful pre-examination and monitoring, when safety standards are strictly tracked, HBOT can be considered a suitable procedure to treat severe infections sustained by MDR bacteria, with an acceptable rate of complication.

3.4.1. Clinical Application of HBOT in Infections

Several studies have evidenced that HBOT, either alone or as an accessory treatment, can be a valuable therapeutic option to cure patients with several diseases, difficult to treat infections included (Table 12).

Table 12. Diseases treatable with HBOT.

Disease	Cause */Circumstances °	Ref
CO poisoning		[233]
Acute anemia		[234]
Rheumatoid arthritis Cell hypoxia	Polarization of Th17 cells to T reg *	[235]
Inflammatory disorders (by ↓ inflammatory mediators) Microcirculatory disorder	Ischemic circumstances ° Compartment syndrome °	[227]
↓ Leucocyte chemotaxis and adhesion ↑ Proliferation of neutrophiles		[236]

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Autoimmune syndrome Immune reactions in antigens Autoimmune symptoms.	Proteinuria ° Facial erythema ° Lymphadenopathy °	[237,238]
↓ Lymphocytes and leukocytes		[234]
↑ Mitochondrial Function		[239]
Chronic skin damage healing (by angiogenesis)		[240]
Recalcitrant infections	Necrotizing fasciitis ° Osteomyelitis ° Chronic soft tissue infections ° Infective endocarditis ° Acute or chronic wounds ° Diabetic foot ulcers °	[227,241–245] [246,247]
Ocular disorders	Cystoid macular edema ° Scleral thinning ° Necrosis faced after pterygium surgery ° Nonhealing corneal edema ° Anterior segment ischemia ° Some blinding diseases °	[248,249]
Brain/cerebral injuries	Ischemic-reperfusion damage °	[250]
Cancer		[251]
Complications by radiotherapy	Radiation-induced skin necrosis °	[252]

↑ Means increase, improvement; ↓ means decrease/reduction.

Basically, HBOT strongly improves the levels of O_2 concentration in blood and the oxygen pressure both in blood and tissues (2000 mmHg and 500 mmHg, respectively), determining hyperoxia conditions, which provide beneficial effects in patients suffering from several diseases as reported in Table 12. HBOT was used to adjust immunology, to maintain the durability of an allograft [253]. HBOT has demonstrated beneficial effects on vascular endothelium, thus promoting angiogenesis, and to induce partial high tensions of O_2 in circulating plasma, thus stimulating O_2 dependent collagen matrix formation, which is an essential phase in wound healing [254]. On the other hand, HBOT effects on sepsis, urinary tract infections and meningitis are not well known, so far. Unequivocally. the most frequent clinical application of HBOT remains for several skin soft tissue infection and osteomyelitis infections, which are associated with hypoxia, caused by anaerobic infections due antibiotic resistant bacteria [255,256]. Currently, HBOT is considered both alone and in combination with different antibacterial treatment a relevant option for solving several cute or chronic diseases [249,251,257,258]. Although animal studies have shown the inhibitory effect of HBOT on inflammation and apoptosis after cerebral ischemia [259,260], clinical trials on humans have not shown any significant benefit. Anyway, it has been indicated that HBOT can improve some neuropsychological and inflammatory outcomes, especially in stroke patients, within the first few hours [261,262]. Furthermore, studies on animals have shown that HBOT is associated with reduced blood–brain barrier breakdown, reduced cerebral edema, improved cerebral oxygenation, decreased intracranial pressure, reduced oxidative burden, reduced metabolic derangement, and increased neural regeneration [260,261,263]. The following Table 13 contains an overview of some clinical studies investigating the application of HBOT for different infections, while Table 14 collects the most relevant studies concerning specifically, the clinical application of HBOT in Surgical Site Infections (SSI) categorized based on the type of SSI or surgery. Sternal wound infections following cardiac surgery, SSIs following neuromodulation or neuro-muscular surgery, and SSIs following the male-to-female gender affirmation surgery (urogenital surgery) have been included.

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Table 13. Overview of some clinical studies investigating the application of HBOT for different infections [227].

Infections	Study papulation	Treatment sessions *	Pressure **	Exposure Time (min)	Main findings	Ref
Burns	53	Based on outcomes	2.5	90	All patients survived	[264]
Burn	40	10	2.5	80	Faster healing, shorter hospitalization	[265]
Brain abscess	41	4–52	2.5–2.8	25–	↓ Treatment failures, ↑ outcomes	[266]
SSIs	42	30	2.4	90	↓ Post-surgical deep infections in CSD	[267]
SSIs	32	Based on outcomes	2-3	90	Valuable AT for PO organ/space S-SSI	[268]
SSIs	6	28–106	2.5–2.8	75	AT for early PO deep infections	[269]
NSTI	48		3	90	Not ↓ mortality rate, number of debridement, hospital stay, antibiotic use	[270]
NSTI	44	Based on outcomes	2.8	60	↑ Survival and limb salvage	[271]
NSTI	32		2.8	45	AT	[272]
NSTI	37		2.5	45	Doubtful advantage of using HBOT as AT for NF in ↓ mortality and morbidity	[273]
DFIs	100	20 to 30	2–3	90	Useful AT for nonhealing DFIs	[274]
DFIs	42	Group1:<10 Group 2:>10	2.5	120	↓ Amputation rate	[247]
DFIs	94	40	2.5	85	Facilitates healing of chronic DFIs	[275]
DFIs	35	38±8	2.2–2.5	90	↓ Amputations	[276]

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DFIs	28	20	2.5	90	↑ Healing rate of nonischemic chronic DFIs	[277]
DFIs	36		2.5	90	Healing response in chronic DFIs	[278]
DFIs	38	40–60	2.5	90	↑ Healing rate ↓ Amputation rate	[279]
DFIs	18		2.4	90	AT when reconstructive surgery is not possible	[280]
Osteomyelitis	6		2.0–2.4	30	Effective following failure of primary therapy of osteomyelitis	[281]
Osteomyelitis	14	30	2.5	120	Effective and safe for chronic refractory osteomyelitis	[282]
Osteomyelitis	1		2	–	Early use of HBOT for a compromised host who develops recurrent osteomyelitis	[283]
Osteomyelitis	12	Based on outcomes	2.5	90	AT for patients who develop S-SSI and osteomyelitis after CTS	[284]

* Days; ** ATA = atmospheres absolute; DFIs = diabetic foot infections; HBOT = hyperbaric oxygen therapy; NSTI = necrotizing soft tissue infections; SSIs = surgical site infections; S-SSIs sternal surgical site infections; NF = neurofibromatosis; ↑ means increase, improvement, improved, high, higher; ↓ means decrease/reduction, low, lower, less; CTS = cardiothoracic surgery; CSD = complex spine deformity; PO = postoperative; AT = adjuntive treatment.

Table 14. Studies finalized to investigating the application of HBOT in treatment of different surgical site infections (SSI) [229].

Study	Surgery	SSI	Population ***/#	ATA Time (min)	Outcomes and Conclusion	Refs.
Case report	CTS	S-SSI	1	2.40 90	Rapid healing and epithelialization *	[285]

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Retrospective	Sternotomy	S-SSI	55	2.50 90	S-SSI cured in all patients within an average of 8 weeks No in-hospital death ↑ Clinical outcome in patients with sterno-mediastinis and post-sternotomy wound infection after CTS	[286]
Prospective trial	CTS	S-SSI	32; 14/18	2–3 90	*S. aureus* was the most common pathogen #, *** Infection duration was similar #,***,** Infection relapse rate was significantly ↓ in *** Intravenous antibiotic use duration ↓ in *** Total hospital stay ↓ in *** HBOT could be a valuable AT for treating PO organ/space S-SSI	[268]
Case report	CTS	S-SSI	1	2.50 90	Sternal wounds totally healed and epithelized in 9 weeks HBOT with TNP dressing is a good alternative method for patients who cannot tolerate or refuse any surgical reconstruction	[287]
Retrospective	NMSS	DWI	6	2.50 3 × 25	All infections resolved Wound healing in an average of 3 months Minor side effects of HBOT HBOT is a safe AT for early deep PO infections in case of spinal implants in HR pediatric patients	[288]
Retrospective	CTS	S-SSI	12; 6/6	2.50 90	No treatment-related complication in *** Length of stay in ICU ↓ in *** Invasive and noninvasive positive pressure ventilation ↓ in *** Hospital mortality ↓ in *** HBOT may be used as a safe AT to ↑ clinical outcomes in patients with S-SSI and osteomyelitis after sternotomy and CTS	[284]
Retrospective	CABS	Mediastinitis	18	2.50 90	1 HBOT-unrelated death caused by sepsis HBOT was well-tolerated Favorable clinical outcomes using HBOT as AT for treating mediastinis patients after CABS	[289]

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Retrospective	NMSS	DWI	42; 18/24	2.40 90	11.9% (5/42) Incidence of infection in both ***,# 5.5% (1/18) Infection rate in *** 6.6% (4/24) Infection rate in # HBOT significantly ↓ PO infections in NMSS patients HBOT is a safe AT to prevent PO deep infections in complex spine deformity in HR NM patients	[267]
Retrospective	CTS	S-SSI	10	2.50 92	70% complete wound healing with fibrous scar formation in 4 weeks HBOT *** HBOT had 80% success as AT in DSWI *** No complications were observed	[290]
Retrospective	NM	HRI	14	2.0–2.8 75	86% of HRI successfully treated without hardware removal *** Hardware was removed following HBOT failure in 2 infections Observed intrathecal pump malfunction caused by HBOT (2.8 bars) HBOT has potential as AT in the treatment of HRI in NM Diminished need for hardware removal and treatment interruption	[291]
Retrospective	CTS	S-SSI	53		Readmitted with infected sternotomies discharged in 2–39 days Time for wounds healing using NPWT alone was 21–42 days in # Time for wounds healing using NPWT+HBOT was 28–42 days in *** HBOT was 5–35 days HBO treatments were 22.6 (+11.06) Restore time 21–98 days in *** (NPWT+HBOT) Multimodality therapy of incision and drainage using NPWT+HBOT+antibiotics is successful for treat complex deep sternal wound infections in the pediatric population after congenital heart surgery	[292]

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Retrospective	MtF-GAS	SSI	33; 15/18	2.2–3.0/90	100% Complete wound healing *** 94.4% Complete wound healing # ↓ Duration of antibiotic therapy *** ↓ Perineal drain time *** ↓ Bladder catheter time *** ↓ Hospital stay *** HBOT as effective adjuvant treatment for SSIs in patients undergoing MtF GAS	[293]

* First reported case of HBOT use for the treatment of deep sternal (surgical site infections) SSI in a heart transplant recipient; S-SSI = sternal surcical site infections; ** 31.8 ± 7.6 vs. 29.3 ± 5.7 days, respectively, $p = 0.357$); ↑ means increase, improvement, improved, high, higher; ↓ means decrease/reduction, low, lower; MtF-GAS = male-to-female gender affirmation surgery; *** in HBOT group; # in not HBOT group, AT = adjuvant therapy; PO = postoperative; TNP = topical negative pressure; CTS = cardiothoracic surgery; CABS = coronary artery bypass surgery; DSWI = deep sternal wound infections; HRI = hardware-related infections; NMSS = neuromuscular sclerosis surgery; NM = neuromodulation; DWI = deep wound infections; ICU = intensive cure units; HR = high risk; NPWT = negative pressure wound care therapy.

4. Possible Novel Methods to Induce ROS Formation: Our Proposal

4.1. Environmental Persistent Free Radicals (EPFRs)

Environmentally persistent free radicals (EPFRs) are defined as long-living organic free radicals stabilized on or inside particles [294]. EPFRs are persistent because of the protection by the particles containing them and the presence of transition metals, thus having lifetimes exceptionally longer (from days to years) than other free radicals. These surface-bound radicals are found in contaminated soil, tar balls, and cigarette smoke, and primarily form during thermal processes such as pyrolysis and combustion of organic materials, waste incineration and photoactivation [295]. In fact, the byproducts from these processes, such as phenols and aromatic polycyclic hydrocarbons provide a breeding ground for EPFR generation. In fact, EPFRs are also found in the matrix of ultrafine and airborne fine particulate matter (PM), which is emitted into the environment by both natural and anthropogenic processes, including coal combustion emissions (16.8%), vehicular emission (32.1%), industrial processes (11.7%), dust storm (27.2%), and nitrates (3.4%). PM is a mixture of organic, inorganic species, solid and liquid components of metals which have the tendency to form radicals with or without the sunlight photoactivation [295,296]. The environmental factors affecting the EPFRs' formation, lifetime and abundance such as precursors, temperature, light irradiation, presence of metals, temperature, pH, humidity, thermal processing time, and oxygen [297]. EPFRs are categorized as no decay, low decay, and fast decay radicals. The no decay EFFRs are unpaired electrons of delocalized over aromatic bonds entrapped inside PM. Phenoxyl radicals are fast decay EPFRs, while semiquinone radicals are subjected to slow decay [298]. EPFRs can produce reactive oxygen species, including hydroxyl radicals, which induce oxidative stress in living organisms, posing adverse environmental and human health effects. The atmospheric oxygen is the only sink for stable free radicals converting them into not particles and/or metal stabilized molecular species thus decaying [295]. The EPFRs decay in atmosphere depends on reaction of EPFRs with molecular oxygen. By reaction with atmospheric oxygen, they generate high levels of reactive oxygen species (ROS), via electron transfer, such as hydroxyl radicals and superoxide anion radicals, thereby inducing cellular oxidative stress [23,24]. For this reason and their possible redox recycling, EPFRs are emerging as environmental pollutants with a ROS-dependent significant toxicity to organisms, including humans, plants, animals, and microorganisms. Anyway, recent research has also explored their potential for degrading organic environmental contaminants. By activating hydrogen peroxide or persulfate EPFRs produce ROS species such as superoxide, singlet oxygen, or OH−, thus inducing the degradation of environmental organic pollutants [295]. Collectively, although EPFRs are long-lived environmental pollutants, harmful to environment and living beings, their capacity of

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activating ROS generation, if properly controlled, can make them a wide range tool to environment remediation.

4.2. Biochar-Derived Persistent Free Radicals (PFRs)

Biochar (BC) is a carbonaceous material obtained by the pyrolysis of different vegetable and animal biomass feedstocks at 200–1000 ◦C in limited presence or in absence of O_2. BC has demonstrated a broad prospective use in the treatment of environmental pollutants, in soil amendment. It has been used in photocatalytic and photothermal systems, for photothermal conversion, to construct electrical and thermal device, as well as 3D solar vapor-generation devices for water desalination [24]. All these potentials are due to its high surface area and rich pore structure, which determines great physical absorptivity [23]. Additionally, they also depend on the chemical characteristics of BC, which in turn depend on the type of biomass used to produce BC, on the original biomass chemical composition, and pyrolysis conditions [299,300]. Whereas, starting from the year 2014 (Figure 7), the presence of persistent free radicals (PFRs) in BC deriving from lignocellulosic biomasses, like the radicals previously detected in combustion-generated particulate matter (PMs), sediments, and contaminated soils, known as environmental persistent free radicals (EPFRs), have been reported.

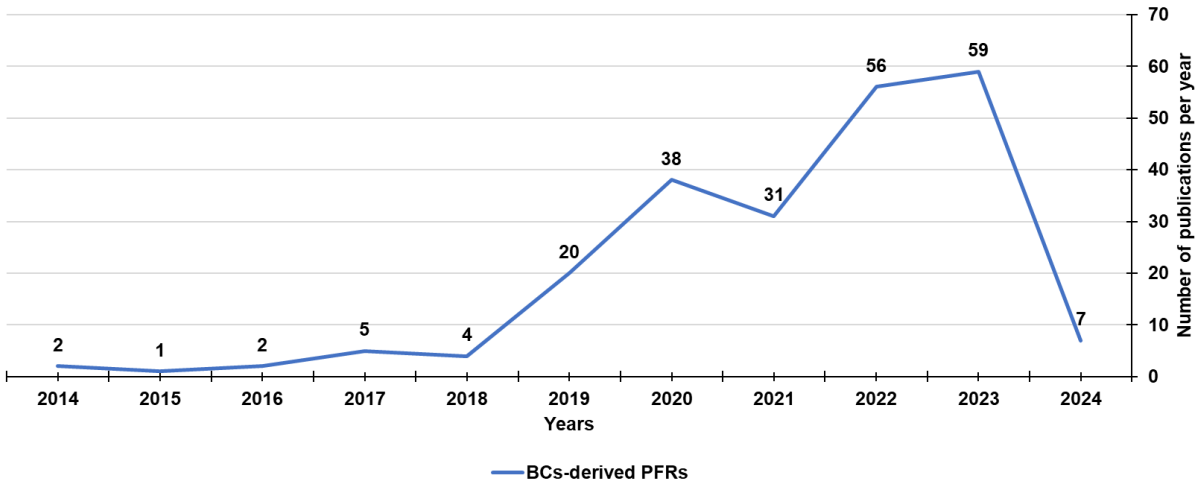

Figure 7. Number of publications on BCs-derived PFRs from 2014 according to Scopus dataset (reviews and chapters in books included). The survey used the following keywords: persistent AND free AND radicals AND biochar [24].

As EPFRs, such reactive species can remain stable for months or years and play a crucial role in the capacity of BC to degrade different types of xenobiotics and pollutant by oxidative reactions via ROS formation. Unlike other free radicals, including ROS, PFRs are resonance-stabilized since they are bound to the external or internal surface of solid particles of BC [24]. If BC is conserved under vacuum, lifetime of PFRs could be infinite (no decay radicals), while when air-exposed they react with molecular oxygen in the air, decay over time, thus producing ROS. Similarly, in aqueous systems, PFRs act as transition metals like Fe^{2+}, forming ROS, as well [301–304]. PFRs are categorized into three classes, i.e., oxygen-centered PFRs (OCPFRs), carbon-centered PFRs (CCPFRs), and oxygenated carbon-centered radicals (CCPFRs-O). The possible presence of PFRs onto a BC, their type and concentrations are significantly affected by pyrolysis conditions, biomass types, the elemental composition of pristine biomass, and the presence of external transition metals as detailed in Table 15.

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Table 15. Factors influencing PFRs formation in BC.

Parameter	Influencing Factors	Specifications	Observations	Ref.
PFRs concentration	Biomass type	Cow manure, rice husk, others (< 500°C)	≠ Concentrations	[305,306]
		Non-lignocellulosic biomass with ⇓ H/C and O/C	⇓ Concentration	[307]
		Lignocellulosic biomass	⇑ Concentration	
	Temperature	300°C, 700°C	≠ Concentrations	[305]
		Maximum concentration at 600°C		[308]
		Maximum of concentration at 500-600°C		[309]
	Transition metals	Adsorb onto biomass and transfers electrons from polymer to metal center during pyrolysis	⇑ Concentration	[25]
Type of PFRs	Temperature	200-300°C	OCR	[309]
		400°C	OCR + CCR	
		500-700°C	CCR	

OCR = Oxygen centered radicals; CCR = carbon centered radicals [24].

4.1.1. Proposed Mechanisms for PFRs Formation During Biomass Pyrolysis

The actual mechanism by which PFRs form during pyrolysis remains not fully clarified. However, transition metals capable of electron transferring and substituted aromatics molecules present in the lignin component of pristine biomass have been recognized to be essential for PFRs formation. Anyway, high concentration of PFRs have been detected also in products obtained by the pyrolysis of non-aromatic cellulose in absence of transition metals [308]. Collectively, PFRs can form by different pathways including or not the presence of transition metals, and once formed PFRs, could be either only surface-stabilized or surface-stabilized in metal-radical complexes [310]. Scheme 1 (concerning lignin) and Scheme 2 (concerning cellulose and emicellulose) report the possible chemical paths by which PFRs may form.

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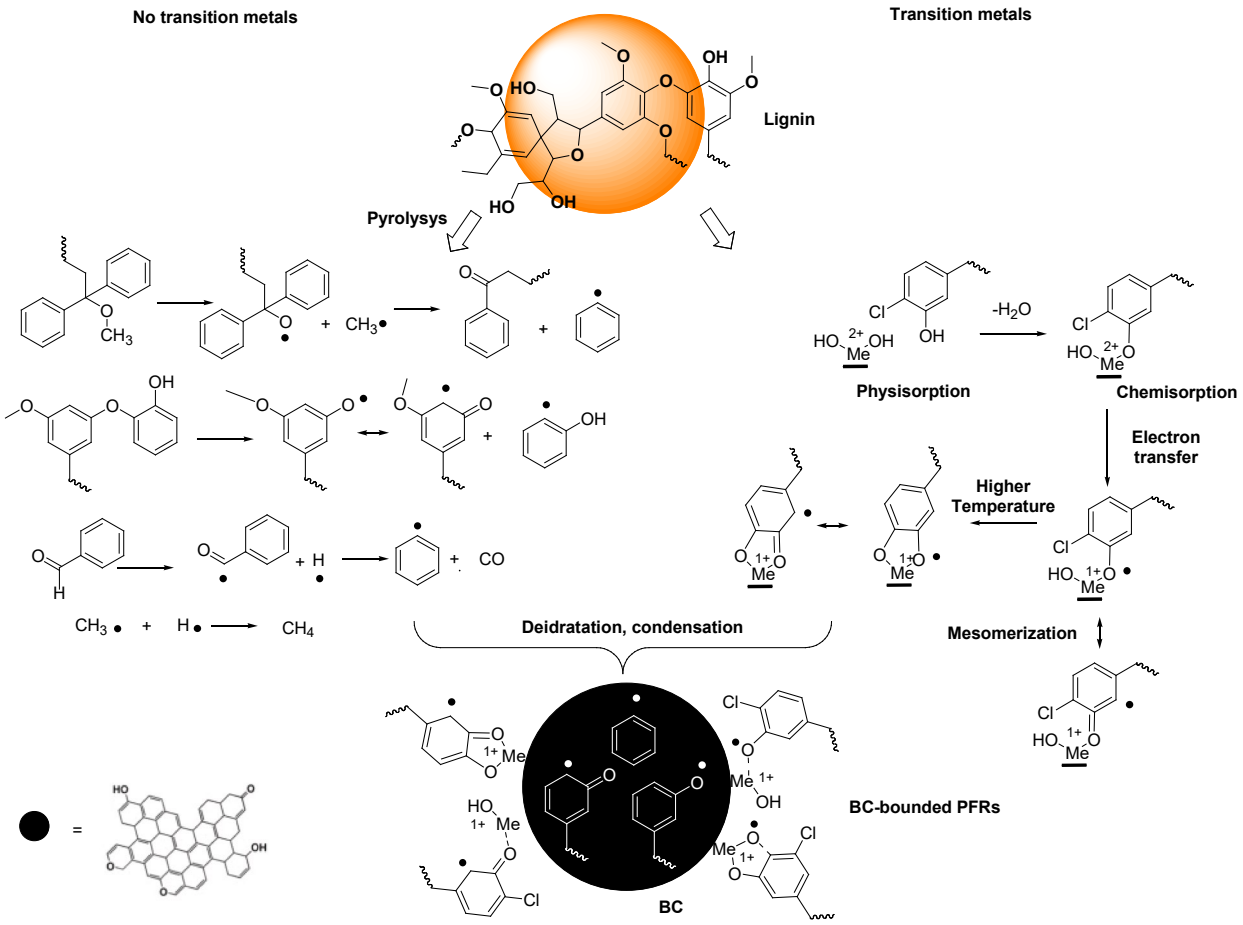

Scheme 1. Possible mechanisms leading to the formation of BC-bounded PFRs from lignin. The orange sphere represents biomass, while the black sphere represents BC whose hypothetic structures depending on pyrolysis condition have been shown at the bottom of the Scheme [24].

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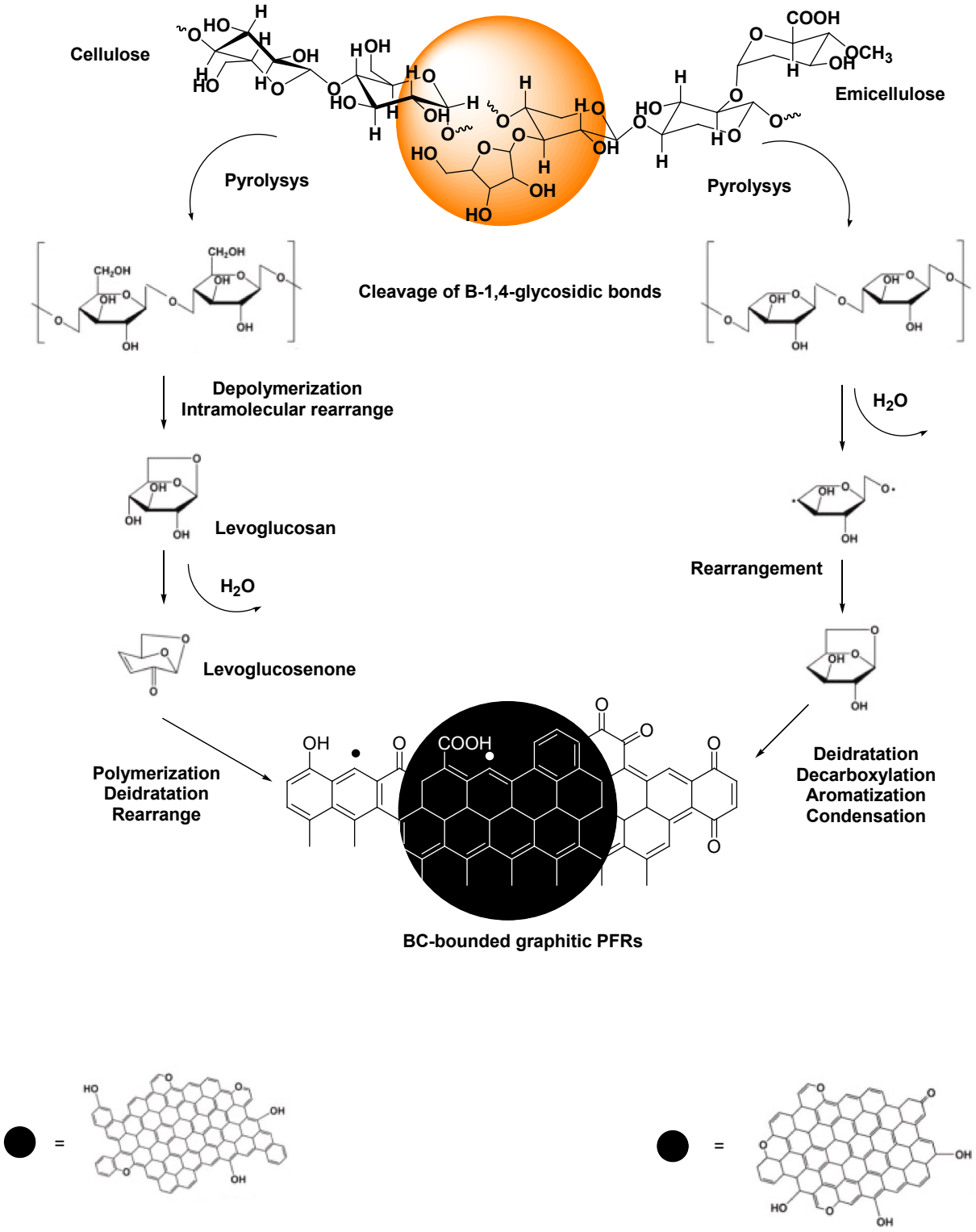

Scheme 2. Possible mechanisms leading to the formation of BC-bounded graphitic PFRs from cellulose (left side) and emicellulose (right side). The orange sphere represents biomass, while the black sphere represents BC whose hypothetic structures depending on pyrolysis condition have been shown at the bottom of the Scheme [24].

Since it is out of scope of this paper, a detailed discussion on the mechanisms reported in Scheme 1 and 2 has been avoided. Readers particularly interested can find major information in a very recent review [24].

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4.1.2. Possible Activities of PFRs and Our Proposal

PFRs formed in BC during combustion of lignocellulosic biomasses either in the presence or absence of external transition metals could promote several beneficial reactions, such as PFRs-mediated remediation and degradation of organic and inorganic pollutants by different actions and mechanisms, including oxidative and reductive processes. PFRs on BC can activate hydrogen peroxide (H_2O_2) or oxygen (O_2), as well as persulfate ($S_2O_8^{2-}$) to produce different radical and not radical oxygenated species (ROS) capable of efficiently degrade organic contaminants by oxidative mechanism, as ROS generated by the previously reported antimicrobial therapies are bactericidal to pathogens inducing OS via ROS stimulation. Therefore, we thought that ROS induction using BC-derived PFRs, whose type, concentration and reactivity can be tunable varing pyrolysis conditions could be a novel method to form ROS for a possibly more selective BC-based antibacterial oxidative therapy. In this regard, in a recent review on BC-derived PFRs, a random selection of the main experimental works regarding the applications of PFRs found in BCs conveyed in the last five years (2019-2023) has been reported. Among the reported PFRs applications, three regarded their use as antibacterial agents (Table 16), thus supporting our idea.

Table 16. BC-derived PFRs and their applications as ROS- forming antibacterial agents reported in the years 2019-2023.

Biomass	Pyrolysis °C/Time	BC-name	Active radicals	Radical Mechanisms	Degraded compound	Refs.
Anaerobic digestion sludge	400°C 600°C 800°C 1000°C	ADSBC 400 ADSBC 600 ADSBC 800 ADSBC 1000	$SO_4^{\bullet-}$ PFRs $OH^{\bullet}$	BC-mediated activation of PDS	Dyes, Estrogens Sulfonamides, *E. coli* Others	[30]
Caragana korshinskii	650 °C/3h	ACB-K-gC3N	PFRs h^+ $^{\bullet}OH$ O_2^-	Electron photogeneration and PFRs mediated H_2O and O_2 activation	*S. aureus*, *E. coli* RhB, TC, NOR, CAP	[31]
Pinewood	600°C	Ag^0-PBC	PFRs $^{\bullet}OH$ O_2^-	UV-light promoted excitation of the electron-hole pairs and subsequently, the production of ROS Enhanced ROS generation by PFRs	MB, *E. coli*	[32]

BCs = biochar; TC = tetracycline; RhB = rhodamine B; PDS = peroxydisulfate; CAP = chloramphenicol; MB = methylene blue; NOR = norfloxacin.The employed BC was derived from pyrolysis of sludge, *Caragana korshinskii* plant and pinewood. In these processes, the electron transfer promoted by PFRs of diverse nature, generated ROS such as $SO_4^{\bullet-}$, $^{\bullet}OH$, $^{\bullet}O_2^-$, $^{\bullet}O_2H$, which carried out the oxidative degradation of different organic pollutants, including drugs, dyes, antibiotics, hormones, and showed antibacterial effects against *E. coli* and *S. aureus*.

5. Conclusions

Upon the colonization of the host cell during infection, the maintenance of redox homeostasis (RH) is a key process for bacterial survival and for escaping the oxidative stress physiologically generated by macrophages to opposing the development of the infection. Pathogens succeed in strictly controlling RH by a mechanism based on different redoxins and low molecular weight-thiol molecules. Therapeutic strategies based on the capacity of different compounds or methods to cause ROS and RNS hyper-generation during phagocytosis to unbalance bacterial redox defences and stop host cell colonization have a great potential to solve the increasing problem of antibiotic-resistant

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infections. ROS have demonstrated to be effective in inhibiting clinically important microbial pathogens, by lipid peroxidation thus damaging their membranes, by harming DNA and impairing protein functions. It has been observed that certain traditional antibiotics and alternative antimicrobials, including nanomaterials, as well as their combination, induce ROS as a secondary or main mechanism of antibacterial effect. Additionally, on the worrying scenario of an increasing global emergence of difficult-to-treat infections due to bacterial resistance to available antibiotics, old procedures that inhibit microbial growth by forming ROS, such as HBOT and medical honey, have been reinvigorated. Together with the more recent PDT, they are in fact currently successfully employed for the treatment or prevention of soft tissue infections and chronic ulcerations. The development of resistance to these methods has not been reported, but unfortunately, since ROS-mediated OS is destructive also on eukaryotes, their clinical application to treat systemic infections is at present impracticable. Further studies, aimed at identifying novel delivery techniques for using ROS with superior selectivity for microbial pathogens is required. As our contribution to this challenge, we have now proposed BC-associated PFRs as a promising novel low-cost and eco-friendly method for ROS generation to be studied for the oxidative inhibition of MDR pathogens and as a potential treatment for a wide range of infections. A greater knowledge concerning the proper pyrolysis conditions employed to obtain the type of PFRs more suitable for this purpose and in optimized concentration could make this ROS-delivering method more selective for bacterial pathogens.

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