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CHARACTERIZATION OF A NOVEL ANTIMICROBIAL AGENT INSPIRED BY PEROXIDASE-CATALYZED SYSTEMS

A Thesis Submitted to the National University of Ireland, Galway for the Degree of Doctor of Philosophy

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SEPTEMBER 2017
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I, Lilit Tonoyan, certify that this Thesis is all my own work and that I have not obtained a degree in this University or elsewhere on the basis of any of this work.

Signed:  

Date: 29 September 2017
Acknowledgments

I would like to express my sincere gratitude and respect to my research guide Prof. Vincent O’Flaherty for his continuous support, vast knowledge, endless motivation, true kindness and immense generosity. This work would not have been possible without your guidance and involvement. I am honored to have had you as my supervisor. Special thanks to my PhD co-supervisor Dr. Gerard Fleming for his great experience, patience and unfailing humor. Thank you for encouraging me along these years. I am very grateful to late Dr. Paul Mc Cay for providing me guidance and support. I will never forget you. I wish to thank NUI Galway and all the good people of the Microbiology Department. Special thanks to almighty technicians Maurice and Mike.

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Abstract

The phenomenon of antibiotic resistance is expanding and the threat regarding our future ability to combat infection is increasing. Thus, key challenges for society and for researchers are to address microbial drug resistance and to develop non-antibiotic based therapies and disinfectants that can avoid induction of resistance.

A novel antibacterial complex was developed, drawing the inspiration from naturally occurring peroxidase-catalyzed systems that play a role in immune defense against invading microbes. In the peroxidase system, a particular peroxidase enzyme catalyzes the oxidation of a halide/pseudohalide, at the expense of hydrogen peroxide (H₂O₂), to generate reactive products with broad antimicrobial properties. However, producing enzymes in quantities widely usable for antibacterial treatments would be both expensive and impractical. In the new peroxidase-like antibacterial complex, it is H₂O₂ that oxidizes the two halide/pseudohalide substrates (iodide and thiocyanate) in the absence of a peroxidase. This enzyme-free iodo-thiocyanate complex (ITC) is a mixture of highly reactive oxygen and iodine species that can damage bacterial cells, resulting in their death.

The objective of this thesis was, firstly, to evaluate the antibacterial properties of ITC. The antibacterial potential of the novel ITC was tested on two Gram-negative and two Gram-positive bacterial strains, including the multidrug-resistant Staphylococcus aureus, in both planktonic and biofilm forms. The results of this study showed that the iodo-thiocyanate complex caused rapid bacterial death in all tested strains, both in biofilms and planktonic cells. Furthermore, the attempts to introduce resistance in these bacteria towards the “killer cocktail”, employing a sequential passage of bacteria in the presence of a sub-lethal concentration of ITC, proved to be not successful. Though the knowledge on the mode of action of the antimicrobial complex is still incomplete, there are indications that its antimicrobial activity is most likely the combinational effect of powerful species capable of oxidizing the essential biomolecules of bacteria, and, perhaps, is a result of simultaneous events.
It is essential to take into account the emergence of bacterial resistance when designing a novel antimicrobial. Thus, the next step in this thesis was to elaborate the studies on the emergence of resistance. A continuous culturing was used to generate antimicrobial resistant mutations, coupled with whole-genome sequencing (WGS) to identify those mutations underpinning resistance. An attempt was made to generate de novo resistance in antimicrobial-sensitive *Escherichia coli* ATCC 25922 during 20-days of continuous culturing when exposed to gradually increasing concentrations of the newly described ITC and a common antibiotic, levofloxacin (LVX). In contrast to antibiotic LVX, the long-term exposure of *E. coli* to ITC did not induce resistance to ITC, nor cross-resistance to LVX, and no mutational pattern was evidenced during WGS-based comparisons between exposed and unexposed bacterial populations.

To derive a biocompatible novel antibacterial agent, both the bacterial and mammalian toxicities must be taken into consideration. For biocompatibility testing, *in vitro* cytotoxicity, in parallel with antimicrobial activity, hemolytic activity and genotoxicity evaluations were carried out. The cytotoxicity of ITC towards human epithelial HeLa cells was evaluated by comparison with some of the oldest, and most widely used, antiseptics hydrogen peroxide, povidone-iodine (PVP-I) and Lugol’s iodine. The cytotoxic concentrations of ITC were equivalent to those resulting in potent bactericidal activity. By contrast, the cellular toxicities of H$_2$O$_2$, PVP-I and Lugol were apparent at sub-bactericidal levels. The activity of ITC was not quenched by organic matter, whereas the activities of the other antiseptics were suppressed. Hemolytic activity was also assessed as another measure of cytotoxicity. ITC, PVP-I and Lugol had dose-dependent effects on the viability of horse erythrocytes, while H$_2$O$_2$ showed no hemolytic impact. HeLa DNA damage caused by ITC was evaluated by *in vitro* comet assay as a measure of genotoxicity. ITC did not generate DNA breakage, while H$_2$O$_2$ resulted in extensive single-strand DNA breaks.

Overall, this research indicates that the novel iodo-thiocyanate complex exhibits a broad-spectrum bactericidal activity against pathogenic bacteria in planktonic and biofilm forms, without triggering the emergence of resistance. The use of this composition may provide an effective and efficient method for killing potential pathogens, as well as for disinfecting and removing biofilm contamination.
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<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BEDtools</td>
<td>browser extensible data tools</td>
</tr>
<tr>
<td>BKC</td>
<td>benzalkonium chloride</td>
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<tr>
<td>BLASTN</td>
<td>basic local alignment search tool for nucleotides</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BWA-MEM</td>
<td>Burrows-Wheeler aligner multi experiment matrix</td>
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<tr>
<td>ca.</td>
<td>around (circa)</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>c-di-GMP</td>
<td>3',5'-cyclic diguanylic acid</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CL</td>
<td>confidence limit</td>
</tr>
<tr>
<td>CTR</td>
<td>control</td>
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<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSMZ</td>
<td>German Collection of Microorganisms and Cell Cultures</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5′-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>e.g.</td>
<td>for example (exampli gratia)</td>
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<tr>
<td>EPI</td>
<td>eflux pump inhibitor</td>
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<tr>
<td>EPO</td>
<td>eosinophil peroxidase</td>
</tr>
<tr>
<td>et al.</td>
<td>and others (et alii)</td>
</tr>
<tr>
<td>etc.</td>
<td>and so forth (et cetera)</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration of the United States</td>
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<tr>
<td>G</td>
<td>glucose</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GADPH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GC</td>
<td>growth control</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic deoxyribonucleic acid</td>
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<tr>
<td>GOD</td>
<td>glucose oxidase</td>
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<td>GSH</td>
<td>glutathione</td>
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<tr>
<td>hRBC</td>
<td>horse red blood cell</td>
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<tr>
<td>HRPO</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>i.e.</td>
<td>that is (id est)</td>
</tr>
<tr>
<td>indel</td>
<td>insertions and deletions</td>
</tr>
<tr>
<td>ITC</td>
<td>iodo-thiocyanate complex</td>
</tr>
<tr>
<td>LA</td>
<td>Lennox agar</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
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<td>LMPA</td>
<td>low melting point agarose</td>
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<td>log</td>
<td>logarithm</td>
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<td>LPO</td>
<td>lactoperoxidase</td>
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<td>levofloxacin</td>
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<td>MATE</td>
<td>multidrug and toxin efflux</td>
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<td>minimum bactericidal concentration</td>
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<tr>
<td>MBEC</td>
<td>minimum biofilm eradication concentration</td>
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<tr>
<td>MF</td>
<td>major facilitator</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MMR</td>
<td>methyl-directed mismatch repair</td>
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<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MRD</td>
<td>modified Robbins’ device</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSA</td>
<td>methanesulfonic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MUMmer</td>
<td>maximal unique matches</td>
</tr>
<tr>
<td>n</td>
<td>replicates</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NCIMB</td>
<td>National Collections of Industrial, Marine and Food Bacteria</td>
</tr>
<tr>
<td>NMN</td>
<td>nicotinamide mononucleotide</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
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<td>Abbreviation</td>
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<tr>
<td>NUCmer</td>
<td>nucleotide MUMmer</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PAβN</td>
<td>Phe-Arg-β-naphthylamide dihydrochloride</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>pH</td>
<td>potential of hydrogen</td>
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<tr>
<td>pK\textsubscript{a}</td>
<td>negative base-10 logarithm of the acid dissociation constant</td>
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<tr>
<td>PMB</td>
<td>polymyxin B</td>
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<tr>
<td>p-value</td>
<td>significance value</td>
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<tr>
<td>PVP-I</td>
<td>povidone iodine</td>
</tr>
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<td>RC</td>
<td>revived culture</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RND</td>
<td>resistance-nodulation-division</td>
</tr>
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<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SAMtools</td>
<td>sequence alignment map tools</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SMR</td>
<td>small multidrug resistance</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
<td>SOS</td>
<td>save our souls</td>
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<tr>
<td>SPO</td>
<td>salivary peroxidase</td>
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<tr>
<td>SV</td>
<td>structural variants</td>
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<td>TC</td>
<td>thawed culture</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
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<td>TNB</td>
<td>5-thio-2-nitrobenzoic acid</td>
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<td>tRNA</td>
<td>transfer ribonucleic acid</td>
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<tr>
<td>TSA</td>
<td>trypticase soy agar</td>
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<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible</td>
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<td>v/v</td>
<td>volume/volume</td>
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<td>vs.</td>
<td>versus</td>
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<td>w/v</td>
<td>weight/volume</td>
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<tr>
<td>WGS</td>
<td>whole-genome sequencing</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZOI</td>
<td>zone of inhibition</td>
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CHAPTER 1

Introduction
1.1 A little bit of antibiotic history

BACK in the “pre-antibiotic era”, the endless tug of war between human beings and microorganisms that cause infections and disease was characterized by some major human advances, including the implementation of sanitation, but mainly by the devastating consequences of infectious disease on human development. It appeared that humans had finally defeated bacterial pathogens and revolutionized the world by discovering antibiotics. Indeed, the discovery of penicillin in 1928, and the isolation of streptomycin in 1943, launched the “golden age of discovery of natural antibiotics” (1940-1960). During the following two decades, several new classes of natural antibiotics were discovered by mining the specialized metabolism of microorganisms. This era was replaced with the “golden age of antibiotic medicinal chemistry” (1960-1980), during which semisynthetic antibiotics were designed by means of chemical modifications of natural platforms (Wright, 2007; Walsh and Wencewicz, 2014; Brown and Wright, 2016). The introduction and development of antibiotics has had an enormous impact on human life. Antibiotics literally have been the lifesavers. Nowadays, antibiotics represent the major therapeutic tool to control and treat a variety of infectious diseases and are a key foundation stone of modern medicine, allowing it to accomplish advanced surgical procedures.

Bacterial pathogens have, however, launched a counter-revolution. As microbes have always possessed the intrinsic tools to engineer resistance, almost simultaneously with the introduction of an antibiotic drug, a new resistant strain was emerging (Figure 1.1). “It didn’t take very long for bacteria to figure out how to outsmart us,” said Dr. Robert Weinstein. While the emergence of resistance in bacteria has been sharply increasing with increasing antibiotic use, the pace of antibiotic discovery has been decreasing since the 1960s. There are several reasons for this, including the challenge of finding new natural drugs, the shift in focus to chemical modification, the lack of industry productivity, competition on drug pricing, challenging regulatory requirements and others (Projan and Shlaes, 2004; Wright, 2007). Moreover, the use, misuse and abuse of antibiotics have led to the phenomenon of multidrug-resistant bacteria – the inhabitants of the “resistance era” – a case of natural selection played fast forward. The rise of “superbugs” leaves the worldwide clinical community with very few options to treat infectious bacterial
diseases. The World Health Organization (WHO) report (2014) has suggested that the world is headed for the “post-antibiotic era”. WHO’s Assistant Director-General for Health Security Dr. Keiji Fukuda warned: "A post-antibiotic era – in which common infections and minor injuries can kill – far from being an apocalyptic fantasy, is instead a very real possibility in the 21st century". In fact, for some patients and pathogens, we are already there. The situation is even more terrifying because superbugs may gain virulence characteristics and cause epidemics (Fernandes, 2006).

Figure 1.1. The timeline of antibiotic introduction and reported resistance. Inhibitors of: cell wall synthesis ( ), nucleic acid synthesis ( ), protein synthesis ( ) and cell membrane integrity ( ). Data taken from Centers for Disease Control and Prevention (CDC, 2013).
1.2 Antibiotics “at work”: Bacterial targets

To understand why bacteria become resistant to antibiotics, a brief description of how antibiotics work is required. Antibiotics inhibit certain elements of the physiology and biochemistry of the microbe causing cell death (bactericidal) or preventing growth (bacteriostatic). Since the discovery of antibiotics, we have learned much about how they work on bacteria. The main classes of antibiotics inhibit five classical targets [Figure 1.2; reviewed in (Džidić et al., 2008; Kohanski et al., 2010b; Fair and Tor, 2014)].

Figure 1.2. Modes of action of antibiotics and biochemical mechanisms of resistance. Major mechanisms of antibiotic action include: inhibiting cell wall synthesis, membrane integrity, protein synthesis, nucleic acid synthesis and essential metabolite synthesis. A few examples for each class are given. The major biochemical mechanisms of bacterial resistance include: stopping the entrance of antibiotic into the cell; pumping the antibiotic out of the cell; modifying the antibiotic target; and modifying the antibiotic itself.

(i) Cell wall synthesis. β-lactam antibiotics, such as penicillins, carbapenems and cephalosporins, block the cross-linking of peptidoglycan units by inhibiting the peptide bond formation reaction catalyzed by transpeptidases (also known as penicillin-binding proteins). Glycopeptides (vancomycin, teicoplanin, oritavancin)
target the bacterial cell wall by binding to the D-alanyl-D-alanine termini of the peptidoglycan chain, thereby preventing the addition of new units to peptidoglycan.

(ii) **Cell membrane integrity.** Polymyxins (polymyxin B, colistin) exert their antibacterial effect by increasing bacterial membrane permeability and causing a leakage of the bacterial content. Daptomycin affects structural integrity via its ability to be inserted into the cell membrane and induce depolarization.

(iii) **Protein synthesis.** Aminoglycosides (gentamicin, tobramycin, amikacin, streptomycin, kanamycin) target the 30S ribosomal subunit, most commonly, the A-site within the 16S rRNA, causing a misreading of the genetic code and perturbing the elongation of the nascent polypeptide chain. Tetracyclines (chlortetracycline, oxytetracycline) bind to the 30S subunit of the ribosome blocking the ribosome-tRNA interaction and again preventing the elongation. Macrolides (erythromycin, tylosin) reversibly bind to the 50S subunit of the ribosomes inhibiting the transpeptidation and translocation processes and interfering with the elongation of nascent polypeptide chains. Chloramphenicol irreversibly binds to the 50S ribosomal subunit blocking peptidyl transferase reaction and preventing protein chain elongation.

(iv) **Nucleic acid synthesis.** Quinolones (nalidixic acid, oxolinic acid, ciprofloxacin, levofloxacin, gemifloxacin) bind with type II and IV topoisomerase-DNA complex, induce DNA breaks and disrupt DNA replication. Rifampicin interferes with a DNA-directed RNA polymerase, thus inhibiting RNA synthesis.

(v) **Essential metabolite synthesis.** Sulfonamides (such as sulfamethoxazole) and trimethoprim each block one of the key steps in folic acid synthesis, which is a cofactor in the biosynthesis of nucleotides, the building blocks of DNA and RNA.

1.3 **Bacteria “strike back”: Antibiotic resistance**

Antibiotic/antimicrobial resistance is the ability of microbes to resist the effects of drugs, meaning the microbes are not killed, and their growth is not stopped (CDC, 2015). Antimicrobial resistance is currently one of the biggest threats to global health, therefore, it is important to understand and study the mechanisms, emergence and spread of antibiotic resistance.
1.3.1 Biochemistry of resistance

Though bacteria acquire resistance through a variety of processes, resistance is performed by bacteria employing only a few biochemical mechanisms. An overview of these mechanisms is presented in Figures 1.2 and 1.3, and brief descriptions, and examples, of the mechanisms are given below. Note that, these are examples and not an exhaustive review, as antibiotic resistance more often will occur via the combination of multiple mechanisms.

(i) Stop the entry of antibiotic. Certain changes in bacterial membrane permeability make it more difficult for the antibiotic to pass through. In this way, less of the antibiotic gets into the bacterial cell to exert its effect. For example, antibiotics, such as β-lactams, chloramphenicol and quinolones, use porins to penetrate through the outer membrane of Gram-negative bacteria. Thus, changes resulting in porin loss, copy number, size or selectivity will alter the rate of diffusion of these antibiotics and increase the level of resistance (Nikaido, 2003; Džidić et al., 2008; Blair et al., 2015).

(ii) Pump the antibiotic out. Active efflux pumps can transport antibiotics out of the bacterium, thus lowering the antibiotic concentration inside the cell and limiting the access of the antibiotic to its target. Efflux pumps affect all classes of antibiotics [reviewed in detail in (Poole, 2005)]. Although some efflux pumps are drug-specific, many efflux systems are multidrug transporters, capable of expelling a wide variety of structurally dissimilar drugs, thus contributing to the phenomenon of multidrug resistance (Džidić et al., 2008; Blair et al., 2015).

(iii) Modification of antibiotic target. This is one of the most prominent mechanisms of resistance. A change in the target of the antibiotic by mutation will change the binding affinity of the antibiotic to the target. For example, fluoroquinolone antibiotics, such as levofloxacin, target the type II topoisomerase (gyrase) involved in the relaxation of supercoiled DNA at replication forks and in separation of daughter strands during bacterial DNA replication. Single mutations at positions 83 or 87 (Escherichia coli numbering) in gyrA provide a high level of resistance (Wright, 2011). The target modification can also be catalyzed by enzymes. For example, erythromycin ribosome methylase methylates 16S rRNA and alters the
drug-binding site, thus inhibiting the binding of macrolides, lincosamines and streptogramins (Blair et al., 2015).

(iv) Direct modification of antibiotic. Bacteria can produce enzymes that destroy, or modify, the antibiotic itself, thereby resisting their action. The enzyme-catalyzed modification of antibiotics is arguably the major strategy in bacterial countermeasures to thwart “enemy” action (Wright, 2011). Biochemical strategies include hydrolysis, group transfer and redox mechanisms. The classical hydrolytic enzymes are the β-lactamases that cleave the β-lactam ring of the penicillin and cephalosporin antibiotics. Other enzymes, such as transferases, inactivate antibiotics (aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin) through chemical substitution (adenylyl, phosphoryl or acetyl groups are added to the periphery of the antibiotic molecule) by this affecting their binding to a target. The oxidation or reduction of antibiotics is the least reported, but one example is the oxidation of tetracycline antibiotics by the TetX enzyme (Džidić et al., 2008).

Figure 1.3. Schematic representation of the biology of the antimicrobial resistance, showing the biochemical and genetic aspects of resistance. Figure was redrawn after Džidić et al., 2008.
1.3.2 Genetics of resistance

Figure 1.3 summarizes the biochemical and genetic aspects of antibiotic resistance. From the genetic point of view, resistance to a certain antibiotic can be an intrinsic property of the bacterium or, can be acquired. The intrinsic resistance of a bacterial species to a particular antibiotic is the ability to resist the action of that antibiotic as a result of inherent structural or functional characteristics (Blair et al., 2015). For example, the glycopeptide antibiotic, vancomycin, is effective only against Gram-positive bacteria, as in Gram-negative organisms it cannot cross the outer membrane and access its target D-alanyl-D-alanine peptide in peptidoglycan.

Bacteria also can acquire resistance either by: (i) de novo mutations in their genome, which are passed on to subsequent generations (vertical gene transfer); or by: (ii) genetic exchange with other bacteria (horizontal gene transfer).

(i) Resistance by mutation. Bacteria are continuously evolving and sometimes mutations in their genomes can cause them to become resistant to a particular antibiotic. Mutational resistance can occur randomly through replication errors or an incorrect repair of a damaged DNA in actively dividing cells; these are called growth-dependent or spontaneous mutations (Džidić et al., 2008). Many biochemical mechanisms of antibiotic resistance are based on mutational events (Eliopoulos and Blázquez, 2003). Specifically, resistance to some antibiotics emerges by the mutation in the genes encoding their targets, rendering the antibiotic unable to inhibit its target (for example, rifamicin and fluoroquinolone resistance via mutations in the genes rpoB and gyrA, respectively). Changes in gene expression of antibiotic uptake, or efflux, systems may also result from mutations, leading to increased resistance to antibiotics (for instance, mutations leading to reduced expression or absence of the OprD porin of Pseudomonas aeruginosa reduce the permeability of the cell wall to carbapenems). Uptake and efflux-associated resistant phenotypes are also caused by mutations in regulatory genes or their promoter regions. Likewise, overproduction of antibiotic-modifying enzymes may result from mutational events (for example, mutations in chromosomal ampC leading to the overproduction of β-lactamases result in resistance to cephalosporins).
When a microbial population is subjected to certain non-lethal selection, however, useful mutants arise among the non-growing cells, while useless mutants do not. This phenomenon, known as *adaptive, directed or selection-induced mutation*, challenged the traditional understanding that mutations only arose randomly and independently from an exposure to a selective pressure (Foster, 1993). Thus, antibiotic resistance can also emerge by *adaptive mutation* in the presence of non-lethal antibiotic pressure that favors them (Krasovec and Jerman, 2003). In fact, adaptive mutation can make a major contribution to the emergence of antibiotic resistance. Sub-lethal levels of antibiotics can generate genetic changes in several ways, such as the production of reactive oxygen species (ROS) within the bacterial cell, direct DNA damage and induction of error-prone DNA polymerases mediated by SOS response [reviewed in (Rodríguez-Rojas et al., 2013)]. The production of ROS has been proposed as a common mechanism in antibiotic-mediated killing (Kohanski et al., 2007). ROS can cause DNA damage directly or indirectly, which if not repaired, lead to the accumulation of mutations. Treatment of bacteria with diverse classes of bactericidal antibiotics at sub-lethal doses stimulated the production of ROS, and induced mutagenesis leading to multidrug resistance (Kohanski et al., 2010a). Another mechanism is through activation of the SOS response. Several functionally unrelated antibiotics induce the SOS system, which in turn triggers the expression of error-prone DNA polymerases able to bypass DNA lesions with reduced fidelity, thus fueling the mutagenesis.

Antibiotic sub-lethal pressure also selects for bacteria with an elevated mutation rate (“hypermutators” or “mutators”). Hypermutation is mainly produced by alterations in the genes of methyl-directed mismatch repair (MMR) system (*mutS, mutL, mutH* and *uvrD*). The MMR system controls the fidelity of DNA replication by eliminating biosynthetic errors. It was suggested that the *hypermutation* is another factor contributing to antibiotic resistance (Eliopoulos and Blázquez, 2003). Acquisition of a mutator phenotype will increase the chances of acquiring antibiotic resistance by mutational events. Hypermutators may also enable multidrug-resistant phenotypes.

(ii) *Resistance by genetic exchange.* Though antibiotic resistance can be acquired via spontaneous mutations, adaptive mutations and hypermutations, there is an
alternative process, key for the rapid spread of multidrug resistance. Resistance can occur by acquisition of resistance conferring genes via horizontal gene transfer, which is considered to be the most important factor in the current pandemic of antimicrobial resistance (von Wintersdorff et al., 2016). Of the various mechanisms that may facilitate horizontal gene transfer, conjugation is the most commonly studied. Conjugation is the transfer of DNA through a process requiring cell-to-cell contact via cell surface pili or adhesins. The acquired material can be incorporated into the recipient’s chromosome or the plasmid. Another way that bacteria acquire new genes is to look in their neighbors’ body when they die and degrade. This process is known as transformation or as some pathologists have dubbed it “the funeral grab”. During this process, bacteria are in a state of competence when they can scavenge foreign pieces of DNA, sometimes, with ready-to-go antibiotic resistance genes. The third route by which bacteria share genetic material is via passing viruses, a process known as transduction. During this activity viruses transfer genes, among others antibiotic resistance, from one bacterium to another. Subsequently, acquired genes can be passed to the progeny by vertical gene transfer or passed on to other species via horizontal gene transfer spreading the antibiotic resistance.

1.4 Antibacterial systems from nature: Peroxidase-catalyzed systems

“There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring ’fastness’ (resistance).”

—Alexander Fleming, 1946

History has shown that the introduction of any novel antibiotic has been rapidly followed by the emergence and spread of resistance (Figure 1.1). In fact, resistance has emerged to every antibiotic class introduced to date. The antibiotic resistance crisis spurred global efforts to develop novel antibacterial alternatives. Naturally occurring peroxidase-catalyzed systems are among the potential candidates. In mammals, phagocytic cells, such as neutrophils, monocytes and eosinophils, as well as exocrine secretions, such as saliva and milk, contain peroxidase-catalyzed systems that comprise part of the innate host defense system. In the peroxidase system, a
particular peroxidase enzyme catalyzes the oxidation of a halide/pseudohalide, at the expense of hydrogen peroxide (H$_2$O$_2$), to generate reactive products with broad antimicrobial properties (Kussendrager and van Hooijdonk, 2000).

1.4.1 Peroxidase-mediated antimicrobial systems of phagocytic cells

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

Myeloperoxidase system of neutrophils. Neutrophils are the first responders of host defense towards invading bacteria and fungi, and MPO is the key component of neutrophils’ antimicrobial armory (Davies et al., 2008). Neutrophils are rich in MPO, which constitutes approximately 5% of the total neutrophil protein and occurring in the cytoplasmic granules at very high concentrations, making up about 25% of the granule proteins (Segal, 2005).

Circulating neutrophils are passive, but they can be quickly activated by components of opsonized bacteria, which bind to the receptors of neutrophils and trigger phagocytic machinery. The binding increases the oxygen uptake, called respiratory burst, and triggers the activation of membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which reduces the molecular oxygen to superoxide radical (O$_2^-$). O$_2^-$ is further converted to H$_2$O$_2$ spontaneously or catalyzed by the superoxide dismutase (Klebanoff, 1991; Henderson and Heinecke, 2003). Although, H$_2$O$_2$ can be directly toxic to microorganisms, its reactivity is increased many orders of magnitude by another mechanism. Neutrophils employ MPO and H$_2$O$_2$ generated during the respiratory burst to oxidize preferentially chloride (Cl$^-$) to hypochlorous acid (HOCl) as the initial product. HOCl is in equilibrium with hypochlorite ion and can react with excess Cl$^-$ to generate chlorine (Klebanoff, 1991). Bacterial targets of these powerful bleaching agents include iron-sulfur proteins, membrane transport proteins, adenosine triphosphate (ATP)-generating systems and the origin of replication site for DNA synthesis, which appears to be the most sensitive (Hampton et al., 1998). The process of MPO-mediated bacterial killing of neutrophil is illustrated in Figure 1.4.
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**Figure 1.4.** Neutrophil killing of bacteria is mediated by the myeloperoxidase system. The bacterium is opsonized and thus recognized by a neutrophil. The pathogen is then engulfed (phagocytosis), triggering the respiratory burst, and killed within the phagosome by the bleaching agents formed via the “toxic triad” (MPO/H$_2$O$_2$/Cl$^-$).

In general, MPO also oxidizes iodide (I$^-$) and bromide (Br$^-$) halides, but not fluoride (F$^-$), and the pseudohalide thiocyanate (SCN$^-$). Cl$^-$ is considered as the main physiological substrate for MPO because of its occurrence at high concentrations in plasma (100 – 140 mM), whereas, SCN$^-$ (20 – 120 μM), Br$^-$ (20 – 100 μM) and I$^-$ (<1 μM) are present in relatively low concentrations (Thomas and Fishman, 1986; van Dalen et al., 1997; Furthmüller et al., 1998).

*Myeloperoxidase system of monocytes.* Monocytes share the phagocytic function of neutrophils, but they also play a central role in the coordination of innate and adaptive immunity, presenting the antigens to T lymphocytes. Monocytes eventually leave the bloodstream and become tissue macrophages attacking microorganisms. They function also as scavengers removing dead cell debris. Monocytes contain
considerably less peroxidase than neutrophils (one third of the peroxidase in neutrophils), and their enzyme is structurally and functionally related to MPO (Lehrer, 1969; Klebanoff, 1991)

Monocytes, as neutrophils, respond to stimulation by the respiratory burst, forming \( \text{H}_2\text{O}_2 \). MPO and \( \text{H}_2\text{O}_2 \) oxidize \( \Gamma^- \) to form iodinating species (a more detailed discussion on iodinating species follows in Section 1.4.6), and oxidize \( \text{Cl}^- \) to chlorinating agents, although in general, not at the same level as in neutrophils (Klebanoff, 1991). When monocytes transform to macrophages they lose their granule MPO and do not utilize MPO/\( \text{H}_2\text{O}_2 \)-dependent antimicrobial mechanism (Klebanoff, 1991; Davies et al., 2008).

Eosinophil peroxidase system of eosinophils. Eosinophils are important in allergic reactions and host defense against parasites (Davies et al., 2008). Eosinophils, while activated, release EPO, and similar to MPO, use NADPH oxidase-derived \( \text{H}_2\text{O}_2 \) to oxidize preferentially \( \text{Br}^- \) and generate a brominating agent hypobromous acid [\( \text{HOBr} \); (DeChatelet et al., 1977)]. EPO can also oxidize \( \Gamma^- \) and SCN\(^-\), as well as \( \text{Cl}^- \) at acidic pH (Seifu et al., 2005).

1.4.2 Peroxidase-mediated antimicrobial systems of saliva

Human saliva contains two peroxidases, salivary peroxidase (SPO) and MPO. SPO is synthesized and secreted by the major salivary glands, whereas MPO is derived from neutrophils that enter the oral cavity during normal extravasation (Grisham and Ryan, 1990; Ihalin et al., 2006). In stimulated whole saliva of healthy adults, the overall concentration of SPO and MPO ranges from 2 – 13 \( \mu \text{g ml}^{-1} \) (Ihalin et al., 2006), the major proportion responsible for peroxidase-catalyzed reactions often being MPO [75%; (Thomas et al., 1994)].

The major source of \( \text{H}_2\text{O}_2 \) in the oral environment is a class of catalase-negative lactic acid bacteria, primarily Streptococci, which are the predominant microorganisms in the oral environment (Thomas and Fishman, 1986). This participant of the peroxidase-catalyzed system may be generated also endogenously, i.e. by neutrophils in the process of phagocytosis and activation of oxygen metabolism (Thomas and Fishman, 1986; Kussendrager and van Hooijdonk, 2000).
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H₂O₂ can also be supplied exogenously, by addition of H₂O₂-producing systems, such as glucose/glucose oxidase (G/GOD), which may produce a more effective antimicrobial system than in the case of directly added H₂O₂ (Kussendrager and van Hooijdonk, 2000).

Human salivary peroxidases catalyze the oxidation of halides I⁻, Br⁻, and pseudohalide SCN⁻ to respective hypo(pseudo)halites in the presence of H₂O₂. In addition, Cl⁻ is oxidized by MPO, but not by SPO. Based on the relative concentrations of halides present, it was considered that Cl⁻ was the leading substrate for MPO in all MPO-related antimicrobial systems in vivo because of its major role as a substrate for MPO in the oxidative killing of microbes in neutrophils (Ihalin et al., 2006). However, antimicrobial studies showed that SCN⁻ is more easily oxidized by MPO in saliva-like conditions. Thomas and Fishman (1986) investigated the oxidants produced by neutrophils in the presence of Cl⁻ and SCN⁻. They concluded that, under conditions similar to those of saliva, where SCN⁻ is present at 0.1 – 3 mM and Cl⁻ at 20 mM, hypothiocyanite (OSCN⁻) was the dominant oxidant formed.

### 1.4.3 Peroxidase-mediated antimicrobial systems of milk

Lactoperoxidase (LPO) is an enzyme that has been found in milk from many mammalian species, and also in many types of secretions e.g., in tears, nasal fluid, airway surface fluid, uterine luminal fluid and vaginal secretions (Ciccognani, 2006). Human milk contains two peroxidase enzymes – LPO secreted from the mammary gland and MPO originated from milk leukocytes. The relative amounts vary widely from sample to sample and depend on the stage of lactation (Pruitt et al., 1994). However, our knowledge of LPO in human milk is limited. The properties of human LPO are similar to bovine milk LPO, thus, surrogate bovine LPO has been generally used to study peroxidase-derived antimicrobial properties of milk. In bovine milk, LPO is, parallel to xanthine oxidase, the most abundant enzyme. Its concentration is approximately 30 mg l⁻¹, constituting about 0.5% of the whey proteins (Kussendrager and van Hooijdonk, 2000).

The primary role of LPO system is to protect the lactating mammary gland and the intestinal tract of the newborn infants against invading bacteria, fungi and viruses.
LPO catalyzes the oxidation of SCN$^-$, also I$^-$ and Br$^-$, but not Cl$^-$. In a way, it is important for LPO not to be able to catalyze the Cl$^-$ oxidation, because LPO occurs extracellularly in mucosal secretions and the oxidation products must not be toxic to the host organism.

Another component of LPO system, hydrogen peroxide, is not normally detected in raw milk. Many milk bacteria, Lactococci, Lactobacilli and Streptococci can produce sufficient H$_2$O$_2$ under aerobic conditions. The source of H$_2$O$_2$, as in the oral environment, can be endogenous by leukocytes in the process of phagocytosis, as well as, exogenous by the addition of H$_2$O$_2$-generating systems, such as G/GOD, sodium percarbonate, etc. (Seifu et al., 2005).

The thiocyanate anion is another major component of the LPO system. It is secreted from mammary, salivary, lacrimal and gastrimal glands. Its concentration is partly dependent on the eating and smoking habits of a human. In human milk, it has been reported that average values range from 0.021 – 0.122 mM (Pruitt et al., 1994). SCN$^-$ can originate from several sources. The major source is the detoxification reaction of cyanide (CN$^-$) by the enzyme thiosulfate cyanide sulfurtransferase, which primarily occurs in the liver. The enzyme catalyzes the transfer of a sulfur atom from thiosulfate to cyanide ion resulting in less toxic thiocyanate. Certain vegetables from Brassica cruciferae (cauliflower, cabbage, kale, etc.) contain a notable amount of thiocyanate precursors, such as the glucosinolates, which upon hydrolysis produce SCN$^-$, and/or isothiocyanate and nitriles (Pruitt et al., 1994; Kussendrager and van Hooijdonk, 2000).

### 1.4.4 Mechanism of halide oxidation

The proposed layout of reactions generating possible enzyme intermediates is illustrated in Scheme 1.1 (Furtmüller et al., 1998; Kussendrager and van Hooijdonk, 2000; Hernandez-Ruiz et al., 2001; Furtmüller et al., 2002; Davies et al., 2008). Halide oxidation starts with the reaction of peroxidase with hydrogen peroxide. The active site of the native peroxidase enzyme contains a ferric heme, which is oxidized with H$_2$O$_2$ to form compound I (Equation 1.1). Compound I is not an enzyme-substrate complex, but a reactive intermediate with a higher oxidation state (+5 compared with +3 for the native enzyme). Compound I is converted back to the
native enzyme through direct two-electron reduction of a (pseudo)halide (X⁻) generating (pseudo)hypohalous acid (HOX) (halogenation cycle; Equation 1.2). Alternatively, compound I is able to oxidize a range of reducing substrates (AH₂) by a mechanism involving two sequential single-electron steps. The first step leads to the generation of a second enzyme intermediate, compound II (oxidation state +4), which is subsequently reduced to the native enzyme by a second molecule of reducing AH₂ substrate, releasing free radicals (AH’⁻) (peroxidase cycle; Equations 1.3, 1.4). Phenolic acids, aromatic phenols, indoles, amines and sulfonates are typical reducing substrates (Veitch, 2004). It is considered that compound II is inactive in (pseudo)halide oxidation (Furtmüller et al., 1998). The agent that oxidizes the (pseudo)halide is thus compound I.

\[
\text{native enzyme} + \text{H}_2\text{O}_2 \rightarrow \text{compound I} + \text{H}_2\text{O} \quad (1.1)
\]

\[
\text{compound I} + \text{X}^- + \text{H}^+ \rightarrow \text{native enzyme} + \text{HOX} \quad (1.2)
\]

\[
\text{compound I} + \text{AH}_2 \rightarrow \text{compound II} + \text{AH}^- \quad (1.3)
\]

\[
\text{compound II} + \text{AH}_2 \rightarrow \text{native enzyme} + \text{AH}^- + \text{H}_2\text{O} \quad (1.4)
\]

**Scheme 1.1.** Generation of catalytic intermediates of the peroxidase enzyme (Furtmüller et al., 1998; Kussendrager and van Hooijdonk, 2000; Hernandez-Ruiz et al., 2001; Furtmüller et al., 2002; Davies et al., 2008).

Although the catalytic mechanism of different peroxidases is similar, they are different in their ability, or inability, to oxidize various (pseudo)halides. Compound I oxidizes halides at different rates (I⁻ > Br⁻ > Cl⁻); however, compound I of different peroxidases may have different redox potential. Thus, although all peroxidases will catalyze the oxidation of I⁻, MPO will oxidize Cl⁻, while SPO and LPO will not, and none of these enzymes is capable of oxidizing F⁻. The pseudohalide SCN⁻ will be oxidized also by LPO, MPO and SPO. The reason behind this variation needs to be clarified, but it is considered that they are due to significant variation in oxidation potential of different peroxidase-peroxide derivatives, rather than limitations on the peroxidase-donor interactions (Morrison and Schonbaum, 1976).
1.4.5 Oxidants generated by oxidation of thiocyanate with MPO, EPO and LPO, their antibacterial mechanism of action, bacterial resistance and mammalian cytotoxicity

Peroxidase oxidation of SCN\textsuperscript{−}. MPO, EPO and LPO are all able to convert SCN\textsuperscript{−} into products with antibacterial activity. In particular, the chemistry of SCN\textsuperscript{−} oxidation by LPO has been widely studied. The chemical species which are responsible for the antimicrobial activity of the LPO/H\textsubscript{2}O\textsubscript{2}/SCN\textsuperscript{−} system is assumed to be one or even more of the thiocyanate oxidation products (Modi et al., 1991).

The proposed mechanism for the peroxidase-catalyzed oxidation of SCN\textsuperscript{−} is depicted in the Scheme 1.2 (Aune and Thomas, 1977; Thomas and Aune, 1978b; Thomas, 1981; Pruitt et al., 1982). Oxidation can go in two different ways, resulting in intermediate oxidation products, which are responsible for the antimicrobial activity. The first pathway is oxidation of SCN\textsuperscript{−} yielding thiocyanogen (SCN\textsubscript{2}) (Equation 1.5), which is unstable in aqueous solution and rapidly hydrolyzes to hypothiocyanous acid (HOSCN) and its ion, hypothiocyanite (OSCN\textsuperscript{−}) (Equations 1.6, 1.7). HOSCN exists in equilibrium with OSCN\textsuperscript{−} (Equation 1.7). The pKa (the negative log of the acid dissociation constant – the greater the value of pKa, the smaller the degree of dissociation) of HOSCN is 5.3, indicating that conjugate base hypothiocyanite predominates in most physiologic fluids (Chandler and Day, 2012). Alternatively, the second pathway is the direct production of OSCN\textsuperscript{−} (Equation 1.8). Nevertheless, OSCN\textsuperscript{−} is the major product observed over SCN\textsuperscript{−} oxidation at neutral pH (Thomas, 1981). For ease of reading, within this thesis the terms OSCN\textsuperscript{−} or HOSCN will be used interchangeably to represent the physiological mixture composed of OSCN\textsuperscript{−}/HOSCN.

The reaction profile is, however, complex and depending upon reaction conditions other short-lived intermediates may be formed in varying amounts. Pruitt et al. (1982) reported that, at neutral pH, addition of excess H\textsubscript{2}O\textsubscript{2} or LPO/H\textsubscript{2}O\textsubscript{2} to an OSCN\textsuperscript{−} generated from the LPO system results in the formation of other highly reactive, short-lived antimicrobial products in addition to OSCN\textsuperscript{−}, which represent higher oxy acids of SCN\textsuperscript{−} (O\textsubscript{2}SCN\textsuperscript{−} and O\textsubscript{3}SCN\textsuperscript{−}) (Equations 1.9, 1.10). The authors
assumed that these higher oxy acids would have better oxidizing properties and, accordingly, would be more effective microbial inhibitors than OSCN$^{-}$.

$$H_2O_2 + 2SCN^- + 2H^+ \xrightarrow{peroxidase} (SCN)_2 + 2H_2O \quad (1.5)$$

$$(SCN)_2 + H_2O \rightarrow HOSCN + H^+ + SCN^- \quad (1.6)$$

$$HOSCN \rightleftharpoons H^+ + OSCN^- \quad (1.7)$$

$$H_2O_2 + SCN^- \xrightarrow{peroxidase} OSCN^- + H_2O \quad (1.8)$$

$$OSCN^- + H_2O_2 \rightarrow O_2SCN^- + H_2O \quad (1.9)$$

$$O_2SCN^- + H_2O_2 \rightarrow O_3SCN^- + H_2O \quad (1.10)$$

**Scheme 1.2.** Reactions of peroxidase-catalyzed oxidation of SCN$^-$ (Aune and Thomas, 1977; Thomas and Aune, 1978b; Thomas, 1981; Pruitt et al., 1982).

Chung and Wood (1970) proposed that the species responsible for the antibacterial activity of LPO/H$_2$O$_2$/SCN$^-$ system may be the cyanide ion. Subsequently, Modi and co-authors (1991) reported that CN$^-$ can be formed at a ratio of [H$_2$O$_2$]/[SCN$^-$] > 2, which was confirmed by $^{15}$N NMR method and by changes in the optical spectrum of LPO. The authors showed that the activity of the system was at its maximum when [H$_2$O$_2$]/[SCN$^-$] ratio was 1 at a pH of 6. The formation of OSCN$^{-}$ was also observed to be the greatest when the ratio of [H$_2$O$_2$]/[SCN$^-$] was 1 at pH < 6. They concluded that the potential bactericidal or bacteriostatic activity of the LPO/H$_2$O$_2$/SCN$^-$ system may be related to the formation of HOSCN/OSCN$^{-}$ species rather than CN$^-$ and (SCN)$_2$, since these species were not present in solution when the bactericidal activity of the system was maximum [i.e. when the [H$_2$O$_2$]/[SCN$^-$] was 1; (Modi et al., 1991)].

*Mechanism of action of peroxidase-oxidized SCN$^-$. The key to the antimicrobial action of the LPO/H$_2$O$_2$/SCN$^-$ system is the oxidation of sulphydryl (SH) groups of microbial proteins and other low molecular weight species of the cytoplasmic thiol pool. The reaction products HOSCN, (SCN)$_2$ and OSCN$^{-}$ react rapidly with sulphydryl groups yielding sulfenyl thiocyanate derivatives (R-S-SCN) (Scheme 1.3;
Equations 1.11 – 1.13). The R-S-SCN can react with another sulfhydryl group to form a disulfide bond (Equation 1.14; though steric constraints may prevent this reaction in proteins) or can undergo further modification, such as reversible hydrolysis to yield sulfenic acid (Equation 1.15).

\[
\text{OSCN}^- + \text{R-SH} \rightarrow \text{R-S-SCN} + \text{OH}^- \quad (1.11)
\]

\[
\text{HOSCN} + \text{R-SH} \rightarrow \text{R-S-SCN} + \text{H}_2\text{O} \quad (1.12)
\]

\[
(\text{SCN})_2 + \text{R-SH} \rightarrow \text{R-S-SCN} + \text{SCN}^- + \text{H}^+ \quad (1.13)
\]

\[
\text{R-S-SCN} + \text{R-SH} \rightarrow \text{R-S-S-R} + \text{SCN}^- + \text{H}^+ \quad (1.14)
\]

\[
\text{R-S-SCN} + \text{H}_2\text{O} \rightleftharpoons \text{R-S-OH} + \text{SCN}^- + \text{H}^+ \quad (1.15)
\]

**Scheme 1.3.** Oxidation of peptide/protein sulfhydryls by oxidation products of SCN\(^-\) (Thomas and Aune, 1978b).

Not all sulfhydryls are, however, sensitive to SCN\(^-\) oxidation products; cysteine (Cys), mercaptoethanol, dithiothreitol, glutathione (GSH), 5-thio-2-nitrobenzoic acid (TNB) and albumin are all readily oxidized, but β-lactoglobulin is poorly oxidized. HOSCN, in addition to sulfur species, can oxidize selenol species, thus selenocysteine residue in proteins is also a target. Under some conditions, such as the presence of LPO, sufficient H\(_2\)O\(_2\) and SCN\(^-\), and when all the sulfhydryls are oxidized, modification of other targets like aromatic amino acid residues (tyrosine, tryptophan, and histidine) can also occur (Pattison et al., 2012; Bafort et al., 2014).

When microbial cytoplasmic membranes are damaged by the oxidation of SH-containing proteins, it leads to the leakage of potassium ions, amino acids and polypeptides. Similarly, uptake of amino acids, purines and pyrimidines by the cell, and, thus, the synthesis of proteins, DNA and RNA are also impeded (Reiter and Härmulv, 1984). However, unlike oxidants like H\(_2\)O\(_2\) and superoxide, OSCN\(^-\) has not been reported to cause DNA damage (Sermon et al., 2005). The alteration of bacterial cell membranes and transporters also hinder glucose and oxygen uptake, thus inhibiting the glucose transport and respiration. The antimicrobial species generated by LPO-catalyzed oxidation of SCN\(^-\) can also inhibit critical cysteines in
several glycolytic enzymes, such as hexokinase, glyceraldehyde-3-phosphate dehydrogenase (GADPH), aldolase and glucose-6-phosphate dehydrogenase (Naidu, 2000). Indeed, it has been hypothesized that HOSCN’s effect on bacterial growth is glycolysis-mediated (Chandler and Day, 2012).

**Bacterial resistance to peroxidase-oxidized SCN**. Despite the importance of the peroxidase/SCN systems in host defense against pathogens, the bacterial stress response and resistance mechanisms to this specific form of oxidative stress have not been exhaustively characterized (Sermon et al., 2005). Bacterial resistance to LPO/SCN- mediated killing has been reported to occur via enzymes and substrates that inactivate and reduce the generated oxidizing agents, as well as by reversing the oxidation of sulfhydryl groups (Madureira et al., 2007). The availability of H2O2 is an important factor for peroxidase-mediated oxidant generation, thus, bacteria which produce H2O2-consuming enzymes (e.g. catalase) show low susceptibility to peroxidase/SCN- mediated inhibition (Davies et al., 2008).

Peroxidase/SCN- systems can cause both reversible and irreversible effects, displaying bacteriostatic and bactericidal activity. Several factors influence their reversible versus irreversible character. Irreversible inhibition is associated with long-term incubation, high OSCN- concentrations and particular bacterial species. Increased concentrations of reducing agents, such as glutathione and cysteine, can reverse the inhibition through buffering OSCN- and can ease the oxidative damage by reversing sulfhydryl modification. It was shown that, in *E. coli*, under the stress induced by LPO system, cysJ was activated (Sermon et al., 2005). Together with CysI, CysJ forms the NADPH-dependent sulfite reductase, which catalyzes a key step in the production of SH compounds such as cysteine, glutathione and coenzyme A. The authors suggested that this operon was induced to boost the *E. coli*’s own sulfhydryl production and that the activation of the sulfite reduction pathway was involved in the protective response against the LPO-mediated challenge (Sermon et al., 2005). Earlier, Mickelson and Anderson (1984) reported that a NADPH-dependent cystine reductase may account for increased resistance against the LPO system in *Streptococcus agalactiae*. Another resistance mechanism was used by *S. cremoris*, which recruited NADH2 oxidase enzyme to catalyze the oxidation of HADH2 with OSCN- by this lowering the inhibitory effect. Another bacterial strain,
Chapter 1

*S. sanguinis* had developed high expression level of NADH-OSCN oxidoreductase that reduced OSCN\(^{-}\) back to SCN\(^{-}\) (Naidu, 2000).

**Mammalian cell cytotoxicity of peroxidase-oxidized SCN\(^{-}\).** There are numerous reports that a variety of dissimilar cells are subject to peroxidase-mediated toxicity. A peroxidase together with H\(_2\)O\(_2\) and a halide (dubbed as “cytotoxic triad”) can exert toxic effects *in vitro* on bacteria, fungi, viruses, tumor cells, erythrocytes, sperm cells and many other mammalian cells. This non-specific toxicity of peroxidase systems is not surprising, given their activity is expressed by hypohalous acids/hypohalite, which are small, inorganic, highly reactive molecules and do not have a preference for reacting with one cell type over the other (Everse et al., 1990). However, long-held debate exists as to the mammalian cytotoxicity of peroxidase/SCN\(^{-}\) system. This system is often regarded as a mild alternative to other peroxidase products, particularly HOCl and HOBr, based on the following characteristics: (i) conversion of SCN\(^{-}\) to HOSCN by a peroxidase is viewed as a detoxification mechanism to remove potentially more damaging oxidants, such as H\(_2\)O\(_2\) and HOCl; (ii) its component SCN\(^{-}\) is a scavenger, in particular for HOCl and HOBr, under physiological conditions; (iii) its product HOSCN reacts preferentially with free sulfhydryl groups (free Cys) (Barrett and Hawkins, 2012).

The literature indicates that the peroxidase/SCN\(^{-}\) system is specifically safe to mammalian cells, which are associated with the oral cavity and the airway. The oxidants generated from this system inhibit bacteria, fungi and viruses in the oral cavity, while at the same time the conversion of SCN\(^{-}\) to HOSCN limits the production of potentially tissue damaging oxidizing species, such as H\(_2\)O\(_2\) and HOCl. Similarly, in airways this system facilitates the bacterial clearance and scavenges deleterious oxidative species (Pattison et al., 2012).

However, other evidence shows that peroxidase/SCN\(^{-}\)-derived oxidants are cytotoxic to other mammalian cells like erythrocytes, macrophages and endothelial cells. For instance, Grisham and Ryan (1990) found that peroxidase-generated HOSCN lysed human erythrocytes at a pH of 6, oxidized the hemoglobin to methemoglobin, and together with OCSN\(^{-}\) oxidized also the glutathione. As with bacterial cells, HOSCN can target critical thiol proteins with high specificity also in mammalian cells. In another study, red blood cells exposed to increasing
concentrations of EPO/H2O2/SCN− oxidation products were first depleted of GSH, after which GSH S-transferase, GADPH and ATPases underwent SH reductant-reversible inactivation, building up the hemolysis. The oxidants inactivated red blood cell membrane ATPases 10 – 1000 times more potently than either HOCl, HOBr and H2O2 did (Arlandson et al., 2001). Similarly, Lloyd et al. (2008) showed that HOSCN induced apoptosis and necrosis of macrophages (J774A.1) with greater efficacy and at lower concentrations than HOCl or HOBr, due to selective targeting of critical thiol residues on mitochondrial membrane proteins. Love et al. (2016) reported that the cellular targets of HOSCN in J774A.1 macrophages were multiple thiol-containing proteins involved in metabolism and glycolysis (fructose bisphosphate aldolase, triosephosphate isomerase, GAPDH and creatine kinase), together with a number of chaperones, antioxidants and structural proteins. They concluded that the ability of HOSCN to inhibit glycolysis and perturb energy production contributed to the cell death. HOSCN also induced apoptosis of human coronary artery endothelial cells by the increase of mitochondrial membrane permeability, the release of cytochrome c from the mitochondria and externalization of phosphatidylserine (Barrett and Hawkins, 2012).

1.4.6 Oxidants generated by oxidation of iodide with/without MPO, EPO and LPO, their antibacterial mechanism of action, bacterial resistance and mammalian cytotoxicity

Oxidation of I−. Iodide ion too can be oxidized by MPO, SPO and LPO. I− is the most readily oxidizable of all halides in vitro, and the peroxidase-catalyzed oxidation of I− yields in molecular iodine (I2) and, depending on I− concentration and pH, hypoiiodous acid (HOI), hypoiodite (OI−) or other iodine species are also present (Kussendrager and van Hooijdonk, 2000). Some authors have suggested that I2 is the major agent, which is able to damage the cells (Thomas and Aune, 1977; 1978c; a), but HOI/OI− has also been identified as possessing antimicrobial properties (Galley et al., 1997; Ihalin et al., 1998; Bosch et al., 2000; Ihalin et al., 2003). However, the active agent responsible for peroxidase/I−-mediated bacterial killing is believed to be a mixture of iodine species that are not fully detailed due to the complex iodine chemistry (Bafort et al., 2014).
Chemists earlier suggested that the reaction between iodide and hydrogen peroxide (without any peroxidase) takes place through a series of short-lived intermediates (such as HOI, OI\(^-\)) and observable intermediates [I\(_2\), I\(_3^-\); (Liebhafsky, 1934)]. Oxidation of \(I^-\) by H\(_2\)O\(_2\) involves two reactions, one slow and one fast: the first reaction forming HOI (Equation 1.16) and the second forming free iodine by the reaction of the hypoiiodous acid with more iodide ion (Equation 1.17). The slowness of the first reaction controls the overall rate (McAlpine, 1945). Triiodide ions (I\(_3^-\)) also can be present in equilibrium with iodide anion and molecular iodine (Equation 1.18).

\[
\begin{align*}
H_2O_2 + I^- + H^+ &\rightarrow HOI + H_2O & (1.16) \\
HOI + I^- + H^+ &\rightleftharpoons I_2 + H_2O & (1.17) \\
I_2 + I^- &\rightleftharpoons I_3^- & (1.18)
\end{align*}
\]

Gottardi (1999), on the other hand, specified ten iodine species (I\(_2\), I\(_3^-\), I\(_5^-\), I\(_62^-\), HOI, OI\(^-\), HI\(_2\)O\(^-\), I\(_2\)O\(^-\), H\(_2\)O\(_2\)\(^+\) and IO\(_3^-\)) for inorganic iodine-water system (Scheme 1.4; Equations 1.19 – 1.27):

\[
\begin{align*}
I_2 + H_2O &\rightleftharpoons HOI + I^- + H^+ \text{ (hydrolysis; 1.19)} \\
HOI &\rightleftharpoons OI^- + H^+ \text{ (dissociation of HOI; 1.20)} \\
I_2 + I^- &\rightleftharpoons I_3^- \text{ (triiodide formation; 1.21)} \\
HOI + H^+ &\rightleftharpoons H_2O I^- \text{ (protonization of HOI; 1.22)} \\
I_3^- + I_2 &\rightleftharpoons I_5^- \text{ (pentaiodide formation; 1.23)} \\
2I_3^- &\rightleftharpoons I_62^- \text{ (dimerization of I}_3^-\text{; 1.24)} \\
OI^- + I^- + H_2O &\rightleftharpoons HI_2O^- + OH^- \text{ (iodination of OI}^-\text{; 1.25)} \\
HI_2O^- &\rightleftharpoons I_2O^- + H^+ \text{ (dissociation of HI}_2\text{O}^-\text{; 1.26)} \\
3HOI &\rightleftharpoons IO_3^- + 2I^- + 3H^+ \text{ (disproportionation; 1.27)}
\end{align*}
\]

**Scheme 1.4.** Iodine species generated in iodine-water system (Gottardi, 1999).

Gottardi has emphasized that the equilibria in the system are governed by H\(^+\) and I\(^-\) ions, which imply that pH value and the additional iodide influence on species concentrations. In the most common case, with iodine in the presence of additional
iodide at pH \( \leq 6 \), only \( I_2 \) and \( I^5^- \) possess the main oxidation power. In the absence of additional iodide, at pH 8 – 9 HOI accounts for the major oxidation capacity. At high iodide concentration (e.g. Lugol’s solution), \( I^5^- \) and \( I_6^{2^-} \) species become important. \( H_2O_2^+, OI^- \) and \( HI_2O^- \) are prominent only at pH >10. \( IO_3^- \) has no oxidative activity in neutral and basic pH conditions, but can act as an oxidant in a form of \( HIO_3 \) at pH <4 (Gottardi, 1999; 2014).

**Mechanism of action of oxidized \( I^- \).** Thomas and Aune (1977) have proposed that the antimicrobial activity of the peroxidase/\( H_2O_2/I^- \) system is due to the oxidation of \( I^- \) to \( I_2 \), followed by rapid iodination of cell components, namely, protein sulphydryls. Oxidation of cell components yields in the reduction of \( I_2 \) back to \( I^- \), so that as a result \( I^- \) is not consumed. Released \( I^- \) can be reoxidized and take part again in the oxidation of other protein sulphydryls. Therefore, one iodide ion can oxidize many cell components (Thomas and Aune, 1978c). In such a way, \( I^- \) acts as a cofactor in the transfer of oxidizing equivalents from \( H_2O_2 \) to cell components (Thomas and Aune, 1978a).

Oxidation of 1 mole of sulphydryl per mole of \( H_2O_2 \) or \( I_2 \) to selenyl iodide (R-S-I) corresponds to the Equations 1.28, 1.29 in the Scheme 1.5. The release of \( I^- \) from selenyl iodide may occur through hydrolysis to yield a sulfenic acid derivative (Equation 1.30) or through disulfide formation (Equation 1.31).

\[
\begin{align*}
H_2O_2 + 2I^- + 2H^+ & \xrightarrow{\text{peroxidase}} I_2 + 2H_2O \quad (1.28) \\
R-SH + I_2 & \rightarrow R-S-I + I^- + H^+ \quad (1.29) \\
R-S-I + H_2O & \leftrightarrow R-S-OH + I^- + H^+ \quad (1.30) \\
R-S-I + R-SH & \rightarrow R-S-S-R + I^- + H^+ \quad (1.31)
\end{align*}
\]

**Scheme 1.5.** Peroxidase-catalyzed oxidation of \( I^- \), and subsequent oxidation of protein sulphydryls (Thomas and Aune, 1977; 1978c).

According to this reaction scheme, the antimicrobial action of peroxidase/\( H_2O_2/I^- \) system depends only on \( H_2O_2 \) and is independent of \( I^- \) concentration. However, the amount of \( I_2 \) formed during the first part of the sequential reactions will depend on the \( H_2O_2 \) or \( I^- \) concentrations, whichever is lower. If \( I^- \) concentration is lower, a small amount of \( I_2 \) will be formed and excess unreacted \( H_2O_2 \) will remain and fewer
cell components will be oxidized (Thomas and Aune, 1978a). Iodination and oxidation produce apparent denaturation of the proteins and enzymes, which are crucial for bacterial viability.

The mode of action of oxidized iodide resembles that of thiocyanate, but differs in the following aspects: (i) all sulfhydryl groups are oxidized by I\(^{-}\) oxidation products; (ii) oxidized I\(^{-}\) species are directed against a broader range of molecules – they can oxidize thioether (R-S-R) and amine (NH\(_2\)) moieties of proteins, NADPH and reduced nicotinamide mononucleotide (NMNH); (iii) due to the cofactor role of I\(^{-}\), a greater extent of cell components are oxidized; (iv) cells do not recover after removal of the oxidized iodide. These indicate that peroxidase/I\(^{-}\) systems have an irreversible bactericidal effect.

Gottardi (2014) has indicated that, although the exact details of microbial killing by I\(_2\) or the reaction products occurring in inorganic aqueous solutions are not known, these oxidants have following consequences: (i) oxidation of SH group of cysteine amino acid results in failure to connect protein chains by disulfide bonds, impeding protein synthesis (this is probably the most important reaction); (ii) iodination of phenolic (OH) and imidazolic (NH···N) groups of hystidine and tyrosine amino acids, and iodination of cytosine and uracil pyrimidine derivatives can increase the bulk of molecules, leading to a form of steric hindrance in hydrogen bonds; (iii) iodine can react with the carbon-carbon double bond (C=C) of unsaturated fatty acids, leading to a change in the physical properties of the lipids and cause membrane destabilization.

*Bacterial resistance to oxidized I\(^{-}\).* An extensive review of the literature suggests that evidence of resistance to peroxidase-mediated iodide oxidants is lacking. Moreover, to date, there are also no reports of resistance development against iodine-based disinfectants. Most researchers interpret this phenomenon as being due to the strong bactericidal activity, expressed by multiple modes of action, that include the disruption of microbial metabolic pathways and destabilization of cell membrane components, causing irreversible damage to the pathogen.

*Mammalian cell cytotoxicity of peroxidase-oxidized I\(^{-}\).* There are several reports on the cytotoxicity of peroxidase/I\(^{-}\) systems to normal, as well as tumor cells. It was
reported that LPO, in the presence of H$_2$O$_2$ and $\Gamma^-$, was cytotoxic for human and mouse lymphoid cells and human erythrocytes. This effect was rather rapid and highly efficient leading to 85 – 90% cell death within 90 min (Edelson and Cohn, 1973). MPO or LPO, when combined with G/GOD (as a H$_2$O$_2$-generating system) and iodide (as an oxidizable cofactor), hemolysed human erythrocytes (Klebanoff and Clark, 1975). Hemolysis of this iodide-dependent system was associated with the iodination of erythrocyte cell components (membrane proteins, hemoglobin). Clark et al. (1975) demonstrated the cytotoxicity of I$^-$-dependent system on mouse ascetic lymphoma cells using 4 cytotoxicity tests ($^{51}$Cr release, trypan blue exclusion, inhibition of glucose C-1 oxidation and loss of oncogenicity for mice). Stanislavski and co-workers (1989) used antibody/glucose oxidase/lactoperoxidase conjugate to target murine plasmacytoma tumor cells. Cytotoxicity was generated when antibody/GOD/LPO targeted cells were incubated in a medium supplemented with glucose and sodium iodide.

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

Combination of Cl$^-$ and Br$^-$. The ultimate activity of species generated by peroxidase/H$_2$O$_2$/ (pseudo)halide systems may be affected because of the reaction of the initial products with other (pseudo)halides. As an example, these set of reactions can generate transhalogen species. More than a century ago, inorganic chemists proposed the existence of interhalogens, which are combinations of different halogens. The general formula of most interhalogen compounds is $XX_n^-$, where $n = 1, 3, 5$ or $7$, and $X$ is the less electronegative of the two halogens. Both binary (BrCl, IBr, and ICl) and ternary (ICl$_3$) interhalogens have been since characterized. Anions of interhalogens and polyhalides are also known; they include Cl$_3^-$, Br$_3^-$, I$_3^-$, Br$_2$Cl$^-$, and BrCl$_2^-$. One pathway for their formation requires hypohalous acid (HOX) and halide ion (X$^-$), Equation 1.32 (Henderson et al., 2001).

$$\text{HOX} + n\text{X}^- + \text{H}^+ \rightarrow XX_n^- + \text{H}_2\text{O} \quad (1.32)$$

Employing this mechanism, HOCl can react with Br$^-$ to yield molecular bromine chloride (BrCl). Henderson et al. (2001) showed that MPO at acidic pH via reaction of HOCl and Br$^-$ generated reactive BrCl interhalogen gas that oxidized nucleobases.
Whereas, Spalteholz et al. (2006) reported a direct formation of BrCl in halide oxidation by compound I of MPO, but not a formation via hypohalous acids.

**Combination of Cl\(^{-}\) or Br\(^{-}\) with SCN\(^{-}\).** Chemically, interhalogens are extremely corrosive species that attack a wide range of compounds and can be implicated in mutagenesis, so it is critical that scavengers govern their reactive properties. One such potential scavenger is SCN\(^{-}\), an endogenous inorganic anion in human physiological fluids, with concentrations ranging from micromolar in plasma to millimolar in saliva. Ashby et al. (2004) have suggested that SCN\(^{-}\) might limit host tissue damage by restricting the lifetime of the more detrimental oxidant HOCl. They have hypothesized that SCN\(^{-}\) acts as a redox buffer via the mechanism of non-enzymatic transfer of oxidizing equivalents from HOCl to SCN\(^{-}\) and, thus, the oxidizing equivalents of HOCl are preserved in OSCN\(^{-}\), which is considered not lethal to mammalian cells (Equation 1.33).

\[
\text{HOCl} + \text{SCN}^- \rightarrow \text{OSCN}^- + \text{Cl}^- + \text{H}^+ \quad (1.33)
\]

Likewise, Nagy et al. (2006) showed that HOBr reacted rapidly with SCN\(^{-}\) to yield HOSCN, and have proposed that SCN\(^{-}\) is a highly efficient scavenger of HOBr, which limits the ability of HOBr to cause biological damage to the mammalian cells. Whether this is true remains to be established, as the formed HOSCN/OSCN\(^{-}\) may be as, or more, damaging than HOBr or HOCl. As discussed earlier, SCN\(^{-}\) oxidation species can exert considerable biological damage because they have greater specificity, particularly for thiols.

**Combination of SCN\(^{-}\) and I.** Long and Skoog (1966) showed that a complex having the probable formula I(SCN)\(_2\)^\(-\) was formed by H\(_2\)O\(_2\) oxidation of iodide ion or elemental iodine in solutions containing potassium thiocyanate. They presented the reasonable equations for those reactions as:

\[
\text{H}_2\text{O}_2 + \text{I}^- + \text{SCN}^- + 2\text{H}^+ \rightarrow \text{I(SCN)}_2^- + 2\text{H}_2\text{O} \quad (1.34)
\]

\[
\text{H}_2\text{O}_2 + \text{I}_2 + 4\text{SCN}^- + 2\text{H}^+ \rightarrow 2\text{I(SCN)}_2^- + 2\text{H}_2\text{O} \quad (1.35)
\]

Schoneshbfer and Henglein (1970) studied the transient complexes which could have been formed between the thiocyanate and halogen ions. Pulse radiolysis of
aqueous solutions containing SCN\(^-\) and I\(^-\) under oxidizing conditions led to the formation of ISCN\(^-\). During the disappearance of the various complexes in addition I\(_2\)SCN\(^-\) was formed. I\(_2\)SCN\(^-\) could be oxidized by H\(_2\)O\(_2\) in the presence of excess SCN\(^-\) and form I(SCN)\(_2\)^- (Bowmaker and Rogers, 1981). Like the I\(_2\)SCN\(^-\) ion, I(SCN)\(_2\)^- is unstable in aqueous solution, but its stability is enhanced in solutions of high acidity, high ionic strength and low temperature (Bowmaker and Rogers, 1981).

In chemical literature, the reaction between I\(_2\) and SCN\(^-\) in aqueous solution under normal conditions has been known for a while, which is characterized as:

\[
4\mathrm{I}_2 + \mathrm{SCN}^- + 4\mathrm{H}_2\mathrm{O} \rightarrow 7\mathrm{I}^- + 8\mathrm{H}^+ + \mathrm{ICN} + \mathrm{SO}_4^{2-} \quad (1.36)
\]

These literature reports all present reactions and species generated without the involvement of a peroxidase enzyme. A more recent study investigated the species formed by the LPO/H\(_2\)O\(_2\)/I\(^-\)/SCN\(^-\) system utilizing \(^{13}\)C nuclear magnetic resonance spectroscopy and gas chromatography-mass spectrometry (Schlorke et al., 2016). They identified the inter(pseudo)halogen cyanogen iodide ICN as a yet unknown LPO product. This product was also formed in MPO or an enzyme-free system. However, it remains unknown as to what extent ICN or other pointed species contribute to the antimicrobial activity of peroxidase-mediated systems.

**Antagonist versus synergist role of SCN\(^-\) and I\(^-\) combination in peroxidase-catalyzed antimicrobial activity.** In general, the use of multiple drugs with different mechanisms of action may affect multiple targets and multiple organisms simultaneously. Antibiotic combinations have been widely used to treat multidrug-resistant bacteria. The reasons why combination therapy is practiced are the following: (i) broadening antibacterial spectrum – ensures that at least one agent will cover the infecting pathogen; (ii) polymicrobial infections – require more than one antibiotic to cover all bacterial pathogens; (iii) synergy – increase the efficacy of the therapeutic effect, decrease the dosage while increasing/maintaining the efficacy and minimizing toxicity; (iv) emergence of resistance – chances of emergence of resistance against two drugs are lower as compared with a single drug (Ahmed et al., 2014). Thus, incorporating two substrates (SCN\(^-\) and I\(^-\)) simultaneously into the peroxidase/H\(_2\)O\(_2\) system, hypothetically, may generate multiple oxidants from both substrates, with multiple mechanisms of action, directed against multiple cellular
targets and multiple bacterial species, with little possibility of resistance development.

The SCN⁻/I⁻ substrate couple was investigated by several researchers who reported contradictory results about the role this coupling played in the antimicrobial action of peroxidase-catalyzed systems; some are supporters of antagonistic, the others synergistic interactions.

(i) Antagonism of the concomitant presence of SCN⁻ and I⁻ in peroxidase systems. Klebanoff (1967) observed that thiocyanate ions were inhibitory to MPO/H₂O₂/I⁻ system. Although he found this paradoxical, considering thiocyanate ions when combined with MPO and H₂O₂ exerted an antibacterial effect.

Ihalin et al. (1998) investigated the effect of both LPO and MPO systems on Actinobacillus actinomycetemcomitans with different (pseudo)halide substrates, thiocyanate, iodide, chloride and their combinations. They demonstrated that the oxidation of I⁻ had the highest antimicrobial ability followed by Cl⁻ and SCN⁻. However, the addition of SCN⁻ into either MPO/H₂O₂/Cl⁻ or MPO/or LPO/H₂O₂/I⁻ system abolished the bactericidal activity of the oxidized halide. Cl⁻, on the other hand, did not affect the bactericidal effects of the MPO/H₂O₂/I⁻ system, but when all three (pseudo)halide substrates were present, no antimicrobial effect was recorded.

Subsequently, Ihalin and co-workers (2001) studied the effects of I⁻, Cl⁻ and SCN⁻, and their combinations, with LPO and MPO on the viability of Porphyromonas gingivalis, Fusobacterium nucleatum, Streptococcus mutans and Streptococcus rattus. The oxidation products of I⁻ were again found to be the most potent, followed by the oxidation products of Cl⁻ (with MPO) and SCN⁻ (with MPO and LPO) against all the bacteria tested. The effects were much weaker on the Streptococcus species. They reported that physiological concentrations of SCN⁻ abolished the effects of LPO/H₂O₂/I⁻ or MPO/H₂O₂/I⁻ or MPO/H₂O₂/Cl⁻ combinations, whereas, Cl⁻ had no effect on MPO/H₂O₂/I⁻ system.

Ahariz and Courtois (2010) studied the susceptibility of Candida albicans to LPO/G/GOD system when both SCN⁻ and I⁻ substrates were simultaneously present. They observed that LPO/I⁻/G/GOD reduced the colony forming unit count to zero, but the addition of SCN⁻ (0.25, 2, 3 and 4 mM) progressively decreased this
antifungal effect. Thus, their results also demonstrated the competition between SCN\(^{-}\) and I\(^{-}\), or the scavenging effect of the SCN\(^{-}\).

(ii) **Synergism of the concomitant presence of SCN\(^{-}\) and I\(^{-}\) in peroxidase systems.** The incorporation of I\(^{-}\) as an additional substrate for the commercial LPO/SCN\(^{-}\)/G/GOD preservative system was carried out aiming to extend the spectrum of activity of the system – OI\(^{-}\) being much more effective against yeast and molds than OSCN\(^{-}\), and the latter efficient against bacteria (Galley et al., 1997). The authors proposed to use this antimicrobial system as a preservative or as an antimicrobial agent in oral hygiene, deodorant and antidandruff products.

Bosch et al. (2000) investigated the change in antimicrobial activity during storage of the peroxidase-catalyzed antimicrobial system, which was similar to the commercially available enzyme system described above (Galley et al., 1997) in that both SCN\(^{-}\) and I\(^{-}\) were utilized as substrates for LPO, and the system relied on G/GOD to generate the H\(_2\)O\(_2\). The effect of iodide addition to LPO/SCN\(^{-}\)/G/GOD system, the chemical stability and the change in antimicrobial effectiveness during storage was studied. The addition of I\(^{-}\) with SCN\(^{-}\) increased the fungicidal and bactericidal effect against *C. albicans*, *E. coli* and *Staphylococcus aureus* confirming the synergistic action between SCN\(^{-}\) and I\(^{-}\), with the SCN\(^{-}\)/I\(^{-}\) ratio of 10:60. Whereas, the inhibition of *P. aeruginosa* growth was at the same level when the system contained or lacked the iodide. The antimicrobial stability of the LPO system was examined over the 18-month period. In general, the aged samples showed activity that was comparable to the freshly prepared solutions, although, with some organisms, a longer contact time was needed for the aged system to exert the antimicrobial effect. Thus, the authors suggested the use of this antimicrobial complex as a preservative in foods and pharmaceuticals (Bosch et al., 2000).

In their other study, Ihalin et al. (2003) targeted the antimicrobial effect of horseradish peroxidase (HRPO)/H\(_2\)O\(_2\)/I\(^{-}\) system on *F. nucleatum*. They showed that in saliva (implying the presence of SCN\(^{-}\)) HRPO/H\(_2\)O\(_2\)/I\(^{-}\) combination reduced the number of viable bacteria to 37\%, compared to 87\% live bacteria in the saliva/HRPO/H\(_2\)O\(_2\) system. These results suggested that saliva (thiocyanate ions) did not inhibit antimicrobial activity of HRPO/H\(_2\)O\(_2\)/I\(^{-}\) system.
1.4.8 Application of peroxidase-catalyzed systems

The use of peroxidase systems as antimicrobial agents has been somewhat limited, mostly because the cost of the enzyme purification is often higher than traditional preservatives, and sourcing large quantities of enzymes can be a problem. Large-scale purification of peroxidases from human leukocytes (myeloperoxidase) or from human milk (lactoperoxidase), or from human saliva (salivary peroxidase) is difficult and very expensive for use in commercial purposes. These enzymes have been purified but only for research objectives. Instead, LPO purified from bovine milk has been extensively used for research and commercial reasons, as it is readily available and is structurally and catalytically similar to human SPO and LPO.

Lactoperoxidase has found many applications because of its broad antimicrobial activity and presence in different body fluids. LPO systems have broad-spectrum of actual and potential applications as natural biopreservatives in oral healthcare, milk industry, food, feed specialties, cosmetics, and related products, which were extensively reviewed elsewhere (de Wit and van Hooydonk, 1996; Kussendrager and van Hooijdonk, 2000; Seifu et al., 2005; Ciccognani, 2006). Here, I will briefly illustrate a few of the many examples.

Enhancement of antimicrobial activity of saliva by peroxidase system.
Commercially available oral health care products, including Biotene®, BioXtra®, Zendium Saliva® and Oralbalance®, used LPO along with its substrate SCN− to enhance or restore saliva's intrinsic antimicrobial ability. The H2O2 component of the system was generally formed in situ in the mouth by a G/GOD system (Tenovuo, 2002). Many of these commercial products are available in the form of toothpaste but also chewing gum, mouth-rinse, moisturizing gel, etc. The question is, whether these products are functioning in vivo. Lenander-Lumikari et al. (1993) reported that commercial toothpaste Biotene® elevated the human salivary levels of HOSCN/OSCN− within 20 min after brushing, although, no bactericidal effect was observed. However, these products have been improved by replacing SCN− with more potent substrate for peroxidase, such as I−. As discussed before, Ihalin and co-authors have shown that oxidation products of iodide are more powerful antimicrobials against periodontal pathogens, such as A. actinomyctemcomitans.
Introduction

(Ihalin et al., 1998), \textit{P. gingivalis} and \textit{F. nucleatum} (Ihalin et al., 2001), rather than products of thiocyanate oxidation. The major problem of LPO/H$_2$O$_2$/I$^-$ system was that the salivary concentrations of SCN$^-$ abolished the bactericidal effect of the system, as SCN$^-$ is the preferred substrate for oral peroxidases \textit{in vivo}. However, it was suggested to replace LPO by HRPO in oral hygiene products, to overcome the blocking effect of SCN$^-$ (Ihalin et al., 2003).

Enhancement of antimicrobial activity of milk by peroxidase system. LPO system has an antimicrobial effect against a diversity of milk-borne pathogenic and spoilage bacteria. A summary of the studies, which targeted the influence of the LPO system on various milk-borne pathogens, is demonstrated in the report of the FAO/WHO technical meeting (2006). The most recommended industrial application of the LPO system is in the dairy industry for the preservation of raw milk during storage and transportation [reviewed in (Naidu, 2000; Seifu et al., 2005)]. The LPO system also has wide application in cheese production, as it can eliminate pathogens on the cheese and extend the shelf life of fresh cheese (Denis and Ramet, 1989; Earnshaw et al., 1989). Another beneficial application of the LPO system is the rearing of calves. Usually, dairy calves are fed with milk substitutes and are prone to infections, such as enterotoxigenic \textit{E. coli}. The addition of a preparation based on LPO system along with lactoferrin in the milk replacements have been shown to decrease the severity and duration of enteric colibacillosis in calves, even when calves were already infected (Still et al., 1990).

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

The consensus in the literature is that the LPO system has mainly a bacteriostatic effect against the common udder pathogens \textit{S. agalactiae}, \textit{S. dysgalactiae}, \textit{S. uberis},
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E. coli, P. aeruginosa and S. aureus (FAO/WHO, 2006). Moreover, even if the peroxidase system is bactericidal to mastitis pathogens in synthetic medium, the addition of the milk to the reaction mixture inhibits the bactericidal properties of the system, suggesting that milk proteins interfere with the bactericidal agents and form adducts with them (Cooray and Björck, 1995). A study reported that the LPO system in mastitic milk was less effective compared to the healthy milk because of a higher concentration of catalase enzyme and reductive agents (FAO/WHO, 2006). However, a WO/2012/140272 patent application (O’Flaherty and McCay, 2012) described a biocidal antimicrobial composition for use in the treatment of mastitis, which comprised a peroxidase, a glycoside hydrolase (to break down disaccharide sugars into monosaccharides), an oxidoreductase enzyme (to react with the monosaccharide sugars and release H₂O₂) and a substrate for peroxidase (dependent on which peroxidase will be used). In any case, further studies are required prior to introducing the LPO system as a suitable mean for in vivo treatment of bovine mastitis.

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

The report by van Hooijdonk et al. (2000) described the potential of the peroxidase system for use in fish farming. Their peroxidase system was comprised of LPO, both SCN⁻ and I⁻ as substrates, and G/GOD as the source of peroxide. They used LPO system to feed the rainbow trout fry and monitor the mortality during the weaning period, which is normally high, mainly because of infections caused by Flexibacter psychrophilus and Octomis salmonis. There was 30% decrease in accumulated mortality in the test LPO group. Elotmani and Assobhei (2004) studied the combined antimicrobial effects of LPO system (LPO/SCN⁻/G/GOD) and nisin against the bacterial strains isolated from sardines. They reported that nisin inhibited only Gram-positive bacteria, whereas LPO system inhibited all strains studied, and even more, the combined effect was significantly higher. The authors suggested that
the combination of these two could be a possible biopreservative for fish and fish products.

The LPO system with both, SCN\(^-\) and I\(^-\) substrates, was successfully incorporated into edible films. Min and Krochta (2005) showed that the incorporation of LPO/H\(_2\)O\(_2\)/SCN\(^-\)/I\(^-\)/G/GOD antimicrobial system into edible whey protein isolate (WPI) films inhibited the growth of *Penicillium commune* and have suggested that the LPO system with WPI films have the potential to be used in complex food systems. A similar study was carried out to develop antimicrobial edible films, by combining the defatted soybean meal, the LPO system (LPO/H\(_2\)O\(_2\)/SCN\(^-\)/I\(^-\)/G/GOD) and heat pressing (Lee and Min, 2014). The authors proposed that the antimicrobial edible films and coatings can be applied to ready-to-eat products to minimize or prevent the growth of pathogenic microorganisms, including Salmonella, during storage.

*Application in wound treatment.* Wound infection can be a challenging problem, especially in the context of growing resistance to antibiotics. Topical application of peroxidase-catalyzed systems and generation of powerful antimicrobials is a promising tool for wound treatment. Several patents were filed describing the use of peroxidase systems for wound healing. US 4576817 patent (Montgomery and Pellico, 1986) proposed an organic absorbent material for body contact, such as a bandage and a pad, incorporating dry enzymes (oxidoreductase and, optionally, peroxidase) which will be activated upon contact with serum. As an oxidoreductase can be used glucose oxidase, generating H\(_2\)O\(_2\) using the glucose from serum, and as for peroxidase can serve LPO, which will interact with produced hydrogen peroxide and an oxygen-accepting anion (thiocyanate, chloride and iodide) in serum to produce oxidized species and inhibit bacteria. Whereas, US 7731954 B2 patent (Davis and Austin, 2010) proposed a wound dressing, comprised of an oxidoreductase enzyme and, optionally, peroxidase, wherein the enzyme(s) are present in hydrated condition, e.g. being present in one or more hydrated hydrogels. The third similar US 7927588 B2 patent (Davis and Austin, 2011) described skin dressings comprised of two dressings: a first dressing incorporating dry oxidoreductase enzyme; and a second dressing carrying a source of water, such that when the both are in contact water migrates from the second dressing towards the
first and hydrates the enzyme. The first dressing is placed on top of the second one, and the dressings are kept separately before use. Alternatively, the embodiment includes also a peroxidase enzyme, preferably present in the hydrated condition. WO/2012/140272 patent (O'Flaherty and McCay, 2012), mentioned in the mastitis section, also suggested using their antimicrobial composition for wound treatment.

1.5 Research motivation and aims

Peroxidase-catalyzed systems are a powerful part of the host defense network against pathogenic bacteria, fungi and parasites, which made the use of these systems in practical applications very valuable. Approaches simulating these natural antimicrobial systems have already found application in many fields and further development of the research field is both promising and necessary, given the global antibiotic crisis. One such imitation of nature is the simultaneous presence of iodide and thiocyanate ions in the peroxidase systems. As highlighted previously, in the presence of two substrates numerous oxidants can be generated which can enhance the antimicrobial effect of the system, target diverse groups of bacteria and avoid the possibility of resistance emergence. However, these two substrate/enzyme systems have been poorly studied and their role in the enhancement of antimicrobial activity remains controversial. Besides, in previously mentioned systems there may be a competition between the two substrates for oxidation by the enzyme, leading to the loss of the active cidal oxidants. It is with these prospects and limitations in mind that the present research was carried out.

The main objective of this research was to explore the properties of the non-enzymatic hydrogen peroxide/iodide/thiocyanate complex. My co-workers and I named this combination iodo-thiocyanate complex or shortly ITC. My specific objectives were: (i) to evaluate the antibacterial properties of ITC; (ii) to reveal the potential for induction of resistance and cross-resistance; (iii) to investigate its potential cytotoxicity in comparison with common antiseptics. In particular, Chapter 2 concentrates on the examination of whether the involvement of two (pseudo)halides and the omission of the enzyme from a peroxidase-like system form an effective and efficient antimicrobial. This study aimed to evaluate the antibacterial properties of the new antimicrobial combination, elucidate potential
reactive species involved in its antimicrobial performance and illustrate its effect on bacterial cell ultrastructure.

It is essential to take into account the bacterial "back-talk" (emergence of resistance) when designing a novel antimicrobial. Among the different mechanisms of resistance development, increased mutation rates in bacteria induced by the sub-lethal levels of antimicrobials and active efflux of antimicrobials have gained much recognition in recent years. In Chapter 3, therefore I estimated whether a near-lethal concentration of the new antimicrobial ITC increased the mutation rate of *E. coli* as measured by fluctuation assays. Additionally, in Chapter 3 I used an efflux pump inhibitor to examine if ITC was a specific substrate for efflux pumps, to investigate whether the efflux systems present in *P. aeruginosa* might play an essential role in adaptation to ITC.

The possibility of the emergence of resistance in bacteria to an antimicrobial therapeutic, upon prolonged exposure to it, is another important factor in determining the antimicrobial's potential. Of an increasing concern are also the extended and extensive usage of one antimicrobial and the selection of cross-resistance to existing antibiotics. In Chapter 4, I used a chemostat continuous culturing technique in an attempt to generate *de novo* resistance to ITC and cross-resistance to bactericidal antibiotic levofloxacin (LVX), in a model organism. *E. coli* underwent 20-day parallel adaptive evolution routes under gradually increasing ITC and LVX selection pressure. Whole-genome sequencing was applied to uncover the genetic basis of this laboratory adaptation to antimicrobials and the development of antimicrobial resistance.

Chapter 2, 3 and 4 suggested that ITC has the evident potential to treat wound infections (refers to an antiseptic) and decontaminate surfaces (refers to a biocide) without triggering the emergence of resistance or cross-resistance. However, to derive a biocompatible antibacterial agent both the bacterial and mammalian toxicities must be taken into consideration. Chapter 5, thus, was focused to investigate the *in vitro* cytotoxicity in parallel with antimicrobial activity, hemolytic activity and genotoxicity of the novel antimicrobial ITC. The suitability of ITC as an antiseptic was then considered and compared with the established antiseptics hydrogen peroxide, povidone-iodine and Lugol’s iodine.
Chapter 6 summarizes the results achieved in this research and indicates the future recommendations.
CHAPTER 2

Antibacterial potential of an antimicrobial agent inspired by peroxidase-catalyzed systems

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Author Contributions
LT designed and completed the experiments, wrote and reviewed the manuscript. PMC, RF and VOF contributed to the conception and design of the experimental work and the interpretation of the results. VOF, RF and GF critically reviewed and edited the manuscript.
Abstract

Antibiotic resistance is an increasingly serious threat to global health. Consequently, the development of non-antibiotic based therapies and disinfectants, which avoid induction of resistance, or cross-resistance, is of high priority. We report the synthesis of a biocidal complex, which is produced by the reaction between ionic oxidizable salts – iodide and thiocyanate – in the presence of hydrogen peroxide as an oxidation source. The reaction generates bactericidal reactive oxygen and iodine species. In this study, we report that the iodo-thiocyanate complex is an effective bactericidal agent with activity against planktonic and biofilm cells of Gram-negative (Escherichia coli and Pseudomonas aeruginosa) and Gram-positive (Staphylococcus aureus and methicillin resistant S. aureus) bacteria. The minimum bactericidal concentrations and the minimum biofilm eradication concentrations of the biocidal composite were in the range of 7.8 – 31.3 and 31.3 – 250 µg ml\(^{-1}\), respectively. As a result, the complex was capable to cause a rapid cell death of planktonic test cultures at between 0.5 and 2 h, and complete eradication of dual and mono-species biofilms between 30 s and 10 min. Furthermore, the test bacteria, including a MRSA strain, exposed to the cocktail failed to develop resistance after serial passages. The antimicrobial activity of the iodo-thiocyanate complex appears to derive from the combinational effect of the powerful species capable of oxidizing the essential biomolecules of bacteria. The use of this composition may provide an effective and efficient method for killing potential pathogens, as well as for disinfecting and removing biofilm contamination.
2.1 Introduction

The phenomenon of antibiotic resistance is expanding and the threat regarding our future ability to combat infection is increasing (WHO, 2014). Thus, key challenges for society and for researchers are to address microbial drug resistance and to develop non-antibiotic therapies.

The antimicrobial properties of naturally occurring peroxidase systems are well known. Human exocrine secretions such as milk, saliva, tears, seminal, vaginal and gastrointestinal fluids; as well as human phagocytic cells, such as neutrophils, monocytes and eosinophils, contain peroxidase enzymes, which comprise part of the innate host defense system (Ihalin et al., 2006; Davies et al., 2008). Peroxidases alone have no antibacterial effect. However, a peroxidase exerts an antimicrobial effect indirectly by catalyzing the transformation of a substrate with low antimicrobial properties into one with high antimicrobial effects (Klebanoff, 1967). The complete antimicrobial peroxidase system requires three components: a particular peroxidase enzyme, hydrogen peroxide (H₂O₂) and an oxidizable substrate such as a halide or a pseudohalide (Ihalin et al., 1998). Peroxidase-catalyzed oxidation of (pseudo)halides yields reactive agents which oxidize microorganisms, damaging essential structural and functional components and causing inhibition of microbial metabolism and growth (Thomas and Fishman, 1986).

Different peroxidases preferably oxidize different (pseudo)halides, generating distinct antimicrobial species. For example, myeloperoxidase (MPO) of neutrophils employs chloride as a substrate and forms hypochlorous acid as the main product (Klebanoff, 2005). Lactoperoxidase (LPO) of milk and salivary peroxidase (SPO) of saliva readily oxidize thiocyanate (SCN⁻) and generate hypothiocyanous acid or its conjugate base hypothiocyanite (OSCN⁻), the latest being predominant in most physiological fluids (Chandler and Day, 2012). Iodide (I⁻) can also be oxidized by MPO, LPO and SPO and it is the most readily oxidizable of all halides in vitro. The peroxidase-catalyzed oxidation of I⁻ yields molecular iodine (I₂) and, depending on I⁻ concentration and pH, hypoiiodous acid and hypoiiodite (OI⁻), or else, other iodine species may be present (Kussendrager and van Hooijdonk, 2000).
The antimicrobial properties of peroxidase systems arouse much interest towards their *in vitro* and *in vivo* applications. The use of peroxidase systems as antimicrobial agents has, however, been somewhat limited, because the cost of the enzyme purification is often higher than traditional preservatives, and sourcing large quantities of enzymes can be a problem. Large-scale purification of peroxidases from human leukocytes (MPO) or from human milk (LPO) and human saliva (SPO) is relatively difficult and expensive. These enzymes have been purified but only for research objectives. LPO purified from bovine milk has been extensively used for research and commercially as it is readily available. LPO systems have broad-spectrum of actual and potential applications as natural biopreservatives in oral healthcare, milk industry, food, feed specialties, cosmetics, and related products, which were extensively reviewed elsewhere (de Wit and van Hooydonk, 1996; Kussendrager and van Hooijdonk, 2000; Seifu et al., 2005; Ciccognani, 2006).

We have developed an iodo-thiocyanate complex (ITC), which has similarities to peroxidase systems in that it is based on halide oxidation, yet has two differences – it is comprised of two substrates, iodide and thiocyanate, and does not employ a peroxidase enzyme. Indeed, though the literature indicates that the peroxidase enzyme is required to catalyze the reaction between $\Gamma^-$, SCN$^-$ and low concentrations of $\text{H}_2\text{O}_2$ in order to exert antimicrobial activity, results from our *in vitro* research indicates that $\text{H}_2\text{O}_2/\Gamma^-/\text{SCN}^-$ combination rapidly generates antimicrobial species, even at low concentrations, without the presence of any peroxidase enzyme. Antimicrobial combinations represent a therapeutic option in the treatment of infections. In general, the combination therapy is used to avoid the development of antimicrobial resistance (as the use of two or more antimicrobials with different targets decreases the possibility that an organism will possess the features necessary for survival) and to enhance the efficacy of the individual antimicrobials through synergistic interactions. Whether a combinational approach could be adopted to increase the antimicrobial potency of a peroxidase-like system by involvement of two halides, was examined in this study. In addition, the study evaluated the antibacterial properties of the new antimicrobial combination, elucidated potential reactive species involved in its antimicrobial performance and illustrated its effect on bacterial cell ultrastructure.
2.2 Materials and Methods

2.2.1 Antimicrobial agents and preparation

All the materials were purchased from Sigma-Aldrich unless otherwise stated. The antimicrobial agents evaluated in this study included: H$_2$O$_2$ and its combinations with potassium iodide (H$_2$O$_2$/KI), with potassium thiocyanate (H$_2$O$_2$/KSCN) and with both (H$_2$O$_2$/KI/KSCN, named ITC). The stock solutions of H$_2$O$_2$/KI and H$_2$O$_2$/KSCN were prepared by combining H$_2$O$_2$ and KI or KSCN, respectively, at a ratio of 1:1 (v/v) to reach 1% final concentrations for each agent. These solutions were considered as 1%, according to the concentration of H$_2$O$_2$ present in the mixtures, since KI and KSCN alone do not possess antimicrobial properties. ITC stock solution was a mixture of H$_2$O$_2$/KI/KSCN at a 1:1:1 ratio (v/v/v) with 1% final concentration of each component (this solution was considered as 1% ITC). Due to high reactivity the stock solution of H$_2$O$_2$/KI was prepared freshly before use and used within 15 min, whereas, H$_2$O$_2$/KSCN and ITC were stable for a long time period (over six months) and were stored at 4°C. Stock solutions (1%) of H$_2$O$_2$ and three test mixtures were diluted to appropriate working concentrations using sterile deionized water (dH$_2$O) or nutrient broth of choice. Antibiotics polymyxin B (PMB), levofloxacin (LVX); antiseptics povidone iodine (PVP-I) and Lugol’s iodine (Lugol) were also used in this study. Their working dilutions were prepared using sterile dH$_2$O or LB (for LVX).

2.2.2 Bacterial strains and inoculation

Bacteria used in this study included: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* NCIMB 10421, *Staphylococcus aureus* DSM 15676, *S. aureus* BH1CC (methicillin-resistant *S. aureus* (MRSA) clinical isolate), *Streptococcus uberis* (mastitis isolate). All bacterial strains were cultured in lysogeny broth (LB) and Lennox agar (LA), and incubated aerobically at 37°C throughout the study. Trypticase soy agar (TSA) supplemented with 5% sheep blood (Fannin, Dublin, Ireland) was used for the differential characterization of Streptococcus and Staphylococcus species.
2.2.3 Antibacterial screening by disc diffusion method

For initial comparison of the antimicrobial activity of \( \text{H}_2\text{O}_2 \) with its three different combinations, the susceptibility screening of four test strains (\( \text{E. coli} \), \( \text{P. aeruginosa} \), \( \text{S. aureus} \) and MRSA) to the antimicrobial combinations was performed using standard technique (CLSI, 2012b). Aliquots (100 µl) of freshly prepared phosphate-buffered saline (PBS) suspension of each bacterial strain at optical density of \( \text{OD}_{625} = 0.1 \), corresponding to \( 10^8 \) colony forming units per ml (cfu ml\(^{-1} \)), was used to lawn the LA plate. The Whatman filter discs (6 mm) were impregnated with 10 µg of antimicrobial mixtures (estimated by the amount of \( \text{H}_2\text{O}_2 \) only) and incubated for 24 h at 37\(^\circ\)C. Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zones (ZOI). Likewise, for concentration-dependent effect of ITC, discs were charged with various doses of ITC (10, 20, 40 and 80 µg disc\(^{-1} \)) and ZOI were measured. Each strain, each antimicrobial and each concentration of ITC were tested in duplicates on three separate occasions.

2.2.4 Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) by broth microdilution method

The MICs and MBCs of \( \text{H}_2\text{O}_2 \), \( \text{H}_2\text{O}_2/\text{KI} \), \( \text{H}_2\text{O}_2/\text{KSCN} \) and ITC were determined against \( \text{E. coli} \), \( \text{P. aeruginosa} \), \( \text{S. aureus} \) and MRSA test strains using a broth microdilution method according to standard guidelines (CLSI, 1999; 2012a). Bacteria at \( 5 \times 10^5 \) cfu ml\(^{-1} \) final density were seeded in 96-well plates and challenged with the specified compounds at serial 2-fold dilutions. The growth of the strains was monitored in microtiter plate reader (Tecan GENios, Salzburg, Austria). \( \text{OD}_{595} \) measurements were recorded at 15 min intervals over 24 h at 37\(^\circ\)C. The MIC was defined as the lowest concentration of antimicrobial agent preventing the appearance of turbidity. MBCs of the antimicrobials were determined by sub-culturing the content of the no growth wells from the above MIC test onto solid media. The MBC values were defined as the lowest concentrations which produced no colonies on the agar plates. The experiments were performed on five occasions. MICs and MBCs of the test compounds from independent experiments varied in the quintuple but only by one concentration higher or lower in the dilution series. Accordingly, the modes of each dataset were reported as MIC or MBC. The
antimicrobial was considered to exhibit bactericidal activity when the MBC/MIC ratio was ≤4 (Pankey and Sabath, 2004).

### 2.2.5 Microbial killing rates by time-kill assay

Time-dependent killing of H$_2$O$_2$ and its three derivatives were identified against *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA strains using standard technique (Moody and Knapp, 2004). Test cultures at $5 \times 10^5$ cfu ml$^{-1}$ density were exposed to all antimicrobial combinations at the concentration of 31.3 µg ml$^{-1}$ in 5 ml LB. In order to have comparative results, for all the test strains and for all the test antimicrobials 31.3 µg ml$^{-1}$ concentration was chosen as it was the most frequently occurring MBC value (Table 2.1). Inoculum in broth without any antimicrobial was considered as a control. After the inoculation all the suspensions were incubated at 37°C under continuous shaking conditions. At predetermined time points (0, 0.5, 1, 2, 4, 8 and 24 h), aliquots (25 µl) were aseptically removed, serially diluted in PBS and plated on LA plates. The plates were incubated at 37°C, and cell survival was determined by colony counts. The determinations were done in duplicates for two occasions.

### 2.2.6 Biofilm growth on modified Robbins’ device (MRD) and determination of minimum biofilm eradication concentrations (MBEC) by viable cell count

Susceptibility of H$_2$O$_2$/KI/KSCN combination (ITC) was determined for mono and dual-species biofilm bacteria. Mono-species biofilm modes of *E. coli* ATCC 25922, *P. aeruginosa* NCIMB 10421, *S. aureus* DSM 15676 and MRSA BH1CC strains, and dual-species biofilm of *S. aureus* DSM 15676 and *S. uberis* were grown as a batch culture coupled with MRD, wherein, the bacterial cells were allowed to attach and proliferate on the surface of polyurethane coupons with their “face” exposed to the recirculating flow over time (Figure 2.1) (Kharazmi et al., 1999). In brief, MRD is an acrylic multiport sampling chamber containing twelve ports in a linear array (Tyler Research Corporation, Edmonton, Canada). Each port accepts a press-fit plug holding a polyurethane coupon (surface area 50 mm$^2$). Coupons were placed on top of the flow, without disturbing flow characteristics. The MRD was connected to peristaltic pump (Watson-Marlow 205S, Falmouth, UK) and growth media reservoir by silicone tubing. The device was disinfected with 1% (w/v) Virkon (Anachem Ltd,
Leicester, UK), the plugs and the coupons were exposed to 100% ethanol, the media bottle and the tubing were autoclaved before and after each run. To establish the mono-species biofilms, the media reservoir containing 300 ml LB was seeded with test bacteria at 10^6 cfu ml\(^{-1}\) density. For co-culturing \textit{S. aureus} and \textit{S. uberis}, 300 ml LB reservoir was seeded with two strains at final density of 10^6 cfu ml\(^{-1}\) each. Broth media, containing given bacteria, was pumped through the device at a rate of 0.1 h\(^{-1}\) under 37°C conditions. After 24 h individual coupons protruding into the flow channel were taken out and prewashed with PBS (removing any unbound cells), exposed to ITC treatment at serial 2-fold dilutions upon 10 min for mono-species biofilms and 30 s for dual-species biofilm and washed again (washing away the antimicrobial agent). The coupons were then transferred to fresh 1 ml PBS in a 2 ml mini-centrifuge tubes. The biofilm cells were removed from the coupons by vortexing 1 min and sonicating in a sonication bath for 10 min (Branson Ultrasonics Corporation, Danbury, USA). Viable cell count on LA plates was used to establish bacterial numbers of mono-species biofilms posttreatment. For differential selection of Staphylococcus and Streptococcus species, aliquots were plated on TSA + blood plates and were enumerated according to the type of hemolysis. For single-species biofilms the results were expressed as log\(_{10}\) cfu ml\(^{-1}\) values, while for mixed biofilms results were relative proportion of each bacterial component expressed at the % of the total population in mixed biofilms. At each concentration of the antimicrobial treatment cfu values were counted from four coupons. The MBEC of ITC was determined as the minimum concentration that prevents the growth in the recovery medium used to collect biofilm cells (0 cfu coupon\(^{-1}\) on plate counts) (Macia et al., 2014).
Antimicrobial activity of an iodo-thiocyanate complex

Figure 2.1. The system for biofilm growth. The modified Robbins’ device is an acrylic multiport flow chamber that contains twelve ports in a linear array. Each port accepts a press-fit plug holding a polyurethane coupon. The growth media containing the given bacteria is pumped at a rate of 0.1 h\(^{-1}\) from a reservoir through MRD and back to the reservoir. The biofilm is grown on the surface of the coupons protruding into the MRD chamber with their “face” exposed to the recirculating flow. The entire system is placed in 37°C incubator.

2.2.7 Potential for resistance using sequential passage in the presence of the antimicrobial

The persistence of antimicrobial susceptibility in experimental populations of bacteria was tested over 20 passages by broth microdilution method in accordance with the procedure described elsewhere (Friedman et al., 2006; D’Lima et al., 2012). Multipassage resistance studies using H\(_2\)O\(_2\), ITC and LVX were performed for \(E.\) \(coli\), \(P.\) \(aeruginosa\), \(S.\) \(aureus\) and MRSA strains. At day one, LB containing twelve 2-fold dilutions of each of antimicrobial drugs – H\(_2\)O\(_2\), ITC (starting at 500 \(\mu\)g ml\(^{-1}\)) and LVX (starting at 8 \(\mu\)g ml\(^{-1}\)), and LB without drug [growth control (GC)] were seeded with bacteria at final density of \(5 \times 10^5\) cfu ml\(^{-1}\) in 96-well plates. Cultures were incubated 24 h in 37°C incubator. For each subsequent daily passage, for each test antimicrobial and strain, aliquots were taken from the wells with concentrations one to two dilutions below the MIC (that matched the turbidity of a GC well) and were used to inoculate the dilution series for the next day, so that the bacteria were
again seeded at $5 \times 10^5$ cfu ml$^{-1}$. H$_2$O$_2$, ITC and LVX were added to the wells containing these bacterial suspensions at 2-fold dilutions. These cultures represented the exposed groups. In a same manner, bacterial culture from the GC well (well cultured without antimicrobial agent from the previous passage) was diluted to $5 \times 10^5$ cfu ml$^{-1}$ into fresh media, dispersed into the wells and H$_2$O$_2$, ITC and LVX were added at the same 2-fold dilutions, representing the unexposed groups. The process was continued for 20 passages and the MIC values for exposed and unexposed groups were recorded. The relative MIC was calculated for each passage from the ratio of MIC obtained from an exposed culture to that obtained from an unexposed culture.

### 2.2.8 Identification of the reactive species

A number of analytical methods were used to identify and quantify the chemical species generated within the antimicrobial mixtures used in this study. Solutions (1% – according to the concentration of H$_2$O$_2$ present) of H$_2$O$_2$/KSCN and H$_2$O$_2$/KI/KSCN were prepared the day before the measurements and their peroxide, hypoiodite/hypothiocyanite, hydroxyl radical and iodine contents were measured. H$_2$O$_2$/KI was prepared freshly for each measurement, due to its weak stability and rapid loss of antimicrobial activity. Iodine content was also measured in 1% solutions of PVP-I and Lugol’s iodine. All the measurements were performed on duplicate solutions for each test antimicrobial.

**Detection of hydrogen peroxide.** Quantofix Peroxide 100 semi-quantitative test strips were used to detect the concentrations of unreacted H$_2$O$_2$ in the test antimicrobial solutions. The tests were carried out in accordance with manufacturer’s instructions.

**Measurement of hydroxyl radical ($\cdot$OH).** The concentrations of $\cdot$OH in 1% solutions of test antimicrobials were detected using a dimethyl sulfoxide (DMSO) as a molecular probe (Babbs and Steiner, 1990). Chemically, DMSO traps $\cdot$OH and is oxidized to a single, stable, non-radical product, methanesulfonic acid (MSA). The measurement of MSA accumulation in DMSO pretreated systems provides a potential mean to capture and count the $\cdot$OH generated therein. Further, MSA can be assayed colorimetrically based on the reaction with diazonium salts, particularly, fast
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blue BB salt. The product is a colored diazosulfone, which can be selectively extracted into an organic solvent and measured spectrophotometrically at 425 nm. The color reaction was carried out according to the assay developed by Babbs and Steiner (1990). The concentration of sulfinic acid was calculated from an MSA standard curve.

Measurement of hypothiocyanite/hypoiodite. Concentrations of OSCN\(^{-}\)/OI\(^{-}\) were measured based on the oxidation of sulphydryl compound 5-thio-2-nitrobenzonic acid (TNB) which absorbs at 412 nm (Aune and Thomas, 1977; Bosch et al., 2000; Cegolon et al., 2014). Firstly, DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] is reduced to TNB with sodium borohydride (NaBH\(_4\)). When mixing yellow-colored TNB with OSCN\(^{-}\)/OI\(^{-}\), these will reoxidize TNB to colorless DTNB. Each mole of DTNB will yield 2 moles of TNB, and each 2 mole of TNB are reoxidized to DTNB by 1 mole of OSCN\(^{-}\) (or OI\(^{-}\) or OSCN\(^{-}\)/OI\(^{-}\)). Particularly, the reactive solution was prepared by mixing 40 mg of DTNB with 20 mg of NaBH\(_4\) and brought to 100 mL with 0.5 M Tris–HCl buffer pH 7. The absorbance of the samples (0.25 ml reactive in 2.65 ml dH\(_2\)O and 0.1 ml sample antimicrobial solution) was measured and compared to the absorbance of reference (0.25 ml reactive in 2.75 ml dH\(_2\)O). OSCN\(^{-}\) or OI\(^{-}\), or OSCN\(^{-}\)/OI\(^{-}\) concentration was calculated by employing the following formula:

\[
[\text{OSCN}^{-}/\text{OI}^{-}] (\text{mM}) = \frac{(\Delta \text{OD}) \times 3 \times 1000}{13600 \times 2 \times 0.1}
\]

where, \(\Delta \text{OD} = (\text{OD}_{\text{reference}}) - (\text{OD}_{\text{OSCN}^{-}/\text{OI}^{-}\text{solution}})\); 3 = total volume; 0.1 = sample volume; 13600 M\(^{-1}\) cm\(^{-1}\) = molar extinction coefficient of DTNB; 2 = correction factor for the stoichiometric reaction (2 moles TNB reacts with 1 mole OSCN\(^{-}\)/OI\(^{-}\) to produce 1 mole DTNB); 1000 = M bring to mM.

Measurement of iodine. I\(_2\) content in all test antimicrobial aqueous solutions was measured by the iodometric titration method as described by Sullivan et al. (1995) which is based on titration with sodium thiosulfate (Na\(_2\)S\(_2\)O\(_3\)). Free iodine is consumed by Na\(_2\)S\(_2\)O\(_3\) and converted to iodide according to the equation:

\[
\text{I}_2 + 2\text{S}_2\text{O}_3^{2-} \rightarrow 2\text{I}^- + \text{S}_4\text{O}_6^{2-}
\]
Briefly, 500 µl of 1% antimicrobial mixtures were titrated with a 5 mM Na₂S₂O₃ standard solution until colorless solutions were obtained. Near the end-point, a 100 µl of 1% starch (BDH Laboratory Supplies, Poole, England) solution was added as an external indicator of this reaction, which reacts with I₂ to produce a blue color and to enhance the color change, and titration was continued further. The amount of iodine present is proportional to the amount of thiosulfate used and was calculated according the formula:

\[
C (I_2) = \frac{C (Na_2S_2O_3) \times V (Na_2S_2O_3)}{2 \times V (I_2)}
\]

where, \(V (I_2)\) is the initial volume of the test solution, \(V (Na_2S_2O_3)\) is the volume of thiosulfate added, \(C (Na_2S_2O_3)\) is the concentration of thiosulfate.

**Detection of iodine species.** Iodine species \([\Gamma^-, I_2 \text{ and } I_3^- \text{ (tri-iodide)}]\) in 1% aqueous solutions of H₂O₂/KI and H₂O₂/KI/KSCN combinations were also qualitatively observed using UV-Vis spectrometry (Gazda et al., 2004) and compared to spectra of commercially available iodine containing mixtures – PVP-I and Lugol’s iodine. UV-Vis absorption spectra over a range of 190 – 700 nm were obtained at room temperature on a Varian Cary 50 Scan UV-visible spectrophotometer, using a quartz cuvette of 1 cm optical length. Where there were overlapping bands or oversaturation, 1% stock solutions of different antimicrobials were diluted at various dilution factors (1/2, 5, 10, 100, 200, 500, 1000).

**2.2.9 Transmission electron microscopy (TEM) to detect structural changes in bacteria**

TEM was used to assess the effect of ITC on bacterial cell ultrastructure. *E. coli* cells were grown to exponential phase and 2. 5 × 10⁸ cfu ml⁻¹ were treated with ITC at 30 and 300 µg ml⁻¹, and H₂O₂ at 300 µg ml⁻¹ for 2 h at 37°C. Before proceeding to TEM sample preparation, aliquots from the treated and untreated cultures were aseptically removed and plated on agar plates for viable cell counts. Next, the cell pellets were washed with PBS and fixed with 2.5% glutaraldehyde (Serva, Heidelberg, Germany) in PBS for overnight. Afterwards, the pellets were washed and post-fixed with buffered (PBS) 1% osmium tetroxide for 2 h. Samples were then dehydrated in a graded series of ethanol (50, 75, 90 and 100%), followed by 1 h incubation with
propylene oxide. They were further infiltrated with epoxy resin (TAAB Laboratories Equipment Ltd, Aldermaston, UK) according to manufacturer’s protocol. Samples were polymerized at 65°C for 72 h. Ultrathin sections were cut at 90 nm (Reichert-Jung Ultramicrotome equipped with diamond knife) and deposited on 200-mesh copper grids. Specimens were post-stained with 0.5% uranyl acetate and 3% lead citrate with automated contrasting apparatus (Leica EM AC20). Sections were observed in a transmission electron microscope (Hitachi H7000) at 75 kV under 10000× and 20000× magnifications.

2.2.10 Agarose gel electrophoresis to detect bacterial DNA damage

The potential of ITC as an agent that would induce bacterial DNA breakage (DNA smear) was examined by agarose gel electrophoresis (Yoon et al., 2002; Kasibhatla et al., 2006). Briefly, exponential phase *E. coli* cells were treated with ITC and H₂O₂ at 30 and 300 µg ml⁻¹ for 30 min, and genomic DNA of each sample was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. gDNA samples were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide (10 µg ml⁻¹) (Promega Corporation, Medison, USA). gDNA samples were electrophoresed at 40 V until the tracking dye (Blue/Orange 6x Loading Dye, Promega, Madison, USA) front reached the bottom of the gel and then visualized under the UV illuminator (Azure c200, Azure Biosystems).

2.2.11 Live/dead staining and fluorescent microscopy to evaluate bacterial outer membrane damage

The impact of ITC on membrane integrity of planktonic and biofilm *E. coli* cells was observed by LIVE/DEAD BacLight staining (Molecular Probes Inc, Eugene, USA), which is widely used assay to demonstrate membrane damage (Ercan et al., 2014). Planktonic cells of *E. coli* in exponential phase were treated with 125 µg ml⁻¹ concentration of ITC for 1 h, washed and stained according to manufacturer’s indications. As red-labeled staining control polymyxin B at 1 µg ml⁻¹ (2 × MIC) concentration was used, as it acts on bacterial membranes, making it more permeable (Storm et al., 1977). Prior to staining, aliquots were taken from untreated, ITC and PMB-treated planktonic cultures for standard plate counts. For assays with biofilms,
E. coli cultures were grown on microscope coverslips over 24 h than washed to remove media and planktonic cells. Established biofilms were then treated with three different concentrations (7.8, 31.3 and 125 µg ml\(^{-1}\)) of ITC mixture for 10 min, washed and stained accordingly. Bacteria were observed under Nikon Eclipse E600 microscope with a 100× oil immersion objective lens, equipped with a QICAM Fast 1394 camera. Live/dead composite fluorescent images were acquired using QCapture Pro 5.1 software.

2.3 Results

2.3.1 Antibacterial profile of new antimicrobial mixtures

Initial antibacterial screening of H\(_2\)O\(_2\) and its combinations with KI, or KSCN, or both KI and KSCN, towards two Gram-negative (E. coli and P. aeruginosa) and two Gram-positive (S. aureus and MRSA) bacteria was carried out by disc diffusion assays (Figure 2.2A, B). Antimicrobial susceptibility discs, loaded with 10 µg of each antimicrobial combination (estimated according to the amount of H\(_2\)O\(_2\) only) showed that ITC had antimicrobial activity towards all the test organisms. By contrast, H\(_2\)O\(_2\), H\(_2\)O\(_2\)/KI and H\(_2\)O\(_2\)/KSCN did not cause inhibition of E. coli growth and induced only very slight inhibition of P. aeruginosa. The antimicrobial combinations under test had similar efficacies for S. aureus and MRSA strains. The preliminary screening by disk diffusion demonstrated that combination of two substrates has a synergistic antimicrobial effect. Subsequently, the dose-dependent inhibition effect of ITC was examined, demonstrating that the ZOIs increased with increasing concentration of ITC for all four test strains (Figure 2.2C, D).

MIC and MBC values of tested antimicrobial compounds are listed in Table 2.1. ITC showed moderate to high activity towards all test organisms with MIC and MBC values ranging 7.8 – 31.3 µg ml\(^{-1}\). In comparison to the other test antimicrobials, MICs and MBCs were lower for ITC, against E. coli and P. aeruginosa, and were similar against S. aureus and MRSA. This outcome is in agreement with disk diffusion results presented in Figure 2.2. MBC/MIC ratios indicated that all antimicrobials exerted a bactericidal effect on the studied strains.
**Figure 2.2.** Antibacterial activity of H$_2$O$_2$, H$_2$O$_2$/KI, H$_2$O$_2$/KSCN and ITC against *E. coli* ATCC 25922, *P. aeruginosa* NCIMB 10421, *S. aureus* DSM 15675 and *S. aureus* BH1CC (MRSA) strains (A, B). Representative sample agar plates showing the zones of inhibition formed by antimicrobials (10 µg of each antimicrobial combination was applied) (A). Mean diameter of the ZOI (in mm, including the 6 mm diameter of the disc) of different antimicrobials recorded in three independent assays performed in duplicates, error bars indicate +SD (B). Dose-dependent antimicrobial activity of ITC against the test strains (C, D). Representative sample agar plates showing the ZOIs around the discs impregnated with different amounts of ITC (10, 20, 40 and 80 µg disc$^{-1}$) (C). Mean diameter of the ZOI of different amounts of ITC recorded in 3 independent assays performed in duplicates (+SD) (D). Dotted lines represent level of no activity.
**Table 2.1.** Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) (expressed as µg ml⁻¹) of test antimicrobial combinations against representative strains determined by broth microdilution method. Data are shown as the mode of n=5.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>H₂O₂</th>
<th>H₂O₂/KI</th>
<th>H₂O₂/KSCN</th>
<th>ITC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>31.3</td>
<td>31.3</td>
<td>31.3</td>
<td>31.3</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NCIMB 10421</td>
<td>62.5</td>
<td>125</td>
<td>62.5</td>
<td>125</td>
</tr>
<tr>
<td><em>S. aureus</em> DSM 15676</td>
<td>7.8</td>
<td>7.8</td>
<td>7.8</td>
<td>7.8</td>
</tr>
<tr>
<td><em>S. aureus</em> BH1CC (MRSA)</td>
<td>15.6</td>
<td>31.3</td>
<td>15.6</td>
<td>31.3</td>
</tr>
</tbody>
</table>

A time-kill assay was used to further evaluate the bactericidal activities of the antimicrobials. The time-course of bacterial viability was determined after bacterial four test strains were treated with H₂O₂ and its derivatives at the concentration of 31.3 µg ml⁻¹ (which was the most frequent MBC value within the strains and the antimicrobials (Table 2.1)) (Figure 2.3). The results showed that *E. coli*, *P. aeruginosa* and *S. aureus* were killed within 30 min after the addition of ITC. Moreover, *P. aeruginosa* and *S. aureus* exposed to ITC showed reduced bacterial load compared with the unexposed control, even at initial sampling time (T₀), indicating that killing of this strains by ITC occurred immediately. Killing of MRSA was achieved after 2 h of exposure to ITC. *S. aureus* was also sensitive towards the rest of the test antimicrobials and its complete killing was observed within 30 min of exposure. H₂O₂ at the tested concentration (31.3 µg ml⁻¹) could not inhibit *E. coli*, or required longer time to eliminate *P. aeruginosa* and MRSA strains, within 4 and 24 h, respectively. H₂O₂/KI and H₂O₂/KSCN combinations could not arrest the growth of *E. coli*, *P. aeruginosa* and MRSA (Figure 2.3).
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Figure 2.3. Time-kill curves of representative strains treated with H$_2$O$_2$, H$_2$O$_2$/KI, H$_2$O$_2$/KSCN and ITC. Concentration of each antimicrobial mixture is 31.3 µg ml$^{-1}$. Mean values of duplicate cfu ml$^{-1}$ measurements (±SD) are plotted.

The total colony forming unit counts obtained from mono-species biofilms formed within 24 h on untreated coupons were $10^6 - 10^7$ cfu ml$^{-1}$ for all strains (Figure 2.4A). The anti-biofilm activity of ITC increased with increased concentration, eventually achieving the killing of *E. coli*, *S. aureus* and MRSA mono biofilms below detection level with MBEC values of 125, 31.3 and 31.3 µg ml$^{-1}$, respectively. ITC was the least effective towards *P. aeruginosa* biofilm, as the highest tested concentration (125 µg ml$^{-1}$) was not sufficient for eradication, although it achieved a 5 log$_{10}$ reduction in cell numbers. The individual contribution of *S. aureus* and *S. uberis* in the formation of dual-species biofilm was about 47 and 53%, respectively (Figure 2.4B). The treatment with a 15.6 µg ml$^{-1}$ dose of ITC
decreased the proportions of both bacterial species to negligible levels within 30 s. The MBEC against mixed biofilm was recorded at 250 µg ml⁻¹.

**Figure 2.4.** Anti-biofilm activity of ITC towards single-species biofilms of *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA strains at different concentrations upon 10 min interaction (A). Mean values of tetrad coupon log₁₀ cfu ml⁻¹ measurements (±SD) are plotted. Anti-biofilm activity of ITC towards dual-species biofilm of *S. aureus* and *S. uberis* at different concentrations at exposure time of 30 s (B). The relative proportion of each bacterial component is expressed at the % of the total population in mixed biofilm. Mean % from tetrad coupons (±SD) are plotted.

The evolution of MICs after successive exposures of *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA strains to sub-MIC concentrations of H₂O₂, ITC and LVX was assessed in this study (Figure 2.5). No significant increase and only 0.5 to 2-fold shifts of the ITC MICs were observed for all the test organisms. For H₂O₂, the largest increase in MIC was the 4-fold elevation towards MRSA, however, this change was reversed during subsequent passages. As for LVX, it showed maximum 4-fold increase in MIC towards *E. coli*, which was observed as a fluctuation rather than a permanent MIC rise. By contrast, *S. aureus* acquired resistance towards LVX. In the presence of sub-inhibitory concentrations of LVX, MIC of LVX rose 16-fold immediately after the first passage and arrived to 64-fold difference at passage three, which stayed stable over the next passages. On the other hand, LVX initially had a 64-fold higher MIC level (4 µg ml⁻¹) towards MRSA compared to counterpart *S. aureus* strain (0.0625 µg ml⁻¹), however it did not increase during *in vitro* evolution.
Figure 2.5. In vitro resistance acquisition of representative strains during 20 serial passaging in the presence of sub-inhibitory levels of H\textsubscript{2}O\textsubscript{2}, ITC and LVX tested by broth microdilution. The relative MIC was the normalized ratio of the MIC obtained for the exposed sub-culture to the MIC that was obtained from unexposed culture; the insets show the expanded y-axis.

2.3.2 Analysis for reactive species in the test antimicrobial mixtures

The antimicrobial solutions were analyzed using a number of analytical methods. The content analysis of 1% H\textsubscript{2}O\textsubscript{2} and KI or/and KSCN combination mixtures is given in Table 2.2. The semi-quantitative test for peroxide presence showed that H\textsubscript{2}O\textsubscript{2} was depleted in H\textsubscript{2}O\textsubscript{2}/KI and H\textsubscript{2}O\textsubscript{2}/KI/KSCN (ITC) mixtures, whereas H\textsubscript{2}O\textsubscript{2}/KSCN yet contained very small amount of unreacted peroxide. The hydroxyl radical content was the highest in H\textsubscript{2}O\textsubscript{2}/KI mixture, though it was also detected in the other combination mixtures. A high O\textsubscript{SCN}\textsuperscript{−}/O\textsuperscript{I}\textsuperscript{−} level was observed in H\textsubscript{2}O\textsubscript{2}/KSCN and H\textsubscript{2}O\textsubscript{2}/KI/KSCN solutions. Though the literature implies that O\textsuperscript{I}\textsuperscript{−} (Bosch et al., 2000), as well as other hypohalous acids (Landino et al., 2008; Gau et al., 2015) can be measured by TNB-to-DTNB oxidation method we detected little
amount of $\text{O}_2^-$ in the $\text{H}_2\text{O}_2$/KI mix. $\text{H}_2\text{O}_2$ itself is oxidizing the TNB (Landino et al., 2008) giving a false positive results for OSCN$^-$/OI$^-$ content consideration. However, most of the $\text{H}_2\text{O}_2$ in the mixtures were reacted and any oxidation of TNB in the mixtures was counted for OSCN$^-$/OI$^-$. Iodine, as it was expected, was detected in $\text{H}_2\text{O}_2$/KI, $\text{H}_2\text{O}_2$/KI/KSCN, PVP-I and Lugol’s mixtures. ITC contained considerable amount of molecular iodine, surpassing even PVP-I. Among all the test antimicrobials ITC was the one most possessing an assembly of reactive oxygen and iodine species in substantial quantities. The content analysis of ITC mixture on other occasions exhibited similar compositional profile.

Table 2.2. Composition of 1% antimicrobial mixtures. Data are given as mean values ($\pm$ SD) of duplicates

<table>
<thead>
<tr>
<th>Antimicrobial solution</th>
<th>$\text{H}_2\text{O}_2$, $\pm$ e</th>
<th>$\cdot\text{OH}$, mM</th>
<th>OSCN$^-$/OI$^-$, mM</th>
<th>I$_2$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_2\text{O}_2$</td>
<td>+</td>
<td>0.14 ± 0.03</td>
<td>0.63 ± 0.03</td>
<td>–</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$/KI</td>
<td>–</td>
<td>1.04 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$/KSCN</td>
<td>+</td>
<td>0.13 ± 0.04</td>
<td>1.24 ± 0.00</td>
<td>–</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$/KI/KSCN</td>
<td>–</td>
<td>0.19 ± 0.06</td>
<td>1.24 ± 0.00</td>
<td>9.7 ± 0.00</td>
</tr>
<tr>
<td>PVP-I</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.9 ± 0.14</td>
</tr>
<tr>
<td>Lugol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>30.75 ± 0.35</td>
</tr>
</tbody>
</table>

As iodine was believed to be the most considerable contributor of ITC antimicrobial action, the presence of different iodine species (I$^-$, I$_2$ and I$_3^-$) in iodine containing mixtures of $\text{H}_2\text{O}_2$/KI, $\text{H}_2\text{O}_2$/KI/KSCN, PVP-I and Lugol was also visualized by UV-Vis spectrometry (Figure 2.6). Scanning of different dilutions of 1% ITC in the range of 190 – 700 nm wavelengths showed that 1/2 and 1/5 diluted solutions had two peaks at about 288 and 354 nm, which are known to be bands of tri-iodide ions (Gazda et al., 2004; Wei et al., 2005; Mazumdar et al., 2010; Mertes et al., 2012; Kireev and Shnyrev, 2015). However, characteristic bands of I$_2$ at about 203 (Wei et al., 2005) and 460 nm (Wei et al., 2005; Kireev and Shnyrev, 2015) were not distinguishable in all the measured dilutions of ITC. Gazda et al. (2007) and Punyani et al. (2007) indicated that the peak at 460 nm had a very low energy, whereas, Kireev and Shnyrev (2015) described I$_2$ peaks at 203 and 460 nm as very large bands, consequently, I$_2$ peaks could have been hidden because of spectral interference or of weak intensity. Moreover, at 1/1000 dilution of ITC we could detect a band applying to iodide ion with absorption maximum of 226 nm. This
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suggested that KI was not completely depleted in H₂O₂/KI/KSCN system and iodine present in the solution could occur in I₃⁻ form. H₂O₂/KI mixture showed two peaks of I₃⁻ at 1/1, 2, 5, 10 dilutions and only I⁻ peak at 1/1000 dilution. Similarly, Lugol displayed two I₃⁻ bands at 1/100, 200, 500 dilutions, I⁻ peak at 1/1000 dilution, and none of I₂ characteristic bands at 460 or 203 nm. In contrast to published evidence (Oster and Immergut, 1954; Mazumdar et al., 2010), we could not acquire iodine bands from PVP-I solution, possibly, because of a spectral interference from PVP polymer.

![Figure 2.6](image)

Figure 2.6. UV-Vis absorption spectra of aqueous solutions of H₂O₂/KI, H₂O₂/KI/KSCN (ITC), PVP-I and Lugol’s iodine at different dilutions. Absorption peaks of I⁻ (226 nm) and I₃⁻ (288 and 354 nm) are shown with dotted arrowlines.

2.3.3 Effect of ITC on cellular components

TEM was used for direct observation of ITC-induced ultrastructural alterations in *E. coli*. Visualization of the micrographs revealed that the untreated control specimen contained more cells with electron-dense (appears dark) cytoplasm, rich in ribosomes (dark granules), which form a riboplasm (Figure 2.7A, B). The DNA
(electron-light material) of the untreated cells was distributed randomly within the riboplasm. While *E. coli* cells in ITC 30 µg ml\(^{-1}\) treated group appeared to have more internal translucent areas – ribosomal grains looked light-colored and DNA appeared as swirls in the middle of the cells, however, cells looked intact. Cells in ITC 300 µg ml\(^{-1}\) treated sample appeared to have more dissolved picture – DNA appeared to be fragmented and ribosomal grains were more scarcely distributed, but then again cells were intact. In addition, the image of H\(_2\)O\(_2\) 300 µg ml\(^{-1}\) treated group resembled the scene from the control sample, composed of mostly cells with “dark matter”. Whilst, viable cell counts showed complete eradication of 10\(^{13}\) cfu ml\(^{-1}\) bacterial load with ITC at high, and even low concentrations within 2 h exposure (Figure 2.7C). H\(_2\)O\(_2\) at 300 µg ml\(^{-1}\) concentration resulted only in 4 log\(_{10}\) reduction of *E. coli*.

**Figure 2.7.** Effect of ITC on cellular components of *E. coli*. Transmission electron micrographs of untreated control, treated with ITC at 30 µg ml\(^{-1}\) and 300 µg ml\(^{-1}\), and H\(_2\)O\(_2\) at 300 µg ml\(^{-1}\) for 2 h, and visualized at 10000× (A) and at 20000× magnifications (B). Cell counts (log\(_{10}\) cfu ml\(^{-1}\)) of viable *E. coli* cells from the same samples prior to preparation for TEM (C). Bacterial DNA damage detection by...
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agarose gel electrophoresis (D). Samples 1, 2, 3 and 4 *E. coli* cells were treated 30 min with ITC at 30 and 300 µg ml⁻¹, H₂O₂ at 30 and 300 µg ml⁻¹, respectively. gDNA was extracted and gel electrophoresis was performed for examination of DNA cleavage (DNA smear). C is gDNA from untreated cells, M is a 1 kb DNA marker.

To confirm the observations from TEM microscopy and to show DNA cleavage in bacterial cells, *E. coli* cells were treated with low (30 µg ml⁻¹) and high (300 µg ml⁻¹) concentrations of ITC and H₂O₂, followed by extraction of gDNA and agarose gel electrophoresis. DNA smears were detected in *E. coli* cells treated with ITC and H₂O₂ at their highest tested concentrations – 300 µg ml⁻¹ (Figure 2.7D, samples 2 and 4, respectively).

![Image](image_url)

**Figure 2.8.** Influence of ITC on *E. coli* membrane integrity assayed by live/dead staining. Fluorescence micrographs of *E. coli* planktonic cells in exponential phase treated with ITC at 125 µg ml⁻¹ and polymyxyn B at 1 µg ml⁻¹ upon 1 h and stained with LIVE/DEAD BacLight stain (A). Cell counts (log₁₀ cfu ml⁻¹) of viable *E. coli* planktonic cells prior to live/dead staining (B). Representative images of *E. coli* biofilm grown on microscope coverslips upon 24 h, than treated 10 min with different concentrations of ITC and stained with LIVE/DEAD BacLight stain (C). Membrane is expected to be intact in green-fluorescent and compromised in red-fluorescent cells.

To further support TEM results and to exhibit that ITC was not compromising membrane integrity of *E. coli*, live/dead co-staining technique was used. It is a two-color fluorescence assay based on membrane integrity that simultaneously
determines live and dead cells. The membrane-permeable SYTO-9 (green-fluorescent nucleic acid stain) labels all bacteria in a population (intact and ruptured), by contrast, propidium iodide (red-fluorescent nucleic acid stain) penetrates only bacteria with damaged membranes, causing a reduction in the SYTO-9 stain fluorescence when both dyes are present (Stocks, 2004). Fluorescent micrographs of live/dead stained *E. coli* planktonic cells showed no difference between ITC-treated and untreated control groups, displaying mostly green stained cells (Figure 2.8A), yet no viable cells were recovered on agar plates from ITC-treated cultures (Figure 2.8B). Whereas, red control PMB-treated group displayed mostly red cells and a 4 log₁₀ reduction in cfu numbers when exposed to 2 × MIC (Figure 2.8A, B). In addition, *E. coli* biofilm cells treated with different concentrations of ITC showed predominantly green-fluorescent cells, similar to the untreated control, with some red-fluorescent cells in the aggregates (Figure 2.8C).

### 2.4 Discussion

Multicellular organisms have developed different enzyme systems, which offer antimicrobial activity and play a significant role in the defense of the host organism. Approaches simulating the natural antimicrobial systems have already found application in many fields and further development of the research field is both promising and necessary, given the global antibiotic crisis. We have developed an easy-to-prepare antimicrobial composition ITC, inspired by naturally occurring peroxidase systems, but with distinctive antimicrobial activity. A biocidal composite is formed from the combination of hydrogen peroxide, iodide and thiocyanate salts at a ratio of 1:1:1 (v/v/v). Incorporation of I⁻ and SCN⁻ into peroxidase systems have been poorly investigated and contradictory results have been reported about the antimicrobial action of this combination; some are supporters of synergistic, the others, antagonistic interactions. For example, Ihalin and group successively reported that the addition of SCN⁻ into the peroxidase/H₂O₂/I⁻ system abolished the bactericidal activity of the oxidized halide (Ihalin et al., 1998; Ihalin et al., 2001). Similarly, Ahariz and Courtois (2010) demonstrated a competition between I⁻ and SCN⁻, reporting that the addition of SCN⁻ to peroxidase/G/GOD/I⁻ system (G/GOD: glucose/glucose oxidase as source of hydrogen peroxide) decreased the antifungal effect of the system.
On the contrary, Galley et al. (1997) suggested the incorporation of I\(^-\) in their commercial peroxidase/SCN\(^-\)/G/GOD preservative system, aiming to extend the spectrum of activity of the system – OI\(^-\) being much more effective against yeast and molds than OSCN\(^-\), and the latter efficient against bacteria. Likewise, Bosch et al. (2000) confirmed the synergistic effect between I\(^-\) and SCN\(^-\).

However, all these reported antimicrobial systems involved a peroxidase enzyme and there could be a competition between two substrates for the oxidation by the enzyme. Whereas, in our system it is H\(_2\)O\(_2\) that is oxidizing both substrates, generating a “cocktail” of various halogenating species. The potent antimicrobial activity of ITC mixture is not solely attributed to the presence of one but rather to the combination of different antimicrobial species, nominating it for an attractive weapon to kill pathogenic microorganisms.

Here, we examined the synergism of antimicrobial activity between a mixture of H\(_2\)O\(_2\) with two substrates (I\(^-\) and SCN\(^-\)) against representative Gram-negative and Gram-positive organisms. The disc diffusion and broth microdilution methods used in our study suggested a putative cooperation between two substrates. Specifically, the observations with these two methods indicated synergistic antimicrobial action against two strains tested – *E. coli* and *P. aeruginosa*, whereas, comparable effects towards *S. aureus* and MRSA (Figure 2.2A, B; Table 2.1). One of the features of ITC was that it was the most rapidly bactericidal among the antimicrobials surveyed. ITC killed bacteria very rapidly, and did not just arrest their growth (Figure 2.3). Whereas, other test antimicrobials showed slow killing kinetics or were bacteriostatic. Since the H\(_2\)O\(_2\)/KI/KSCN triple combination is successfully eliminating pathogenic bacteria and what is more, causing the death of bacteria, it could be considered as a potential antimicrobial for treatment of bacterial infections. These observations will lead us to further investigate the potential of ITC on an extended list of antibiotic-resistant clinical isolates.

Microorganisms commonly attach to organic or inorganic surfaces and exist in a biofilm mode. Biofilms are ubiquitous and can occur on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, natural aquatic systems, household surfaces, etc. (Donlan, 2002). Biofilms are responsible for a number of infectious diseases. Cystic fibrosis, native
valve endocarditis, otitis media and chronic prostatitis all emerged from biofilm-associated microorganisms (Donlan and Costerton, 2002). Microbial biofilms developed on or within medical indwelling devices play a central role in pathogenesis of patient infection (Trautner and Darouiche, 2004). Oral biofilms can form a dental plaque on the surfaces of teeth. Further development of dental plaque can lead to serious complications, such as caries, gingivitis, and periodontitis (Hope and Wilson, 2004). Furthermore, bacterial biofilms play a significant role in chronic wound infections and are major barrier to wound healing (Phillips et al., 2010). While biofilm development is not a prerequisite for persistent infection, eradication of biofilm-based infections is particularly difficult (Archer et al., 2011). It is commonly accepted that biofilms are less susceptible towards antimicrobial treatment than planktonic cells, and many antimicrobial agents show reduced efficacy in eliminating biofilms. Our study showed that H$_2$O$_2$/I$_{-}$/SCN$_{-}$ mixture was able to effectively eradicate mono-species biofilm mode of Gram-negative and Gram-positive pathogenic strains within a short 10 minutes exposure (Figure 2.4A). Though biofilms can be formed by a single bacterial species, more commonly biofilms represent complex mixed communities (Elias and Banin, 2012). Studies showed that multi-species biofilms tend to withstand antimicrobial treatment or disinfection more efficiently (Leriche et al., 2003; Burmølle et al., 2006; Kara et al., 2006). In our study, however, ITC could readily eliminate even established dual-species biofilm community within just 30 seconds (Figure 2.4B). The treatment of dual-species biofilm for 10 min at the tested concentrations (15.6 – 250 µg ml$^{-1}$) resulted in no culturable cells (data not shown), thus, the effect of 30 s contact time is presented. Oppositely, the treatment of P. aeruginosa mono-species biofilm over 10 min resulted in viable counts, thus, the effect of 30 s on mono-species biofilm was not assessed. Anyhow, relatively short contact times and low concentrations were required to eliminate mono and dual-species biofilms. We shall also note here that biofilms were established over recirculating batch culture, thus, considering nutrient limitation and bacterial waste accumulation, they were grown for 24 h. Since biofilms tend to become less susceptible as they age, testing the antimicrobial efficacy of ITC on 1-day old biofilms may be a limitation, and future research can potentially address it. In any case, the novel antimicrobial complex could be
considered a useful approach in the fields of medical, food and water microbiology to prevent and control biofilm-associated contaminations.

Successful inhibition of potential pathogenic bacteria would allow possible use of the new iodo-thiocyanate system as a potential therapeutic or disinfectant. The possibility of the development of resistance to an antimicrobial therapeutic is an important factor in determining its potential. Therefore, ITC was tested against four test organisms to assess whether emerging resistance could be developed using multiple exposures to ITC. *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA were cultured for 20 consecutive passages in the presence of ITC, and compared with LVX (Figure 2.5). No increase was recorded for ITC MIC towards all the test organisms, whereas, LVX MIC increased 64-fold for *S. aureus*. These results imply that ITC is unlikely to introduce a resistance in bacteria typically observed with antibiotic usage. This is probably due to a multiplicity of targets, requiring simultaneous mutations within the organism. Similar cases of “resistance resistant” antimicrobial approaches are photodynamic therapy and cold atmospheric plasma that avoid triggering resistance emergence by hitting multiple targets simultaneously (Tavares et al., 2010; Vatansever et al., 2013; Matthes et al., 2014).

While the study presented here would suggest that ITC could be considered as a biocidal agent suitable for disinfection applications, its use in infection treatment will of course be based on whether it can be used without serious harm to the human or animal. The cytotoxic aspects of ITC should thus be evaluated in future research.

Analysis of the chemical composition of the ITC mixture revealed that a bactericidal property of it is due to coupling chemistry of various cidal species. The reactive mixture contained hydroxyl radical, hypo(pseudo)halite ions and iodine, the latter being the dominant antimicrobial component (Table 2.2). Iodine in the form of tri-iodide ions were detected also by UV-Vis spectrometry (Figure 2.6). Individually, these species found in ITC mixture possess antimicrobial properties and target different sites in microorganisms. The hydroxyl radical can directly oxidize all biomolecules, however, the DNA is an especially favored target of it (Farr and Kogoma, 1991; Imlay, 2003). Biological activities of oxidized SCN$^-$ and I$^-$ are summarized in the review of Bafort et al. (2014). The review highlights that OSCN$^-$ targets sulphydryl group of peptides and proteins, inhibiting glycolysis, respiration
and glucose transport in bacteria. However, not all thiols are sensitive towards OSCN$^-$, so the reversible inhibition is occurred, indicating the bacteriostatic effect of OSCN$^-$. Products of oxidized iodide (OI/I$_2$) can affect thiol groups, NAD(P)H, and thioether groups, inhibiting bacterial glycolysis, respiration, glucose transport and pentose phosphate pathways. In contrast, the oxidized $\Gamma^-$ species are reactive towards all thiol groups coming up as bactericidal. Moreover, Thomas and Aune (1977) reported that oxidation of cell components with I$_2$ yielded in the reduction of I$_2$ back to $\Gamma^-$, so that as a result $\Gamma^-$ was not consumed. Released $\Gamma^-$ could be reoxidized and take part again in the oxidation of other protein sulfhydryls. Therefore, one iodide ion could oxidize many cell components. In any case, although, reactive oxygen and iodine species were present in the mixture of ITC, the full chemistry and other possible reactive species generated therein are yet to be clarified.

TEM was used in order to gain an insight on the bactericidal effect of ITC. Similarly, Schreier et al. (1997) carried out TEM study to elucidate the molecular effect of PVP-I on E. coli, S. aureus and Candida albicans cell ultrastructures. They recorded rapid partitioning of riboplasm and pronounced coagulation of nuclear material without visible cell rupture or lysis in E. coli and S. aureus. Under our observations, significant morphological changes occurred in E. coli cells subjected to ITC treatment (Figure 2.7A, B). Cells treated with low concentration (30 µg ml$^{-1}$) of ITC were more translucent in peripheral ribosome-rich cytoplasmic region and contained light-colored ribosomal grains, as compared to the untreated control. ITC may have bound with ribosomal protein thiol groups and “loosened” ribosomes. These groups are involved in molecular interactions that maintain the integrity of the ribosomes, keeping them in a compact configuration (Beeley, 1970). In addition, DNA swirls emerged as a notable bright electron zones in the center of the cells, suggesting that ITC could have an effect on DNA condensation (Figure 2.7A, B). Similar DNA swirling and condensation was observed in E. coli and S. aureus cells treated with silver ions claiming that Ag$^+$ treated cells lost their replication ability (Feng et al., 2000). Increase in the concentration of ITC (300 µg ml$^{-1}$) created even more translucent cytoplasmic region and scarcely distributed ribosomal granules (Figure 2.7A, B). The increase of ITC concentration in the cells also brought to disappearance of distinctive central nuclear zone, indicating the fragmentation of DNA swirls. Likewise, DNA cleavage was observed in DNA gel electrophoresis.
study – ITC was inducing DNA smear when cells were treated at 300 µg ml\(^{-1}\) concentration (Figure 2.7D, sample 2). However, at both concentrations the cells exhibited notable alterations in the cell cytoplasm without losing the cell membrane integrity (Figure 2.7A, B). Live/dead staining assay supported these observations that ITC was not compromising membrane integrity in \textit{E. coli} (Figure 2.8A, C). We should note that at high (300 µg ml\(^{-1}\)) and even low (30 µg ml\(^{-1}\)) concentrations ITC was totally eliminating bacteria within 2 h of exposure (Figure 2.7C), meaning that observable morphological changes should be the representatives of its bactericidal action. The above discussion led to the suggestion that the antimicrobial effect of ITC is perhaps a result of simultaneous events.

### 2.5 Conclusions

Herein, we report on an antimicrobial composition, which exhibits a broad-spectrum activity against pathogenic bacteria in planktonic and biofilm forms, rapidly eliminating them. Moreover, our \textit{in vitro} study showed that the novel antimicrobial does not promote the acquisition of resistance in bacteria. Possession of low MIC, rapid bactericidal activity and little possibility for triggering resistance emergence are positive characteristics for potential therapeutics or biocides. Therefore, it is anticipated that ITC might in future find use as a novel antimicrobial agent, to treat infections and/or to decontaminate surfaces. However, further biocompatibility studies will be necessary to establish its safety as an antimicrobial agent.

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CHAPTER 3

Mutation rate and efflux response of bacteria exposed to the novel biocidal agent

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Author Contributions
LT designed and completed the experiments, wrote and reviewed the manuscript. VOF critically reviewed and edited.
Abstract

Increased mutation rate in bacteria is one of the important mechanisms of acquired drug resistance. Antimicrobials, at sub-lethal concentrations, can act as selectors and promoters of resistance by increasing the mutation rates. Thus, the role of mutation rate needs to be considered in the assessment of potential new antimicrobials. In this study, we measured the mutation rate, by fluctuation analysis, when multiple parallel cultures of *Escherichia coli* were grown in the presence, and absence, of a novel biocidal iodo-thiocyanate complex (ITC). Then, the number of levofloxacin (LVX) resistant-bearing cultures were counted and compared to the total cell number. In this study, *E. coli* did not show elevated mutation rate based on the development of LVX resistance, when it was grown under a near-lethal challenge of ITC. Another relevant factor affecting the appearance of antimicrobial resistance is the role of efflux pumps. Consequently, we evaluated whether the ITC mixture, which contained reactive oxygen and iodine species, could potentially be a substrate for efflux pumps, and thus that efflux-related resistance could arise towards ITC. To screen the effect of efflux on ITC, minimum inhibitory concentration determinations were carried out against *Pseudomonas aeruginosa* in the absence, and presence, of the model efflux pump inhibitor phenylalanine-arginine β-naphthylamide. The results demonstrated that ITC was not affected by the presence of active efflux pumps and that efflux-mediated resistance emergence to it was thus less likely than for some other antimicrobials.
3.1 Introduction

A novel antimicrobial iodo-thiocyanate complex (ITC), inspired by the peroxidase-catalyzed system of phagocytes, and based on the oxidative reaction between hydrogen peroxide and two oxidizable substrates, iodide and thiocyanate, was previously reported (Tonoyan et al., 2017). The complex was described as a mixture of reactive oxygen (ROS) and iodine species, which possessed biocidal properties, meaning it had multiple target sites in the cell and the overall damage to these target sites resulted in broad-spectrum bactericidal effect (Maillard, 2002).

Bacteria have developed different strategies to cope with oxidative stress. A variety of mechanisms can increase the rate at which mutations occur and, consequently, may increase the chances of the emergence of a phenotype able to survive under the stressful conditions (Eliopoulos and Blázquez, 2003). “Hypermutable” (or “mutator”) bacteria are those that have elevated mutation rates due to defects in DNA repair or error avoidance systems (Oliver and Mena, 2010). The term mutation rate simply reflects the chance of mutation to a particular phenotype occurring during a particular period of time. For example, the chance of acquiring a mutation-based resistance to an antibiotic, or to a bactericidal antimicrobial, during a specific period of incubation will increase as the mutation rate increases. Thus, being a mutator will increase the chances of acquiring antimicrobial resistance mediated by mutational events. Many in vitro and in vivo studies have been reported in the literature indicating that antibiotic treatment can contribute to the selection of mutators [reviewed in (Denamur and Matic, 2006)]. The selection should occur at doses close to lethal because low concentrations of the antibiotic will not induce the hypermutation, and high concentrations will kill the population (Eliopoulos and Blázquez, 2003). The presence of mutators is a risk factor in the treatment of infections, as they might be responsible for therapeutic failures (Denamur and Matic, 2006).

The importance of hypermutation in the emergence of antimicrobial resistance prompted us to question whether the exposure of bacteria to a near-lethal level of ITC would increase their mutation rate. In vitro detection of mutator strains can be
done using standard susceptibility testing methods, such as the disc diffusion assay and the Etest, when antibiotic-resistant mutator subpopulations of bacteria can be identified within the inhibition zones. Alternatively, the hypermutation may be detected by determination of mutation rates (Jolivet-Gougeon et al., 2011).

Active efflux of toxic compounds (such as antibiotics and biocides) from cells is another important mechanism that bacteria employ to protect themselves. Bacterial efflux transporters have been grouped into five main families: major facilitator (MF), multidrug and toxin efflux (MATE), resistance-nodulation-division (RND), small multidrug resistance (SMR) and ATP binding cassette (ABC) (Webber and Piddock, 2003). Decreased intracellular accumulation of antibiotics due to active efflux is another mechanism that contributes to the failure of therapy with many currently used antibiotics (Lomovskaya et al., 1999). Of the various bacterial resistance mechanisms against antimicrobial agents, multidrug efflux pumps also comprise a major cause of multiple drug resistance. Moreover, efflux-related resistance can affect many drug classes, including biocides.

In their review, Blanco et al. (2016) reported that the expression of efflux pumps was linked to a decrease in the efficiency of several classes of biocides, such as chlorhexidine digluconate, benzalkonium chloride, chloroxylenol, triclosan, quaternary ammonium compounds, phenolic parabens, DNA intercalating agents, as well as iodine compounds and hydrogen peroxide. Another review from Alcalde-Rico and co-authors (2016), highlighted that efflux pumps were relevant elements in the defense against the oxidative stress produced in the host during phagocytosis. For instance, Bogomolnaya et al. (2013) demonstrated that the ABC type macrolide-specific MacAB pump was induced upon exposure to H₂O₂ and was critical for the survival of Salmonella enterica serovar Typhimurium in the presence of peroxide. They concluded that the MacAB drug efflux pump played a role in the protection of Salmonella against oxidative stress. Likewise, Lin et al. (2015) showed that RND efflux pump smeYZ deletion mutant of Stenotrophomonas maltophilia was more susceptible to redox compounds (such as H₂O₂ and menadione), and human serum and neutrophils, concluding that this efflux pump was involved in the protection against ROS.
Efflux pumps are also known to be involved in resistance to antibiotics through exposure to biocides. For example, Fraud and Poole (2011) examined the impact of ROS (H₂O₂) on mexXY (RND type multidrug efflux system in *P. aeruginosa*) expression and the subsequent development of MexXY-OprM-dependent aminoglycoside resistance. They demonstrated that, while H₂O₂ did not enhance aminoglycoside resistance in vitro, long-term (8-day) exposure of *P. aeruginosa* to H₂O₂ increased the frequency of resistance, dependent upon MexXY. However, the MexXY-OprM efflux system did not contribute to peroxide resistance itself.

Given the contribution of efflux pumps to the rise of resistance to antibiotics and biocides, it was relevant to examine whether ITC was a specific substrate for efflux pumps. Our main aim was to establish if efflux-mediated resistance could be developed towards ITC. A basic in vitro screening test of antimicrobial extrusion from bacteria by efflux pumps is the determination of the antimicrobial MIC change in the absence, and presence, of efflux pump inhibitors (EPI) (Laudy et al., 2016).

In this study, we thus examined the spontaneous and ITC-induced mutation rates in *E. coli* that confer resistance to levofloxacin. Additionally, we investigated whether ITC was a specific substrate for efflux pumps in *P. aeruginosa*, and thus, if ITC exposure had the potential to select for ITC-resistant mutants through efflux pump action.

### 3.2 Materials and Methods

#### 3.2.1 Bacterial strains, growth conditions, antimicrobials and efflux pump inhibitor

The strain used for fluctuation assays was *Escherichia coli* ATCC 25922, and the strain used for efflux-related antimicrobial sensitivity testing was *Pseudomonas aeruginosa* NCIMB 10421. The bacterial strains were grown in lysogeny broth (LB) and Lennox agar (LA), and incubated aerobically at 37°C.

Stock solutions of the antibiotic levofloxacin (Fluka) and the efflux pump inhibitor Phe-Arg-β-naphthylamide dihydrochloride (PAβN; Sigma-Aldrich) were prepared by dissolving the powder in sterile deionized water (dH₂O) and then filter-sterilizing the solutions. The disinfectant, benzalkonium chloride (BKC), was
obtained in a powder form (Sigma-Aldrich) and routinely stored in a desiccator at room temperature. The stock solution of BKC was prepared using sterile dH2O and was filter-sterilized. The stock solution of the novel antimicrobial ITC was prepared by mixing aqueous hydrogen peroxide, potassium iodide and potassium thiocyanate at 1:1:1 (v/v/v) ratios (Tonoyan et al., 2017). The stock solutions of the compounds were kept at 4°C and reused for the duration of the tests; their working concentrations were prepared by dilutions in sterile dH2O or LB.

For the fluctuation assays, LVX was added in the agar media at 0.05 µg ml⁻¹ concentration, and ITC was added in broth media at 3.9 µg ml⁻¹ concentration. For efflux-related antimicrobial sensitivity testing, PAβN was applied in broth media at a concentration of 20 µg ml⁻¹.

3.2.2 Fluctuation assay and determination of the mutation rate by the Poisson distribution (p0) method

The mutation rate was estimated using the p0 method, which calculates the mutation rate based on the distribution of mutants in a number of parallel cultures (Pope et al., 2008). The p0 method requires large number of cultures. Briefly, E. coli ATCC 25922 was cultured in LB at 37°C to an optical density of OD₆₀₀ = 0.5, corresponding to approximately 4 × 10⁸ colony forming units per ml (cfu ml⁻¹). A dense culture of wild-type, levofloxacin-susceptible, E. coli was diluted in LB to yield a 500 ml master inoculum culture, containing approximately 500 cfu ml⁻¹. Thus, 5 ml aliquots were dispensed into 50 culture tubes, to generate independent cultures. To determine the effect of ITC on mutation rate, E. coli was grown in a parallel broth with ITC added. More specifically, ITC was added to the remaining 250 ml of E. coli suspension at concentration equivalent to 0.25 × MIC (3.9 µg ml⁻¹), and again 5 ml aliquots were dispensed into 50 culture tubes. A near-lethal concentration of ITC was chosen such that within the first 6 h of incubation there was an observable effect on growth, which was followed by “recovery” of the culture to near untreated cell density within 24 h treatment (Kohanski et al., 2010a). Each culture was grown overnight at 37°C to the high density (ca. 10⁸ cfu ml⁻¹) needed to obtain LVX-resistant mutants. The average final number of cells (Nᵣ) per treatment was determined by plating an appropriate dilution of two cultures on non-
selective LA plates. An aliquot (1.5 ml) of culture from each tube was transferred to a sterile micro-centrifuge tube. The tube was centrifuged for 10 min, at 5000 × g, the supernatant was aspirated, and the bacterial pellet was suspended in 50 µl of sterile PBS. The entire volume was spread onto a LA plate containing 0.05 µg ml⁻¹ of LVX, which will inhibit the growth of susceptible cells, leaving only resistant mutants. The plates were incubated for 2 days at 37°C. This procedure was expected to produce mutants derived from independent mutational events in each culture.

### 3.2.3 Calculation of mutation rates and statistical evaluation

The multiple parallel cultures were carried out and scored positive if one yielded a resistant mutant. The average number of mutations per culture (m) was calculated based on the number of cultures that yielded no mutants (p₀) using the Poisson distribution, 
\[ p₀ = e^{-m}. \]
By rearranging the formula m can be derived, 
\[ m = -\ln p₀. \]

As only a portion of each parallel culture was plated (1.5 ml out of 5 ml), the observed mutation number per culture were corrected to actual mutation number by the correction factor, 
\[ m_{\text{act}} = m_{\text{obs}} \frac{z-1}{z \ln(z)}, \]
where z is the fraction of cultures plated (Foster, 2006).

The \( \mu \) mutation rate per cell per generation was calculated from the equation, 
\[ \mu = \frac{m}{N\ell}. \]
95% confidence intervals on m and \( \mu \) were calculated using Equations 23 and 24 from Foster (2006). The significant differences were defined by non-overlapping 95% confidence intervals.

### 3.2.4 Determination of MICs of antimicrobials in the absence and presence of efflux pump inhibitor by broth microdilution assay

PAβN is an efflux pump inhibitor, which can also be toxic to bacteria (Askoura et al., 2011). It was thus necessary to define the concentration sufficient to exert an effect on efflux pump activity, but not enough to exert general toxicity to the cell. For that reason, \( P. \text{aeruginosa} \) was grown in LB in the absence, and presence, of the doubling dilutions of PAβN, and growth was monitored in microtiter plate reader (Tecan GENios). The highest PAβN concentration (20 µg ml⁻¹), which did not
change the growth curve of *P. aeruginosa* by comparison to unexposed culture was chosen for further experiments.

To detect the effect of efflux on the antimicrobial activity of ITC and BKC in *P. aeruginosa*, the minimum inhibitory concentrations of ITC and BKC were measured in the presence and absence of PAβN. The MICs were estimated in LB on multiple occasions as detailed previously (Tonoyan et al., 2017). At least a 4-fold decrease in the MIC values after the addition of PAβN was considered significant (Laudy et al., 2016).

**3.3 Results and Discussion**

**3.3.1 Mutation rate of *E. coli* exposed to near-inhibitory concentration of ITC**

Reactive oxygen species can directly damage DNA, leading to the accumulation of mutations. It was expected that the exposure of bacteria to the ITC reactive mixture would lead to an elevated mutation rate, which may have promoted the rise of resistance to ITC and cross-resistance to other antibiotics (Kohanski et al., 2010a). In this study, we used fluctuation analysis to compare the rate of mutations in *E. coli* ATCC 25922 causing resistance to LVX when exposed to 0.25 × MIC of ITC, to the rate of unexposed culture. Quinolones are among the most suitable antibiotics for mutation rate measurements, because resistance to them arises most importantly as a result of point mutations in chromosomal genes (Pope et al., 2008). Resistance to LVX arises, firstly, from alterations in the target enzymes, DNA gyrase and topoisomerase IV. Additional mutations in the next most susceptible targets drug entry and efflux, as well as in genes controlling drug accumulation, augment resistance further. However, resistance to quinolones can also be mediated by plasmids (Jacoby, 2005).

Our results showed that, in the absence of any antimicrobial stressor, the mutation rate of *E. coli* was approximately $1.0 \times 10^{-9}$ mutations/cell/generation [95% confidence limits (CL): $0.7 \times 10^{-9}$ and $1.6 \times 10^{-9}$; (Figure 3.1)]; and that exposure to a near-lethal concentration of ITC did not significantly elevate the mutation rate $2.4 \times 10^{-9}$ mutations/cell/generation (95% CL: $1.49 \times 10^{-9}$ and $2.83 \times 10^{-9}$).
3.3.2 The effect of efflux pump inhibitor on the susceptibility of *P. aeruginosa* to ITC

*P. aeruginosa* is intrinsically resistant to multiple antimicrobial agents, which is attributed to the synergy between the outer membrane impermeability and, most importantly, multidrug efflux pumps (Poole, 2001). So far, six efflux pumps have been identified in *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexJK-OprM and MexVW-OprM, which expel a variety of dissimilar compounds (Kriengkauykiat et al., 2005). One could speculate that these efflux pumps would be involved in adaptation to different types of antimicrobials, including oxidant-based antimicrobial ITC.

An indication that a test antimicrobial is an efflux pump substrate is the increased antimicrobial activity (i.e. lower MIC) of the antimicrobial in the presence of EPI (Dreier and Ruggerone, 2015). The most widely used EPIs for Pseudomonas are the group of peptidomimetic molecules with PAβN as a leading compound (Askoura et al., 2011). PAβN, formerly known as MC-207,110, was reported to be broadly active against MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM in *P. aeruginosa* (Dreier and Ruggerone, 2015). The mechanism of action of PAβN is through competitive inhibition, when PAβN is recognized by the efflux pumps as a
substrate instead of the other compound of interest (Askoura et al., 2011). The pumps expel the PAβN outside the cell, whereas, the antimicrobial remains inside the cell, thus, lower concentration of the antimicrobial is needed to exert its effect.

**Figure 3.2.** Minimum inhibitory concentrations (MIC; expressed in µg ml⁻¹) of ITC and BKC against *P. aeruginosa* NCIMB 10421 in the presence, and absence, of an efflux pump inhibitor (EPI) determined by broth microdilution method. Each experiment is represented by a single point (n=10 for ITC and ITC + EPI, n=5 for BKC and BKC + EPI). The median for each group is indicated on the graph by horizontal bars and by values.

In this work, we investigated the tolerance of a *P. aeruginosa* to ITC and BKC in the presence, and absence, of PAβN. Prior to the screening of the efflux effect, it was established that the addition of 20 µg ml⁻¹ of PAβN to a *P. aeruginosa* culture was not affecting growth. BKC was used for comparative reasons, considering the reports that MexCD-OprJ multidrug efflux pump in wild-type *P. aeruginosa* was induced by the sub-inhibitory concentrations of BKC (Morita et al., 2003) and overexpression of both MexAB-OprM and MexCD-OprJ pumps contributed to adaptation of *P. aeruginosa* to BKC (Mc Cay et al., 2010). The results of this study showed that, when inhibiting efflux activity there was 32-fold decrease in the MIC of BKC, indicating that BKC is a target for efflux pumps and is expelled by *P. aeruginosa* (Figure 3.2). By contrast, the MIC change of ITC was not significant (2-fold decrease), suggesting that ITC was not a substrate for the targeted efflux pumps in *P. aeruginosa*. Being a poor substrate for efflux pumps, ITC thus has reduced risk for the development of efflux-mediated resistance in *P. aeruginosa*, since the
overexpression of such pumps would be unlikely to be effective as a resistance mechanism. However, to make a confident claim only one screen and one organism are not reliable. To understand the transport and resistance mechanisms more progressive tools and systematic studies are required.

### 3.4 Conclusions

We have found that the *E. coli* exposed to the near-lethal level of ITC had a slight, but not significant increase in mutation rate as compared to unexposed culture, suggesting that ITC usage most likely will not promote resistance development *via* increased mutation rates. Additionally, we have shown that ITC was not a specific substrate for multidrug efflux pumps in *P. aeruginosa*, thus, has a low risk for resistance development *via* increased efflux.

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CHAPTER 4

Continuous culture of *Escherichia coli* ATCC 25922, under selective pressure by a novel antimicrobial complex, does not result in development of resistance

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**Author Contributions**

LT designed and completed the experiments, wrote and reviewed the manuscript. GF and VOF contributed to the conception and design of the experimental work and the interpretation of the results. VOF, RF and GF critically reviewed and edited the manuscript.
Abstract

We attempted to generate de novo resistance in antimicrobial-sensitive *E. coli* ATCC 25922 towards a newly described biocidal complex, ITC (iodo-thiocyanate complex), and towards a common antibiotic levofloxacin (LVX), during continuous culture in a chemostat. We measured resistance using the minimum inhibitory concentration metric. The *E. coli* underwent 20-day parallel adaptive evolution routes, under gradually increasing ITC and LVX selection pressure. Long-term exposure of *E. coli* to ITC did not induce resistance to ITC, nor cross-resistance to LVX, and no mutational pattern was evidenced during whole genome sequence (WGS)-based comparisons between exposed and unexposed bacterial populations. Moreover, the exposed *E. coli* population could not survive a 2 × MIC challenge of ITC. By contrast, resistance to LVX was rapidly induced (on day 1 the MIC had increased 16-fold), selected for (by day 14 the MIC had increased 64-fold) and enriched, with a highly characteristic genome mutational pattern. WGS of this evolved population revealed that the majority of mutations appeared in the genes of LVX target proteins (GyrA, ParC, ParE) and drug influx (OmpF). This study suggests that the usage of ITC may not trigger the emergence of facile resistance or cross-resistance, in contrast to common antibiotics.
4.1 Introduction

Antibiotic resistance is a major global health problem. Preventing the emergence of resistance requires a thorough understanding of the underlying causes. Mechanisms of bacterial resistance to antibiotics, or other antimicrobial agents, have been well documented. Some bacteria possess intrinsic resistance towards antibiotics as natural structural and functional properties, while many others acquire resistance, mediated by several biochemical mechanisms (e.g. to stop its entrance, to pump it out, to break it down and/or to inactivate its target), by gene sharing between bacteria, or de novo as a result of specific gene mutations [spontaneous or induced by an agent; (Walsh, 2000)]. Though development of antibiotic resistance is a natural phenomenon and inevitable, the emergence of widespread, multi-agent resistance in many bacteria, including important pathogens, has been accelerated by the use, misuse and abuse of antibiotics and other antimicrobial agents, including triclosan, triclocarbon and others (FDA, 2013). Preservation of antibiotics, underpinned by the availability of novel antimicrobial biocides that avoid the emergence of antibiotic cross-resistance, is thus an important societal goal.

Recently, we described a new biocidal complex, which we named the iodo-thiocyanate complex (ITC), and reported on its antibacterial properties, which suggested the feasibility for use of ITC as a biocide/antiseptic (Tonoyan et al., 2017). Generally, a biocide is described as a chemical agent, usually broad-spectrum, that inactivates microorganisms (McDonnell and Russell, 1999). The term biocide is mainly defined by its usage (Rossmore, 2012) and includes disinfectants, antiseptics and preservatives (Fraise, 2002; Chapman, 2003a). Most biocidal agents have multiple targets in the bacterial cell and the cell death is caused by combined detrimental effects (Maillard, 2002). Moreover, the target sites can differ according to the biocide concentration applied and the interactions resulting the cell death are not always clearly defined (Fraise et al., 2008). Our study showed that ITC is an oxidative mixture containing reactive oxygen (ROS) and iodine species that possess biocidal properties (Tonoyan et al., 2017). These reactive species appeared to interact with bacterial DNA and ribosomes, however, similar to other biocidal agents, the exact cause of cell death induced by ITC was difficult to pinpoint. Taking
into account that such reactive species provide direct and indirect mechanisms for mutagenesis (Dwyer et al., 2009), the potential for the development of bacterial resistance toward ITC, and other antimicrobials, is of concern in the context of its potential application. Indeed, there is increasing concern that extensive biocide usage can be responsible for the selection and maintenance of antibiotic-resistant bacteria in various environments (Russell, 2002).

In recent years, substantial research efforts have been directed towards bacterial adaptation and resistance to different types of biocide (Russell, 2004). As the modes of action of biocides are generally difficult to distinguish, the detailed mechanisms underpinning bacterial resistance to biocides also remain uncertain (Russell, 2002). From the genetic point of view, biocide resistance can arise via mutation or by the acquisition of genetic elements bearing resistance genes. From the mechanistic point of view, as a result of the multiplicity of target sites, resistance to biocides is less common acquired via target inactivation mechanisms. More commonly, biocide resistance can be developed due to the inactivation of the biocide and the reduction of its intracellular concentration to a level that is not harmful to the bacterial cell, that is to say, reduced influx, or enhanced efflux of a biocide (Poole, 2002; Chapman, 2003a; Maillard, 2005). Considering the common shared mechanisms for resistance towards antibiotics and biocides, there is a likelihood of cross-resistance between biocides and antibiotics. Furthermore, according to “radical-based” theory, all bactericidal antibiotics share a common mechanism to kill bacteria, mediated by ROS accumulation in the cells (Kohanski et al., 2007). Treatment with sub-lethal levels of a bactericidal antibiotic could thus generate resistance to other bactericidal antibiotics via ROS-mediated mutagenesis (Kohanski et al., 2010a). In this case, it is expected that exposure of the cells to the sub-lethal level of a ROS-based biocide (ITC) will lead to cross-resistance to antibiotics.

Since resistance is a response adaptation of bacteria to strong natural selection imposed by the application of antibiotics and biocides, understanding the adaptive processes in pathogen populations and, in particular, characterizing the genetic changes that turn drug-sensitive bacteria into resistant variants, is essential to address the problem of resistance. Laboratory evolution experiments have been successfully used to study pathogen adaptation to antibiotics during the evolutionary process, in
real time and under precisely controlled conditions (Jansen et al., 2013). Chemostat-based continuous flow systems are one of the generally applied experimental evolution techniques. In a chemostat vessel, the bacterial population can be maintained in exponential growth phase continuously by the regular inflow of fresh media and simultaneous outflow of waste culture (Ziv et al., 2013). Although mutations in chemostat populations will occur naturally in the presence, or absence, of a mutagenic agent, the evolution may be “directed” by the addition of a selector (such as an antimicrobial drug) and the population may be enriched with a desired phenotype [such as antimicrobial-resistant; (Fleming et al., 2002)]. In such settings, the evolving pathogen population may be continuously challenged by a temporal gradient of drug concentrations to promote the sequential emergence and fixation of multiple resistance mutations leading to increasingly higher resistance levels (Palmer and Kishony, 2013; Lukačišinová and Bollenbach, 2017). Controlled chemostat continuous culture systems may thus simulate bacterial evolution in the host environment, in response to an antimicrobial treatment. As would be expected at an infection site, the pathogens in continuous cultures are grown at high densities and antimicrobials are supplied in a continuous manner (Udekwu and Levin, 2012).

Microbial experimental evolution studies have been revolutionized by recent advances in nucleic acid sequencing technologies. It is now technically and financially feasible to associate the phenotypic response to the underlying genetic changes during an experimental evolution study. Combining experimental evolution with whole-genome sequencing of evolved populations may uncover the genetic basis of laboratory adaptation to antimicrobials and the emergence of antimicrobial resistance (Köser et al., 2014). This approach may reveal how many mutations underlie antimicrobial resistance, how are they distributed across the genome (coding vs. noncoding, synonymous vs. nonsynonymous mutations) and through time (mutations rise and fix at early or later stages of adaptation) and what target genes are contributing to resistance (Dettman et al., 2012)?

In this study, we used chemostat continuous culture systems in an attempt to generate de novo resistance to the ITC biocide and to assess its relationship to cross-resistance to a bactericidal antibiotic in a model organism. *E. coli* ATCC 25922 was used as the model organism, as it is well-characterized at a molecular and
physiological level and a complete annotated genome sequence is available. Levofloxacin was selected as the antibiotic of choice as increased resistance to LVX may be acquired in E. coli under chemostat settings (Fleming et al., 2002) and the mechanisms of resistance toward LVX are well documented. Starting with an isogenic drug-sensitive E. coli ATCC 25922, we exposed the evolving population to increasing levels of ITC or LVX. To implement selective pressure, while maintaining a sizeable population, the concentrations of the antimicrobials were increased in a stepwise manner, with increases applied when the density of the culture had almost completely returned to the pre-antimicrobial treatment level. We monitored the development of ITC and LVX-resistant phenotypes by changes in minimum inhibitory concentration profiles. In parallel, we followed ITC and antibiotic resistance emergence in unexposed and LVX-exposed populations. Next, we performed WGS of polymorphic populations sampled over time and compared starter, intermediate and final populations to reveal possible mutational determinants of ITC and LVX resistance.

4.2 Materials and Methods

4.2.1 Bacteria, growth media and antimicrobials

The parental strain used in this study was the drug-susceptible E. coli ATCC 25922. All the other cultures were derivatives generated by means of chemostat culturing. The lyophilized strain was resuscitated on fresh Lennox agar (Sigma) and the growth from a single colony was transferred into the nutrient broth of choice (lysogeny broth, LB; Sigma). This single colony suspension was used to inoculate three parallel chemostat vessels in order to ensure that the parental strain utilized throughout the continuous culturing was genetically homogeneous at the start. The continuous cultures were grown in quarter-strength LB medium to ensure nutrient limitation. The developed cultures were stored at −20°C in 20% glycerol.

LVX (Fluka) stock solution was prepared from powder in sterile deionized water (dH₂O) and was filter-sterilized; ITC stock solution was prepared as previously described (Tonoyan et al., 2017). Briefly, to prepare 1% stock solution of ITC, hydrogen peroxide, potassium iodide and potassium thiocyanate aqueous solutions
were mixed at 1:1:1 (v/v/v) ratio. Stock solutions of ITC and LVX were kept at 4°C and reused for the duration of the experiments.

4.2.2 Chemostat setup, continuous culturing and enrichment of antimicrobial-adaptive variants

A schematic diagram of the chemostat setup is illustrated in Figure 4.1. Continuous cultures were performed in glass culture vessels (200 × 45 mm) with 100 ± 2 ml working volume equipped with the medium break, by which the media and the air were supplied. The oxygen was delivered into the vessel by pumping filter-sterilized air at a rate of ca. 100 ml min⁻¹. The cultures were mixed by bubbling with sterile air. Medium flow from the reservoir to the culture vessel was controlled by a peristaltic pump (Watson-Marlow 505U). The working volume of the chemostat was fixed by the height of the central overflow tube. Fresh feed was introduced at constant flow rate and culture waste was removed at the same rate according to hydraulic principles. Chemostats were operated at a dilution rate of 0.4 hr⁻¹. This rate of dilution will result in a generation time of 1.73 h per generation (Dykhuizen and Hartl, 1983). Samples were taken using a sterile syringe attached to the sampling port. Chemostat vessels were submerged in a temperature-controlled water bath and operated at 37°C.

Three individual vessels (designated as selection-free, ITC and LVX selection chemostats) were brought to their working volumes and inoculated from a single colony suspension of *E. coli* to obtain a final cell concentration of 10⁶ colony forming units per ml (cfu ml⁻¹). Cultivations were started in batch mode for 24 h to reach culture saturation, and then switched to chemostat mode by initiating the feeding. Meantime, the MICs of ITC and LVX toward this isogenic pre-culture were determined, and these values were used to infer the initial antimicrobial concentrations to be added in the chemostat feeds. Subsequently, two media feeds were supplemented separately with ITC and LVX at their 0.5 × MIC (7.81 and 0.024 µg ml⁻¹, respectively). Antimicrobial concentrations in the media feed were increased stepwise by 0.5 × MIC increments when optical densities (OD₆₀₀) of ITC and LVX-exposed cultures reached to the value recorded prior the addition of the antimicrobial. Each chemostat culture was continuously operated for 20 days (282
generations). Increases in the feed concentrations of ITC and LVX to $3 \times$ MIC and $3.5 \times$ MIC, respectively, were made in the respective feeds during the chemostat course. Daily 2 ml samples were taken from chemostat cultures and used to test for a variety of parameters, such as OD$_{600}$ readings, viable cell counts, broth MIC determinations, culture preservation and WGS of mixed population samples.

Figure 4.1. Schematic representation of the chemostat experimental setup. The air supply system is colored in blue, the media supply system is colored in orange, while the outflow system is colored in grey.

4.2.3 Culture density, cell number and minimum inhibitory concentrations

The cell density of 1 ml chemostat-derived culture was measured using a spectrophotometer (Spectronic 20 Genesys) at a wavelength of 600 nm. Viable cell number was determined by colony counts on antibiotic-free agar plates after appropriate dilutions in phosphate-buffered saline. MICs for LVX and ITC in
quarter-strength LB were measured by following growth in 96-well plates, as described previously (Tonoyan et al., 2017). The MIC was defined as the lowest concentration of antimicrobial agent preventing the appearance of turbidity. All measurements were performed in triplicate and the mode was reported as an MIC. For each of the chemostat cultures, the MIC change was calculated as the ratio of the MIC at sampling day to the MIC of starting day. Chemostat-derived *E. coli* cultures were considered to be resistant to ITC and LVX when a sustained >4-fold MIC increase from initial MIC was noted (D'Lima et al., 2012).

### 4.2.4 Whole-genome sequencing of chemostat-derived polymorphic populations and mutation calling

The focal populations for WGS were unexposed chemostat culture from day 1, 14 and 20, ITC-exposed culture from day 14 and 20, and LVX-exposed culture from day 14 and 20. Aliquots (1 ml) of frozen glycerol stocks of polymorphic population samples (and not single colonies) collected from three chemostat cultures were thawed and immediately pelleted for genomic DNA isolation [further referred as thawed culture (TC)]. The samples represented approximately 1% of the total population in the chemostat. Likewise, a small aliquot was used to revive the frozen samples via overnight growth in 1 ml LB at 37°C and pelleted for gDNA extraction [further referred as revived culture (RC)]. We could not revive the day 14 ITC-exposed culture as a result of the total clearance of bacteria by ITC, and the culture kept at 4°C was used for downstream analysis instead (further indicated as 4°C culture (4C)]. gDNA was extracted using QIAamp DNA Mini Kit (Qiagen), DNA concentration was quantified by Qubit Fluorometer (Life Technologies) and DNA integration was assessed by 0.75% agarose gel electrophoresis.

The genomes were sequenced using the Illumina-based platform of the MicrobesNG service ([http://www.microbesng.uk](http://www.microbesng.uk)) to generate 2 × 250 bp paired-end reads. For fourteen sequenced samples (6 control, 4 ITC and 4 LVX), the average coverage (over the 5130767 bp of the reference genome) ranged from 53 – 200×. Reads were adapter trimmed using Trimmomatic and the quality assessed using in-house scripts combined with SAMtools, BEDtools, and BWA-MEM via MicrobesNG. These reads were then aligned onto the *E. coli* ATCC 25922 reference genome (accession no. CP009072) and putative mutations were identified with the
BRESEQ re-sequencing pipeline. This pipeline can detect single nucleotide polymorphisms (SNP), small insertions and deletions (indel), as well as structural variations (SV), such as large indels, duplications, mobile element insertions or other rearrangements, in polymorphic population samples (Barrick and Lenski, 2009; Barrick et al., 2014; Deatherage and Barrick, 2014; Deatherage et al., 2014). As the samples were “metagenomic”, that is to say a mixture of DNA from different individuals, BRESEQ was run at polymorphism mode with default parameters. BRESEQ uses a Kolmogorov-Smirnov test for bias in the qualities of bases supporting the new variant, and Fisher's exact test for bias in the strands of reads supporting the new variant, in both cases rejecting the mutation if it was biased at a p-value = 0.05 significance level. The variation in reads at a given position was classified as a true population polymorphism (as opposed to sequencing error) when ≥5% of the reads differed from the others for a given allele.

The initial lists of predicted mutations by BRESEQ were manually edited. Mutations in repeat regions may not be fully predicted due to limitations of short-read DNA sequencing data. For that reason, we identified the positions of repeat regions in the reference genome using NUCmer (alignment of multiple closely related nucleotide sequences in MUMmer software) and BLASTN (alignment of somewhat similar sequences in NCBI website) to perform self-comparison. SNPs and small indel (≤20 nt) mutations, which had a BLASTN match in several locations, as well as large indels within repetitive regions or genes with multiple copy numbers were ignored to avoid any concern about inaccurate read alignment. We also discarded the mutations that were present to 100% in all samples (which were likely to be the difference between the reference *E. coli* ATCC 25922 and the starting isolate), considering only the mutations that accumulated throughout the selection experiment.

### 4.3 Results

#### 4.3.1 Growth and phenotypic resistance profiles of chemostat-derived cultures

A continuous culture of *E. coli* ATCC 25922 was gradually adapted to increasing concentrations of ITC and LVX during a 20-day experiment, with the aim of
enriching the population with antimicrobial-resistant bacteria. The emergence of drug resistance in these exposed cultures was then compared with unexposed cultures.

**Figure 4.2.** Growth and MIC profile of continuous cultures of *E. coli* ATCC 25922: control (A), exposed to ITC (B) and exposed to LVX (C). The optical density (600 nm), viable cell counts (cfu ml⁻¹) and minimum inhibitory concentrations (MICs; µg ml⁻¹) of ITC and LVX were measured daily for parallel populations evolving under no antimicrobial selection, selection with ITC and LVX. On the last day, the
concentrations of ITC and LVX added to the medium reservoirs corresponded to 3 × and 3.5 × their original MICs, respectively. The fold change in MIC was the ratio of the MIC obtained on the particular sampling day to the MIC recorded on day 1.

The growth and MIC profiles of three cultures are summarized in Figure 4.2. The cell density, and the number of cells, increased during the first 8 days in culture not exposed to antimicrobial selection, subsequently reaching a relative steady state with 0.1 OD unit and 2 log₁₀ fluctuations (Figure 4.2A). The MIC values of ITC and LVX towards the unexposed continuous culture did not alter significantly between the first and last day of cultivation. This indicated that the culture, which was not exposed to any antimicrobial agent, did not develop resistance toward the ITC and LVX during the 20 days of evolution (Figure 4.2A).

The cell density and cell count measurements of the ITC-exposed culture (Figure 4.2B) indicated that, despite the administration of a media containing 0.5 and 1 × MIC of ITC into the chemostat vessel, the *E. coli* ATCC 25922 grew exponentially up to day 8. The *E. coli* population declined in response to a 1.5 × MIC supplementation of ITC on day 8, but resumed growth during the following days. Subsequently, a 2 × MIC of ITC was applied into the vessel on day 12, which caused growth to abruptly cease – the culture density decreased progressively reaching zero on day 15, while no viable cell numbers were detectable on either day 14 or 15. On day 15, the feeding of the ITC-exposed culture was paused overnight. Following this, the culture resumed growth, possibly as a result of wall-adhering survivors reseeding the planktonic population (Udekwu and Levin, 2012). There was no increase in the ITC MIC profiles in samples tested throughout the culture experiment, despite prolonged exposure to ITC. However, a decrease in MIC was noted on days 13, 14 and 15 compared to the samples tested on days 9 and 12, which may have been an artifact created as a result of culture decline and low starting inoculum density for MIC determinations. No increase was recorded either in the MIC of LVX in the ITC-exposed culture, although 2 to 4-fold fluctuations were noted on days 9 and 12. These observations indicated that exposure to ITC did not induce resistance to it, nor did it induce cross-resistance to LVX. In addition, ITC at a concentration of 2 × MIC was able to eliminate the planktonic bacterial population despite 12 days of prior exposure to ITC (days 14, 15; Figure 4.2B).
On day one, feeding the *E. coli* ATCC 25922 culture with medium containing 0.5 × MIC of LVX, inhibited the growth of the culture and had also triggered a 16-fold increase in LVX MIC by day 2 (Figure 4.2C), indicating a rapid adaptation of the culture to LVX. Subsequently, the culture continued to grow exponentially and the MIC against LVX increased further (32-fold that of the inoculum by day 5), indicating that the culture was being enriched with more resistant variants. The subsequently applied stepwise challenge with LVX caused the culture density and cell number to fluctuate in a saw-tooth pattern – the introduction of a 2 × MIC LVX selected for higher resistance, leading to a 64-fold increase in LVX MIC by day 13 of culture. The MIC of ITC towards the LVX-exposed culture did not alter substantially during the experiment (Figure 4.2C). These results indicated that a stepwise challenge of an *E. coli* ATCC 25922 culture, with increasing concentrations of LVX, rapidly selected for LVX-resistant phenotypes, failed to eliminate the planktonic population and did not induce cross-resistance against ITC.

### 4.3.2 Genotypic profile of chemostat-derived cultures

We performed whole-genome sequencing on samples of the polymorphic populations isolated over the course of the chemostat selection experiments and determined the allelic frequencies of all mutations that reached at least 5% frequencies within an individual population. We sequenced, using the Illumina platform, the genomes of mixed populations from unexposed chemostat culture from day 1 (start), day 14 (intermediate) and day 20 (end), ITC-exposed culture and LVX-exposed culture on days 14 and 20, and then screened for mutations using the BRESEQ pipeline. Our principal focus was on data obtained from thawed cultures (denoted TC), but we also sequenced revived cultures (denoted RC) for quality assurance. Mutations identified as being shared among thawed and revived cultures are available as Venn diagrams in Figure 4.3.
Figure 4.3. The pattern of mutation sharing between thawed (TC) and revived (RC) cultures depicted as Venn diagrams. The exception is the ITCD14 sample, which we could not revive, instead, the mutations from 4°C culture are shown (ITCD144C). Untreated populations are in grey, ITC populations are in yellow, and LVX are in blue.

The total number and the type of genetic alterations identified in the chemostat-derived thawed cultures are shown in Figure 4.4, while the detailed information on predicted mutations is provided in Table 4.1. BRESEQ detected several large insertions and amplifications in the samples, but as all occurred in repeat regions, they were not considered in this analysis to avoid any concern about inaccurate short-read alignment. The number of total mutations in all treatment groups increased over time, reaching the highest totals in the LVX and ITC-exposed day 20 populations. The counts of nonsynonymous substitutions also increased over time in all the test populations.
Figure 4.4. Mutational spectrum of chemostat-derived *E. coli* ATCC 25922 polymorphic population samples. “Metagenomes” of selection-free (CTR day 1, 14, 20), ITC (day 14 and 20) and LVX-exposed (day 14 and 20) thawed cultures were sequenced using the Illumina platform and polymorphisms were identified by BRESEQ. The total number of mutations (N) in each culture is shown above each column. The types of mutations present in each culture are presented below the columns. Base substitutions are presented as nonsynonymous (a point mutation altering the amino acid sequence of a protein), synonymous (a point mutation not altering the amino acid), nonsense (a point mutation resulting in a premature termination codon), noncoding (a mutation in noncoding RNA genes) and intergenic mutations; indels are deletions and insertions of \( \leq 20 \) nt size.

The inoculum population, which was started from a single isogenic colony and was grown in a batch mode for 24 h (CTRD1TC) already bore a collection of mutations by comparison with the reference *E. coli* ATCC 25922 isolate (Figure 4.4; Table 4.1). The majority of these mutations occurred in noncoding (6/15), or intergenic regions (6/15). One synonymous and, most interestingly, two nonsynonymous SNPs were also observed. Notably, the CTRD1TC population was already polymorphic containing subpopulations with a common levofloxacin conferring mutation at position 87 for the GyrA subunit of DNA gyrase – the same codon in *gyrA* was a subject to two mutations: D87G at 64.3% and D87H at 31.8% frequencies (Table 4.1). These two mutations were also present in the unexposed
population on days 14 and day 20, however, their frequencies fluctuate and we cannot predict whether the mutations would have ultimately been lost or retained. Similarly, the population revived from the unexposed day 1 culture (CTRD1RC) contained subpopulations bearing repeated \textit{gyrA} D87G and D87H mutations at relatively similar frequencies (Table 4.1). In any case, according to the MIC profile, the unexposed polymorphic population did not display or develop resistance towards LVX throughout the culture history (Figure 4.2A). In addition, these two nonsynonymous \textit{gyrA} D87 mutations were not present in the ITC and LVX-exposed populations on days 14 and 20. The unexposed culture on days 14 and 20 (CTRD14TC and CTRD20TC) had a slightly different set of mutations by comparison with day 1. Of interest, perhaps, are the nonsynonymous A221P substitution in \textit{malT} gene (MalT, the transcriptional activator of the maltose regulon), 6 nt deletion in \textit{treR} coding (99 – 104/948 nt) region (TreR, the repressor of operons involved in trehalose transport and degradation under osmotic stress) and an A→G substitution in the intergenic region between the dicarboxylate symporter family protein/inner membrane protein Alx (Table 4.1). These changes were present only in the CTRD14 and CTRD20 samples (in both thawed and revived cultures), at >15% frequencies.

Although the ITC-exposed culture did not show decreased susceptibility towards ITC or LVX, we sought genomic alterations that could potentially contribute to the development of ITC resistance, or antibiotic cross-resistance. We examined the mutation list (Table 4.1) of ITC-exposed populations to identify genetic determinants, which may represent possible mutational targets in this evolved population. In most cases, however, we do not know if a particular mutation inactivates, activates, or otherwise acts on the activity of the gene product. The \textit{E. coli} ATCC 25922 population, which was under selection pressure through exposure to ITC, accumulated a higher number of nonsynonymous and synonymous SNPs over time compared to the other cultures (Figure 4.4). The frequencies at which these mutations occur in the ITC-exposed populations are, however, generally low (Table 4.1). Distinctively, the ITC-exposed populations contained subpopulations, which had nonsense mutations in genes, such as the PTS system mannose/fructose/sorbose IID component family protein gene (W264* change in all ITC-exposed populations at 7 to 12% frequencies), or the conserved hypothetical protein under DR76_2342
locus tag gene (W105* change in ITCD20TC&RC populations at 7.7% and 5.8% frequencies, respectively), or in the DNA mismatch repair endonuclease MutH gene (E148* change in ITCD14FC at 9.5%, in ITCD144C at 8.3% and ITCD20FC at 33.2% frequencies, respectively). The ITCD20TC&RC populations harbored a small proportion of subpopulations with an additional W108R mutH substitution (at 7.9% and 6.2% frequencies, respectively). MutH is the endonuclease of the DNA methyl-directed mismatch-repair (MMR) pathway, which is a pathway critical for the avoidance of mutations and maintenance of replicative fidelity. MMR defective bacteria have reduced ability to repair DNA damage, and are thus more prone to develop and accumulate mutations, including those conferring antibiotic resistance (Woodford and Ellington, 2007). These mutations only existed in a small fraction of the population, however, and it is unknown whether finally they would become fixed in a population.

The primary mechanisms of resistance to LVX are chromosomal mutations that result in alteration of the A and B subunits of the target proteins, DNA gyrase (encoded by \( \text{gyrA} \) and \( \text{gyrB} \)) and topoisomerase IV (encoded by \( \text{parC} \) and \( \text{parE} \); and decreased intracellular drug accumulation due to increased drug efflux (mutations in \( \text{acrRAB} \), \( \text{marRAB} \) and \( \text{soxSR} \) operons) (Zayed et al., 2015); or changes in outer membrane proteins [such as OmpF, OmpC, OmpD and OmpA; (Correia et al., 2017)]. In our study, the population that was evolved under LVX selection carried a point mutation in the \( \text{gyrA} \) gene at a frequency of 100% on days 14 and 20 (Table 4.1). The nonsynonymous G to A substitution resulted in a S83L amino acid change, which is the most frequent mutation observed in quinolone-resistant \( E. \ coli \) (Ruiz, 2003). This was a unique mutation, which occurred only in LVX-exposed whole populations (including the revived cultures). In addition, all the LVX-exposed populations uniquely possessed a \( \text{parC} \) G78D nonsynonymous substitution (LVXD14TC at 12.7%, LVXD14RC at 21.4%, LVXD20TC at 18.2% and LVXD20RC at 13.3% frequencies; Table 4.1). The G78D change was an amino acid position (amino acid positions numbered according to \( E. \ coli \)) also known to be associated with LVX resistance in both Gram-positive and Gram-negative bacteria, although reports on this change are less frequent than changes in S80 or E84 of \( \text{parC} \) (Weigel et al., 2002). LVXD20TC also had other mutations occurring in the genes specific to quinolone resistance, such as nonsynonymous A426V, R432S
substitutions and a 3 nt insertion in coding (1373/1893 nt) region of parE, as well as
a 4 nt insertion in the coding (34/1089 nt) region of ompF gene (Table 4.1). The role
that reduced expression, or the complete absence, of the OmpF porin plays in
confering fluoroquinolone resistance has been reported previously (Tavío et al.,
1999; Fàbrega et al., 2010). Our results indicated that decreased susceptibility and
resistance to LVX in the LVX-exposed polymorphic culture were associated with
multiple mutations in the gyrA, parC, parE and ompF genes. It is worth noting that
LVXD20TC had a F36C nonsynonymous change in mutS gene (9.2%). MutS is
another protein of the MMR system, which recognizes and binds to mispaired
nucleotides and enables the further action of the MMR machinery. It was shown that,
as a result of mutS F36A substitution, MutS failed to recognize the mismatch site
thus failing to initiate the repair (Junop et al., 2003). In addition, LVXD20TC had
elevated numbers of mutations, perhaps, as a result of this mutS F36C modification.
The LVX-exposed populations also had an increased number of indels. Interestingly,
LVX-subjected populations also had several nucleotide insertions in the yhjK
(LVXD20TC&RC) and yhjH (LVXD14TC, LVXD20TC&RC) genes (Table 4.1).
YhjK is a predicted c-di-GMP phosphodiesterase (PDE), while YhjH is a c-di-GMP
PDE involved in regulation of the switch from flagellar motility to sessile behavior.
Deletion of the yhjH gene and an insertion in yhjH gene was shown to impair the
swimming motility of E. coli (Girgis et al., 2007) and contribute to the transition
from planktonic to biofilm lifestyle. The 6 nt deletion in coding region (727 –
732/2706 nt) of malT gene, is also noteworthy, perhaps as a modification to facilitate
the adaptation of E. coli to nutrient limiting environment in chemostat [Table 4.1;
(Notley and Ferenci, 1995)].
Resistance to the iodo-thiocyanate complex: Part 2

Table 4.1. Predicted mutations in all chemostat-derived polymorphic population samples.

<table>
<thead>
<tr>
<th>Position*</th>
<th>Mutation</th>
<th>CTR</th>
<th>CTRIC</th>
<th>D14TC</th>
<th>D14RC</th>
<th>D20TC</th>
<th>D20RC</th>
<th>ITC</th>
<th>ITCIC</th>
<th>LUX</th>
<th>LUXIC</th>
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<tr>
<td>1,037,811</td>
<td>C→T</td>
<td>1.4%</td>
<td>1.4%</td>
<td>1.4%</td>
<td>1.4%</td>
<td>1.4%</td>
<td>1.4%</td>
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<td>1.4%</td>
<td>1.4%</td>
</tr>
<tr>
<td>1,046,560</td>
<td>C→T</td>
<td>7.4%</td>
<td>7.4%</td>
<td>7.4%</td>
<td>7.4%</td>
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*Genome position in the ancestral reference strain *E. coli* ATCC 25922 [GenBank: CP009072]. The lengths of colored data bars represent the % of mutations in the populations (unexposed in light grey, ITC-exposed in yellow and LVX-exposed in blue). Nonsynonymous mutations are in blue, synonymous: in green and nonsense: in red.
Table 4.1. Predicted mutations in all chemostat-derived polymorphic population samples (continued).

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*Genome position in the ancestral reference strain E. coli ATCC 25922 [GenBank: CP009072]. The lengths of colored data bars represent the % of mutations in the populations (unexposed in light grey, ITC-exposed in yellow and LVX-exposed in blue). Nonsynonymous mutations are in blue, synonymous: in green and nonsense: in red.

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*Genome position in the ancestral reference strain E. coli ATCC 25922 [GenBank: CP009072]. The lengths of colored data bars represent the % of mutations in the populations (unexposed in light grey, ITC-exposed in yellow and LVX-exposed in blue). Nonsynonymous mutations are in blue, synonymous: in green and nonsense: in red.
4.4 Discussion

The potential for the development of resistance to an antimicrobial is an important factor in determining its suitability for therapeutic or environmental applications. In this study, we used continuous culturing to generate antimicrobial resistant mutations, coupled with WGS to identify those mutations underpinning resistance. Antimicrobial resistance can develop and increase via the gradual accumulation of multiple mutations (Toprak et al., 2012). To study this, we gradually challenged the evolving population in the chemostat with antimicrobials. Three separate enrichment continuous cultures of drug-susceptible *E. coli* ATCC 25922 were operated for 20 days to select for ITC and LVX-resistant mutants, which were then compared with selection-free culture. We measured the resistance using the MIC metric – an increase in resistance occurs when the population is able to grow in the environment of higher concentration of an antimicrobial (Lukačišinová and Bollenbach, 2017). Chemostat cultures grown under no selection pressure and under ITC selection pressure yielded populations with no altered susceptibility towards either ITC or LVX (Figure 4.2A, B). These results indicate that ITC did not cause the emergence of resistance to itself, or the emergence of cross-resistance to the antibiotic LVX. Moreover, ITC at 2 × MIC dose was able to eradicate a $10^{20}$ cfu ml$^{-1}$ load of bacteria (Figure 4.2B), a concentration much higher, for example, than the bacterial concentration in wound fluid allowing the progression of wound healing [$<10^{6}$ cfu ml$^{-1}$; (Bowler et al., 2001)]. This signifies that ITC is suitable to disinfect heavily contaminated sites without risk of the facile emergence of resistance, even at very high cell densities. By contrast, the derivative chemostat culture, grown under LVX selection pressure, rapidly gained resistance towards LVX (MIC of LVX increased 64-fold by day 20), but no cross-resistance was observed towards ITC (Figure 4.2C). LVX also failed to eradicate the bioburden at a 3.5 × MIC concentration. Complete genome sequencing of these populations provided information about the mechanisms underlying the acquired phenotypes.

ITC is a biocidal mixture of reactive oxygen and iodine species. These reactive species can damage a variety of cellular macromolecules and thus trigger adaptive oxidative stress responses in bacteria intended to protect them from these stressors.
Moreover, these agents can provoke their own resistance-promoting responses. Expression of multidrug efflux systems is positively impacted by oxidative agents. Similarly, antioxidant mechanisms are recruited in response to antimicrobial exposure, which are known to generate ROS as key to their lethal effects. As such, oxidative stress responses may contribute to antimicrobial resistance in several ways (Poole, 2012). Poole has reviewed, in detail, the evidence supporting links between the oxidative stress response and antimicrobial resistance in bacteria, and summarized the mechanisms of oxidative stress-induced resistance (Poole, 2012). These include, amongst others, the redox-responsive regulator SoxSR of multidrug efflux system AcrAB-TolC in *E. coli*; e.g. reactive agents can cause direct mutations leading to constitutive *soxS* expression and elevated *acrAB* expression and, thus, antimicrobial resistance. We examined the genome sequences of ITC-exposed populations (Table 4.1) for significant (nonsynonymous, nonsense) nucleotide variations, including those involving amino acid change or amino acid sequence termination, and compared with stress-inducible antimicrobial resistance mechanisms [Table 1 from reference (Poole, 2012)]. We could not identify any determinants, which could have contributed to oxidative (or stress in general) response-mediated resistance of the ITC-exposed culture to ITC and/or LVX.

On the other hand, cross-resistance may occur when different antimicrobial agents hit the same target, initiate a common pathway to cell death, or share a common path to access their respective targets. As a result, the development of resistance to one antibacterial agent is accompanied by the emergence of resistance to another agent (Chapman, 2003b). LVX is a member of the fluoroquinolone class of antibiotics with broad antimicrobial activity against clinically relevant bacteria causing respiratory, skin and genitourinary tract infections. Following regulatory approval in the mid-1990s, LVX became one of the most widely prescribed antibiotics in the world (Noel, 2009). The heavy prescription of LVX has led to increased resistance. LVX resistance is thought to be a result of gradually acquired changes, and several mutations are required to produce a high level of resistance (Fàbrega et al., 2009). In Gram-negative bacteria, resistance mutations firstly occur in *gyrA*, with “hot spots” for mutation at amino acid positions 83 and 87. Once this initial mutation has reduced the susceptibility of resistant bacteria to LVX, additional mutations in target genes *parC, parE*, or else, mutations causing reduced drug accumulation, can further
augment the resistance (Jacoby, 2005). Indeed, in our study, the day 14 LVX-exposed population was entirely composed of \textit{gyrA} S83L mutants (occurrence 100%), while a proportion of the population also carried the \textit{parC} G78D mutation. By day 20, the LVX-exposed population was further enriched with \textit{parE} and \textit{ompF} mutants. These results indicate that, the acquisition of high level LVX resistance (e.g. a 64-fold increase in MIC) was related to mutations in genes encoding the three protein targets and mutations in uptake protein. In general, combining chemostat and WGS was a productive approach to generate, follow and understand the evolution of LVX resistance in \textit{E. coli} ATCC 25922. ITC-exposed populations, by contrast, did not possess any common mutations conferring LVX resistance (Table 4.1), indicating that, although ITC has the potential to generate random mutations; perhaps, randomly those mutations did not occur in the target genes of LVX and genes providing reduced drug accumulation.

The MMR pathway safeguards the genome by correcting the base mismatches arising as a result of replication errors (Hsieh and Yamane, 2008). Recent studies suggest that MMR may also be important in the response to oxidative DNA damage (Bridge et al., 2014). Defects in MMR result in increased rates of mutations in organisms ranging from bacteria to humans. MMR-defective bacteria are considered to be “hypermutable” and to express a “mutator” phenotype. Mutator phenotypes, in general, result from alterations in genes coding for DNA repair enzymes (\textit{mutS}, \textit{mutL}, \textit{mutH} and \textit{mutU}). The genes affected in the studied mutator strains are \textit{mutS}, \textit{mutL}, \textit{mutH} and \textit{mutU} in decreasing order (Eliopoulos and Blázquez, 2003). The \textit{E. coli} ATCC 25922 population, which was grown under ITC selection, harbored a nonsense mutation in \textit{mutH} gene on day 14 [detected in thawed culture (ITCD14TC) at a frequency of 9.5% and in 4°C culture (ITCD144C) at a frequency of 8.3%] and on day 20 [detected at a frequency of 33.2% in thawed culture (ITCD20TC), but not in a revived culture (ITCD20RC)]. The LVXD20TC population also harbored the F36C nonsynonymous change in \textit{mutS} gene at a frequency of 9.2%. The ITCD20TC and LVXD20TC cultures, indeed, had an elevated number of mutations by comparison to the other samples; however, we cannot definitively ascribe this to \textit{mutH} or \textit{mutS} mutations. These mutations only represented a small fraction of the total; but to be able to produce a mutator phenotype they must be genetically dominant. It is worth noting here that, in Chapter 3, we measured the mutation rate
conferring LVX resistance in *E. coli*, which was grown for 24 h in the presence of a near-lethal concentration of ITC, and showed no increase in the mutation rate by comparison to the unexposed culture. At present, there are mixed views about the link between hypermutation and emergence of antimicrobial resistance (Eliopoulos and Blázquez, 2003). Schaaff et al. (2002) found that vancomycin resistance could be reached much more quickly in a mutator background (*mutS* knockout mutant) vs. nonmutator (5 vs. 19 passages, respectively). However, in our study during the timeline of the selection experiments, the ITC-exposed population did not develop elevated MIC values for ITC and LVX (Figure 4.2B), while the LVX-exposed population showed elevated MIC values towards LVX (which was due to other primary mutations rather than *mutS*), but not toward ITC. These mutations may have appeared too late in the selection to become common in the populations. However, other investigators have concluded that, despite the fact that a *mutS*-inactive strain of *S. aureus* had 78-fold higher mutation frequencies compared to wild type, neither stable hypermutation nor transient increases in mutation frequency were likely to play a significant role in the development of antibiotic resistance in *S. aureus* (O’Neill and Chopra, 2002).

### 4.5 Conclusions

The overall conclusions of this study are that we were unable to generate *de novo* resistance to ITC in a drug-sensitive *E. coli* ATCC 25922, despite 20 days of selective evolution, and, thus, we could not identify mutations that would have yielded ITC resistance. Mutations in the gene of DNA mismatch repair endonuclease *MutH* may be implicated in elevated mutation numbers and result in mutator phenotype, thus contributing to antimicrobial resistance; however, these mutations did not become prominent in the evolved ITC-exposed population. By contrast, resistance to levofloxacin quickly built up in the *E. coli* ATCC 25922 culture exposed to increasing concentrations of LVX, apparently driven by mutations in the genes coding for the target proteins (GyrA, ParC, ParE) and reduced drug accumulation (OmpF). Judging from the example of LVX, combining experimental evolution and whole-genome sequencing can provide a deeper understanding of the principles of how resistance emerges under different selective pressures.
The lack of emergence of facile resistance to ITC in *E. coli* ATCC 25922, and the lack of cross-resistance to LVX are encouraging with respect to the potential for applications of ITC as a biocidal agent in a variety of settings. An understanding of the possible mechanisms, if any, of ITC resistance, however, requires further longer-term investigations. The dynamics and determinants of potential ITC resistance should also be tested in numerous parallel cultures of a range of other bacteria, and the potential for cross-resistance studied on a range of important antibiotics.

### 4.6 Acknowledgments

Financial support from Irish Research Council Government of Ireland Postgraduate Scholarship Scheme (GOIPG/2013/584) is gratefully acknowledged. The authors would like to acknowledge MicrobesNG ([http://www.microbesng.uk](http://www.microbesng.uk)) team for excellent genome sequencing service. The authors wish to sincerely thank Dr. Leighton Pritchard (The James Hutton Institute, Dundee, Scotland) and Dr. Fiona Brennan (Teagasc, Johnstown Castle, Co. Wexford, Ireland) for advice on genome sequencing and for valuable scientific discussion. Huge thanks to Nick Waters (NUI, Galway) for his assistance in bioinformatics.
CHAPTER 5

In vitro comparative cytotoxicity study of a novel biocidal iodo-thiocyanate complex

The concise version of this manuscript is submitted to Toxicology In Vitro.

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Author Contributions
LT designed and completed the experiments, wrote and reviewed the manuscript. PMC, CG, AB and VOF contributed to the conception and design of the experimental work and the interpretation of the results. VOF, AB, GF and RF critically reviewed and edited the manuscript.
Abstract

Novel biocides, which avoid the induction of cross-resistance to antibiotics, are an urgent societal requirement. Here, we compared the cytotoxic and bactericidal effects of a new antimicrobial agent, the iodo-thiocyanate complex (ITC), with those of the commonly used antiseptics, hydrogen peroxide (H₂O₂), povidone iodine (PVP-I) and Lugol’s iodine (Lugol). The antimicrobials were co-incubated for 10 min with HeLa and *Escherichia coli* cells in the presence and absence of organic matter (Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum). The cytotoxic concentrations of ITC were equivalent to its bactericidal concentrations (<7.8 µg ml⁻¹). By contrast, cytotoxic effects of H₂O₂, PVP-I and Lugol were apparent at concentrations lower than their bactericidal concentrations (250, 125 and 31.3 µg ml⁻¹, respectively). The cellular effects of ITC were not quenched by organic matter, unlike the other antiseptics. ITC, PVP-I and Lugol had dose-dependent effects on the viability of horse erythrocytes, while H₂O₂ showed no hemolytic impact. ITC, at 30 or 300 µg ml⁻¹, did not cause DNA breakage in HeLa cells, as assessed by *in vitro* comet assay, whereas H₂O₂ caused extensive single-strand DNA breaks. The pronounced antimicrobial potency of ITC and its favorable cytotoxicity profile suggests that ITC should be considered for antiseptic applications.
Cytotoxicity of the iodo-thiocyanate complex

5.1 Introduction

The increasing occurrence of antibiotic resistance in pathogenic bacteria, coupled with a dramatic decline in the number of newly approved antibiotics, represents a major societal challenge. In the context of growing resistance to antibiotics, wound infection control and management, along with the control of potentially pathogenic bacteria in healthcare environments, is becoming challenging and represents a major healthcare burden (WUWHS, 2008).

Nature suggests a possible future direction in the form of peroxidase-catalyzed antimicrobial systems. A peroxidase enzyme together with hydrogen peroxide, inorganic ion substrate and generated oxidized products is known as a peroxidase system (Kussendrager and van Hooijdonk, 2000). These naturally occurring systems are part of the host defense network and provide an immediate defense against invading microorganisms (Davies et al., 2008). A large number of studies have investigated and asserted the antimicrobial potential of peroxidase-catalyzed systems, as extensively reviewed elsewhere (Kussendrager and van Hooijdonk, 2000; Naidu, 2000; Seifu et al., 2005; Davies et al., 2008; Bafort et al., 2014). Peroxidase/hydrogen peroxide/halide systems have a dual role, however, acting as both a friend and a foe (Klebanoff, 2005; Davies et al., 2008). As they possess a non-specific, broad-spectrum target mechanism, aside from mediating bacterial cell killing, destroying invading parasites, combating fungal infections and inactivating viruses, they can also attack a variety of mammalian cells (Edelson and Cohn, 1973; Clark and Klebanoff, 1977), including tumor cells (Clark and Klebanoff, 1975; Jong and Klebanoff, 1980; Henderson et al., 1981).

Peroxidase-catalyzed systems may also inspire the development of a new generation of antimicrobial agents. Our previous research evaluated three such antimicrobial agents (Tonoyan et al., 2017). The agents, all produced without a peroxidase enzyme, were formed by the reactions of $\text{H}_2\text{O}_2$ with iodide ($\Gamma^-$) salt ($\text{H}_2\text{O}_2/\text{KI}$); or $\text{H}_2\text{O}_2$ with thiocyanate ($\text{SCN}^-$) salt ($\text{H}_2\text{O}_2/\text{KSCN}$); or $\text{H}_2\text{O}_2$ with both ion substrates ($\Gamma^-$ and $\text{SCN}^-$). The latter reaction forms the iodo-thiocyanate complex (ITC). In the ITC, $\text{H}_2\text{O}_2$ serves as a source of oxidation for two substrates, generating reactive oxygen and iodine species within the reaction mixture, which possess...
powerful antimicrobial properties. The mixture was shown to contain low concentrations of antimicrobial species such as hypoiodite, hypothiocyanite and hydroxyl radical; with molecular iodine being the major cidal component. The biocidal ITC formulation effectively and rapidly killed Gram-negative and Gram-positive bacteria, including multidrug-resistant *Staphylococcus aureus*. It was shown that ITC was capable of eradicating mono and dual-species bacterial biofilms within short exposure times (30 s). In addition, we were unable to induce resistance in bacteria to the ITC, likely due to the presence of multiple cellular targets (Okano et al., 2017). ITC, therefore worked in agreement with Paul Ehrlich’s famous guidance to “hit hard and hit fast” (Tonoyan et al., 2017). These characteristics suggested the potential for use of ITC as a new antimicrobial to prevent, and, possibly, treat bacterial infections.

In addition to antimicrobial activity, however, the cytotoxic potential of the new agent must also be evaluated. A variety of approaches can be used to evaluate and screen the *in vitro* toxicity of antimicrobial substances. Testing the effects of compounds on the viability of mammalian cells grown in culture (*in vitro* cytotoxicity tests) is widely used as an indicator of potential toxic effects in whole animals (Riss et al., 2011). Among the different cytotoxicity tests, the methyl tetrazolium (MTT) viability assay is the most popular, low-cost and convenient method (Fotakis and Timbrell, 2006). Considering that antimicrobials may potentially come into direct contact with blood, an evaluation of hemotoxicity is also worthwhile. The standard measure of blood compatibility is hemolytic activity or the lysis of red blood cells (Li et al., 2012). Genotoxicity testing is also an important aspect of the safety assessment of broad-spectrum substances, including pharmaceuticals and biocides (Corvi and Madia, 2016). Our previous research indicated that the ITC contains reactive species, which may have the potential to induce bacterial DNA breakage; the detection of DNA breaks by single cell gel electrophoresis (comet assay), would thus be informative.

The aim of the present study was to investigate the *in vitro* cytotoxicity, hemolytic activity and genotoxicity of the novel antimicrobial ITC in relation to its antimicrobial activity. The potential suitability of ITC as an antiseptic was then considered and compared with H$_2$O$_2$, PVP-I and Lugol’s iodine.
5.2 Materials and Methods

5.2.1 Antimicrobial agents and preparation

All the materials were purchased from Sigma-Aldrich, unless otherwise stated. The antimicrobial agents evaluated in this study were H2O2/KI, H2O2/KSCN and H2O2/KI/KSCN [ITC; (Tonoyan et al., 2017)]. The stock solutions were prepared by combining concentrated solutions of H2O2 (v/v) with KI (w/v) and/or KSCN (w/v) to obtain 1% stock solutions for each agent. These solutions were considered as 1% according to the concentration of H2O2 present in the mixtures, as KI and KSCN do not possess antimicrobial activity alone (data not shown). The antiseptics H2O2 [30% (v/v) stock solution], PVP-I [10% (w/v) available iodine stock] and Lugol’s iodine [1.25% (w/v) available iodine stock] were also used in this study. The stock solutions were diluted to the desired working antimicrobial concentration range using sterile deionized water (dH2O).

5.2.2 Test bacteria, cell cultures, nutrient solutions and growth conditions

The bacterial strain used in this study was Escherichia coli ATCC 25922 (from the American Type Culture Collection) which was cultured aerobically on Lennox agar and lysogeny broth at 37°C. HeLa human cervical epithelial cell lines were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures) and were used between 20 – 50 passages. HeLa cells were grown as a monolayer in complete DMEM [Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin] at 37°C, 5% CO₂.

5.2.3 Cytotoxicity studies

5.2.3.1 Determination of simultaneous cellular and bacterial toxicities of antimicrobials towards human and bacterial cells

The cellular toxicity of the test antimicrobials and antiseptics towards human epithelial HeLa cells was assessed utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, i.e. estimation of cell viability and
metabolism based on mitochondrial succinate dehydrogenase activity (Fotakis and Timbrell, 2006), whereas, their antimicrobial potential towards bacterial *E. coli* cells was assessed using viable bacterial cell counts.

The simultaneous cytotoxic and bactericidal effects of antimicrobials were determined by co-incubation of HeLa and *E. coli* cells, for 10 min, with the antimicrobials, at a range of concentrations, in two different media [phosphate-buffered saline (PBS) and DMEM supplemented with 10% FBS without addition of antibiotics (DMEM + FBS)]. HeLa cells were seeded at a concentration of $8 \times 10^3$ cells well$^{-1}$ in 96-well plates in complete DMEM media, to a final volume of 200 µl, and then incubated at 37°C in a humidified environment containing 5% CO$_2$. After 72 h, the media was aspirated and the confluent cell monolayer was washed with PBS. After the wash, the cell monolayer in each well was covered with 200 µl PBS, or DMEM + FBS, containing *E. coli* cells [at $10^6$ colony forming unit per ml (cfu ml$^{-1}$) final density] and the antimicrobial agent (at a specific concentration). Eight 2-fold dilutions of the selected antimicrobials were tested, and the concentrations attained in the wells ranged from 7.8 to 1000 µg ml$^{-1}$. The HeLa and *E. coli* cells were exposed to the antimicrobials in PBS or DMEM + FBS for 10 min at room temperature, after which the supernatant was removed from the cell monolayers. It was shown previously (Tonoyan et al., 2017) that ITC at a specified concentration range, could eradicate *E. coli* biofilm within 10 min, and this was thus considered an appropriate duration of exposure to demonstrate any antimicrobial activity. Following the 10 min incubations, 10 µl aliquots were taken from the supernatant and 10-fold serially diluted in PBS. Aliquots (5 µl) of the dilutions were then replica (three per sample dilution) spot-plated on agar plates to enumerate viable *E. coli*. This method can be used to enumerate bacteria on a high-throughput scale in a fast, easy-to-use, labor-efficient and cost-efficient manner (Sieuwerts et al., 2008; Beck et al., 2009; Harrison et al., 2010). Bactericidal activity (expressed as a minimum bactericidal concentration; MBC) was defined as a $>3 \log_{10}$ reduction (99.9% kill) in colony forming units per ml (cfu ml$^{-1}$) from the starting *E. coli* inoculum concentration (Rose and Poppens, 2009).

The HeLa monolayers were washed twice with PBS and treated with complete DMEM supplemented with MTT dye (0.6 mg ml$^{-1}$) for 4 h at 37°C. The medium
Cytotoxicity of the iodo-thiocyanate complex

containing MTT was aspirated, formazan crystals were dissolved in 150 µl dimethyl sulfoxide (DMSO) and the absorbance (A) was measured at 595 nm (Tecan GENios, Salzburg, Austria). Cellular viability was expressed as a percentage of the untreated controls, implying the formula:

\[ \text{Viability, \%} = \frac{A_{\text{Treated}} - A_{\text{Blank}}}{A_{\text{Untreated}} - A_{\text{Blank}}} \times 100\% \]

where an untreated control sample contained cells and DMSO only, while blank sample contained DMSO only. All the treatments and their concentration range were tested in duplicate, and all experiments were repeated at least twice. HeLa viability results were demonstrated graphically as mean % viability ± standard deviation (SD), *E. coli* viability results as mean log_{10} cfu ml^{-1} ±SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test (SigmaPlot Version 11, Systat Software, Inc., San Jose, USA).

5.2.3.2 Visualization of morphological changes in Hela cells

The impact of the antimicrobial treatments on HeLa cells was also evaluated microscopically. Untreated cells, cells lysed with 10% Triton X-100, and cells exposed to the highest concentrations of the antimicrobials (1000 µg ml^{-1}) in either PBS, or DMEM + FBS, were visualized in 96-well plates, after 10 min antimicrobial treatments, using bright-field optic microscopy on an inverted microscope (Leica DM IL LED, Leica Microsystems Ltd.) fitted with a DFC420C digital camera using LAS at 20× objective magnification.

5.2.3.3 Recovery of HeLa cells after withdrawal of antimicrobials

To assess whether the cytotoxic effects of the antimicrobials on human cells were reversible or irreversible, cytotoxicity was measured either immediately after 10 min exposure of HeLa cells to antimicrobials, or after recovery based on a previously published protocol (Müller and Kramer, 2007). Briefly, HeLa cells were exposed to antimicrobials in PBS or DMEM + FBS media for 10 min and the MTT assay was performed. Subsequently, another set of HeLa cells were exposed to antimicrobials in PBS or DMEM + FBS for 10 min, carefully washed twice to remove the
antimicrobials, and then cultured for a further 24 h. Following incubation, the MTT assay was performed and cell viability was determined. Data were represented graphically as mean % viability ± SD, from two independent experiments, conducted in duplicate. To estimate the significant recovery of cells exposed to different concentrations of antimicrobials, the data from HeLA cells exposed for 10 min and then immediately tested was compared to data from the revived HeLA cells by two-way ANOVA, followed by Tukey’s pairwise multiple comparison test (SigmaPlot).

5.2.4 Hemolytic activity studies

5.2.4.1 Determination of hemolytic activity of antimicrobials towards horse red blood cells (hRBC)

The hemolytic activity of the antimicrobials was tested by determining the release of hemoglobin in horse erythrocyte suspension as a measure of red blood cell lysis (Evans et al., 2013).

The horse RBCs (hRBCs) were prepared by centrifugation of defibrinated horse blood (TCS Biosciences; packed cell volume 40%) at 3000 × g for 5 min. Plasma, white blood cells and platelets were removed as a supernatant by washing the blood with PBS. After obtaining a clear supernatant, the hRBC pellet was resuspended in PBS to 4% (v/v) hRBC suspension. 900 µl of hRBC suspensions were then added to micro-centrifuge tubes containing 100 µl of antimicrobial solutions to give final concentrations of antimicrobials at a range of 7.8 – 1000 µg ml⁻¹. PBS and 10% Triton X-100 were used as 0% and 100% hemolysis controls, respectively. Considering that dH₂O can be hemolytic and the concentration range of antimicrobials was prepared in dH₂O, 100 µl of dH₂O (maximum added dH₂O) in 900 µl hRBC was included as a reference for water-based samples. After incubation, the tubes were centrifuged at 3000 × g for 5 min and 20 µl aliquots from supernatant were transferred into 96-well plates containing 180 µl PBS. Hemoglobin release was monitored by measuring the absorbance at 540 nm (Tecan GENios, Salzburg, Austria). Hemolysis was calculated as a percentage of the maximum hemolysis achieved by Triton X-100, implying following formula:

\[
\text{Hemolysis, } % = \left( \frac{A_{\text{Treatment}} - A_{\text{PBS}}}{A_{\text{Triton}} - A_{\text{PBS}}} \right) - \left( \frac{A_{\text{Water}} - A_{\text{PBS}}}{A_{\text{Triton}} - A_{\text{PBS}}} \right) \times 100%
\]
Cytotoxicity of the iodo-thiocyanate complex

where $A_{\text{Treatment}}$, $A_{\text{PBS}}$, $A_{\text{Water}}$ and $A_{\text{Triton}}$ are the absorbances of the supernatants from samples incubated with the antimicrobials, from untreated control PBS, from reference control dH2O and 100% lysis control 10% Triton X-100, respectively. All the treatments were carried out in duplicates and experiments were repeated at least twice. The results were depicted graphically as mean % hemolysis ± SD. The statistical significance between different concentrations, by comparison to untreated cells, was assessed using one-way ANOVA with follow-up Tukey’s post hoc test (SigmaPlot).

5.2.4.2 Visualization of morphological changes in horse erythrocytes

To assess erythrocyte lysis, hRBCs, exposed to the highest concentration of the antimicrobials (1000 µg ml$^{-1}$) for 1 h, were visualized microscopically. Aliquots (10 µl) from the antimicrobial-treated hRBC suspensions were transferred to a hemocytometer chamber and viewed by inverted microscope (Leica DM IL LED, Leica Microsystems Ltd.) at 40× objective magnification.

5.2.5 Genotoxicity studies

5.2.5.1 Assessment of ITC-induced DNA breaks in HeLa cells

The comet assay was used to analyze and quantify any DNA strand breaks caused by ITC at the individual cell level. The assay was performed as described elsewhere, but with some modifications (Olive and Banath, 2006). Specifically, 80% confluent HeLa cells were detached and resuspended in PBS at a density of $5.75 \times 10^5$ cells ml$^{-1}$. ITC was added to cell suspensions, at final concentrations of either 30 or 300 µg ml$^{-1}$, and incubated for 30 min. Exposure of cells, not treated with ITC, to either H$_2$O$_2$ at 300 µg ml$^{-1}$ or UV-C irradiation (performed under the Philips TUV 64T5/SP UV-C disinfection lamp of laminar SterilGard Hood, producing UV radiation of 253.7 nm) for 30 min were used as two positive controls, while untreated cell suspensions in PBS represented the negative controls. After treatment, a 50 µl aliquot of each cell suspension was added to 450 µl 1% low melting point agarose in PBS (LMPA) and 150 µl cell suspension/LMPA mix were subsequently placed onto a microscope slide, which had been previously coated with 1% normal melting point agarose (200 µl slide$^{-1}$) and dried at 50°C. Thus prepared, each slide contained approximately 10000 cells. Coverslips were added to the slides and the preparations
were allowed to gel a 4°C. Once gelled, the coverslips were removed and slides were submerged in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% sodium lauroyl sarcosine, 1% Triton X-100 and 10% DMSO). Lysis was performed for 2 h at 4°C. Afterwards, the microgels were rinsed with, and then placed in, cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13) to equilibrate for 30 min. Electrophoresis was conducted in the same buffer at 20 V (1 V cm⁻¹) and about 200 mA (adjusted by raising or lowering the buffer level) for 30 min. Immediately after electrophoresis, the slides were neutralized in cold 0.4 M Tris pH 7.4 (3 × 5 min washes) and then washed with cold dH₂O. Microgels were further stained with 200 μl of 1 μg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) for 10 min and then washed with dH₂O. All steps were performed under dimmed light to reduce additional light-induced DNA damage. The slides were then observed by inverted fluorescent microscope (Leica DMI3000 B, Leica Microsystems Ltd.) at 20× objective magnification and comets were scored using Comet Assay IV image analyzing software (Perceptive Instruments Ltd.). To quantify DNA damage, the results were expressed in the average extent tail moment ± SD of 50 randomly selected comets per treatment.

\[
\text{Extent tail moment, } \mu\text{m} = \text{tail length} \times \text{tail DNA %}
\]

where tail DNA % is the tail DNA intensity/cell DNA intensity × 100, in %, and tail length is the distance from tail start to the end of the tail, in μm. The significance of difference between control and treated groups was statistically analyzed by one-way ANOVA, followed by Tukey’s post-hoc test (IBM SPSS Statistics 21 Inc., Chicago, USA).

5.3 Results

5.3.1 Cytotoxic and antibacterial activities of antimicrobials

The cytotoxic and antibacterial effects of the antimicrobials were determined simultaneously by co-incubation of human HeLa, bacterial E. coli cells in different concentrations of antimicrobials, for 10 minutes, in either PBS, or in DMEM + FBS, and then applying the MTT assay and viable cell counting. When incubated in PBS (Figure 5.1A), H₂O₂/KI and H₂O₂/KSCN were cytotoxic at all concentrations tested.
Cytotoxicity of the iodo-thiocyanate complex (7.8 – 1000 µg ml⁻¹) with very slight antimicrobial activity, with the exception of the highest dose (1000 µg ml⁻¹) of H₂O₂/KSCN, which caused a 5 log₁₀ reduction in E. coli (Figure 5.1A). ITC, when incubated in PBS, was highly toxic towards both cell types after 10 min exposure; even at the lowest test concentration of ITC (7.8 µg ml⁻¹) no viable E. coli and HeLa cells were detected (Figure 5.1A). Amongst the antiseptics, the antimicrobial potential of H₂O₂ increased in a concentration-dependent manner, with a bactericidal effect noted at a concentration of 250 µg ml⁻¹ (the MBC value). It was, however, highly cytotoxic at all concentrations tested (Figure 5.1A). The cellular and bacterial toxicities of PVP-I also increased in a manner depending on its dose. PVP-I exhibited the least cytotoxic performance among the tested antimicrobials – around 20% cytotoxicity at 31.3 µg ml⁻¹. However, the bactericidal concentration of PVP-I (125 µg ml⁻¹) was accompanied by toxicity towards HeLa. Lugol was cytotoxic even at concentrations below the MBC (31.3 µg ml⁻¹; Figure 5.1A).

Microscopic observations supported the results from the MTT cytotoxicity assay. With respect to the PBS incubations (Figure 5.2A), H₂O₂/KI, H₂O₂/KSCN and H₂O₂, at 1000 µg ml⁻¹, completely lysed and “dissolved” HeLa cells after a 10 min contact time. However, upon exposure to ITC, PVP-I and Lugol, the cells seemed to not be fully lysed, but looked unhealthier in comparison to untreated control (Figure 5.2A).

The antimicrobial and cytotoxic effects of the antimicrobials were notably different in the presence of the cell culture media supplemented with 10% fetal bovine serum (DMEM + FBS; Figure 5.1B), by comparison to the PBS incubations. H₂O₂/KI had a similar modest antimicrobial activity profile, but its cytotoxic impact decreased (Figure 5.1B). H₂O₂/KSCN had no antimicrobial activity at all test concentrations and was also less cytotoxic towards HeLa cells (Figure 5.1B). ITC was still toxic towards both cell types in a similar manner to that observed in PBS, but the toxicity concentration thresholds increased 2-fold (from 7.8 to 15.6 µg ml⁻¹; Figure 5.1B). H₂O₂ displayed almost no antimicrobial activity and reduced cytotoxicity in the presence of organic matter. PVP-I displayed decreased antimicrobial and cytotoxic potential. Lugol had very small antimicrobial activity, except at the highest concentration tested, while cytotoxicity was considerable even at the concentrations without any antimicrobial effect (Figure 5.1B).
In agreement with the trends recorded from the MTT assay, microscopic observations revealed that, when incubated in DMEM + FBS (Figure 5.2B), the HeLa cells exposed to H₂O₂/KI looked intact, healthier but with a reduced population when exposed to H₂O₂/KSCN and H₂O₂, whereas, the impact of ITC, PVP-I and Lugol on HeLa morphology was comparable to that observed with cells incubated in PBS (Figure 5.2A).

Figure 5.1. Cytotoxic and bactericidal effects of different antimicrobials on human epithelial HeLa cell line and bacterial *E. coli* ATCC 25922 cells at different concentrations upon 10 min exposure in PBS (A) and DMEM + FBS (B) media. White circles and white triangles represent HeLa % viability and *E. coli* log₁₀ cfu ml⁻¹ viability, respectively, in PBS (A); grey circles and grey triangles are HeLa and *E. coli* viabilities, respectively, in DMEM + FBS (B). Data are presented as mean.
values from two independent experiments conducted in duplicate, ±SD. Statistical significance between different concentrations in comparison to untreated HeLa cells was assessed using one-way ANOVA with follow-up Tukey’s post hoc test. * denotes to p-value <0.05, ** to p-value <0.01.

![Image](image.png)

**Figure 5.2.** Morphology of HeLa cells exposed to different antimicrobials at 1000 µg ml⁻¹ for 10 min in PBS (A) and DMEM + FBS (B). Controls include: PBS as untreated and Triton X-100 as lysed. Cells were observed with bright-field light microscopy, objective magnification: 20×, scale bar: 100 µm.

### 5.3.2 Recovery of HeLa cells from cytotoxic effect of antimicrobials

The potential for recovery of HeLa cells from the cytotoxic effects of the test antimicrobials was assessed using MTT assays. In these experiments, antimicrobials were removed by washing after 10 min of cell exposure, and the cells were then cultured in fresh complete culture medium for 24 h. The recovery study in PBS (Figure 5.3A) with H₂O₂/KI showed that the dose-response curve was shifted to a less cytotoxic response range, indicating that H₂O₂/KI cytotoxicity to HeLa cells was, at least partially, reversible. There was no recovery of cells after exposure to
H$_2$O$_2$/KSCN and H$_2$O$_2$, however, indicating an irreversible detrimental effect against HeLa cells. ITC, PVP-I and Lugol showed significant recovery when cells were exposed to the lowest test concentrations. Interestingly, concentrations of PVP-I and Lugol between 15.6 and 62.5 µg ml$^{-1}$ were significantly more cytotoxic after the removal of these antimicrobials and further culturing of the cells than after the initial 10 min exposure.

**Figure 5.3.** Recovery of HeLa cells in PBS and DMEM + FBS media from cytotoxic effects of antimicrobials after withdrawal of the agents. HeLa cells were exposed to indicated antimicrobials for 10 min at various concentrations in PBS (A) or DMEM (B).
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+ FBS (B), cell viability was measured by MTT assay and the results were expressed as dose-response curves (indicated by white or grey circles, respectively). Additionally, another set of cells were exposed in a same manner in PBS (A) or DMEM + FBS (B), but antimicrobials were withdrawn after 10 min, cells were further incubated in drug-free medium for additional 24 h, viability was measured by MTT assay and the results were expressed as dose-response curves (indicated by white or grey squares, respectively). Two-way ANOVA followed by the Tukey’s post-hoc test was used for statistical analysis. * denotes to p-value <0.05, ** denotes to p-value <0.01 significant difference between different treatment groups. Note that the 10 min data in this figure are the same as corresponding data in Figure 5.1.

In contrast to the observations in PBS, in the presence of the cell culture media and additional serum (Figure 5.3B), there was generally no significant recovery of HeLa cells after the exposure and withdrawal of any antimicrobial at any concentration, with the exception of the lowest test concentration of ITC. This could be explained by the generally lower cytotoxicity of antimicrobials in the presence of DMEM + FBS.

5.3.3 Hemolytic effect of antimicrobials

The hemolytic effect of all the test antimicrobials on mammalian red blood cells was investigated by assessing their ability to lyse horse red blood cells following 1 h of co-incubation (Figure 5.4). H2O2/KI and H2O2 did not show any hemolytic effect within the measured concentrations. The microscopic images of hRBC exposed to the highest test concentration (1000 µg ml⁻¹) of H2O2/KI and H2O2 supported the results from spectrometric evaluation, with no morphological changes in the cells evident (Figure 5.5). The exposure of hRBC to H2O2/KSCN, ITC and PVP-I at dosage levels of 7.8 to 1000 µg ml⁻¹ resulted in dose-dependent hemolysis (Figure 5.4). Micrographs of the hRBCs, exposed to the highest concentration of these antimicrobials, confirmed that at 1000 µg ml⁻¹ ITC ruptured the cells, whereas H2O2/KSCN and PVP-I reduced the number of unimpaired cells (Figure 5.5). Lugol exhibited the highest hemolytic activity at lower concentrations of all the tested antimicrobials, with 30% hemolysis at a 62.5 µg ml⁻¹ concentration (Figure 5.4). At concentrations of Lugol above 500 µg ml⁻¹ an apparent drop of activity was observed, but visualization of the actual color of the supernatants (Figure 5.4, inset in Lugol) explained that this decrease was due to the color interference of Lugol and hemoglobin. These observations were additionally confirmed by microscopic images.
of extensive cell debris and complete lysis caused by Lugol at the highest tested concentration (Figure 5.5). As the concentration range of all antimicrobials was prepared in dH2O, the effect of water was additionally examined. Micrographs of the water-treated sample showed that 100 µl dH2O (which was the maximum amount of water-based sample added to hRBC) in 900 µl hRBC-PBS mixture was neither impeding cell integrity, nor reducing the number of healthy cells (Figure 5.5).

**Figure 5.4.** Hemolytic effect of different antimicrobials on horse red blood cells (final concentration 4%) as a function of antimicrobial concentration (0 – 1000 µg ml⁻¹) at 37°C upon 1 h incubation. Each point represents mean of % hemolysis of duplicate samples from two experiments, ±SD. One-way ANOVA followed by the Tukey’s test was used for statistical analysis. * denotes to p-value <0.05, ** denotes to p-value <0.01 as compared with the untreated control. Insets are the representative assay plate wells containing the diluted supernatants of different treatments at various concentrations.
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Figure 5.5. Morphology of hRBCs exposed to different antimicrobials at 1000 µg ml⁻¹ throughout 1 h. Controls include PBS as untreated, Triton X-100 as lysed and H₂O as water-based sample. Cells were observed with bright-field microscopy, objective magnification: 40×, scale bar: 50 µm.

5.3.4 Genotoxic effect of ITC

The presence of DNA breakage, induced by ITC, was evaluated using the *in vitro* comet assay, by comparison with the known DNA damaging agents active in the absence of S9 metabolic activation: UV-C irradiation and H₂O₂ oxidative stress. HeLa cells were exposed to ITC, at either 30 or 300 µg ml⁻¹ concentrations, for 30 min and then examined for DNA breakage. The concentrations and the duration of ITC exposure were chosen based on our previous study (Tonoyan et al., 2017), which revealed that 30 min treatment of *E. coli* cells with ITC at 300 µg ml⁻¹, but not 30 µg ml⁻¹, produced a bacterial DNA smear, as assessed by gel electrophoresis. In the same study, we also observed a bacterial DNA smear when cells were challenged for 30 min with 300 µg ml⁻¹ H₂O₂. Here, upon inspection of comet assay
micrographs it was clear that ITC apparently did not cause DNA breaks in HeLa cells at either 30 µg ml⁻¹ (Figure 5.6B) or 300 µg ml⁻¹ (Figure 5.6C) concentrations, by comparison to the untreated controls (Figure 5.6A). By contrast, UV-C induced elongated-shaped double-strand breaks of DNA (Figure 5.6D), while H₂O₂ generated droplet-shaped extensive single-strand breaks (Figure 5.6E). The assessment of the extant tail moments showed that there was no difference between the control and the ITC 30 and 300 µg ml⁻¹ treatments, while H₂O₂ 300 µg ml⁻¹ and UV-C treatments were significantly different from the controls and also from each other (Figure 5.6F).

**Figure 5.6.** Comet assay micrographs for detection of DNA damage in HeLa cells induced by ITC. Untreated cells (A), treated cells (30 min) with ITC 30 µg ml⁻¹ (B), ITC 300 µg ml⁻¹ (C), UV-C (D) and H₂O₂ 300 µg ml⁻¹ (E). DNA is stained with DAPI, objective magnification is 20×, scale bar is 50 µm. Quantification of DNA fragmentation in HeLa cells (F). Average of the extent tail moment ± SD of 50 random cells per experimental point is shown. Comets were scored using Comet assay IV image analyzing software. One-way ANOVA followed by the Tukey’s test was used for statistical analysis. * denotes p-value <0.05 difference between samples.

### 5.4 Discussion

Antiseptics are widely used in hospitals and other healthcare settings to inactivate, reduce or eliminate pathogenic microorganisms in, or on, living tissue (Drosou et al., 2003). An ideal antiseptic should have a broad, potent and rapid antimicrobial spectrum with no risk of developing antimicrobial resistance (McDonnell and Russell, 1999). In a previous study (Tonoyan et al., 2017), we characterized three
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antimicrobial formulations (H₂O₂/KI, H₂O₂/KSCN and the novel ITC) that exhibited evident potential as wound antiseptics. From the viewpoint of antimicrobial activity, ITC, out of the three, best met the requirements of an optimal antiseptic. However, an ideal antiseptic needs also to be biocompatible (Müller and Kramer, 2008). The term “biocompatibility” refers to different properties of the antimicrobial and a few of the most important aspects are in vitro cytotoxicity, hemolytic activity and genotoxicity (Fischer et al., 2003).

Unfortunately, many frequently used antiseptics are cytotoxic. Therefore, the biological balance between microbial and cellular toxicities should be taken into consideration. Here, the three newly described antimicrobial compositions were tested for their bacterial and cellular toxicities and compared to commonly used antiseptics. H₂O₂, PVP-I and Lugol are some of the most widely used and oldest antiseptics, despite controversy about their cytotoxicity (Block, 2001; Khan and Naqvi, 2006; Cooper, 2007; Sibbald et al., 2011). Additionally, H₂O₂, being one of the components of ITC mixture, and iodine, being the prominent product of ITC, explain the choice of these antiseptics.

For a more realistic portrayal of “antiseptic at work”, our study employed a simultaneous assessment of the potential cytotoxic effects and bactericidal efficacy of the test antimicrobials, upon 10 min exposure to human and bacterial cells, in two different media. In this work, HeLa cervical epithelial cell lines and E. coli cells were used as representatives of human and bacterial cells, respectively. On the other hand, phosphate-buffered saline and cell culture media supplemented with 10% fetal bovine serum were selected as two media for performance of antimicrobial and cytotoxic activities in the absence and presence of bioburden, respectively. The HeLa cell line serves as a model system not only in cancer research, but also to gain knowledge of every fundamental process that occurs in human cells (Masters, 2002). Besides, HeLa cells are human epithelial cells and so cytotoxicity demonstrated towards them may potentially translate to toxicity towards other epithelial cells. On the other hand, E. coli is another conventional model organism, which is extensively used to study bacterial processes, is a recommended reference strain for antimicrobial susceptibility testing and is one of the common pathogens found in wound infections (Bowler et al., 2001). PBS is widely used in cell and bacterial
culturing, as well as in wound rinsing (Atiyeh et al., 2009), while, nutrient-rich cell culture medium DMEM supplemented with 10% FBS represents conditions simulating wound bioburden (Müller and Kramer, 2008; Kramer et al., 2016; Schedler et al., 2017). Despite the differences in experimental design, conditions and test organisms, several studies examined both the cytotoxic effect and the microbicidal activity of antimicrobial agents (Lineaweaver et al., 1985a; Lineaweaver et al., 1985b; Alaçam et al., 1993; Hidalgo et al., 2002; Müller and Kramer, 2008). However, these studies evaluated cytotoxic and antimicrobial aspects of the chosen antiseptics in separate assays, as opposed to the present work.

In this study, the co-incubation of *E. coli*, HeLa cells and H$_2$O$_2$/KI upon 10 min in PBS (Figure 5.1A) revealed that H$_2$O$_2$/KI had a weak antibacterial effect during the short exposure time, while being cytotoxic at all doses tested. H$_2$O$_2$/KSCN also damaged HeLa cells to a greater extent than *E. coli*. The ITC was shown to be similarly toxic towards both human HeLa and bacterial *E. coli* cells. In our study, the cellular toxicity of H$_2$O$_2$ remarkably exceeded its antibacterial potency in PBS. Lineaweaver and co-workers also reported a higher cellular toxicity of H$_2$O$_2$ compared to its antimicrobial potency when tested at full strength (3%) and when serially diluted in saline (Lineaweaver et al., 1985a; Lineaweaver et al., 1985b). Lugol was also highly cytotoxic at microbicidal concentrations. In our experiments, PVP-I was the only antiseptic that had concentrations non-lethal to human cells, coupled with concentrations causing moderate reduction of bacterial cell numbers. Similarly, Lineaweaver and co-workers identified bactericidal and non-cytotoxic dilutions of PVP-I in saline (Lineaweaver et al., 1985a; Lineaweaver et al., 1985b). By contrast, Sanchez et al. (1988) reported that non-lethal concentrations of PVP-I in PBS allowed significant bacterial survival.

Generally, the antimicrobial activity of biocides is dependent on number of factors, such as biocide concentration, exposure time, presence of organic matter, etc. (Maillard, 2005). It is commonly accepted that H$_2$O$_2$ (WFBSC) and iodine solutions (Khan and Naqvi, 2006; Cooper, 2007) lose their germicidal activity once they come into contact with organic load. Therefore, the questions arise whether publicly accepted antiseptics are adequate as wound antiseptics and if the new antimicrobials described here have more suitable antimicrobial properties. Thus, in
order to more realistically picture the wound environment, the simultaneous assessment of antimicrobial and cytotoxic activities of the antimicrobials under consideration was also conducted in cell culture medium supplemented with fetal bovine serum as an indicator of organic challenge. In the presence of organic load, H$_2$O$_2$/KI, H$_2$O$_2$/KSCN and H$_2$O$_2$ were minimally cytotoxic (Figure 5.1B), by contrast with our observations during culture in PBS (Figure 5.1A). Only modest antimicrobial activity was noted with H$_2$O$_2$/KI and H$_2$O$_2$ (Figure 5.1B), however, while H$_2$O$_2$/KSCN displayed no antimicrobial activity following 10 min exposure, at the concentrations tested. The cytotoxicity of PVP-I was reduced to some extent in DMEM + FBS, but its antimicrobial capacity was also negatively impacted. Similarly, Müller and Kramer (2008) reported that PVP-I was well-tolerated by murine fibroblasts, but had low bactericidal activity against *E. coli* and *S. aureus* when tested in FBS supplemented culture media. ITC remained cytotoxic and bactericidal, matching the performance observed in PBS. Lugol again exhibited a greater cytotoxic influence than an antimicrobial effect, though the two effects were milder in the presence of organic matter.

The antibacterial profiles of H$_2$O$_2$/KI, H$_2$O$_2$/KSCN and H$_2$O$_2$ (Figure 5.1A, B), against *E. coli*, were somewhat surprising, given the minimum inhibitory and bactericidal concentrations (MIC/MBC) for these agents (31.3/31.3 µg ml$^{-1}$) reported in our previous study (Tonoyan et al., 2017). In the present study, however, the antimicrobial susceptibility testing was based on viable cell counts after only a short contact time (10 min) between the antimicrobial and *E. coli* in either PBS or DMEM + FBS; and with an MBC definition of a concentration causing $>3$ log$_{10}$ reduction (99.9% kill). By contrast, in our previous study we used broth microdilution MIC determinations, over 24 h of continuous exposure to the antimicrobials in nutrient broth, with follow-up MBC determination as a concentration causing 100% kill (Tonoyan et al., 2017). Despite this, the antibacterial profile of ITC was comparable in both studies – 100% kill of *E. coli* was detected at 7.8 and 31.3 µg ml$^{-1}$ concentrations after 10 min exposure in PBS and DMEM + FBS, respectively (Figure 5.1A, B), and 100% kill was observed at 15.6 µg ml$^{-1}$ concentration after 24 h exposure in nutrient broth [the MBC value; (Tonoyan et al., 2017)]. These observations indicate that regardless of the test conditions and methods used, ITC demonstrated a fast-acting and effective antibacterial profile.
Mammalian cells and tissues contain defense systems to detoxify reactive intermediates, thus preventing or limiting cell damage. Toxic processes have reversible or irreversible features as a consequence of the interplay with cellular defense and repair mechanisms (Wallace, 1997). Thus, it is not unreasonable to assume that the cytotoxicity of an antimicrobial, based on the action of reactive oxygen species, could be reversible and temporary. To test this hypothesis, we performed drug washout experiments, followed by analyzes of cell viability utilizing MTT assay (Figure 5.3). The MTT test depends on dehydrogenase activity in intact mitochondria, and tests of mitochondrial function are sensitive indicators of oxidant injury (Watson et al., 1994). The experiments showed that the incubation of HeLa cells with H₂O₂/KI, for 10 min in PBS with the subsequent removal of the antimicrobial, allowed for recovery of some cells at treatment concentrations between 7.8 and 125 µg ml⁻¹ and thus indicated, to some extent, a reduced cytotoxic effect (Figure 5.3A). However, this was the only recovery case amongst all the tested antimicrobials. Interestingly, a similar study by Müller and Kramer (2007) showed that cytotoxic effects of PVP-I containing products (in a defined concentration range ensuring >20% cell viability) on epithelial cells and fibroblasts, after 30 min exposure, were completely reversible after washout of the compound and follow-up culturing. By contrast, Watson et al. (1994) concluded that even a mild injury induced by H₂O₂ (0.1 mM H₂O₂ upon 1 h) in human colonic carcinoma epithelial cells was not fully reversible. Seemingly, 10 min exposure of HeLa cells to the other antimicrobials was enough to cause irreversible and permanent cellular destruction, however, the actual machinery involved in oxidative damage and repair cannot be extracted from these observations.

Another conventionally used measure of biocompatibility is the hemolytic activity of the antimicrobial candidate, in particular those nominated for open wound treatment. When ranked according to their hemolytic activities, the antimicrobials tested in this study had the following order: Lugol > ITC > PVP-I > H₂O₂/KSCN > H₂O₂ ≥ H₂O₂/KI (Figures 5.4; 5.5). It was shown that H₂O₂/KI and H₂O₂ were the only two antimicrobials, which did not cause hemolysis of horse erythrocytes. This was somewhat surprising, considering that these two agents lysed HeLa epithelial cells in PBS (Figure 5.2A). However, in the presence of organic matter, and under more representative physiological conditions, H₂O₂/KI and H₂O₂ had considerably
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lesser impact on HeLa membrane integrity (Figure 5.2B). By contrast to the results with H2O2/KI and H2O2, the ITC and Lugol ruptured erythrocytes (Figure 5.5) but not epithelial cells (Figure 5.2A, B). Our previous work (Tonoyan et al., 2017) also showed that ITC did not compromise the membrane integrity of bacterial cells. Possible explanations may be the extremely fragile nature of erythrocytes, as well as the variable composition of different cellular membranes and, hence, distinct susceptibility to lysis by the different agents. The exact mechanism by which the modes of toxicity differ needs further study, but it was notable that the concentration of ITC needed to cause hemolysis was considerably higher compared to concentration required to exert an antimicrobial effect.

The in vitro comet assay is a sensitive, low cost and rapid genotoxicity test. It can be the test of choice for preliminary genotoxicity screening of drug candidates early in the development of new pharmaceuticals (Giannotti et al., 2002; Žegura and Filipič, 2004). In principle, any type of eukaryotic cell may be used for comet assay genotoxicity testing. For the in vitro comet assay, well-characterized cell lines or primary cells are preferred, which are generally exposed to test compounds in the presence and absence of metabolic activation system, such as S9 (Tice et al., 2000; Žegura and Filipič, 2004). In this study, we used the alkaline comet assay tool to assess the genotoxicity of ITC on human HeLa cell line only in the absence of S9 metabolic activation. The alkaline comet assay detects both single and double-stranded DNA breaks (Karbaschi and Cooke, 2014) producing special comet-like appearances. H2O2 is known to primarily produce single-stranded breaks (Cortés-Gutiérrez et al., 2014), on the other hand, double-strand breaks are the major feature of the UV-radiation (Bogdanov et al., 1997). In the present study we have revealed that, indeed, H2O2 induced single-strand breaks in the shape of droplet-like comets (Figure 5.6E), and UV-C irradiation produced double-stranded breaks in the shape of elongated comets (Figure 5.6D). However, the exposure to ITC resulted in round-shaped images (Figure 5.6B, C), suggesting that the new test antiseptic ITC was not causing DNA breaks in HeLa cells at low (30 µg ml⁻¹) or at high (300 µg ml⁻¹) concentrations, as evaluated by in vitro comet assay.
5.5 Conclusions

The *in vitro* cytotoxicity of a novel, biocidal, iodo-thiocyanate complex, ITC, was investigated and compared to commonly used antiseptics. The antimicrobial potency of ITC, together with a comparable cytotoxicity profile to existing antiseptics, suggests that it can be considered for future antiseptic and therapeutic applications. Indeed, the concentrations of ITC resulting in cytotoxicity were equivalent to those resulting in potent bactericidal activity. By contrast, the cellular toxicity of the commonly used antiseptics, \( \text{H}_2\text{O}_2 \), PVP-I and Lugol, towards HeLa cells was apparent at levels too low to cause significant bactericidal effects. The activity of ITC was not quenched by organic matter, while, the activities of commonly used non-selective antiseptics were suppressed by organic load. In addition, ITC did not produce DNA breaks as detected by *in vitro* comet assay at 30 and 300 \( \mu \text{g ml}^{-1} \) concentrations.

5.6 Acknowledgments

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CHAPTER 6

Concluding remarks and future recommendations
6.1 Concluding remarks

The antibiotic doomsday scenario is on the horizon. If we are to keep pace with the rise of drug resistance, we need to refill the antimicrobial pipeline. Peroxidase-catalyzed systems are widespread throughout nature as part of mammalian defenses against invading microorganisms. They are, therefore, inspiring for the development of alternatives to antibiotics.

Antibacterial activity of the iodo-thiocyanate complex

What is the antimicrobial potential of a complex comprised of hydrogen peroxide and two oxidizable ion substrates? This thesis explored this question, and focused on the combined oxidation of iodide and thiocyanate by hydrogen peroxide, in the absence of peroxidase, in order to generate a potent antimicrobial. This approach was novel, as the literature on peroxidase systems: (i) generally reported on the use of one substrate rather than two and, more significantly, (ii) taught the requirement for the presence of a peroxidase enzyme, before active antimicrobial molecules could be formed, at least when dealing with physiologically-relevant (low) reactant concentrations.

What did this research reveal about the antimicrobial potential of the complex? My hypothesis was that H₂O₂ would oxidize both substrates and would generate a broad set of oxidants, which would have: (i) more than one target in the bacterial cell; (ii) target more than one type of bacteria; (iii) and would not trigger the emergence of resistance. Accordingly, I tested the antimicrobial potential of the H₂O₂/KI/KSCN combination against two Gram-negative and two Gram-positive bacteria (including a multidrug-resistant MRSA) and compared its effect with H₂O₂/KI, H₂O₂/KSCN and H₂O₂ using standard in vitro testing methods (Chapter 2). The advantage of the dual-substrate system over other agents tested was apparent against the representative E. coli and P. aeruginosa strains, whereas towards S. aureus and MRSA all the antimicrobials had comparable effects. One of the notable features of ITC was that it was the most rapidly bactericidal among the antimicrobials surveyed. ITC killed the pathogens within minutes, and did not just arrest their growth, while did not require the presence of a peroxidase to do so. By contrast, the peroxide and the one-substrate systems showed slow killing kinetics or
were bacteriostatic. An antimicrobial, which can successfully eliminate pathogenic bacteria by killing them, is a good candidate to treat bacterial infections.

It is becoming imperative that any novel antimicrobial compound be effective not just against planktonic but biofilm forms, the phenotype most often noted in infections (Chen et al., 2013). So far, the literature lacks reports determining the potential of the peroxidase systems in targeting biofilm phenotypes. I therefore examined the anti-biofilm potential of the new complex. Mono-culture biofilms of *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA, and dual biofilms of *Staphylococcus aureus* and *Streptococcus uberis* were established, over 24 h, on the coupons of a modified Robbins’ device, and then treated with a concentration gradient of ITC for 10 min and 30 s duration, respectively. The results of this study demonstrated that ITC at concentrations similar to planktonic MIC could eradicate mono and dual-species established biofilms within short exposure, suggesting that it can be used in the fields of medical, food and water microbiology to prevent and control biofilm-associated contaminations.

It was proposed that ITC was bactericidal due to the combined chemistry of various cidal species. I have detected hydroxyl radical, hypothiocyanite, hypoiodeite and predominantly iodine in the reaction mixture. These species individually target different sites in bacteria. The hydroxyl radical favors the DNA; oxidized SCN\(^-\) species target free thiol groups of proteins and other low molecular weight species, selenothiols; oxidized I\(^-\) species target all thiols, thioether, amine, phenol, imidazole moieties of amino acids, carbon-carbon bonds in fatty acids, nucleotides, HADPH, NMNH and other molecules. All these oxidants collectively can cause DNA damage, inhibition of metabolism, membrane destabilization and altered transport of molecules. Thus, ITC appears to be a powerful “cocktail” of reactive oxidants. To gain an insight into the bactericidal effect of this “cocktail” I looked at morphological changes in *E. coli* when exposed to ITC. I could detect changes in riboplasm, alteration in DNA condensation and DNA fragmentation, without noticeable membrane damage, supporting the idea that the antimicrobial activity of ITC derives from the combinational and simultaneous action of various oxidants. Besides, ITC avoided triggering the emergence of resistance as it hits multiple targets simultaneously. This was evidenced by the fact that repeated exposure of *E.
coli, *P. aeruginosa*, *S. aureus* and MRSA to sub-MIC levels of ITC did not generate resistance. By comparison, the *S. aureus* strain developed resistance to the bactericidal antibiotic, levofloxacin, within three sub-cultures under the same protocol. Resistance training in this study (Chapter 2) was performed using broth microdilution method over 20 serial passages, and the development of resistance was monitored by looking at the change in minimum inhibitory concentrations (resistance occurs when the population is able to grow in the environment of higher concentration of an antimicrobial).

In conclusion, the results of Chapter 2, indeed, revealed that H$_2$O$_2$/KI/KSCN triple combination was a mixture of multiple active oxidants, and exhibited a broad-spectrum bactericidal activity against pathogenic bacteria in both planktonic and biofilm forms, without triggering resistance.

**Bacterial “back-talk” to the iodo-thiocyanate complex**

What was known about the potential for bacterial resistance to peroxidase-catalyzed systems or to active oxidants alone? As noted earlier in this thesis (Chapter 1), bacterial resistance to peroxidase/SCN$^-$-mediated killing happens by means of enzymes and substrates that inactivate and reduce the generated oxidizing agents, as well as by reversing the oxidized thiol targets. The documentation of resistance toward peroxidase-oxidized I$^-$ species, along with iodine-based biocides was, however, lacking.

What was the contribution of this research to the knowledge of the potential for the emergence of bacterial resistance toward ITC? One of the main objectives of this thesis was to reveal the potential of ITC for induction of resistance and cross-resistance, thus, different mechanisms and aspects of resistance were studied in Chapter 2, 3 and 4.

In Chapter 2 I reported no emergence of resistance to ITC in four strains, but this experiment was performed using the serial passage of batch cultures exposed to sub-lethal concentrations of ITC. In Chapter 4, I investigated the potential for resistance to emerge in *E. coli*, using continuous cultures, exposed to gradually increasing concentrations of ITC. In the case of the serial passage, the bacterial population is cultured for a defined period of time (commonly 24 h) covering exponential to
stationary phase populations, followed by a transfer of small bacterial inoculum from sub-inhibitory medium to new culture medium restarting the entire cycle afresh. A typical serial passage experiment may include 5 – 10 transfers; in my study, we carried out 20 transfers. The chemostat technique implies the maintenance of a bacterial population continuously in exponential phase by regular re-introduction of fresh media (that may be supplemented with the desired dose of an antimicrobial) and simultaneous removal of the waste culture. Jansen and co-authors (2013) addressed that each method covers certain situations in the patient and/or clinical environment. They pointed out that serial passage reflects patient-to-patient transfer or spread across tissues within a single patient, where the transmitted inoculum is usually very small and can outgrow after the successful invasion. The chemostat would rather represent infection within a single tissue or systemic bacteremia. When the evolving pathogen population in the chemostat is also continuously challenged by a temporal gradient of an antimicrobial, the chemostat system may simulate bacterial evolution in the host environment, in response to an antimicrobial treatment. During this adaptive evolution, mutations will arise, and those that are beneficial under the antimicrobial selection pressure will be fixed over time in the population. After adaptation, understanding what genetic changes confer the resistance is desirable. Therefore, combining the experimental evolution study with whole-genome sequencing of the evolved population, one can study the evolution of antimicrobial resistance in real time and identify resistance mechanisms to a novel antimicrobial. Often, these analyses involve sequencing the genomes of individual clones isolated from evolving populations at a time. Instead, DNA from polymorphic populations of cells may be analyzed all at once to track genetic diversity. Studies of this kind have revealed the complex evolutionary dynamics of new mutations involving genetic hitchhiking, clonal interference, fixation/extinction events, parallel evolution, and frequency-dependent interactions (Deatherage et al., 2014).

Thus, Chapter 4 describes the 20-day parallel adaptive evolution routes of drug-sensitive E. coli in both the absence of selection pressure, and under gradually increasing ITC biocide, and antibiotic LVX, selection in chemostat culture. Similarly to the serial passage method, the emergence of resistance was measured using the MIC metric. Starter, intermediate and end polymorphic populations were sequenced to reveal the genetic basis of any observed phenotypic changes. I was not able to
generate resistance in *E. coli* to ITC and cross-resistance to LVX under these experimental settings. By contrast, upon exposure to the sub-MIC level of LVX within 2 days of continuous cultivation, *E. coli* rapidly developed resistance to LVX (16-fold increase in MIC). I note that, in the batch multi-passage experiment described in Chapter 2, *E. coli* did not show high degree of resistance when exposed to the sub-MIC of LVX – indeed there was no sustained MIC increase but rather a 2 to 4-fold MIC drift. The sensitivity of continuous culturing over batch passaging may explain the observed differences, thus, it is important to define the experimental approach at the start carefully. Further exposure of the continuous culture to twice the MIC dose of LVX selected for higher resistance (64-fold increase in MIC at the end of the culture history). However, this LVX challenge did not select for ITC resistance.

Another beneficial trait of ITC that I noticed from the study reported in Chapter 4 was that, at 2 × MIC it cleared the culture and we had to switch from the continuous culturing to the batch mode for overnight to continue the experiment, whereas no clearance was achieved using LVX. These results once again confirmed that, under more realistic conditions, mimicking an antimicrobial treatment at the infection site, ITC was able to decontaminate the bacterial infection without leading to the emergence of resistance or cross-resistance.

As no resistance arose to ITC, I was not able to reveal the exact mechanism that could contribute to ITC resistance. Despite the growing advances in next generation sequencing, to link the specific genotypic elements to the phenotype remains difficult. Evolved populations often have multiple mutations, and determining causality for a phenotype, not to say the mechanism underlying the anticipated-but-not observed phenotype, can require significant efforts. LaCroix and co-authors (2015) suggested an alternative approach to ease the identification of causal mutations by performing multiple independent experiments and examining mutations that occur most frequently. Performing multiple experiments, under strictly identical conditions, can help filter causal mutation candidates encountered during adaptive evolution. From my mutation list, of interest were the mutations in DNA mismatch repair endonuclease MutH that, theoretically, can lead to the development of a mutator phenotype, thus, contributing to antimicrobial resistance.
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and cross-resistance. However, the brief experimental study in Chapter 3 (discussed below) indicated that, E. coli exposed to ITC at near the MIC did not obtain a significantly higher mutation rate to LVX than unexposed culture. With respect to LVX resistance, I could assign the high degree of resistance (64-fold increase in MIC; Chapter 4) to several LVX resistance-conferring mutations, which were previously identified in the literature. The mutational repertoire of the LVX-exposed population comprised mutations in the genes encoding for LVX target proteins (GyrA, ParC, ParE) and membrane porin (OmpF). Considering this information, coupling experimental evolution and whole-genome sequencing can provide a deeper understanding of the principles of resistance evolution and enable predictions of antimicrobial resistance. The predictions, however, may be complicated with the antimicrobials that possess multiple targets.

Concluding Chapter 4, drug-sensitive E. coli did not develop de novo resistance to ITC and cross-resistance to LVX when exposed to ITC.

In recent years, much attention has been directed towards the potential for the emergence of antimicrobial resistance via induction of increased mutation rates in bacteria and increased efflux of the antimicrobial drugs from bacteria. Mutation rate determinations are widely considered an important task for the prediction of antimicrobial resistance emergence. Thus, a small study (Chapter 3) was carried out to measure the rate of E. coli mutation from levofloxacin sensitivity to resistance, when it was grown under the near-lethal challenge of ITC. Among the different fluctuation assays, I used the p0 method, which is the simplest method and does not involve complex mathematical calculations. I grew 100 replicate parallel cultures of LVX-sensitive E. coli in the absence, and the presence, of ITC (at 0.25 × MIC concentration) starting from a small inoculum, and I recorded the mutational events, which occurred independently in each culture by plating the fraction of the culture in LVX-selective (at 2 × MIC concentration) solid media. Further calculations of the mutation rates of ITC-exposed vs. unexposed E. coli revealed that there was a modest 2.3-fold increase in the rate, which was not statistically significant. Whereas, hypermutators or mutators, generated because of the defects in mismatch repair system, have elevated mutation rates from 100 to 1000 times (Miller, 1996). Considering there was no significant increase in the mutation rate of E. coli, ITC
may not contribute to the development of LVX resistance. However, this is only a preliminary assessment that needs to be further explored. I note that, the conventional laboratory tests, which measure the mutation rates, are generally based on the development of resistance toward one antibiotic, such as levofloxacin or rifampicin, though the antibiotic-resistant phenotypes do not always reflect the same genotypes in all selected mutants. For instance, the LVX resistance mutation rate is actually the result of the combination of the mutation rates of several target genes (\textit{gyrA, gyrB, parA, parC}) and several different influx and efflux systems. This means that the calculated “phenotypic” mutation rate is the result of several different “genotypic” mutation events, and that the bacterial population may thus have multiple different mutation rates (Martinez and Baquero, 2000). Meantime, comparing the \textit{E. coli} culture exposed to the gradually increasing concentration of ITC (Chapter 4) and the culture exposed to the near-inhibitory concentration of ITC (Chapter 3), I noticed that all the mentioned mutations, which could result in LVX resistance, were absent from the mutation list of ITC-exposed culture (Chapter 4). This indicates that ITC challenge, most likely, would not lead to increased mutation rate conferring LVX resistance. In any case, the mutation rate is not a simple characteristic and various factors can affect it (Martinez and Baquero, 2000).

In Chapter 3, I also addressed the involvement of efflux pumps in possible ITC resistance in \textit{P. aeruginosa} by measuring the ITC MIC in the absence, and the presence, of an efflux pump inhibitor. Theoretically, the presence of an EPI should reduce the tolerance of a strain displaying efflux-mediated adaptation to an antimicrobial. This could decrease the MIC of ITC by a modest 2-fold, (which is usually considered as non-significant) hinting that efflux-related resistance to ITC may not be important. As a note, rather than a strong parallel, the \textit{E. coli} grown under ITC pressure did not carry mutations in the genes of efflux systems or their regulators (Chapter 4). These observations are, however, currently speculative and require further supportive experiments.

\textbf{Comparative cytotoxicity of the iodo-thiocyanate complex}

\textit{What does literature tell us about the mammalian cytotoxicity of peroxidase-catalyzed systems or the active oxidants alone?} It is well documented that peroxidase systems have a dual role, behaving as both a friend and a foe. The highlight is that as.
they have non-specific, broad-spectrum target mechanism, aside from mediating bacterial cell killing, destroying invading parasites, combating fungal infections and inactivating viruses, can attack a variety of mammalian cells, including tumor cells. Thus, I expected cytotoxicity.

What is this research reporting on mammalian cytotoxicity? The excellent antibacterial properties of ITC suggest its use as an antimicrobial therapeutic to decontaminate wound infections. Thus, in Chapter 5 I inspected the cytotoxicity of ITC by comparison with some of the oldest, and most widely used, antiseptics: hydrogen peroxide, povidone-iodine and Lugol’s iodine. Additionally, H₂O₂, being the component, and iodine, being the prominent product of ITC, explain the choice of these antiseptics. For biocompatibility testing, I used in vitro cytotoxicity, hemolytic activity and genotoxicity evaluation.

Typically, in vitro cytotoxicity assays for toxicity screening of new antibacterials imply the incubation of a range of the compound concentrations with a chosen mammalian cell line, for 24 h, followed by the assessment of cell death or viability using an appropriate test. Considering the wide diversity of available cell lines, the choice of a target cell type for cytotoxicity screening may appear a daunting task. However, in their data mining study of a cytotoxicity database of the National Cancer Institute’s Developmental Therapeutics Program Bugelski et al. (2000) concluded that when viewed in the context of the primary screening, cell line choice has a surprisingly minimal effect on the outcome of cytotoxicity screens. Therefore, the cytotoxicity of a new antibacterial can be evaluated using any cell line to which one has access. In my study (Chapter 5), I used HeLa cervical cancer cells, which, aside from their ready availability, are epithelial cells, and thus better suited for cytotoxicity screening for a candidate antiseptic. Regarding the choice of cytotoxicity assay, I selected the MTT viability test, which is the most popular, low-cost and convenient method. Additionally, mentioned previously, the MTT test depends on dehydrogenase activity in intact mitochondria, and tests of mitochondrial function are sensitive indicators of oxidant injury. To more realistically picture the wound environment and antiseptic treatment, I simultaneously incubated human HeLa and bacterial E. coli cells with test antimicrobials, for 10 min, in two different media: phosphate-buffered saline and cell culture media supplemented with 10%
bovine serum. This work showed that, in PBS, the cytotoxic concentrations of ITC were equivalent to those resulting in potent bactericidal activity; by contrast, the cellular toxicity of the commonly used antiseptics H2O2, PVP-I and Lugol was apparent at levels too low to cause significant antibacterial effects. The toxic activities of ITC were not quenched by organic matter (DMEM + FBS), whereas the activities of the other antiseptics were significantly suppressed. The comparison of the antimicrobial profiles of ITC and H2O2 against *E. coli* in Chapter 2 and Chapter 5 indicated that, regardless of the test conditions and methods used, ITC demonstrated a fast-acting and effective antibacterial profile, as opposed to H2O2. In Chapter 2, the antimicrobial susceptibility testing was based on broth microdilution MIC determinations, over continuous 24 h exposure of bacteria to antimicrobials in nutrient broth and follow-up MBC determination as a concentration causing 100% kill. The recorded MICs/MBCs of ITC and H2O2 were 15.6/15.6 and 31.3/31.3 µg ml⁻¹, respectively (Chapter 2). While in Chapter 5, the antimicrobial susceptibility testing was based on viable cell counts after a short 10 min contact time between the antimicrobial and *E. coli* in PBS or DMEM + FBS, and the MBC definition was a concentration causing 99.9% kill. In any case, ITC caused 100% kill at 7.8 (in PBS) and 31.3 (in DMEM + FBS) µg ml⁻¹ concentrations, while, H2O2 resulted in 100% kill at 1000 µg ml⁻¹ in PBS and only 2 log₁₀ reduction (99% kill) at 1000 µg ml⁻¹ in DMEM + FBS (Chapter 5).

In Chapter 5 I also studied whether the cytotoxic effects of antimicrobials could be reversed, considering mammalian cells and tissues contain defense systems to detoxify reactive intermediates by this preventing or limiting the cell damage. I simply applied the antimicrobials in the same manner as when testing for cytotoxicity, but after 10 min exposure the antimicrobials were removed, the HeLa cells were washed and then cultivated further. However, I could not detect a recovery from most of the test antiseptics at most of the test concentrations, suggesting that 10 min exposure of HeLa cells to the antimicrobials was enough to cause irreversible and permanent cellular damage.

The hemolytic activity was also assessed in Chapter 5 as another measure of cytotoxicity. ITC, PVP-I and Lugol had dose-dependent effects on the viability of horse erythrocytes, while H2O2 showed no hemolytic impact. This was surprising,
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considering H\textsubscript{2}O\textsubscript{2} lysed HeLa epithelial cells in PBS. However, in the presence of organic matter, and under more representative physiological conditions, H\textsubscript{2}O\textsubscript{2} had a considerably lesser impact on HeLa membrane integrity. Thus, I thought to link the absence of H\textsubscript{2}O\textsubscript{2}-mediated hemolysis with its inactivation by organic matter. Nevertheless, Klebanoff and Clark (1975) considered the H\textsubscript{2}O\textsubscript{2} non-hemolytic due to erythrocyte catalase. On the other hand, ITC caused hemolysis of horse erythrocytes, but I could not observe lysis of HeLa cells (Chapter 5) or E. coli cells by direct visualization under transmission electron microscope, as well as using live/dead staining and fluorescent microscopy (Chapter 2). In Chapter 5, I ascribed these differences to the extremely fragile nature of erythrocytes and to the variable composition of different cellular membranes and, hence, distinct susceptibility to lysis by different agents.

Finally, in Chapter 5 I studied the genotoxicity of ITC on HeLa cells using an \textit{in vitro} comet assay, which detects DNA strand breaks at the cellular level. ITC did not generate DNA breakage, while H\textsubscript{2}O\textsubscript{2} resulted in extensive single-strand DNA breaks. This outcome was somewhat contradictory to what I reported in Chapter 2 – i.e. that TEM and gel electrophoresis detected DNA fragmentation and smear when E. coli was exposed to the similar concentrations of ITC. Even though ITC possess non-specific toxicity, different types of cells are affected in a different manner.

\textit{What is my overall conclusion?} The pronounced antimicrobial potency of ITC and its comparable cytotoxicity profile with existing antiseptics, suggest that it should be considered for antiseptic and therapeutic applications. This comparative cytotoxicity study has raised questions such as: why use antimicrobials which are not effective for prevention and treatment of infections and, yet, cytotoxic; and why is the use of extremely toxic anticancer drugs is tolerated, but the use of toxic, yet, effective antimicrobials in life threatening infections is feared by general public? In my opinion, in the era of a dry antibiotic pipeline, we need to preserve last resort antibiotics, and consider newly developed and effective antimicrobials even in light of some cytotoxicity, advance in the management of this cytotoxicity and find suitable applications.
6.2 Future recommendations

While the results presented in this thesis suggest the future application of ITC as an antiseptic or a biocide, it is apparent that much basic and applied research remains to be carried out. Thus, the following recommendations are proposed:

- Expand the antimicrobial screening of ITC on an extended list of microorganisms, including multidrug-resistant clinical isolates, fungi and viruses. The general rule regarding antibacterial activity spectrum has been “broader is better”. This is true when treating severe infections, as clinicians do not always know the causative pathogen, and thus usage of broad-spectrum antimicrobial will save the precious time.

- Employ chemical methods (e.g. mass spectrometry) to reveal the molecular identities of all reactive species occurring in the ITC mixture; elaborate the mechanism of action studies to detect multiple targets of those multiple species in various types of microorganisms; link the chemical identities to their targets and antimicrobial actions. To have a fully characterized profile of the antimicrobial mixture one wants to know “who is there and what are they doing there?”.

- Conduct further biofilm testing, incorporating more mature biofilms (between 96 to 120 h), complex biofilms (mixed species), different materials (dentine, enamel, hydroxyapatite) and more sophisticated biofilm model systems (oral, wound). Extensive tests will offer wider possibilities and suggestions for the usage of the new antimicrobial mixture.

- Investigate the therapeutic utility of ITC on animal models, such as in vivo wound, lung, urinary tract, digestive, device-related infection models, dental plaque and many more. ITC has a future, but will it work in real life?

- Conduct future resistance studies with numerous parallel evolving cultures to test the dynamics and determinants of ITC resistance in multidrug-resistant pathogens; follow the cross-resistance on larger sets of antibiotics. If one would replay the tape of adaptation to ITC, would this evolution result the same outcome?
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