Antimicrobial hand rub: An Investigation of the Various Chemicals Used in the Concentrate Skin Formula to Enhance the Product Performance

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Abstract

The following research project was conducted in order to evaluate the antimicrobial efficacy of an alcohol-free skin concentrate formula intended for use on hands. Substitute compounds for alcohol in antimicrobial formulae is receiving much interest due to the tendency of alcohol to dry and irritate the skin after repeated use. As well as assessing the formula's efficacy, the individual components were assessed using a series of certified antimicrobial assays. Chief among the components of the skin concentrate formula was chlorhexidine digluconate, a prominent potent antimicrobial compound. The bacterial strains selected for exposure to antimicrobial susceptibility assays were *Escherichia coli, Staphylococcus aureus*, and *Pseudomonas aeruginosa.* Furthermore, in addition to assessing antimicrobial potency, parallel cytotoxicity studies were performed on a mammalian cell line, to determine the extent of potential toxic behaviour demonstrated by the skin concentrate and its constituents. The chosen mammalian cell viability assay was the MTT (3-4(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium) reduction assay. The selected mammalian cell line for cytotoxicity studies was A549, which is a lung-derived carcinoma cell line. Topical antibacterial compounds are commonplace in the healthcare, domestic and industrial environments; hence the importance of assessing their cytotoxicity cannot be undervalued.

Silver is recognised as an effective antimicrobial, especially in the healthcare setting where it is incorporated into wound dressings to combat external infections. Silver sulfadiazine and silver nitrate represent two of the most common silver-containing antimicrobial compounds. For comparison against silver containing compounds, silver ions were generated in solution using a Dowex® cation exchange resin for subsequent antimicrobial susceptibility and cytotoxicity testing.

The potent antimicrobial efficacy of chlorhexidine digluconate, alone and in combination with other excipients, was observed. All three strains proved to be susceptible. Furthermore, silver displayed similar potent antimicrobial activity against all three strains. However, cytotoxic properties were observed for chlorhexidine digluconate and silver. Therefore, while chlorhexidine diguconate is a distinguished antimicrobial compound, the cytotoxic properties associated with it suggest a replacement compound with reduced cytotoxic properties may be required for incorporation into the formula.

Introduction

Bacteria represent the most abundant organisms on the planet and their biomass by far outweighs that of any other organism. Understandably, one would perceive humans as the dominant species on the planet and consider the current era as a principally mammalian one. However, bacteria have been the most dominant organisms for most of the time life has occupied the planet. Remarkably, there are more bacterial cells within the human body than there are human eukaryotic cells. In addition, bacteria have the ability to endure a wide variety of environments, from the coldest to the hottest locations on earth. As a consequence, bacteria are more adaptable than any other organism.

On a daily basis, the majority of microorganisms encountered by humans are innocuous, thereby allowing peaceful coexistence (Stephens & Murray, 2001). According to 16S ribosomal RNA gene sequencing, the majority of bacterial inhabitants associated with conventional skin flora belong to four phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. In addition, the aforementioned phyla constitute the majority of bacteria inhabiting the gastrointestinal tract and oral cavity. Furthermore, microbes inhabiting the skin can indirectly assist host immunity by competing for nutrients and space, thereby inhibiting the growth of pathogenic bacteria (Sandford & Gallo, 2013).

The distribution of transient bacteria by hands plays a considerable role in the direct and indirect transmission of disease. Furthermore, a single source of infection has the capacity to spread to other people, either by direct means or indirect means. For example, a study analysing the transmission of *Salmonella* from one sick family member to others established that the chance of another family member acquiring the infection was as high as 60 %. Therefore, it is necessary to decrease the number of bacteria that can be transferred to shared inanimate objects,

food, or other people. Moreover, proactive risk management steps are essential to assist in disrupting the transmission of potentially pathogenic microorganisms to oneself, to others, and to inanimate objects (Fischler et al., 2007).

Crystal Clear

The following MSc (By Research) project was conducted with financial support from Crystal Clear International Limited. Crystal Clear International Limited is committed to enhancing treatments in the sphere of skincare. From progressive beauty technology to acclaimed skincare products, Crystal Clear covers an extensive range of requirements. Furthermore, the aim of the project was to evaluate the antimicrobial activity of a current disinfectant hand rub formula, under the ownership of Crystal Clear International Limited, against different test organisms. In addition, antimicrobial ability was evaluated using internationally recognised assays for the assessment of novel antimicrobial compounds. As well as assessing the antimicrobial activity of a hand rub formula, the constituents of the formula were separately assayed in order to compare their individual antimicrobial capabilities. Moreover, alternative formula constituents were assayed in order to identify possible replacements. In addition to examining the antimicrobial activity of the hand rub formula and its individual components, potential cytotoxicity towards mammalian cells was assessed. Furthermore, antimicrobial assays combined with cytotoxicity investigations provided a comprehensive understanding of the overall efficiency of the hand rub formula and its individual constituents.

The hand rub solution consisted of five principal components with varying functions within the formula. The five principal components are water, tetrasodium ethylenediamine tetraacetate (EDTA), didecyldimethylammonium chloride (DDAC), a mixture of benzyl-C12-14-alkyldimethylammonium chlorides collectively referred to as benzalkonium chloride (BAC), and chlorhexidine digluconate. EDTA is included in many cosmetic formulations as a chelating agent and its function is to combine with polyvalent metal ions in solution to generate soluble ring structures. Furthermore, EDTA is utilised in foods, drugs, and cosmetics to neutralise the adverse effects of metal ions on the stability of cosmetic products (Lanigan & Yamarik, 2002).

DDAC and BAC belong to a group of compounds called quaternary ammonium compounds (QACs). The general structure of QACs includes a positively charged nitrogen atom displaying four bonds. In the case of fully substituted QACs, the nitrogen atom can attach to four nonpolar alkyl or aryl chains. In addition, the counter-ion is perceived as less fundamental in many of the applications of QACs, however it can have an impact on solubility. Moreover, the amphiphilic characteristics of QACs contributes to their diverse applications, such as surfactants, dyes, neuromuscular blocking agents, and disinfectants. Furthermore, BAC and DDAC represent two of the most common commercial mono-cationic QACs in today's market (Jennings, Minbiole & Wuest, 2015). Chlorhexidine digluconate represents the principal antimicrobial component in the formula; demonstrating activity against both Gram-positive and Gram-negative bacteria. In addition, chlorhexidine digluconate has been used extensively in surgical hand-wash formulations, as an antiseptic, in topical treatments for wound sepsis, as an anti-plaque agent, and within topical slow release vehicles for the treatment of periodontal disease (Gilbert & Moore, 2005).

Background Information on Bacteria

Bacteria as Prokaryotes

Bacteria are recognised as a biological domain of the living world; a domain is defined as the highest rank in the classification system of organisms. There are three cellular domains: Bacteria, Eukarya, and Archaea. The three domains of life arise from observations regarding differences in cellular structure and biochemical functioning. Furthermore, these distinctive traits include different deoxyribonucleic acid (DNA) replication apparatus for bacteria, unique ether-linked isoprenoid membrane phospholipids in archaea, and the existence of various complex organelles in eukaryotes (López-García & Moreira, 2015).

Bacteria are categorised as prokaryotes; unicellular organisms which lack a membrane-encased nucleus and specialised membrane-bound organelles responsible for carrying out specific functions within a eukaryotic cell. For example, mitochondria generate the energy required to drive activities within animal cells from the degradation of food molecules. Surrounding the cellular contents is a plasma membrane that is composed of fatty acids; molecules that are characterised by having a hydrophilic region and a hydrophobic region. When a large amount of these molecules come into contact they spontaneously self-assemble into two distinct layers, with their hydrophobic regions assembling on the interior of the membrane and their hydrophilic regions assembling on the exterior. Cells are distinct entities and the presence of a boundary in the form of a plasma membrane further strengthens this observation. In addition to a membrane which regulates the passage of molecules across it with the aid of membrane-associated proteins, virtually all bacteria possess a cell wall comprising of sugar moieties cross-linked by polypeptides known as peptidoglycan. The main purpose of the cell wall is to protect the cell from mechanical injury and to prevent cell lysis due to osmotic pressure. Prokaryotic

cells differ greatly from eukaryotic cells with regards to the organisation of intracellular constituents; more specifically the DNA molecules within a typical eukaryotic cell are intricately assembled into multiple, linear chromosomes encased within a nuclear envelope and separate from the plethora of intracellular processes occurring within the cell. In contrast, the genetic material within a typical bacterial cell is assembled into a single, circular molecule which is not contained within a protective nucleus and is free-flowing within the cytoplasm.

DNA Replication

Faithful replication of genetic information, which is incorporated within the double helix of DNA, is crucial for the inheritance of traits that determine the phenotype of cells and the organism. The entire process of genome replication can be divided into a variety of sub-processes; the core of DNA replication is the intracellular machinery that undergoes the process of copying DNA, including the proteins that initiate the entire process and the proteins that generate two copies of DNA from one copy. Furthermore, bacterial genomes are considerably smaller than eukaryotic genomes; a single molecule of DNA within a bacterium that resides in the gut flora of humans is approximately 5 million base pairs in length. In contrast, the single DNA molecule in the largest human chromosome is 245,203,898 base pairs in length, consequently the DNA replication process occurring within both cell types will exhibit different features (O'Donnell, Langston & Stillman, 2013).

A single bacterium ordinarily contains one chromosome with a single location defined as the "origin", where the process of replication begins. In bacteria, origins are well-defined sequences which are the target for replication initiator proteins to bind to and instigate the process of replication (O'Donnell et al., 2013). Bacterial, archaeal, and eukaryotic cells all utilise origin-binding proteins derived from the AAA^+ superfamily; a collection of functionally

diverse NTPases which obtain energy from ATP hydrolysis in order to engage in molecular remodelling events (Snider, Thibault & Houry, 2008). In bacteria, multiple copies of DnaA, an origin-binding protein, assemble together to produce a helical filament which unwinds a dense A/T base-pair region in the origin sequence, thus generating a single–stranded DNA "bubble". The primary objective of origin-binding proteins is to facilitate the loading of helicase proteins, known as DnaB, onto the single strand of DNA. DnaB comprises of six subunits; a feature shared by all three domains of life and migrates along the single strand of DNA representing a wedge to prise the parental duplex apart. DNA polymerase is restricted to being solely capable of covalently linking nucleotides to an existing single strand of DNA, thus the synthesis of a primer is required to initiate cellular DNA replication. In *Escherichia coli,* the single-subunit enzyme, DnaG, catalyses the synthesis of the primer by temporarily binding to the helicase; the newly synthesised primer is approximately 12 nucleotides in length. The replication apparatus responsible for simultaneously generating two daughter duplexes is commonly referred to as a replisome; a multiprotein complex. The bacterial replisome is coordinated by the clamp loader which attaches to three molecules of DNA polymerase III. Furthermore, the clamp loader also binds to the helicase and continually compiles β-clamps onto primed locations as they are generated by primase for DNA polymerase III to interact with in order to elongate a new single strand of DNA. The presence of a sliding clamp in the replisome is to allow DNA polymerase III to catalyse the formation of many covalent links between nucleotides each time it is bound to a DNA molecule; this is made possible by strengthening the association between the enzyme and the DNA molecule (O'Donnell et al., 2013).

Gram-negative & Gram-positive Bacteria

Most types of bacteria can be differentiated into two distinct groups based on the chemical components of their cell walls; a feature shared by plant eukaryotic cells. Furthermore, the Gram stain is a technique which classifies bacteria as either Gram-positive or Gram-negative. Gram-negative cell walls, such as those found in *E. coli*, usually consist of a thin peptidoglycan layer with an outer membrane enclosing it; the outer membrane exhibits a chemically distinct composition from the inner plasma membrane. In contrast, Gram-positive cell walls, such as those found in *Staphylococcus aureus*, are much thicker and Gram-positive bacteria lack an outer membrane.

Both membranes present in Gram-negative bacteria contain proteins that aid in the migration of matter and information. However, both membranes exhibit distinctive structural and functional differences. The lipid content of the inner cytoplasmic membrane is entirely composed of phospholipids, which are uniformly distributed among the inner and outer leaflet. In contrast, the outer membrane of Gram-negative bacteria is highly asymmetric; the inner leaflet exhibits identical lipid composition to the cytoplasmic membrane and the outer leaflet contains lipopolysaccharides. With regards to the functional differences, the outer membrane is significantly leakier than the cytoplasmic membrane due to the pore-forming proteins situated in the outer membrane (Koebnik, Locher & Van Gelder, 2000). Outer membrane proteins (OMPs) are integral membrane proteins which display a β-barrel structure in the membrane, with short loops between strands on the periplasmic side and large protracted loops on the extracellular side. As they are protruding into the outside of the bacterial cell, OMPs have a variety of roles, such as serving as adhesion factors in virulence and acting as channels for the uptake of nutrients (Rollauer, Sooreshjani, Noinaj & Buchanan, 2015).

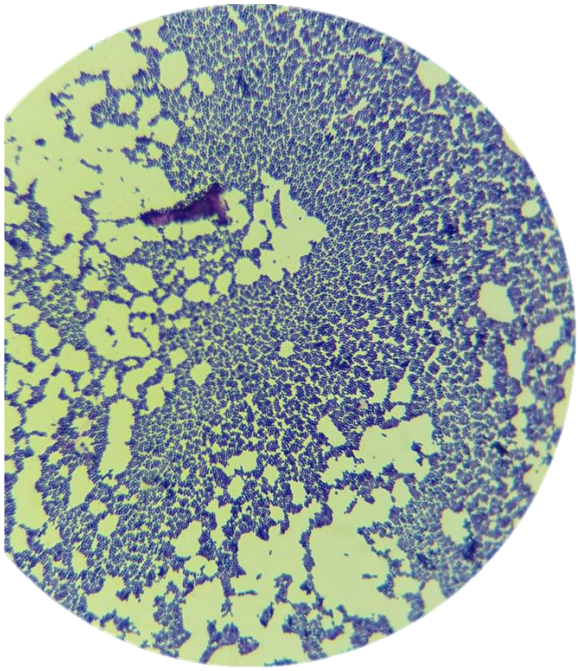

Figure 1. A Gram stain image of a culture of Gram-positive *Staphylococcus aureus.* The cells appear purple after staining due to the retention of crystal violet dye.

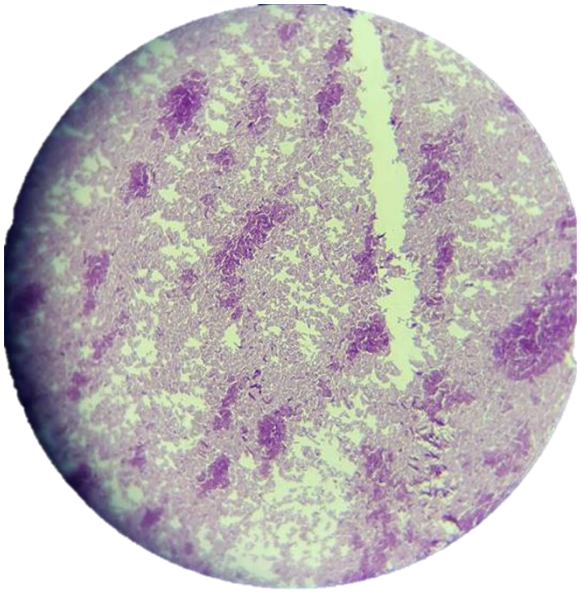

Figure 2. A Gram stain image of a culture of Gram-negative *Escherichia coli*. The cells do not retain the purple colour characteristic of crystal violet following the application of alcohol. Furthermore, the cells appear red due to the retention of the counter dye safranin.

Metabolic Pathways

Despite the more complex structural nature of typical eukaryotes, both cell types share many common attributes, such as possessing a plasma membrane and having a common set of metabolic pathways. Essentially, a metabolic pathway encompasses a sequence of separate reactions, each governed by a specific enzyme, which facilitate a complex chemical transformation. In cells, particular molecules serve as fuel and release chemical energy that is exploited to generate adenosine triphosphate (ATP). In essence, ATP comprises of an adenosine molecule bonded to three phosphate groups and is considered to be a high-energy molecule due to the presence of energy-rich phosphate bonds. Furthermore, ATP is central to the energy transformations of all living organisms and can be hydrolysed in various locations within a cell to liberate free energy to drive endergonic reactions. In non-photosynthetic cells, sugar glucose represents the most common chemical fuel and glucose catabolism involves liberating the energy stored within the covalent bonds.

Glycolysis is an example of a metabolic pathway which occurs in the cytoplasm of both eukaryotes and prokaryotes, with the former transporting the end product of glycolysis to the mitochondrial matrix. Glycolysis is the first step in glucose catabolism which encompasses ten enzyme-catalysed reactions to generate pyruvate; a three-carbon molecule from glucose. Glycolysis liberates relatively little of the energy that is contained within a glucose molecule; following glycolysis is the citric acid cycle and oxidative phosphorylation which both release much more usable energy. Specifically, glycolysis generates four ATP molecules from one molecule of glucose, yet the initial steps of glycolysis consume two molecules of ATP, thereby establishing a net production of two ATP molecules.

Pyruvate oxidation represents the next step in the aerobic catabolism of glucose, which occurs in the plasma membrane of prokaryotes and the mitochondrial matrix of eukaryotes. Pyruvate oxidation links the process of glycolysis and subsequent oxidative reactions to liberate energy from the catabolism of glucose. In general, pyruvate oxidation involves the generation of a two-carbon acetate molecule and carbon dioxide from the oxidation of pyruvate. Following the generation of acetate, coenzyme A is bound to the newly formed acetate to produce acetyl coenzyme A (acetyl CoA). Furthermore, the principal role of acetyl CoA is to donate the acetyl group to oxaloacetate, which initiates the citric acid cycle. Additionally, pyruvate oxidation, the citric acid cycle, and oxidative phosphorylation are collectively referred to as cellular respiration. However, in the absence of oxygen, glycolysis is followed by fermentation which involves the conversion of pyruvate into lactic acid or ethanol, and is associated with the incomplete oxidation of glucose.

The citric acid cycle represents one of life's most significant energy-reaping pathways and completes the oxidation of glucose to carbon dioxide. The citric acid cycle occurs within the cytoplasm of prokaryotes and within the mitochondrial matrix of eukaryotes. The citric acid cycle comprises of eight reactions and concludes with the regeneration of the starting material; oxaloacetate. As well as carbon dioxide, the citric acid cycle generates reduced electron carriers in the form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$).

The process of ATP synthesis is referred to as oxidative phosphorylation and involves the re-oxidation of electron carriers. Oxidative phosphorylation occurs in the mitochondria of eukaryotes and the plasma membrane of prokaryotes. Furthermore, the process of oxidative phosphorylation is divided into two components; electron transport and chemiosmosis.

Electron transport involves the migration of electrons down a wire of protein complexes embedded in the inner mitochondrial membrane called a respiratory chain; stimulating the movement of protons out of the mitochondrial matrix and across the inner mitochondrial membrane by means of active transport, thus establishing a proton concentration gradient. In addition, the imbalance of protons establishes a charge difference between the intermembrane space and the matrix, since protons bear a positive charge. The resulting electric field across the membrane can be likened to a charged-up battery. Furthermore, the proton concentration gradient and the electric charge difference combine to establish a source of potential energy called the proton-motive force. This force drives protons back across the membrane into the mitochondrial matrix through a specific proton channel called ATP synthase. The proton-motive force and ATP synthase couple together in a process known as chemiosmosis. ATP synthase links the movement of protons back across the membrane with the synthesis of ATP.

Pathogenesis

Bacteria as Pathogens

The majority of microorganisms that humans encounter on a daily basis are innocuous; allowing these microorganisms to coexist peacefully with humans. However, some of these microorganisms have siblings that are capable of triggering disease. *E. coli* represents a classic example of a species of bacteria that can give rise to commensal strains and pathogenic strains. *E. coli* is found in the intestines as commensal bacteria; the colonisation of the gastrointestinal tract by large numbers of commensal bacteria protect humans from foodborne bacteria by presenting these pathogenic bacteria with a competitive disadvantage. On the other hand, pathogenic *E. coli* strains are capable of causing a broad range of human diseases, such as watery diarrhoea and haemolytic-uremic syndrome; the latter being associated with the serotype *E. coli* O157:H7 (Stephens & Murray, 2001). Pathogenic bacteria exploit an array of mechanisms to trigger disease in human hosts. In addition, bacterial pathogens express an extensive range of molecules that bind to host cell targets in order to elicit a variety of different host responses. Furthermore, an approach to combating bacterial disease is the identification and characterisation of the diverse molecular strategies utilised by pathogenic bacteria to trigger disease (Wilson et al., 2002).

Substantial influx in data regarding DNA sequences, clever utilisation of polymerase chain reaction (PCR) techniques, and the impact of crystallographic methods indicate that there are common strategies utilised by a bacterium in order to become a pathogen. Microbial proteins, usually in the form of enzymes, which are secreted from pathogenic bacteria and are able to kill host cells at low concentrations are called exotoxins. Exotoxins alone have the capacity to inflict most of the clinical features associated with cholera and tetanus, thereby often

representing a central role in the pathogenesis of microbial disease (Finlay & Falkow, 1997). Proteinaceous toxins can be transported to eukaryotic cells by two different methods: distribution into the surrounding extracellular environment or direct insertion into the cytoplasm of the host cell (Wilson et al., 2002). Toxins are differentiated into groups based on their structural homology and biochemical activity. Moreover, A-B toxins represent a very common type of endotoxin which include two subunits; the B subunit and the A subunit. The B subunit facilitates the attachment of the toxin to the host cell receptor and the subsequent insertion of the toxin into the host cell. The A subunit bears the enzymatic activity which acts on the host cell. The enzymatic activity demonstrated by the A-subunit varies among toxins; ADP-ribosylating activity and proteolytic activity are examples of the activity that can be demonstrated by the A-subunit. The B domain is subject to much variation, presumably to accommodate for attachment specificities associated with different hosts and tissues. Examples of A-B toxins include the pertussis toxin, produced by *Bordetella pertussis*, and the cholera toxin, produced by *Vibrio cholerae*. In addition, proteolytic toxins represent another category of exotoxin and their mode of action includes the degradation of specific host proteins which gives rise to some of the distinctive clinical symptoms of the disease. Furthermore, both *Clostridium tetani* and *Clostridium botulinum* release toxins that target synaptobrevins which in turn inhibits the release of neurotransmitters, thereby leading to different types of paralysis (Finlay & Falkow, 1997).

Microbial pathogens require the means to adhere to host surfaces for successful colonisation and the subsequent production of disease. In addition, identifying the specific role of a particular adhesin molecule is difficult since an individual pathogen can express numerous adherence factors (Finlay & Falkow, 1997). Host surfaces include skin, mucous membranes, and deeper tissues. Furthermore, hosts can deploy numerous mechanical forces to detach

microbes from these surfaces, such as saliva secretion, coughing, sneezing, mucous flow, peristalsis, and blood flow (Wilson et al., 2002). Bacterial adhesins are elements on the cell surface which facilitate the adherence of bacteria to host surfaces. Additionally, they can be divided into two major categories; pili and non-pilus adhesins. The conservation of the molecular machinery associated with the biogenesis and subsequent assembly of pili onto bacterial surfaces constitutes one of the major features among diverse pili. The family of P pili represent one of the best-studied examples of the processes involved in pilus assembly. P pili that are expressed on the surfaces of some *E. coli* strains are thought to be crucial in the pathogenesis of pyelonephritis; a disease characterised by urinary tract colonisation followed by infection of the kidney. The specificity for a certain tissue is possibly dictated by the presence of a host receptor which is recognised by a particular adhesin. Furthermore, interaction with the host receptor is ensured by the position of the adhesin at the distal tip of the pilus. PapG is an adhesin molecule belonging to the P pili family and is located at the distal end of the pilus rod. PapG binds to the α-$_D$-galactopyranosyl-(1-4)-β-$_D$-galactopyranoside (galabiose) receptor; a member of a globoseries of glycolipids which are located on the host cells lining the upper urinary tract (Finlay & Falkow, 1997).

Afimbrial adhesins are protein adherence factors, although they do not assemble into long, polymeric fimbrial structures. In addition, afimbrial adhesins commonly interact more intimately with the host cell (Wilson et al., 2002). *B. pertussis* is a respiratory mucosal pathogen that causes pertussis; a disease characterised by symptoms similar to those produced by the common cold which are followed by extended periods of damaging coughing fits. *B. pertussis* secretes an afimbrial adhesin known as filamentous haemagglutinin (FHA); a 220 kilodalton (kDa) polypeptide which facilitates the attachment of bacteria to ciliated respiratory cells and alveolar macrophages (Sandros & Tuomanen, 1993). FHA contains the arginylglycylaspartic

acid (RGD) sequence; a eukaryotic recognition motif which enables FHA to bind to the cell-surface integrin, CR3, on leukocytes. Subsequent bacterial uptake into macrophages does not initiate oxidative burst; promoting intracellular survival (Finlay & Falkow, 1997). Additionally, FHA contains a carbohydrate recognition domain which mediates the attachment of bacteria to ciliated cells. Furthermore, bacterial strains with in-frame deletions of nucleotide sequences that affect the amino acid sequences defining the carbohydrate recognition domain fail to attach to ciliated cells and macrophages (Sandros & Tuomanen, 1993).

Pathogens can further infiltrate the host in order to continue the infection cycle. The pathogenic principle which is characterised by further permeation into a host cell is referred to as invasion. Invasion can be divided into two categories; extracellular and intracellular. Extracellular invasion arises when a microbe breaches the barriers of a tissue in order to spread within a host while residing outside of host cells. *S. aureus* represents one of the bacterial species which utilises the strategies associated with extracellular invasion. The features of extracellular invasion include the release of several enzymes that degrade host cell molecules, such as hyaluronidase and nuclease. Hyaluronidase cleaves proteoglycans in connective tissue and nuclease digests released DNA. Furthermore, extracellular invasion enables pathogenic bacteria to access niches in tissues from where they are capable of multiplying, progressing to other locations, producing toxins, and generating inflammatory responses. Intracellular invasion arises when a microbe infiltrates the cells of a host tissue and subsequently prospers within the intracellular environment. Some pathogens, such as many species belonging to the Rickettsia genus, rely on intracellular invasion of mammalian cells to sustain their own lifecycles. On the other hand, facultative intracellular pathogens utilise their ability to survive within host cells as a means of proliferation, or dissemination to other tissues. In addition, the genes that allow pathogens to invade non-phagocytic cells have been established to encode an

evolutionarily related type III protein secretion pathway. Furthermore, the type III protein secretion pathway inserts signalling proteins from the microbe into the host cell, which stimulate host cell signalling pathways to coordinate the uptake of the microbe (Wilson et al., 2002).

The invasion system found in *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* represents a well-studied model of bacterial entry into epithelial cells. Both *Y. pseudotuberculosis* and *Y. enterocolitica* have a chromosomal gene that dictates the formation of invasin; an outer membrane protein which facilitates attachment and access into non-phagocytic cells (Finlay & Falkow, 1997). Invasin coordinates the uptake of bacteria into mammalian cells by way of binding to β1 integrins; membrane αβ heterodimeric receptor proteins that facilitate adhesion between cells, the extracellular matrix, and pathogens. Additionally, β1 integrins display a much higher affinity for invasin than the more common extracellular matrix ligands, such as fibronectin, which contributes to the eventual uptake of the microorganism (Gillenius & Urban, 2015). For many intestinal microorganisms, the initial cell type that is encountered is the microfold cell (M cells) which can be found distributed within the epithelium or over lymphoid patches. Consequently, bacterial interaction with M cells instigates the binding of invasin to the β1 integrin followed by internalisation of the microbe (Isberg & Van Nhieu, 1994).

Enteric Pathogenic *E. coli* serotype O157:H7

The serotype *E. coli* O157:H7 represents a distinct antigenic-related variation within the species of *E. coli*. Essentially, serotyping identifies strains of bacteria based on their cell surface antigens. Furthermore, *E. coli* O157:H7 expresses on its cell surface the O157 antigen; the

outermost component of a liposaccharide molecule that protrudes from the outer membrane of the cell (Wolf, 1997). The repeating monomeric unit structure which constitutes the O-polysaccharide exhibits vast diversity with regards to the type of carbohydrate monomer and the presence, or absence of non-carbohydrate substituents. In addition, the position and stereochemistry of the O-glycosidic linkages are subject to much variation. Furthermore, the structure of the O-polysaccharide within an organism determines its serological specificity (Whitfield & Raetz, 2002). Interestingly, Boyer, Sumner, Williams, Kniel & McKinney (2011) demonstrated that the absence of the O157 antigen reduced the cells ability to attach to the surface of lettuce. Moreover, the absence of the O157 antigen increased the hydrophobic characteristics of the cell, which indicates that the hydrophobic properties of a cell may influence the cells ability to attach to food surfaces. Additionally, the physiochemical properties of the cell were altered by the absence of the O157 antigen, yet only hydrophobicity influenced the cells ability to attach to the surface of lettuce. In contrast, the H7 antigen is proteinaceous and incorporated into the structural components of the flagellum; a rigid helical tail which is driven by a molecular motor at its base (Wolf, 1997).

E. coli O157:H7 is a harmful foodborne pathogen that is capable of triggering severe diseases in humans worldwide; this serotype represents a significant public health concern in North America, Europe, and other areas of the world. *E. coli* O157:H7 is categorized as enterohemorrhagic; a subset of pathogenic *E. coli* that are capable of producing Shiga toxins (Stxs). Furthermore, Shiga toxins cause haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS). Pathogenic *E. coli* are divided into categories based on serogroups, pathogenicity mechanisms, clinical symptoms, or virulence factors. Enterohemorrhagic *E. coli* (EHEC) comprise of several pathogenic serotypes that are regularly linked with human diseases; *E. coli* O157:H7 represents the most frequently isolated serotype from ill persons in

the United States, Japan, and the United Kingdom. Despite the total number of case numbers associated with *E. coli* O157:H7 infections being lower than those of other enteric pathogens, such as *Salmonella* spp, the diseases produced by *E. coli* O157:H7 result in higher hospitalisation and fatality rates. Furthermore, cattle are a major reservoir of *E. coli* O157:H7, as the presence of this harmful serotype within cattle does not induce any of the symptoms associated with *E. coli* O157:H7 colonisation within humans (Youn Lim, Yoon & Hovde, 2010). The foremost virulence properties associated with *E. coli* O157:H7 are adherence to mucosal cells and the production of Shiga-like toxins (Griffin & Tauxe, 1991). The colonisation of the intestinal mucosa by *E. coli* O157:H7 is characterised by the generation of the attaching and effacing (A/E) lesion. The A/E lesion is generated by effacement of the microvilli followed by bacterial adherence to the epithelial cell membrane (Youn Lim et al., 2010).

Shiga Toxin

The Shiga toxin (Stx) is among one of the most potent bacterial toxins. Stx occurs in *Shigella dysenteriae* type 1 and some serotypes of *E. coli*; the Shiga toxin found in specific strains of *E. coli* is referred to as Stx1a in order to distinguish it from the toxin produced by *Shigella dysenteria.* In addition, some strains of *E. coli* produce a second, antigenically distinct toxin known as Stx2a. Interestingly, strains that produce Stx2a are more commonly linked with the life-threatening haemolytic uremic syndrome (HUS). Furthermore, Stx is composed of two major subunits; the A subunit and the B subunit. The A subunit consists of 293 amino acids and consists of an enzymatic region that facilitates the cytotoxicity of the toxin. The B subunit is composed of five structurally identical subunits which assemble to form a pentamer; the A subunit and the B subunit interact non-covalently (Melton-Celsa, 2014). The A subunit demonstrates RNA N-glycosidase activity against 28S rRNA; a component of the large subunit

that forms part of eukaryotic ribosomes, ultimately obstructing the process of protein synthesis and inducing cellular apoptosis. In addition, the B subunit targets globotriaosylceramide-3 (Gb3); a glycolipid cellular receptor which is expressed on different cell types depending on the host (Nguyen & Sperandio, 2012). Once the Shiga toxin has been inserted into the gut lumen it targets the Gb3 receptor by means of translocation across the intestinal epithelium into the underlying tissues and bloodstream. Additionally, high concentrations of the Gb3 receptor are present in renal tubular cells and microvascular endothelial cells. The locations of the aforementioned types of cells are commonly found in the kidney, gut, and brain. Furthermore, the locations of these cells explain the clinical manifestations of HUS (Clements, Young & Constantinou, 2012). Furthermore, cattle lack expression of the Gb3 receptor on their vascular endothelium, thus allowing *E. coli* O157:H7 to thrive within cattle without presenting any of the adverse effects associated with the pathophysiology of the Shiga toxin (Nguyen & Sperandio, 2012).

Attaching and Effacing Lesions

The gastrointestinal epithelium represents a significant defence barrier against disease-causing microbes. However, enteric pathogens, such as *E. coli* O157:H7, have developed virulence traits that allow them to colonise and penetrate this barrier. Moreover, several important pathogenic microbes utilise type III secretion systems (T3SS) in order to insert virulence factors into infected eukaryotic cells, where they subsequently hijack cell-signalling apparatus. Furthermore, enteric human pathogens are able to colonise the gut mucosa by way of attaching and effacing (A/E) lesions. In general, A/E lesions are characterised by intimate bacterial attachment to the apical plasma membrane, localised accumulation of F-actin, and effacement of brush border microvilli. In addition, the ability of pathogens to generate A/E lesions is dependent upon the presence of the locus of enterocyte effacement (LEE). The LEE represents

a genomic pathogenicity island which bears the information required for the formation of a T3SS, regulators, chaperones, and effector proteins (Cepeda-Molero et al., 2017).

The configuration of the a T3SS bears a resemblance to a "molecular syringe", which enteric *E. coli* can utilise to insert effector proteins, such as the translocated intimin receptor (Tir), directly into the cytoplasm of target cells. Tir is inserted in the cytoplasmic membrane in a particular arrangement which promotes interaction of its central domain with intimin; a bacterial cell surface protein, thus facilitating an attachment between the bacterial cell and the eukaryotic cell. Furthermore, proteins associated with the regulation of cytoskeletal arrangement are recruited by EspFu; a secreted bacterial effector protein. The aforementioned proteins include Wiskott-Aldrich (N-WASP); an actin nucleation-promoting factor, and insulin receptor tyrosine kinase substrate p53 (IRSp53). Ultimately, this induces the deposit of actin beneath attached bacteria, thereby generating the characteristic pedal-like architecture (Nguyen & Sperandio, 2012).

Staphylococcus Aureus

S. aureus exists as both a human pathogen and a commensal bacterium, with approximately 30 % of the population colonised by *S. aureus*. Furthermore, *S. aureus* is the foremost cause of bacteraemia and infective endocarditis. In addition, *S. aureus* is associated with a wide-range of skin and soft tissue infections; ranging from benign to life-threatening. Moreover, *S. aureus* represents the most common pathogen that is extracted from surgical site infections, cutaneous abscesses, and purulent cellulitis. (Tong, Davis, Eichenberger, Holland & Fowler, 2015).

S. aureus entering the skin stimulates the arrival of neutrophils and macrophages to the site of infection. However, *S. aureus* has an astounding arsenal of evasive techniques, such as

inhibiting chemotaxis of leukocytes, sequestering host antibodies, concealment via generating a polysaccharide capsule, or producing biofilm in order to avoid detection by the immune system, and withstanding destruction after ingestion by phagocytosis (Tong et al., 2015). Receptors situated on the surface of neutrophils play a significant role in the recognition of bacteria by way of stimulating phagocyte chemotaxis and activation. In addition, *S. aureus* produces a molecule called chemotaxis inhibitory protein of *S. aureus* (CHIPS); a secreted molecule that inhibits receptor-mediated recognition of pattern-associated molecular patterns (PAMPs), which are molecules secreted by bacteria and crucial for phagocyte detection of bacterial invaders. Following ingestion, *S. aureus* can deploy even more diverse procedures to undermine immune responses, such as producing catalase and superoxide dismutase in order to neutralise reactive oxygen species (ROS). Furthermore, *S. aureus* produces a series of proteins to combat the oxygen-independent killing aspects of the innate immune responses; such aspects include antimicrobial peptides (AMPs). With regards to neutralising the activity of cationic AMPs, *S. aureus* undertakes upregulation of resistance mechanisms, such as the D-alanylation of teichoic acids and the incorporation of cationic phospholipid lysyl-phosphatidyl glycerol into the cytoplasmic membrane. These mechanisms combine to reduce the net negative charge of the bacterial surface, thus reducing the binding activity of cationic AMPs (DeLeo, Diep & Otto, 2009).

Biofilms

Background

The history of microbiology has largely depicted microorganisms as planktonic, freely suspended cells which flourish in nutritionally rich culture media. The discovery of microorganisms that are able to adhere to exposed surfaces encouraged supplementary studies which revealed that these surface-associated microorganisms displayed a distinctive phenotype, with regards to gene transcription and growth rate. Van Leeuwenhoek observed microorganisms on the surface of teeth using simple microscopes, thus the discovery of microbial biofilms is attributed to Van Leeuwenhoek. Furthermore, these biofilm-associated microorganisms demonstrated specific mechanisms for the attachment to a surface, the establishment of a community configuration, and detachment from a surface. Moreover, applying the confocal laser scanning microscope to examine biofilm ultrastructure and analysing the genes associated with cell adhesion have significantly contributed to the understanding of biofilms (Donlan, 2002).

A biofilm is an accumulation of microbial cells that are adhered to a surface and encased in a matrix of mainly polysaccharide material (Donlan, 2002). In the majority of biofilms, the microorganisms represent less than 10 % of the dry mass, whereas the extracellular matrix can represent over 90 %. The matrix establishes the platform for the three-dimensional architecture of the biofilm; the matrix is responsible for the adhesion to surfaces and cohesion within the biofilm. Furthermore, the matrix contributes to the robust interactions between cells by immobilising them and retaining them in close proximity. In addition, the matrix also exhibits recycling features by retaining all of the components of lysed cells, such as DNA, which can be used in horizontal gene transfer (Flemming & Wingender, 2010). Organisms capable of

biofilm development are unlike their planktonic counterparts with regards to the genes that are transcribed. Moreover, biofilms can develop on a wide assortment of surfaces, such as living tissues, medical devices, industrial water system piping, and natural aquatic systems. Furthermore, depending on the surface, biofilms demonstrate a variable nature which can be demonstrated by scanning electron micrographs. In addition, biofilms associated with water systems are composed of corrosion products, clay material, fresh water diatoms, and filamentous bacteria. On the other hand, biofilms associated with medical devices are configured of single, coccoid organisms and the accompanying extracellular polymeric substance matrix (Donlan, 2002). Bacteria encased within a biofilm exhibit elevated resistance to antibiotics, disinfectants, and to host immune defence systems. Therefore, the significance of biofilms is well acknowledged in medical, environmental, and industrial environments (Stepanović et al., 2007).

Biofilm Structure

Microbial cells and the associated extracellular polymeric substance (EPS) primarily constitute the biofilm entity. In addition, the EPS may represent 50 % to 90 % of the total organic carbon of biofilms. Furthermore, the EPS displays variation with regards to physical and chemical properties, but is predominately comprised of polysaccharides (Donlan, 2002). The EPS generated by microbial cells may comprise of neutral macromolecules, however the majority of the macromolecules found in the EPS are polyanionic; this is due to the presence of either uronic acids or ketal-linked pyruvate (Sutherland, 2001). In addition, the polyanionic nature of these matrices promote association with divalent cations, such as calcium, which increases the mechanical stability of a mature biofilm by cross-linking with the polymer strands (Donlan, 2002). There are two crucial properties concerning the EPS that yield a significant effect on the biofilm; first, the primary conformation of the polysaccharides present in the EPS is

governed by their structure and composition. For example, the inclusion of 1,3- or 1,4-β-linked hexose residues into the backbone of bacterial EPS yields more rigid structures that are not prone to flexibility. Second, the EPS found in the biofilm varies with regards to spatial and temporal features, thereby not existing as generally uniform (Donlan, 2002). Leriche, Sibille & Carpentier (2000) performed a study using the binding specificity of lectins towards sugar moieties in order to characterise and quantify exopolysaccharides produced by bacterial biofilms. Furthermore, various bacterial genera were utilised in the assay in order to establish whether the application of one lectin is enough to observe biofilm exopolysaccharide production over time and to establish which particular strains could be characterised by exploiting the binding specificity of lectins. The assay utilised two lectins which recognise the most common saccharide units found in biofilm matrices; concanavalin A was employed to quantify $_{D}$-glucose and $_{D}$-mannose residues. In addition, wheat germ agglutinin was employed to quantify *N*-acetyl-$_{D}$-glucosamine and *N*-acetylneuraminic acid. Furthermore, Leriche et al. (2000) concluded that different organisms produced differing amounts of EPS and the extracellular contents derived from the biofilm increased over time. Increased availability of carbon substrates will increase the amount of EPS within the biofilm, although a balance between the availability of carbon and the limitation of certain nutrients, such as nitrogen, potassium, or phosphate, is essential for the increased production of EPS (Sutherland, 2001).

Pseudomonas Aeruginosa Biofilm Formation

P. aeruginosa is an aerobic Gram-negative bacterium capable of causing both community-acquired and hospital-acquired infections. Additionally, *P. aeruginosa* is a cause of infection in immunocompromised hosts and patients with cystic fibrosis. Furthermore, *P. aeruginosa* has an extensive repertoire of antimicrobial resistance mechanisms, such as multidrug efflux pumps, β-lactamases, and the ability to downregulate outer membrane porins. In addition, *P.*

aeruginosa utilises various mechanisms of virulence such as the secretion of toxins and the ability to generate biofilms (Driscoll, Brody & Kollef, 2007).

Alginate, an extracellular polysaccharide, can be overproduced in some isolates of *P. aeruginosa*. The overproduction of alginate can be attributed to a mutation in the *mucA* gene, thereby allowing alginate biosynthesis to be induced under the influence of the AlgU gene. Furthermore, the presence of alginate has been observed to hinder infected host immune responses, including the scavenging of free radicals produced by macrophages, generating a physical barrier that obstructs phagocytosis, and preventing neutrophil chemotaxis and complement activation. In addition, alginate is seemingly instrumental in the formation of biofilms associated with *P. aeruginosa* (Driscoll et al., 2007).

The term 'biofilm' is related to the mode of growth exhibited by bacteria that yields a collection of microcolonies embedded within a biopolymer matrix and attached to a surface. Furthermore, biofilms associated with *P. aeruginosa* can be found in the airways of patients suffering with cystic fibrosis. In addition, while undergoing the procedure of attachment, planktonic cells are able to communicate intercellularly using a process known as quorum sensing (Driscoll et al., 2007). Bacteria utilise the mechanism of quorum sensing in order to perceive the density of the surrounding bacterial population and react to this information by regulating various genes. Furthermore, the mechanism of bacterial communication within a colony is achieved by the production of signalling molecules which are received by receptors situated on the surface of bacteria; Gram-negative bacteria produce acylated homoserine lactones, whereas Gram-positive bacteria often produce oligopeptides. The autoinducer signal molecules, *N*-(3-oxododecanoyl) homoserine lactone and *N*-butyryl homoserine lactone, increase as a population of *P. aeruginosa* increases. As a consequence, an intracellular threshold is attained

which stimulates these molecules to bind to and activate their cognate transcriptional regulators (Smith & Iglewski, 2003). In biofilm development, *P. aeruginosa* will generate exopolysaccharides which results in the production of a matrix that is characterised by an intricate assembly of bacterial microcolonies separated by water channels (Driscoll et al., 2007).

Mechanisms of Biofilm Resistance

It is well established that biofilm-associated cells encased in an exopolysaccharide matrix exhibit properties that are distinct from planktonic cells; one of which is an increased resistance to antimicrobial agents. *In vitro* investigations have begun to illuminate the mechanisms underlying how and why surface-attached microbial communities display resistance to antimicrobial agents. Furthermore, there are multiple resistance mechanisms which vary depending upon the species of bacteria and the biocide being applied. In addition, these mechanisms include chemical or physical diffusion barriers to antimicrobial compounds, reduced biofilm growth rate as a result of nutrient restriction, initiation of general stress responses, and the development of a biofilm-specific phenotype (Mah & O'Toole, 2001).

The influence the exopolysaccharide matrix has on the migration of antimicrobial agents to the underlying cells varies with the antimicrobial agent being applied. For example, relating to antibiotics, it has been demonstrated that by cultivating biofilms associated with *P. aeruginosa* on one side of a dialysis membrane, the amount of piperacillin that can permeate into a biofilm can be evaluated. Furthermore, biofilms associated with *P. aeruginosa* obstruct the passage of piperacillin. On the other hand, biofilms associated with *S. epidermis* allowed the penetration of rifampicin and vancomycin across the membrane, thereby indicating that these antibiotics had successfully penetrated the biofilm. Additionally, hydrogen peroxide demonstrated

invasive behaviour against a thin biofilm-covered bead associated with a low colony-forming unit (CFU) count, whereas thicker biofilms demonstrated increased resistance to hydrogen peroxide. Intriguingly, a thick biofilm developed by a mutant strain of *P. aeruginosa*, which lacked one of the fundamental catalase genes, *katA*, was impervious to hydrogen peroxide. Furthermore, restriction of antimicrobial compounds to the cells embedded within the biofilm can be attributed to a reaction with, or sorption to, the components of the biofilm (Mah & O'Toole, 2001).

An increase in resistance can be stimulated by a switch from exponential to slow, or no growth. Due to the fact that the cells developing within a biofilm are likely to experience some form of nutrient limitation, it has been implied that this physiological alteration can be attributed to the resistance of biofilms to antimicrobial agents. Furthermore, by examining the growth rate-induced effects concerning the planktonic cells and biofilm cells of *P. aeruginosa*, *E. coli*, and *S. epidermis*, it was observed that the sensitivities of both types of cells towards ciprofloxacin increased with increasing growth rate. In addition, concerning *P. aeruginosa*, it was observed that as the growth rate increased, the planktonic cells demonstrated more susceptibility than biofilm cells to ciprofloxacin. Additionally, studies have analysed the resistance associated with planktonic and biofilm cells as they transition from exponential growth into stationary growth. Moreover, the results obtained established that resistance increased as both types of cells transitioned into the stationary phase of growth, with biofilm cells exhibiting 15-times more resistance than planktonic cells (Mah & O'Toole, 2001).

An emerging concept associated with biofilm resistance is that a biofilm-specific phenotype is stimulated in a subpopulation of the community to help suppress the adverse effects of antimicrobial agents by the expression of active mechanisms. In addition, this resistance-linked

phenotype might be stimulated by nutrient limitation, particular types of stress, high cell density, or a combination of the aforementioned factors. Furthermore, phenomena such as multidrug efflux pumps, which are capable of expelling chemically unrelated antimicrobial agents from the cell, could be related to the development of resistance to antimicrobial compounds. The generation of a multidrug-resistant phenotype in *E. coli* is stimulated by upregulation of the *mar* operon. As well as multidrug efflux pumps, the membrane-protein composition can be subjected to alterations, thereby leading to changes in cell permeability when exposed to antimicrobial compounds. Moreover, mutations in regulatory genes, such as *ompB,* which is responsible for encoding outer membrane porin proteins, enhanced the resistance of *E. coli* to β-lactam antibiotics. With regards to outer membrane porins, it has been demonstrated that biofilm-associated bacteria exhibit elevated expression levels of *ompC*, an outer membrane protein, and other osmotically regulated genes compared with planktonic bacteria. Furthermore, its plausible that the environmental conditions within a biofilm are linked with elevated osmotic stress, thereby resulting in alterations concerning the cell envelope that afford the bacteria protection from the adverse effects of antimicrobial compounds (Mah & O'Toole, 2001).

Moreover, there are a variety of mechanisms that promote resistance against antimicrobial compounds; mechanisms which depend upon the bacterial complement of the biofilm and the antimicrobial compound being applied to eradicate the biofilm. Such mechanisms include the development of a heterogenous population of cells, thereby giving rise to varying levels of resistance throughout the population. For example, the cells situated in close proximity to the liquid-biofilm interface might be inaccessible, to a small extent, by the generation of enzymes from the exopolysaccharide matrix which neutralise antimicrobial compounds. In addition, the cells situated in an intermediate position might exhibit slow growth and might benefit from

protection from the outermost cells. To conclude, another sub-population of cells might be stimulated by environmental alterations to express a biofilm-specific resistance phenotype (Mah & O'Toole, 2001).

Antimicrobials

Background

In the present day, vigilant hand-washing practices are encouraged to reduce the spread of bacteria from person to person and ultimately prevent the rise of infections within individuals. Ironically, persistent hand-washing practices can produce undesired side effects, especially in the healthcare environment where strict guidelines encourage methodical hand-washing practices (Moadab, Rupley & Wadhams, 2001). In order to maintain the lowest possible microbial counts during surgical procedures, surgical hand scrubbing practices are applied in pre-surgical preparations to remove microorganisms from the hands and forearms. In the event of accidental damage to a surgical team member's glove during surgery, effective hand scrubbing minimises the patient's risk of acquiring a postoperative infection (Paulson, 1994). Contact dermatitis, as well as other skin problems, is connected with routine hand-washing practices and wearing gloves. Healthcare workers with impaired skin can facilitate the transmission of larger numbers of bacterial pathogens compared with healthcare workers with undamaged skin. Additionally, patients with dermatological problems are more likely to be subjected to skin colonisation by organisms such as *Staphylococcus Aureus* (Larson et al., 1998). Undamaged skin provides the body with protection against environmental pathogens; the skin is comprised of the epidermis and the dermis. In addition, the squamous stratified epithelium of the epidermis is compiled of five distinctive layers of cells. Furthermore, the body's internal and external environment are separated by the epidermis. As a consequence of compromised skin, the underlying tissue becomes exposed which greatly increases the risk of infection (Gould, 2012). Consequently, the use of rinse-free hand sanitisers has increased, which are used alongside proper hand-washing techniques with soap and water. Additionally, gels and foams represent the most widely used hand sanitisers, which use alcohol as the

principal antimicrobial agent. The popularity of these rinse-free sanitisers is chiefly based on aspects such as cost, inclusion of emollients, fragrances, delivery vehicle, size, and marketing. In addition, the rationale behind the selection of alcohol-based sanitisers rarely includes the products effectiveness at eradicating bacteria after a single use (Dyer, Gerenratch & Wadhams, 1998). As well as being prominently utilised in healthcare environments in the form of antiseptics and disinfectants, they are also widely employed by the general public due to increasing concerns regarding the potential for microbial contamination in the food and general consumer markets. Furthermore, an extensive range of active chemical agents, or biocides, can be found in these products; many of which have been associated with antimicrobial activity for hundreds of years. In general, biocides are recognised as possessing a broader spectrum of activity than antibiotics, while antibiotics are associated with having specific intracellular targets (McDonnell & Russell, 1999). Furthermore, the overall effectiveness of an antimicrobial agent can be subdivided into three categories; the immediate antimicrobial efficacy, the persistent effectiveness, and the residual antimicrobial efficacy. The immediate antimicrobial efficacy relates to the quantitative measurement of both the mechanical and immediate inactivation of microorganisms on the surfaces of the skin. The persistent antimicrobial effectiveness relates to the quantitative measurement of a topical antimicrobials capacity to prevent the occurrence of microbial recolonisation of the skins surface following the application of the product. In addition, the prevention of microbial recolonisation can be achieved by either microbial inhibition or eradication. The residual antimicrobial efficacy is defined as the measurement of a products cumulative abilities after it has been employed for a period of time. Furthermore, certain antimicrobial agents are absorbed into the stratum corneum of the skin following prolonged use, thus inhibiting bacterial recolonisation on the surface of the skin (Paulson, 1994).

The mechanisms involved in the action of antiseptics and disinfectants against bacteria has received considerable attention, thus yielding significant progress in the understanding of antimicrobial modes of action. Regardless of cell type, it is likely that there is a common sequence of events with respect to the mechanisms of antimicrobial action. Moreover, this sequence of events can be envisaged as an initial interaction of the antimicrobial compound with the cell surface, subsequent permeation into the cell, and action at the target site. In addition, cell viability can be significantly affected by interaction at the cell surface, however the majority of antimicrobial agents deliver their effects intracellularly. Furthermore, the outermost layers of microbial cells can significantly influence whether microbial cells will be susceptible or insusceptible to antimicrobial compounds (McDonnell & Russell, 1999).

Alcohol

The most common alcohols employed in antimicrobial formulations are ethyl alcohol, isopropyl alcohol, and *n*-propanol. Alcohol exhibits rapid broad-spectrum activity against vegetative bacteria, viruses, and fungi. Despite its inability to eradicate spores, alcohol is known to inhibit the growth of spores and the germination of spores. As a consequence, alcohol is widely utilised in hard-surface disinfection and skin antisepsis. In essence, the mode of action regarding alcohol is thought to involve damage to the membrane integrity, rapid denaturation of intracellular materials, such as proteins, followed by obstruction of metabolic processes and eventual cell lysis (McDonnell & Russell, 1999). Succinic acid dehydrogenase, an enzyme present in many species of bacteria, such as *E. coli*, is able to extract hydrogen from a variety of substrates. Furthermore, succinic acid dehydrogenase is prone to denaturation when exposed to alcohol (Sykes, 1939). Studies conducted by Sykes (1939), using methylene blue as a redox indicator to monitor the enzymatic activity of succinic acid dehydrogenase, concluded that various aliphatic monohydric alcohols were capable of comprehensively incapacitating the

enzyme. Alcohol is proven to produce adverse effects against the skin as it removes sebum and lipids that protect the skin from bacterial infections. Moreover, a study conducted by Dyer et al. (1998) on the efficacy of various hand-wash formulae on human subjects revealed, despite all alcohol-containing products exhibiting potent antimicrobial activity, that all subjects who completed the test protocol using alcohol-based hand sanitiser formulae reported palmar pain or discomfort. In addition, following detailed assessment, these subjects were discovered to have noticeable swelling that was, in some cases, supplemented by erythema of the palmar tissues. Furthermore, persistent use of hand sanitisers containing alcohol promotes the skins vulnerability to infection by transient pathogenic bacteria. In the healthcare environment, these circumstances can promote the spread of disease-causing bacteria (Dyer et al., 1998). Therefore, the development of alcohol-free formulae is essential to reducing the spread of disease and protecting the natural barrier functions of the skin.

Chlorhexidine

Belonging to a class of compounds known as biguanides, chlorhexidine is probably the most widely used biocide in antiseptic products, specifically in hand-wash formulae and oral products. Its widespread inclusion in various biocide formulae is attributed to its broad-spectrum efficacy, substantivity for the skin, and low irritation (McDonnell & Russell, 1999). Chlorhexidine comprises of two cationic groups separated by a hydrophobic hexamethylene bridging structure, consequently it is categorised as a cationic antimicrobial agent which exhibits activity towards Gram-positive and Gram-negative bacteria. In general, cationic antimicrobial agents interact strongly with bacterial cells due to the presence of a net negative charge on the outermost surface of the cell. Following strong association with the anionic sites, particularly acidic phospholipids and proteins situated on the surface of bacterial cells; osmoregularity, metabolic catabolism of the cell membrane, and membrane-associated

enzymes are affected. As a consequence, leakage of cellular materials occurs, such as potassium ions, as well as inhibition of respiration (Gilbert & Moore, 2005). Early work proposed that the growth inhibitory effects of chlorhexidine were linked with its ability to impede the activities of membrane-bound ATPase. However, this mode of action has since been disregarded as the concentrations required to yield such effects are substantially greater than those likely to be deployed *in vivo* (Chawner & Gilbert, 1989). Studies conducted by Barrett-Bee, Newboult & Edwards (1994) revealed that the application of chlorhexidine inhibited oxygen uptake by bacteria, such as *E. coli* and *P. aeruginosa*, hence triggering a reduction in ATP levels. However, Barrett-Bee et al. (1994) concluded that the primary antimicrobial action of chlorhexidine was its ability to interfere with the function of cellular membranes. Furthermore, studies performed by Barrett-Bee et al. (1994) revealed that the application of chlorhexidine triggered the release of β-lactamase from the periplasmic space of Gram-negative bacteria, which signifies damage to the integrity of the outer membrane of Gram-negative bacteria. β-lactamase is responsible for promoting antibiotic resistance in Gram-negative bacteria against β-lactam antibiotics, thus repeated exposure to β-lactam antibiotics has generated dynamic and continuous mutations in β-lactamases. In addition, β-lactamase is able to exert its resistance mechanisms against hydrolytically susceptible chemical bonds in antibiotics, thus weakening the activity of antibiotics (Shaikh, Fatima, Shakil, Rizvi & Kamal, 2015). Chlorhexidine and quaternary ammonium compounds (QACs) share a similar mode of action, however the hydrophobic regions of QAC biocides are prone to solubilisation within the hydrophobic core of the cell membrane, whereas those of chlorhexidine are not. In addition, being six carbons in length, the hydrophobic region of chlorhexidine is able to integrate into the lipid bilayer by means of folding, therefore chlorhexidine disrupts the bilayer by bridging between pairs of adjacent phospholipid head groups and dislodging the associated divalent cations. Furthermore, the distance between phospholipid head groups within a

monolayer approximately corresponds to the length of the hexamethylene group in chlorhexidine (Gilbert & Moore, 2005). Loss off membrane fluidity is a consequence of the interaction between chlorhexidine and the phospholipids within the bilayer. Furthermore, the effects of such an interaction can manifest itself into cellular leakage of potassium ions and protons (Elferink & Booij, 1974).

When deployed in a domestic environment, antimicrobial agents are incorporated into formulations alongside several excipients that may serve to increase the overall potency. Antimicrobial formulations are applied across a variety of settings which include healthcare and industrial environments, where their purpose includes pharmaceutical product preservation and hard-surface disinfection. Furthermore, biocides can be included in medical device coatings, such as sutures, wound dressings, and urinary catheters for the purpose of preventing bacterial adhesion which may lead to the production of robust biofilms (Cowley et al., 2015). The universal application of antimicrobial compositions illustrates how important the regulation of bacterial populations, among other microorganisms, inhabiting the skin is to consumers. Understandably, antimicrobial compositions must be able to significantly decrease populations of different microorganisms swiftly, although without demonstrating harmful effects against the skin. Typical antimicrobial cleansing formulae contain an active antimicrobial agent, a surfactant, and various other ingredients such as fragrances. In addition to all the components being possibly retained within an aqueous carrier, pH adjusters may be incorporated into antimicrobial formulae.

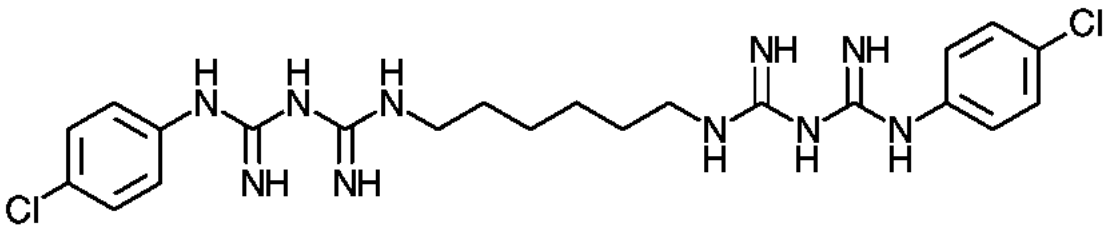

Figure 3. The molecular structure of chlorhexidine.

Quaternary Ammonium Compounds

Quaternary ammonium compounds (QACs) can be incorporated alongside chlorhexidine into antimicrobial formulations to serve primarily as surfactants, as well as demonstrating antimicrobial properties. QACs are examples of cationic surfactants; named accordingly due to the presence of a positive charge on the hydrophilic portion of their molecular structure. Furthermore, QACs are composed of two chemically distinct regions within their molecular structure; a hydrophobic group and a hydrophilic group (McDonnell & Russell, 1999). As a result of their amphiphilic nature, QACs are incorporated into various cosmetic and personal care products. Within these product formulations, they are utilised for their surfactant, preservative, and antimicrobial properties. Furthermore, QACs exhibit a detergent-like mechanism of action when interacting with microbial life, as a consequence of their hydrophobic and hydrophilic components. In general, electrostatic interactions between the positively charged QAC head and the negatively charged bacterial membrane are followed by penetration of the QAC side chains into the intramembrane region. Diffusion into the intramembrane region triggers leakage of cytoplasmic materials followed by cellular lysis. In addition, the additional outer membrane associated with Gram-negative bacteria reduces the antibacterial activity of QACs, whereas the lack of an outer membrane in the structure of Gram-positive bacteria increases their susceptibility to the antimicrobial activity of QACs (Jennings

et al., 2015). Low QAC concentrations interact strongly with anionic sites located on the membrane surface, thus instigating the suppression of osmoregulatory capabilities; triggering the discharge of potassium ions and protons. Intermediate concentrations disrupt membrane-situated cellular processes, such as respiration, solute transport, and cell wall biosynthesis. High concentrations, used in many biocidal formulations, eradicate cells as a consequence of the solubilisation of their membranes, thus triggering the release of all cellular contents (Gilbert & Moore, 2005).

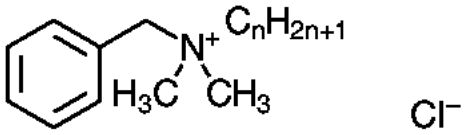

Figure 4. Alkyldimethylbenzylammonium (ADBAC) chlorides represent a mixture of ADBACs where n = 8, 10, 12, 14, 16, and 18.

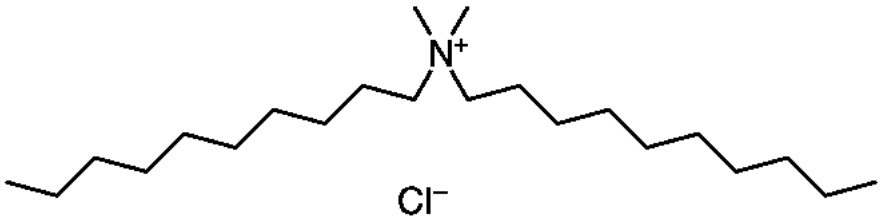

Figure 5. The molecular structure of didecyldimethylammonium chloride (DDAC).

Silver

The antimicrobial properties demonstrated by silver against a broad range of microorganisms has been acknowledged since ancient times. Presently, silver is deployed to regulate the growth of bacteria in a variety of medical applications, such as dental work, catheters, and the healing of burn wounds (Jung et al., 2008). For antimicrobial applications, commonly used silver

compounds are silver sulfadiazine, silver nitrate, and silver acetate. Furthermore, the interaction of silver with thiol groups represents its antimicrobial mechanism of action. Moreover, it has been suggested that bacterial inactivation is instigated by the interaction of silver with thiol groups in enzymes and proteins. In addition, it has been proposed that silver salts exert their antimicrobial effects by binding to key functional groups in fungal enzymes. Silver ions trigger the discharge of potassium ions from microorganisms, thus the cytoplasmic membrane, which many enzymes are linked with, represents a significant target site for the antimicrobial activity of silver ions. Furthermore, silver ions instigate structural abnormalities within cells of *P. aeruginosa*; affected cellular constituents are the cytoplasmic membrane, cytoplasmic contents, and outer cell layers. In addition, silver ions interact with the bases in the structure of DNA in order to exert lethal action (McDonnell & Russell, 1999).

Jung et al. (2008) conducted a study concerning the antimicrobial activity of the silver ion against *Staphylococcus aureus* and *Escherichia coli*. In addition, the mechanism of action of the silver ion was also investigated. Furthermore, transmission electron microscopy (TEM) analysis showed *S. aureus* cells undergoing lysis, thereby resulting in the discharge of their cellular contents, when exposed to a silver ion solution for 2 hours. In contrast, *S. aureus* cells that had not been exposed to a silver ion solution displayed their normal coccal morphology, uniform distribution with regards to electron density within the cytoplasm, intact cell membranes, and cell walls displaying a structurally intact peptidoglycan layer. In addition, *S. aureus* cells treated with a silver ion solution exhibited impaired walls, impaired membranes, and uneven electron distribution within the cytoplasm. Concerning *E. coli* cells that had not been treated with a silver ion solution, Jung et al. (2008) reported no alterations with regards to their normal morphology, with their flagella and fimbriae undamaged. However, *E. coli* cells that had been subjected to a silver ion solution exhibited abnormal morphology. In addition,

TEM analysis of treated *E. coli* cells revealed either localised or complete separation of the cell membrane from the cell wall.

Antimicrobial Susceptibility Testing

Overview

Clinical microbiology laboratories employ antimicrobial susceptibility testing to determine the effectiveness of prospective antimicrobial agents, or to identify bacterial isolates demonstrating resistance (Jorgensen & Ferraro, 2009). Antimicrobial susceptibility testing can be applied in drug discovery, epidemiology, and prediction of therapeutic outcome. The rise of antimicrobial resistance to antibiotics is a growing concern; increased resistance decreases the efficacy of important groups of antibiotics, such as tetracyclines and cephalosporins. As a consequence of rising antimicrobial resistance, the development of novel antibiotics is essential and demands suitable procedures to determine their antimicrobial potency. Natural products such as plants, prokaryotic bacteria, eukaryotic microorganisms, and various animal organisms represent the major sources of new drug molecules today. However, when reviewing published articles examining the antimicrobial efficacy of potential antimicrobial agents, it is difficult to compare different sets of results due to the application of diverse, non-standardised approaches with regards to inoculum preparation techniques, inoculum size, growth medium, incubation conditions, and end points determination. Furthermore, a variety of laboratory methods can be employed to assess the *in vitro* antimicrobial activity of potential antimicrobial compounds. The disk diffusion assay represents one of the most elementary antimicrobial susceptibility methods currently employed today. In addition, in order to obtain more conclusive information concerning the time-dependent or concentration-dependent effects of antimicrobial agents, time-kill studies can be applied. Furthermore, flow cytofluorometric methods can illuminate the extent of cellular damage imposed upon a microorganism (Balouiri, Sadiki & Ibnsouda, 2016).

Preparing dilutions of antimicrobial agents in order to obtain minimum inhibitory concentrations (MICs) is crucial for providing reference values for antimicrobial susceptibility testing. Furthermore, MICs are used in resistance surveillance, evaluating similarities between new agents, determining the susceptibility of organisms that generate ambiguous results in disk assays, further evaluation of organisms where the disk assay may be fallacious, and when quantitative results are required for clinical management. Dilution tests involve evaluating an organism's ability to produce visible growth on a series of agar plates, or in broth, containing dilutions of the antimicrobial agent. Furthermore, the minimum inhibitory concentration (MIC) can be defined as the lowest concentration of an antimicrobial agent that inhibits visible growth of a microorganism within a specified time period and under defined *in vitro* conditions. MIC data influences the chosen course of action taken by a clinician with regards to treatment decisions, as they provide information about the susceptibility of an organism to various antimicrobial agents. However, reproducibility between different laboratories demands careful control and standardisation due to the fact that different methods may significantly affect the results yielded (European Committee for Antimicrobial Susceptibility Testing, 2003).

In the clinical environment, MIC values can be used to categorise microorganisms as either clinically susceptible, intermediate, or resistant to the tested antimicrobial agent. The advisory standards for these groupings are published by different national organisations, such as the Clinical and Laboratory Standards Institute (CLSI) in the USA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Wiegand, Hilpert & Hancock, 2008).

The skin concentrate formula provided by Crystal Clear Limited was subjected to antimicrobial evaluation in accordance with the European standard BS EN 1276. The experimental procedure required by the standard is referred to as a quantitative suspension test. Scientific literature

does contain experimental procedures with regards to the quantitative suspension test. Müller & Kramer (2008) have outlined an experimental procedure for the quantitative suspension test. Despite the procedure not being an exact duplicate of the procedure outlined by the standard, the procedure does share a near identical design in order to achieve log reduction values. Log reduction values are quantitative measurements which outline what percentage of bacteria are eradicated during the course of the test. In general, a bacterial suspension of a known concentration, in colony-forming units per millilitre (CFU/mL), is prepared. From the suspension, an aliquot is transferred into fresh cell culture medium containing a test compound. Organisms in the suspension are exposed to the test compound for a pre-determined amount of time and at a pre-determined temperature. Following the appropriate contact time, an aliquot of the test combination is extracted and the bactericidal activity of the test compound is neutralised by a chosen compound, either alone or in combination with another compound. Following an appropriate time of inactivation, the number of viable cells is determined by way of preparing serial dilutions in fresh broth, followed by plating an appropriate amount of each dilution on agar, and incubating the agar plates for a pre-determined duration. Once the number of viable cells, in CFU, is obtained, the reduction in viability can be obtained (Müller & Kramer, 2008).

Disk Diffusion Assay

The disk diffusion assay is routinely employed for antimicrobial susceptibility testing and represents the official method used in many clinical microbiology laboratories. The disk diffusion assay involves inoculating agar plates with a standardised inoculum of a test microorganism. Following inoculation, paper disks containing the test compound are deposited onto the agar surface and the agar plates are incubated in appropriate conditions for a specified time duration. Essentially, the test compound disseminates into the agar and prevents the

growth of the microorganism on solid media. Furthermore, the diameter of growth inhibition generated by the test compound around the disk is measured. Both EUCAST and the CLSI have published a set of standards which dictate the type of growth media to use, the temperature, the period of incubation, and the required inoculum size. The standards comprised by the CSLI recommend using Mueller-Hinton agar and an inoculum size of 1-2 x 10^8 CFU/mL, which is equivalent to the 0.5 McFarland standard. Additionally, the incubation temperature required is 35 ± 2 °C and the incubation time duration is 16-18 hours (Balouiri et al., 2016).

The inhibition of growth produced by an antimicrobial agent in the disk diffusion assay does not signify bacterial death, hence this method cannot differentiate between bactericidal and bacteriostatic effects. Additionally, the MIC cannot be established using the disk diffusion method as it is unachievable to quantify the amount of antimicrobial agent that has dispersed into the agar medium. Nonetheless, the disk diffusion assay provides numerous advantages over other antimicrobial susceptibility methods, such as the low cost associated with the assay, the simplicity involved in performing the assay, and the straightforward analysis of results. Furthermore, the capacity of the disk diffusion assay to evaluate a substantial number of microorganisms and antimicrobial agents represents another advantage (Balouiri et al., 2016).

Dilution Methods

Dilution methods represent the most appropriate methods for establishing MIC values as they allow the concentration of antimicrobial agents to be estimated in agar or broth medium. Broth and agar dilution methods allow quantitative analysis of the *in vitro* behaviour of antimicrobial agents against bacteria (Balouiri et al., 2016). However, the MIC value obtained using dilution methods does not consistently relate to an absolute value, since the actual MIC value will represent a point between the lowest test concentration that inhibits bacterial growth and the

next lowest concentration (Patel, 2012). Numerous sanctioned guidelines exist for dilution antimicrobial susceptibility testing against fastidious and non-fastidious bacteria. Furthermore, the standards comprised by EUCAST and the CLSI provide standardised procedures for evaluating antimicrobial agents that are practical to apply in the majority of clinical laboratories (Balouiri et al., 2016).

Broth Dilution Method

The tube dilution method represents one of the earliest antimicrobial susceptibility testing methods. The method involves preparing a series of two-fold dilutions of test compound and distributing them into individual test tubes. Each dilution of antimicrobial agent is tested against a suspension of bacteria containing 1-5 x 10^5 CFU/mL. Following incubation, monitoring differences in turbidity is used to differentiate varying degrees of bacterial growth. The tube dilution method provides a quantitative result in the form of the MIC, although it does offer many disadvantages including the tedious task of preparing dilutions, the likelihood of errors while preparing dilutions, the copious amounts of reagents required for the assay, and the necessity for a large workstation to accommodate the practical procedures (Patel, 2012).

In an effort to make the broth dilution method more practical, the test can be conducted in small, disposable plastic trays. The miniaturisation and mechanisation of the test allowed up to twelve different agents, of as little as 100 μL, to be evaluated in a range of eight two-fold dilutions in a single 96-well plate. The typical clinical microbiology laboratory utilises a mechanical dispensing device to aliquot precise volumes of diluted test agent into the individual wells of a plate from large volume vessels. Similar to the tube dilution method, each well of the plate is inoculated with a standardised suspension of bacteria. Following incubation overnight without agitation, multiple MICs are established using a manual or automated

viewing apparatus in order to examine each individual well for growth simultaneously (Jorgensen & Ferraro, 2009). MIC values can be affected by the size of the inoculum, the type of growth medium used, the incubation time, and the method employed to prepare the inoculum. Therefore, the CLSI and EUCAST have both issued approved standardised guidelines for the broth dilution assay. Additionally, both sets of guidelines are very similar in terms of methodology, with differences associated with inoculum preparation and inoculum size. Furthermore, EUCAST guidelines recommend employing spectrophotometric means in order to ascertain MIC values. CLSI guidelines recommend using Mueller-Hinton broth as the growth medium, using an inoculum size equivalent to the 0.5 McFarland standard, and incubating the plates for 18 hours in an incubator set at 35 °C (Balouiri et al., 2016).

The main advantages associated with the microdilution method include the reduced amounts of reagents and space required due to the miniaturisation of the assay. In addition to using spectrophotometric means to examine wells for bacterial growth, colorimetric methods can be employed which act as visual aids in distinguishing between wells exhibiting bacterial growth and wells not exhibiting bacterial growth. Colorimetric methods utilise dye reagents which act as growth indicators in microdilution methods, such as the resazurin dye (Balouiri et al., 2016). Resazurin permeates cells and serves as a redox indicator that can be utilised to measure viable cell number. Furthermore, resazurin is converted to resorufin by viable cells which is characterised by a fluorescent, pink colour. In addition, the general disadvantage of using resazurin to monitor cell viability includes the necessity to incubate the substrate with viable cells at 37°C for a suitable time period in order to generate a signal; incubation increases the probability of artefacts being chemically produced from interactions among assay chemistry, the compounds being assayed, and the biochemistry of the cell (Riss et al., 2013). Furthermore, establishing the minimum bactericidal concentration (MBC) represents the most common

means of measuring the bactericidal activity of antimicrobial agents. The MBC is defined as the lowest concentration of antimicrobial agent required to kill 99.9% of the inoculum after a 24-hour time period in incubation under a standardised set of conditions. Furthermore, following the acquirement of the MIC value, the MBC can be obtained by sub-culturing from wells displaying no visible indicators of bacterial growth on non-selective agar plates to establish the number of surviving cells (CFU/mL) after a further 24-hour incubation period (Balouiri et al., 2016).

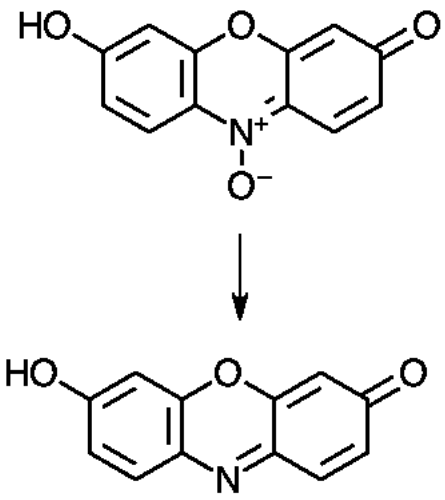

Figure 6. The conversion of resazurin to the fluorescent, pink resorufin product by viable cells. NADH is oxidised to NAD^+ alongside the conversion.

Agar Dilution Method

The agar dilution method entails supplementing molten agar medium with varying concentrations of antimicrobial agent, typically using serial two-fold dilutions, followed by the application of a standardised bacterial inoculum to the surface of the agar medium (Patel, 2012). The MIC endpoint in the agar dilution method is defined as the lowest concentration of antimicrobial agent that completely inhibits bacterial growth under appropriate incubation conditions. Furthermore, antimicrobial agents can react with liquid medium in the broth

dilution method which hinders the detection of bacterial growth. As a consequence, the agar dilution method is often preferred to the broth dilution method in establishing the MIC (Balouiri et al., 2016). An additional advantage of the agar dilution method includes the possibility of evaluating multiple strains of bacteria on the same set of agar plates simultaneously. A disadvantage of the agar dilution method include it requiring significant economic and technical resources if the assay is not automated. Additional disadvantages are the necessity to use agar plates within one week of preparation and the difficulty of confirming the purity of the inoculum due to the persistent risk of contamination (Patel, 2016).

Biofilm Quantification

The *in vitro* investigation of biofilm formation and development over the last decades has generated a wide-ranging series of model systems. Furthermore, the majority of these model systems quantify biofilm biomass by employing traditional plating methods which are laborious and time-consuming. Alternative assays can be categorised into biofilm biomass assays, viability assays, and matrix quantification assays (Peeters, Nelis & Coenye, 2007). Bacteria associated with biofilms demonstrate elevated resistance to antibiotics, disinfectants, and host immune defences. Therefore, the significance of biofilms is appropriately acknowledged in medical, environmental, and industrial environments (Stepanović et al., 2007).

Crystal violet staining represents a well-documented technique for the quantification of biofilm biomass in a 96-well, flat-bottomed plate. Furthermore, crystal violet staining was first described by Christensen et al. (1985) to assess the adherence of coagulase-negative staphylococci to plastic tissue cell culture plates; subsequent modifications to the technique have increased its accuracy by allowing biofilm biomass quantification to be measured in the

entire well (Peeters et al., 2007). In brief, the method involves plating cultures of coagulase-negative staphylococci in fresh Trypticase soy broth (TSB) following an appropriate growth period. Following an appropriate incubation period, the microorganisms that have attached to the plate are fixed in place before being stained by the application of crystal violet dye, whereas any non-adherent cells are discarded appropriately. Following the application of crystal violet dye, the optical densities of the stained bacterial cells are recorded at a wavelength of 570 nanometres (nm). Furthermore, the results obtained are used to categorise bacteria into non-adherent, weakly adherent, and strongly adherent (Christensen et al., 1985).

The advantages of the assay described by Christensen et al. (1985) include it being inexpensive and straightforward to perform. Furthermore, the crystal violet assay is routinely employed for the quantification of biofilms generated by a broad range of microorganisms (Peeters et al., 2007). However, crystal violet dye does not stain the slimy material characterised by acid mucopolysaccharides, thus crystal violet dye is inadequate for indicating the presence of slimy material. Furthermore, Alcian blue can be employed to indicate the presence of slimy material as it selectively stains acid mucopolysaccharides (Stepanović et al., 2007).

Biocompatibility

In the healthcare setting, healthcare professionals are constantly exploring new ways to optimise treatments for difficult-to-heal wounds. Additionally, exposed wounds, particularly in diabetic and immunosuppressed patients, are prone to invading pathogens. Invading pathogens lead to bacterial colonisation which is associated with delayed healing and triggers severe morbidity, such as multi-organ failure. Therefore, topical antiseptics play a key role in the treatment of wounds (Hirsch et al., 2010). Antiseptics are substances with anti-infective properties that, following topical application, eradicate or inhibit the growth of microorganisms in or on living tissue. In addition, to inhibit the development of bacterial resistance, antiseptics are applied at concentrations considerably higher than their maximum bactericidal concentrations (MBCs). However, antiseptic preparations should not exert toxic effects on host tissues. Additionally, antiseptics should be consistent with the concept of biocompatibility of medical products, which states that antiseptics should not impair healing processes (Müller & Kramer, 2008). Therefore, it is important to evaluate potential cytotoxicity of antiseptics as they are routinely administered on human skin for therapy (Hirsch et al., 2010). Chlorhexidine digluconate is well established as a potent antimicrobial agent and its application as an antiseptic is firmly recognised as the gold standard. However, despite chlorhexidine digluconates substantial bactericidal capabilities against a wide-ranging variety of bacteria, evidence has emerged indicating that chlorhexidine digluconate demonstrates adverse effects on mammalian tissues and cells (Giannelli, Chellini, Margheri, Tonelli & Tani, 2008).

Giannelli et al. (2008) conducted an *in vitro* study concerning the effect of chlorhexidine digluconate on different cell types; osteoblastic Saos-2 derived from osteosarcoma cells, murine fibroblasts, and murine endothelioma H-end cells. With regards to cell viability, studies concluded that the concentration of chlorhexidine digluconate contributed to its effect on cell

viability. In addition, the impact of chlorhexidine digluconate on cell viability was time-dependent. Furthermore, Saos-2 cells demonstrated elevated sensitivity towards chlorhexidine digluconate, since their viability was substantially decreased by approximately 57.5 % after exposure to 0.01 % chlorhexidine digluconate for 1 minute. In addition, confocal microscopy confirmed significant disruption to the cytoskeletal organisation, thereby resulting in cell growth arrest and apoptosis. Furthermore, Giannelli et al. (2008) investigated the apoptosis-inducing activity of chlorhexidine digluconate. It was concluded that following 1 minute of exposure to 0.01 % chlorhexidine digluconate, 30 % of Saos-2 cells displayed apoptotic nuclei. Additionally, following 1 minute of exposure to 0.12 % chlorhexidine digluconate, 50 % of fibroblastic cells and endothelial cells displayed apoptotic nuclei. Apoptotic indications are characterised by condensed nuclear chromatin, fragmented DNA, widespread cytoplasmic vacuolisation, and blebbing. However, necrotic cell death indications were present, such as an intact nucleus with dispersed chromatin, cytoplasmic vacuolisation, and the loss of plasma membrane integrity with the accompanying leakage of cytoplasmic content. Giannelli et al. (2008) investigated the effect of chlorhexidine digluconate on mitochondrial function using the membrane-permeant fluorochrome JC-1 dye. Furthermore, it was established that following 1 minute of exposure to 0.01 % chlorhexidine digluconate, osteoblasts exhibited loss of mitochondrial transmembrane polarisation. In addition, the ability of chlorhexidine digluconate to disrupt the mitochondrial membrane potential was observed in both fibroblastic and endothelial cells. Giannelli et al. (2008) investigated the possible signalling pathways triggering chlorhexidine digluconate-induced cell death by analysing the capability of chlorhexidine digluconate to instigate intracellular calcium accumulation within osteoblasts. Furthermore, using the calcium indicator, Fluo-3 AM, it was established that the addition of 0.01 % chlorhexidine digluconate triggered swift increases in both cytoplasmic and nuclear calcium ion concentration after 50 seconds. Additionally, using the oxidative stress indicator,

CM-H_2 DCFDA, Giannelli et al. (2008) concluded that intracellular production of reactive oxygen species (ROS) commenced after 30 minutes of exposure to chlorhexidine digluconate. Furthermore, the cell lines used in the study conducted by Giannelli et al. (2008) were assumed to represent common targets for cytotoxic activity in the surgical procedures employed for the treatment of periodontitis and peri-implantitis. However, the results obtained provide constructive insight into chlorhexidine digluconates toxic effects on eukaryotic cells. Giannelli et al. (2008) concluded that chlorhexidine digluconate demonstrates elevated toxicity in different cell types *in vitro* and provided indication that intracellular signalling molecules contribute to the adverse effects of the compound.

There are many cell-based assays that can be employed to determine whether test compounds exhibit effects on cell proliferation, or exhibit direct cytotoxic effects that will ultimately lead to cell death. Additionally, cell-based assays are commonly employed to measure receptor binding and a wide-range of signal transduction events that may include the expression of genetic reporters, trafficking of cellular components, or monitoring organelle function. Furthermore, tetrazolium reduction assays evaluate some aspects of general metabolism as markers of cell viability, and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium) reduction assay represents the first homogenous cell viability assay developed for a 96-well plate format that was acceptable for high throughput screening (Riss et al., 2013).

A conventional MTT assay includes preparing the MTT substrate in a physiologically balanced solution, administering the substrate to cells in culture media, and incubating the cells for 1-4 hours. Following incubation, the insoluble formazan precipitate gathers inside the cell near the cell surface and within the culture medium. Prior to recording absorbance, solubilisation of the

formazan product is required in order to stabilise the colour; this can be achieved using acidified isopropanol or dimethyl sulfoxide (DMSO). The quantity of formazan generated is presumed to be directly proportional to the number of viable cells and is quantified by measuring shifts in absorbance at 570 nanometres (nm). Furthermore, the ability to convert MTT into formazan is not retained by dead cells (Riss et al., 2013). The conversion of MTT to formazan is facilitated by dehydrogenases located in the mitochondria of living cells, however enzymes and reducing agents occurring in other organelles, such as the endoplasmic reticulum, are also involved (van Tonder, Joubert & Cromarty, 2015). Parameters such as the concentration of MTT, the length of incubation, the number of viable cells, and their metabolic activity contribute to the amount of signal generated. In addition, certain culture conditions can adjust the metabolism of cells which will consequently affect the rate of MTT reduction to formazan. For example, the amount of MTT reduction per cell will decrease when the growth of adherent cells in a culture becomes contact inhibited, thus impeding metabolism. Therefore, the aforementioned circumstance will generate a loss of linearity between absorbance and cell number. Besides heightened confluence, other unfavourable culture conditions, such as altered pH or exhaustion of vital nutrients, such as glucose, may affect the ability of cells to reduce MTT (Riss et al., 2013).

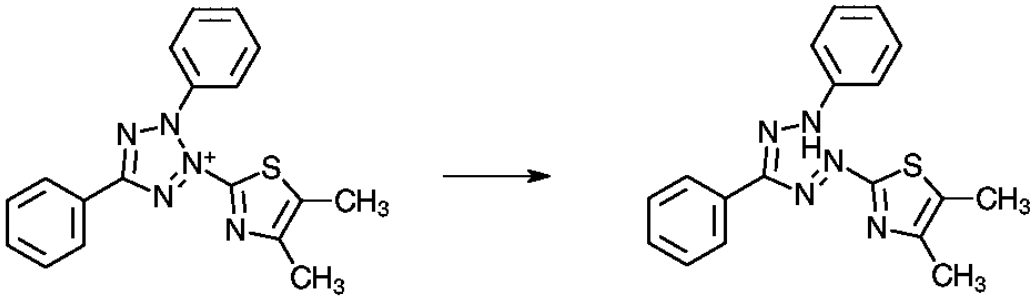

Figure 7. The conversion of MTT to the coloured formazan product by metabolically active cells.

Experimental

Background Information for Experimental Procedures

All practical research was conducted at the University of Salford. Laboratory consumables, chemicals, reagents, and media required for all aspects of the research were obtained from various scientific equipment suppliers. These suppliers included Sigma-Aldrich and Thermo Fisher Scientific. Furthermore, Crystal Clear International Limited financed the procurement of the necessary research materials. Crystal Clear International Limited provided the components used in their skin concentrate formula. Bacterial strains and mammalian cell lines were obtained from the University of Salford. *Escherichia Coli*, *Staphylococcus Aureus*, and *Pseudomonas Aeruginosa* were selected to be used in the antimicrobial susceptibility assays. Initially, all three species were maintained in brain heart infusion (BHI) broth and stored at 4°C. As the practical research progressed, all three species were eventually streaked onto separate Mueller-Hinton agar plates in order to obtain distinct single colonies. The agar plates were wrapped securely in parafilm and stored at 4°C. Whenever it was necessary to obtain fresh cultures, single colonies were isolated from the appropriate agar plates and transferred into a suitable medium. All media associated with antimicrobial susceptibility testing was pre-prepared in bulk, subjected to sterilisation in an autoclave, and safely stored in the relevant laboratory. HepG2 and A549 were the chosen adherent mammalian cell lines used in the cytotoxicity experiments. HepG2 is a cancerous liver-derived immortalised cell line and A549 is a cancerous lung-derived immortalised cell line. Mammalian cell lines were stored in an appropriate cell culture incubator which provided appropriate conditions for the promotion of cellular growth and maintenance. In addition, mammalian cell lines were maintained in T75 cell culture flasks. Maintaining cell lines involved employing the common practices associated with tissue cell culture. Furthermore, routine passaging was required to preserve cellular life,

as overly confluent cultures contain elevated quantities of toxic metabolites and decreased levels of nutrients. Initially, HepG2 was the chosen adherent cell line to be used in the cytotoxicity assays, however an early contamination setback required the need for a different cell line, A549, to be cultivated. Tissue cell culture reagents were stored in the appropriate manner. For long term use, complete media was stored at 4°C, trypsin-EDTA was stored at -20°C, Penicillin-Streptomycin (10,000 U/mL) was stored at -20°C, heat-inactivated fetal bovine serum (FBS) was stored at -20°C, L-glutamine (2 mM) was stored at -20°C, and phosphate-buffered saline (PBS) (1x) at pH 7.2 was stored at 4°C. The chosen media was RPMI-1640. For long term use, MTT dye (3 mg/ml in PBS) was stored at -20°C. Furthermore, all tissue cell culture reagents were handled in a sterile cabinet, and under no circumstances handled outside a sterile environment. Whenever practical research regarding tissue cell culture was performed, it was of the utmost importance to adhere relentlessly to aseptic criteria. On every occasion, the cell culture hood was allowed to establish a sterile environment for three minutes before any work was conducted. Once a sterile environment had been established, the work surface within the hood was thoroughly sanitised with 1 % virkon, followed by 70 % ethanol. All equipment that was placed in the cell culture hood was sprayed thoroughly with 70 % ethanol. Before conducting work in the cell culture hood, gloves were habitually sanitised with 70 % ethanol.

For the antimicrobial susceptibility assays that required 96-well plates, the number used for each occasion varied. Furthermore, as this was a yearlong practical research project, the experimental design varied with regards to the number of repeats of treated cells. In addition, the number of repeats concerning both positive and negative controls varied throughout the research. On every occasion the MTT assay was performed, one 96-well plate was used to minimise the risk of contamination. However, similar to the antimicrobial susceptibility assays,

the experimental design was subjected to slight alterations with regards to the number of repeats of treated cells. In addition, the number of repeats concerning both positive and negative controls varied throughout the research.

Preparation of Crystal Clear Skin Concentrate

The preparation of the skin concentrate was conducted using a batch manufacturing sheet provided by Crystal Clear. The sheet detailed the procedure used by Crystal Clear to produce the skin concentrate, including the ingredients and the quantities required. The procedure followed by Crystal Clear was intended for the production of huge quantities of skin concentrate; a single batch is required to be made up to 1000 litres. Furthermore, it would be unfeasible to replicate the procedure entirely in a university laboratory. Such an approach would require large amounts of resources and demand large amounts of laboratory space for the procedure to be performed. Therefore, an alternative method had to be designed which would be far more economical with regards to laboratory space and resources. To combat this issue, the quantities of each ingredient were reduced, however the appropriate ratio of ingredients within the formula was maintained. The following method represents an adapted version of the procedure employed by Crystal Clear to manufacture the skin concentrate.

To produce skin concentrate (500 mL) as dictated by Crystal Clear, H_2O (382.5 mL) was added to a suitably sized beaker. EDTA (7.5 g) was subsequently added and the contents of the beaker was continually stirred for a duration of 10 minutes. Didecyldimethylammonium chloride (BTC101E) (38.4 mL), benzyl-C12-14-alkyldimethylammonium chlorides (BAC50) (41.6 mL), and chlorhexidine digluconate (30 mL) (20 %) $_{(aq)}$ were added to the beaker in turn; followed by constant stirring for a duration of 10 minutes. The pH of the solution was adjusted to be within a range of 6.5-7.5 using citric acid solution.

Preparation of Silver Ions in Solution

The preparation of silver ions in solution was performed using a rudimentary method. To generate silver ions, a Dowex® G26 hydrogen form cation exchange resin was used. The cation exchange resin was washed with ethanol (3 x 25 mL). The exchange resin was dried using a water aspirator to generate a vacuum. The dried cation exchange resin was stirred continuously for 2 hours with $AgNO_{3\,(aq)}$. Vacuum filtration was used to separate the solution from the cation exchange resin. The resin was subsequently dried in a laboratory oven. The resin was stirred continuously for 2 hours with deionised H_2O. Vacuum filtration was used to separate the solution of silver ions from the cation exchange resin. To confirm the presence of silver ions in solution, both visual and quantitative methods were employed. To quantitatively measure the concentration of silver ions in solution, inductively coupled plasma mass spectrometry (ICP-MS) was used. Visual confirmation was performed using a dilute solution of $HCL_{(aq)}$ (0.5 M) in order to generate the white insoluble precipitate of $AgCl_{(s)}$.

Gram Stain

The presence of a net negative charge on the surface of bacteria allows cationic dyes, such as crystal violet dye, to adhere to them. The Gram stain is used to differentiate bacteria into Gram-positive or Gram-negative. Differentiation is based on cell wall composition, with Gram-positive bacteria possessing a thick peptidoglycan-containing cell wall. In order to prepare the bacterial sample, a fresh single colony from an overnight agar plate was suspended in sterile distilled water. Using a sterile pipette dropper, a drop was placed on a clean slide and cautiously heat fixed using a Bunsen burner. Once the bacterial sample had been successfully heat fixed to the slide, 'ammonium-oxalate-crystal violet' was applied to the slide; ensuring that the entire bacterial film was covered. The dye was left to act for 30 seconds. The dye readily penetrates the cells of Gram-positive bacteria, and the outer membrane of Gram-negative bacteria, by means of associating with the negative elements of the cell. After 30 seconds, the excess dye was carefully removed using water. After the first washing step, Grams iodine was applied; ensuring that the entire bacterial film was covered. Grams iodine was left to act for 30 seconds. Grams iodine associates with crystal violet dye generating a crystal violet-iodine complex. After 30 seconds, the reagent was discarded appropriately, however the slide was not subjected to a thorough washing step. After the excess reagent had been carefully discarded, the entire slide was exposed to ethanol for 15 seconds. The purpose of this step was to remove the crystal violet-iodine complexes from Gram-negative bacteria; this is achieved by removing the outer membrane and leaving the cell wall exposed. The much structurally thinner cell wall of Gram-negative bacteria releases the crystal violet-iodine complexes, whereas the structurally thicker cell wall of Gram-positive bacteria retains the dye. Following treatment with ethanol, the entire slide is swiftly and thoroughly washed with water. Following the washing step, the whole slide was covered with safranin. Following the application of the counterstain, the entire slide was

washed with water to remove any excess dye and allowed to air dry. Once the slide was sufficiently dried, the stained bacterial film could be visualised under a microscope.

Disk Diffusion Assay

The disk diffusion assay was primarily employed to confirm if a particular compound or formulation possessed antimicrobial capabilities. In the disk diffusion assay, antimicrobial capabilities were confirmed by means of visual indications. For every occasion the disk diffusion assay was performed, preparation of antimicrobial compounds and the subsequent assay were performed aseptically, thus requiring the use of a Bunsen burner to establish an aseptic zone. In addition to establishing an aseptic zone, each antimicrobial compound was sterile filtered, using a 0.22 μm filter, before use in the assay. Sterile distilled water was used as the diluent if dilutions were required. Commodities associated with dispensing liquids, such as pipette tips, were sterilised before use in the assay in order to reduce the risk of erroneous results due to contamination.

Following the preparation of test compounds, 20 μL of each was applied to a disk and stored at room temperature for a duration of 1 hour. Following disk permeation, bacterial suspensions were prepared by directly removing single colonies from stored plates and adjusting the turbidity to correspond to that of McFarland 1 in sterile distilled water. Using sterile cotton swabs, Mueller-Hinton agar plates were inoculated with adjusted bacterial suspensions. The disks were transferred to the agar plates with a maximum of three disks per plate and placed in an incubator, set at 37°C, overnight. Antimicrobial capabilities were evaluated by measuring the zone of inhibition around the disk in millimetres (mm).

Optical Density Assay

Although not recognised as an official antimicrobial susceptibility assay, the optical density assay was performed in order to assess the progression of a compounds antimicrobial activity in a real-time setting. Before attempting the assay, it was necessary to select the appropriate media. The rationale behind selecting appropriate media was the tendency of various compounds to chemically react with various types of media. Furthermore, these reactions produced precipitates which would generate erroneous data. For the purpose of selecting appropriate media through trial and error, aseptic techniques were not required as live organisms were not being handled in these experiments. In order for the trial and error experiments to be conducted in an economical sense, the experiments were performed in 96-well plates. By applying 100 μL of test compound, at a relevant concentration, to 100 μL of different types of media in a 96-well plate, it was possible to identify a suitable type of media for the optical density assay. Lysogeny broth (LB) was found to be a suitable liquid medium for the optical density assay as it did not react readily with chlorhexidine digluconate or diluted skin concentrate. Furthermore, if a precipitation reaction was to occur, it would occur at a higher concentration of chlorhexidine digluconate, however the intensity of the precipitate would not be substantial enough to interfere with the optical density assay. In contrast, brain heart infusion (BHI) broth was prone to instantly react with chlorhexidine digluconate, thus it was appropriately disregarded as the liquid growth medium in the optical density assay.

The optical density assay procedure largely resembled the procedure described by Ashraf et al. (2012) and the aforementioned procedure included the use of LB media. Aseptic techniques were rigorously employed throughout the course of the assay. All inoculations were performed in a sterile environment by using a Bunsen burner to provide an aseptic atmosphere and sterilised loops were used. In addition, monitoring absorbance values at regular intervals was

achieved using sterile disposable pipette droppers and adhering to aseptic standards. Additionally, the appropriate blanks were used to calibrate the spectrophotometer before being used to monitor absorbance values. Moreover, the blanks were prepared aseptically in order to eliminate contamination risks if the assay was to be conducted during the course of a day. Commodities associated with dispensing liquids, such as pipette tips, were sterilised before use in the assay to reduce the risk of erroneous results due to contamination. Flasks containing LB media were composed before the assay and subjected to sterilisation. Furthermore, all antimicrobial compounds were subjected to sterile filtration, using a 0.22 μm filter, before use in the assay.

Bacterial strains were prepared by transferring a loopful of stored bacterial suspension into 10 mL of fresh LB media and placed in a shaking incubator, at 37°C, set at 200 revolutions per minute (rpm). Following overnight incubation, 100 μL of bacterial suspension was transferred into 10 mL of fresh LB media and subjected to further agitated incubation at 37°C. When the absorbance of all bacterial suspensions reached 1 at 600 nanometres (nm), all cultures were diluted by transferring 1 mL of culture into flasks containing 99 mL of fresh LB media, thus obtaining dilutions at 1:100. For each strain tested, there were three flasks; a positive control, a negative control, and a test flask. The positive control flask contained a standardised bacterial suspension. The negative control flask contained purely LB media. The test flask contained a standardised bacterial suspension and a test compound. Once 1 mL of test compound was added to the test flask, the absorbance of each flask was recorded at 600 nm, and all flasks were placed in a shaking incubator set at 200 rpm with an internal temperature of 37°C. At regular intervals, the contents of each flask was monitored by recording optical density values at 600 nm.

Broth Dilution Assay

The broth dilution assay was performed in order to establish minimum inhibitory concentration (MIC) values for antimicrobial compounds and formulations. Additionally, the broth dilution assay was performed in order to establish minimum bactericidal concentration (MBC) values. The assay was performed in accordance with the EUCAST guidelines where possible. For every occasion the broth dilution assay was performed, aseptic techniques were employed when preparing antimicrobial compounds and when performing the assay to alleviate contamination concerns. Aliquoting media was performed in a sterile cabinet using disposable sterile pipettes. All stock antimicrobial test compounds were sterile filtered, using a 0.22 μm filter, in a sterile cabinet before the subsequent dilutions were composed using sterile distilled water as the diluent. The two-fold serial dilution technique was the preferred technique employed for the preparation of test compound dilutions. On occasion, the ten-fold serial dilution technique was employed. Inoculations were performed using a Bunsen burner to provide an aseptic atmosphere and standardised bacterial suspensions were plated in a sterile cabinet. Monitoring absorbance values was performed aseptically using sterile pipette droppers in order to dispense aliquots of bacterial suspensions into disposable cuvettes. The treatment of bacterial cell suspensions with test compounds was performed in a sterile cabinet. The attainment of the MBC was performed aseptically, either by a flame or in a sterile cabinet. Furthermore, on every occasion the broth dilution assay was performed, it was necessary to ensure that all equipment associated with dispensing liquids, such as pipette tips, was sterilised before use.

The preparation of bacterial suspensions involved isolating a single colony from each stored strain of bacteria and transferring each colony into separate universal tubes containing 10 mL of sterile Mueller-Hinton broth. The tubes were placed in a shaking incubator, set at 200

revolutions per minute (rpm), for overnight incubation at 37°C. Following incubation, each fresh culture was sub-cultured by transferring 200 μL of each suspension into separate universal tubes containing 10 mL of Mueller-Hinton broth and subjected to further agitated incubation at 37°C. At regular intervals, the absorbance of each suspension was monitored at 600 nanometres (nm) until 0.5 was achieved for all strains. Once an absorbance of 0.5, or thereabouts, had been achieved for each suspension, all suspensions were diluted in fresh double-strength Mueller-Hinton broth to achieve an absorbance of 0.01-0.05 at 600 nm.

Once all bacterial suspensions had been standardised, 100 μL were dispensed into the individual wells of 96-well plates. Appropriate wells were omitted for the purpose of establishing negative controls by dispensing 100 μL of purely sterile broth into them. 100 μL of test compound was dispensed into the appropriate wells containing a standardised bacterial suspension, and wells were left untreated for the purpose of establishing positive controls. Once the appropriate wells had been treated, the 96-well plates were placed in an incubator, set at 37°C, overnight. To allow more dilutions to be evaluated, test compounds were applied to the 96-well plates in a horizontal fashion. In general, three repeats were adequate for each dilution for a given strain of bacteria. In addition, the number of repeats for both sets of controls was endeavoured to be two.

Following overnight incubation, the wells were visually inspected in order to determine the MIC. The MIC was ascertained by highlighting the last well exhibiting no bacterial growth. Furthermore, the corresponding concentration was designated as the MIC value. Once the MIC was ascertained visually, all plates were placed in a microtiter-plate reader and absorbance values were recorded at 600 nm. In some cases, a visual aid was required such as resazurin dye in order to assist in determining the MIC. On every occasion resazurin was required, it was

composed on the day it was required and then appropriately discarded. 0.2 % resazurin dye was composed by combining one resazurin tablet with 12.5 mL of sterile distilled water and homogenising the mixture using a vortex mixer. Resazurin dye was sterile filtered before being applied to the 96-well plates. Each well of a 96-well plate was treated with 30 μL of 0.2 % resazurin dye. All plates that had been treated with resazurin dye were subjected to 1 hour of incubation, at 37°C, to allow the reduction of resazurin in the presence of viable cells to occur. Furthermore, viable cells with their metabolic activity intact will convert resazurin to resorufin, which exhibits a fluorescent, pink colour.

On occasion, it was necessary to obtain the minimum bactericidal concentration (MBC). The MBC was obtained by sub-culturing from three wells upstream from the MIC. Sub-culturing was achieved by extracting 100 μL from the appropriate wells and preparing a ten-fold dilution series of each in Mueller-Hinton broth. From the ten-fold dilution series, 20 μL was dispensed onto Mueller-Hinton agar plates and evenly spread using a sterile spreader. The plates were incubated overnight at 37°C. The number of viable cells, colony-forming units per millilitre (CFU/mL), was calculated by selecting all agar plates that contained between 30 and 300 distinct colonies. The MBC was determined as the minimum concentration corresponding to a ≥99.9 % reduction in viable cells.

Crystal Violet Assay for Biofilm Quantification

The crystal violet assay was performed in order to assess biofilm production by certain bacterial strains. Biofilms represent a significant obstacle in the medical environment and research associated with combating their elevated resistance to disinfectants has received substantial attention. However, it must be firmly established that the purpose of the following assay was to quantify biofilm production by selected bacterial strains when exposed to certain experimental conditions outlined by Bonaventura et al. (2007). The assay described by Bonaventura et al. (2007) was later modified to include treatment of selected bacterial strains with test compound. However, this modification was very rudimentary and limited with regards to the conclusions that could be drawn from it. Furthermore, the modified assay was able to assess a test compounds ability to inhibit biofilm production, however it was not suitable for assessing a test compounds ability to eradicate pre-existing biofilms.

The following assay was performed using the required aseptic practices to minimise the risk of contamination. All inoculations were performed in an aseptic area provided by a Bunsen burner and a sterile cabinet was used for the purpose of plating bacterial strains. On every occasion the assay was performed, pipette tips and media were subjected to sterilisation in an autoclave. Aliquoting media was performed in a sterile cabinet using sterile disposable pipettes. For this particular assay, tryptic soy broth (TSB), supplemented with 1 % glucose, was required. Using distilled water as the diluent, 1 % crystal violet dye was obtained from crystal violet powder and subsequently filtered through a bottle-top sterile filter unit.

To prepare the inoculum, a single colony of each bacterial strain was removed from each stored strain of bacteria and transferred into supplemented TSB for incubation overnight at 37°C. Following overnight incubation, each culture was diluted in fresh supplemented TSB media to

achieve a 1:100 dilution by transferring 200 μL of culture into 19.8 mL of fresh TSB. 200 μL of diluted culture was deposited into the appropriate wells of a flat bottomed 96-well plate. For purely biofilm quantification, the general plate layout consisted of three repeats for each strain and two repeats of negative controls. Negative controls contained purely sterile media. When test compounds were applied, the plate layout consisted of three repeats for each strain tested, two repeats of negative controls, and two repeats of positive controls. Furthermore, the volume of test compound added to the appropriate wells was equivalent to the volume of bacterial suspension. However, it must be established that the pre-determined plate layout was not adhered to on occasion. Inoculated plates were incubated, at 37°C, for a duration of 48 hours without agitation. Following incubation, the absorbance of each well was recorded at a wavelength of 570 nanometres (nm) in a microtiter-plate reader. Once the absorbance values had been obtained, the entire contents of each well were carefully aspirated. The removal of planktonic cells and media received careful consideration due to the high possibility of damaging the biofilm integrity. Furthermore, the following steps in the assay did not require an aseptic environment. Once the contents of each well was carefully removed and appropriately discarded, plates were washed in order to guarantee complete removal of all non-adherent cells whilst preserving the biofilm integrity. Washing plates involved gently submerging them in a container of distilled water and carefully emptying the wells by flicking the plates. Alternatively, the wells could be emptied by gentle aspiration with a pipette to minimise damage to the biofilm layer. However, the latter procedure was exceedingly time-consuming. Individual plates were washed three times in order to ensure complete removal of non-adherent cells. After all wells had been sufficiently washed, all plates were left overnight at room temperature in an inverted position to dry. 200 μL of 1 % crystal violet solution was added to each well and all plates were left at room temperature for a duration of 50 minutes to allow the crystal violet dye to penetrate the biofilm layer. Following a suitable exposure time,

excess crystal violet dye was removed and all wells were subjected to gentle washing as described previously to remove unbound dye. Following the washing procedure, all plates were left to air dry at room temperature for 3 hours. Resolubilisation of the dye was achieved by dispensing 150 μL of 99.8 % methanol into each well. Furthermore, the process of liberating crystal violet dye from adherent cells using methanol enabled indirect but precise measurement of biofilm production. The optical density was recorded for all plates at 570 nm in a microtiter-plate reader.

Tissue Cell Culture

Before an MTT assay could be attempted, mammalian cells were grown and maintained in meticulous fashion in order to avoid bacterial contamination. Mammalian cells were habitually monitored and passaged in order to maintain a healthy cell line. All tissue cell culture work was performed in a sterile cabinet. All reagents required for the composition of complete media were pre-warmed at 37°C for 15 minutes. To compose 100 mL of complete media supplemented with 10 % fetal bovine serum (FBS) and 1 % L-glutamine necessary for the cultivation of mammalian cells, 89 mL of media was dispensed into a sterile flask. 1 mL of L-glutamine and 10 mL of FBS were subsequently added to the flask.

On occasion it was necessary to resuscitate a frozen cell line for cytotoxicity studies. Cell culture stocks are frozen at -80°C and protected from freezing damage by 10 % (v/v) dimethyl sulfoxide (DMSO). Complete media was pre-warmed at 37°C for 15 minutes before being used. To resuscitate a cell line, the cap of the cryogenic vial was carefully loosened to liberate any residual liquid nitrogen. With extreme caution and haste, the cryogenic vial was thawed in a water bath set at 37°C until one or two crystals were visible. Aseptically, the contents of the vial were placed in a tube containing 8 mL of pre-warmed complete media and subjected to centrifugation at 2000 revolutions per minute (rpm) for 5 minutes. The supernatant was discarded and the pellet was thoroughly resuspended in 10 mL of complete media; ensuring thorough dissociation of cells. The cell suspension was subsequently added to 10 mL of complete media in an appropriate tissue flask. Following inoculation, the contents of the flask was gently swirled to ensure even distribution. The flask was placed in an appropriate tissue cell culture incubator set at 37°C.

Sub-culturing is essential for the continuation of an adherent cell line. Before use, complete media, sterile phosphate-buffered saline (PBS) and sterile trypsin-EDTA were pre-warmed at 37°C for 15 minutes. From a pre-existing flask of confluent cells, the media was appropriately discarded and 10 mL of PBS was dispensed into the tissue flask. The flask was gently swirled for a duration of 2 minutes. Following gentle agitation, the PBS was extracted from the flask and discarded. 2 mL of trypsin-EDTA was dispensed into the flask and the flask was subsequently incubated for 5 minutes. To achieve a 1 in 6 passage, 7 mL of media was dispensed into the flask to inactivate the trypsin-EDTA. 1.5 mL of the cell suspension was added to a new flask containing 18.5 mL of complete media. The newly composed flask of cells was placed in a tissue cell incubator at 37°C.

MTT Assay to Evaluate Cytotoxicity

The assessment of cytotoxicity towards a mammalian cell line was achieved using the MTT (3-4(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium) reduction assay. Before application, antibacterial compounds were sterile filtered using a 0.22 μm sterile filter. Dilutions of antibacterial compounds were performed using sterile distilled water as the diluent. Both the two-fold serial dilution method and the ten-fold serial dilution method were used. Pipette tips required for the extracting and dispensing of liquid were sterilised before use in the assay. Before use in the assay, complete media, sterile PBS, and sterile trypsin-EDTA were pre-warmed at 37°C for 15 minutes. The preparation and application of MTT dye solution was performed in a tissue cell culture cabinet.

Once the cells had reached the desired confluency, they were used in the MTT assay. The cell monolayer was washed and trypsinized in accordance with the aforementioned sub-culturing protocol. From the 9 mL of cell suspension, 0.5 mL was dispensed into an Eppendorf tube. From the 0.5 mL of cell suspension, a further 100 μL was extracted and mixed thoroughly with 0.4 % tryphan blue solution in a fresh Eppendorf tube. From the mixture, 20 μL was dispensed into a haemocytometer in order to calculate the concentration of viable cells using an inverted microscope. Furthermore, cells that appeared stained were not viable and disregarded in the subsequent calculations to obtain the viable cell concentration. Once the concentration, per μL, of viable cells was ascertained, the required concentration of cell solution for the assay was obtained. In general, each well of a flat-bottomed, 96-well plate was inoculated with 100 μL of cell suspension with a concentration of 75 cell/μL. Once all the appropriate wells had been inoculated, the 96-well plate was placed in an appropriate tissue cell culture incubator at 37°C overnight to allow the cells to adhere to the base of the wells.

Once it was established that the plate was free from contamination and the cells had adhered successfully to the base of the well, the cells were treated with test compound. 100 μL of test compound was added to each well containing cells. A number of wells were left free of test compound in order to serve as positive controls. Sterile complete media was dispensed into the remaining empty wells to serve as negative controls. In general, 2-4 repeats of treated cells per test compound were plated, two repeats of positive controls were plated, and two repeats of negative controls were plated. Once the cells had been treated with the relevant test compound, the 96-well plate was incubated overnight at 37°C.

Following overnight incubation, the wells were treated with MTT dye solution. In order to compose MTT dye solution (3 mg/mL in PBS), 0.06 grams of MTT powder was dissolved in 20 mL of PBS using a vortex mixer to ensure the solution was thoroughly homogenised. As MTT dye solution is photosensitive, the solution was wrapped in aluminium foil to protect it from light exposure. 30 μL of MTT dye solution was added to all wells and the plate was incubated at 37°C for 3 hours; this was to allow MTT to be converted to formazan during this time period. Furthermore, the formazan crystal appears purple when generated by viable cells. Following incubation, the contents of each well were carefully aspirated using a vacuum; ensuring not to disturb the formazan product. Once the contents from each well had been carefully extracted, 100 μL of dimethyl sulfoxide (DMSO) was added to all wells to solubilise the formazan crystal. The absorbance of each well was recorded at 540 nanometres (nm) and 690 nm using a microtiter plate reader.

Results

Optical Density Assay

The antimicrobial effect of the disinfectant skin concentrate, diluted to 6 % (v/v) as dictated by Crystal Clear, was evaluated against *S. aureus* using the optical density assay. Absorbance values at 600 nanometres (nm) were used to measure bacterial density. In addition, a positive control was included which exclusively contained *S. aureus*. The positive control essentially represents the growth of *S. aureus* over a 7-hour time period. Furthermore, all absorbance values recorded at 2.5 cannot be considered entirely accurate as the spectrophotometer used did not record readings surpassing 2.5.

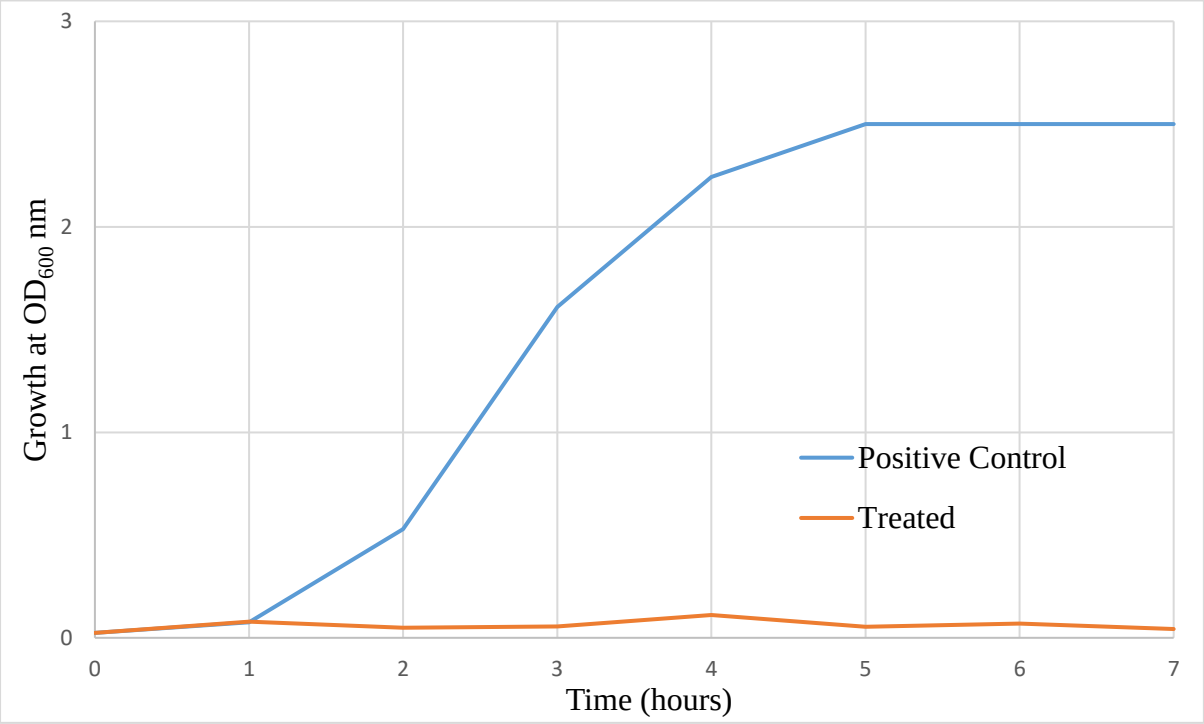

Figure 8. A graph to show the effect of a diluted skin concentrate formula at 6% (v/v) on the growth of *S. aureus* over a 7-hour time period.

Broth Dilution Assay

Standardised cultures of *S. aureus*, *E. coli* and *P. aeruginosa* were subjected to two-fold serial dilutions of chlorhexidine digluconate in order to determine the minimum inhibitory concentration (MIC). Absorbance values at 600 nanometres (nm) were used to measure bacterial density. To visually confirm the presence of a viable culture or not, or to alleviate concerns regarding contamination, the resazurin reduction assay was employed. The starting concentration of chlorhexidine digluconate was 2 % (v/v).

Table 1. A table to show the effects of two-fold serially diluted concentrations of chlorhexidine digluconate on three different strains of bacteria by recording absorbance values at 600 nanometres (nm). All values represented in this table are averages. CHX = chlorhexidine digluconate.

Sample	Dilution	*S. aureus* & CHX (n=2)	Positive Control *S. aureus* (n=1)	*E. coli* & CHX (n=2)	Positive Control *E. coli* (n=1)	*P. aeruginosa* & CHX (n=2)	Positive Control *P. aeruginosa* (n=1)	Negative Control (n=2)
Absorbance (arbitrary units)	1	2.128	1.679	2.25	1.767	2.421	2.326	0.164
	2^{-1}	2.384	1.17	2.367	1.856	2.458	2.086	0.117
	2^{-2}	2.814	1.073	2.764	1.827	2.366	2.533	0.155
	2^{-3}	2.293	1.319	1.579	1.746	1.471	1.518	0.157
	2^{-4}	1.246	1.322	0.672	1.735	0.624	1.413	0.136
	2^{-5}	0.462	1.22	0.305	1.778	0.291	1.223	0.116
	2^{-6}	0.162	1.231	0.076	1.746	0.075	1.244	0.149
	2^{-7}	0.053	1.43	0.052	1.809	0.046	1.344	0.098

The assay was repeated using the same experimental design, however all three strains were subjected to ten-fold serial dilutions of chlorhexidine digluconate to further distinguish the effects between high and low concentrations of chlorhexidine digluconate on standardised bacterial cultures. The starting concentration of chlorhexidine digluconate was 2 % (v/v).

Table 2. A table to show the effects of ten-fold serially diluted concentrations of chlorhexidine digluconate on three different strains of bacteria by recording absorbance values at 600 nanometres (nm). All values represented in this table are averages. CHX = chlorhexidine digluconate.

Sample	Dilution	*S. aureus* & CHX (n=2)	Positive Control *S. aureus* (n=1)	*E. coli* & CHX (n=2)	Positive Control *E. coli* (n=1)	*P. aeruginosa* & CHX (n=2)	Positive Control *P. aeruginosa* (n=1)	Negative Control (n=3)
Absorbance (arbitrary units)	1	1.98	0.406	2.175	1.09	2.627	0.085	0.031 (±0.002)
	10^{-1}	1.238	1.655	1.032	2.762	1.326	0.888	0.038 (±0.006)
	10^{-2}	0.047	1.372	0.042	1.953	0.042	1.55	0.035 (±0.004)
	10^{-3}	0.04	1.12	0.126	2.062	0.038	1.235	0.036 (±0.003)
	10^{-4}	1.083	1.06	1.771	2.322	1.151	1.348	0.038 (±0.002)
	10^{-5}	0.481	1.04	1.822	2.227	1.333	1.661	0.038 (±0.002)
	10^{-6}	0.572	1.2	1.749	1.803	1.341	0.704	0.035 (±0.003)
	10^{-7}	0.604	1.585	1.486	1.867	1.516	0.095	0.036 (±0.006)

The antimicrobial effects of the disinfectant skin concentrate and chlorhexidine digluconate were evaluated against a standardised inoculum of Gram-negative *E. coli*. Both agents were ten-fold serially diluted. In addition, a column of the 96-well plate was used to evaluate the intensity of background signal interference generated by the reaction between chlorhexidine digluconate and Mueller-Hinton microbiological growth medium. The starting concentration of chlorhexidine digluconate was 1 % (v/v).

Table 3. A table to show the effects of ten-fold serially diluted concentrations of chlorhexidine digluconate and skin concentrate on *E. coli* by recording absorbance values at 600 nanometres (nm). All values represented in this table are averages. CHX = chlorhexidine digluconate. SC = skin concentrate.

Sample	Dilution	*E. coli* & CHX (n=3)	*E. coli* & SC (n=3)	Positive Control (n=2)	Negative Control (n=2)	CHX & Mueller-Hinton broth (n=1)
Absorbance (arbitrary units)	1	0.823 (±0.6)	0.196 (±0.03)	0.165	0.052	1.236
	10^{-1}	1.514 (±0.7)	0.401 (±0.05)	0.573	0.153	0.543
	10^{-2}	0.054 (±0.009)	0.058 (±0.006)	1.711	0.176	0.045
	10^{-3}	0.052 (±0.004)	0.045 (±0.002)	1.846	0.104	0.045
	10^{-4}	1.356 (±0.05)	1.444 (±0.09)	1.698	0.083	0.049
	10^{-5}	1.413 (±0.05)	1.366 (±0.05)	1.705	0.067	0.046
	10^{-6}	1.307 (±0.03)	1.267 (±0.02)	1.715	0.077	0.045
	10^{-7}	1.233 (±0.07)	1.214 (±0.02)	1.828	0.067	0.044

The antimicrobial effects of the disinfectant skin concentrate and chlorhexidine digluconate were evaluated against a standardised inoculum of Gram-positive *S. aureus*. Both agents were ten-fold serially diluted. In addition, two columns of the 96-well plate were used to evaluate the intensity of background signal interference generated by chlorhexidine digluconate and skin concentrate reacting with Mueller-Hinton microbiological growth medium.

Table 4. A table to show the effects of ten-fold serially diluted concentrations of chlorhexidine digluconate and skin concentrate on *S. aureus* by recording absorbance values at 600 nanometres (nm). All values represented in this table are averages. CHX = chlorhexidine digluconate. SC = skin concentrate.

Sample	Dilution	*S. aureus* & CHX (n=3)	*S. aureus* & SC (n=3)	Positive Control (n=2)	Negative Control (n=2)	CHX & Mueller-Hinton broth (n=1)	SC & Mueller-Hinton broth (n=1)
Absorbance (arbitrary units)	1	2.056 (±0.6)	0.761 (±0.2)	0.491	0.042	2.363	0.858
	10^{-1}	0.681 (±0.06)	0.253 (±0.04)	1.355	0.187	0.56	0.143
	10^{-2}	0.041 (±0.008)	0.049 (±0.003)	0.956	0.102	0.041	0.043
	10^{-3}	0.040 (±0.002)	0.039 (±0.001)	0.982	0.084	0.05	0.05
	10^{-4}	0.477 (±0.1)	0.552 (±0.1)	0.988	0.061	0.04	0.048
	10^{-5}	0.523 (±0.1)	0.451 (±0.09)	1.056	0.041	0.045	0.045
	10^{-6}	0.564 (±0.1)	0.482 (±0.1)	1.168	0.043	0.04	0.045
	10^{-7}	0.757 (±0.04)	0.677 (±0.1)	1.286	0.106	0.053	0.041

To eliminate background signal interference, two-fold serial dilutions of chlorhexidine digluconate, from a starting concentration of 0.01 % (v/v), were evaluated against *S. aureus* and *E. coli.*

Table 5. A table to show the effects of two-fold serially diluted concentrations of chlorhexidine digluconate on *S. aureus* and *E. coli* by recording absorbance values at 600 nanometres (nm). All values represented in this table are averages. CHX = chlorhexidine digluconate.

Sample	Dilution	*S. aureus* & CHX (n=2)	*E. coli* & CHX (n=2)	Positive Control *E. coli* (n=1)	Positive Control *S. aureus* (n=1)	Negative Control (n=2)
Absorbance (arbitrary units)	1	0.038	0.041	1.717	1.465	0.203
	2^{-1}	0.030	0.036	1.599	1.022	0.156
	2^{-2}	0.035	0.04	1.499	0.93	0.121
	2^{-3}	0.032	0.039	1.683	0.893	0.125
	2^{-4}	0.03	0.039	1.505	0.895	0.121
	2^{-5}	0.031	0.039	1.516	0.846	0.139
	2^{-6}	0.545	1.32	1.644	0.95	0.14
	2^{-7}	0.729	1.506	1.422	0.976	0.155
	2^{-8}	0.655	1.471	1.495	1.083	0.184
	2^{-9}	0.761	1.546	1.492	1.079	0.196
	2^{-10}	0.683	1.54	1.508	0.963	0.171
	2^{-11}	1.359	1.804	1.757	1.482	0.094

Biofilm Assay

The ability of two-fold serial dilutions of chlorhexidine digluconate and hydrogen peroxide to inhibit the development of biofilm generated by a standardised inoculum of *S. aureus* was evaluated. Absorbance values at 570 nanometres (nm) were recorded. In addition, two columns of the 96-well plate were used to evaluate the intensity of background signal interference generated by chlorhexidine digluconate and skin concentrate reacting with tryptone soya microbiological growth medium. The starting concentration of chlorhexidine digluconate was 1 % (v/v) and the starting concentration of hydrogen peroxide was 3 % (v/v).

Table 6. A table to show the effects of two-fold serially diluted concentrations of chlorhexidine digluconate and hydrogen peroxide on the development of biofilm produced by *S. aureus* following the application and solubilisation of crystal violet dye. Absorbance values were recorded at 570 nanometres (nm). All values represented in this table are averages. CHX = chlorhexidine digluconate.

Sample	Dilution	*S. aureus* & CHX (n=2)	*S. aureus* & Hydrogen Peroxide (n=2)	Positive Control (n=2)	Negative Control (n=2)	CHX & Tryptone Soya broth (n=1)
Absorbance (arbitrary units)	1	3.5	0.212	0.268	0.263	3.5
	2^{-1}	3.5	0.204	0.31	0.201	3.5
	2^{-2}	3.461	0.212	0.383	0.207	3.34
	2^{-3}	2.892	0.206	0.458	0.197	3.03
	2^{-4}	2.709	0.218	0.428	0.194	2.4
	2^{-5}	2.673	0.201	0.315	0.228	3.373

The ability of two-fold serial dilutions of two broad spectrum antimicrobial compounds, acetic acid and hydrogen peroxide, on the inhibition of biofilms generated by *P. aeruginosa* was evaluated. The starting concentration of acetic acid was 0.25 % (v/v).

Table 7. A table to show the effects of two-fold serially diluted concentrations of acetic acid and hydrogen peroxide on the development of biofilm produced by *P. aeruginosa* following the application and solubilisation of crystal violet dye. Absorbance values were recorded at 570 nanometres (nm).

Sample	Dilution	*P. aeruginosa* & Hydrogen Peroxide (n=1)	*P. aeruginosa* & Acetic Acid (n=1)	Positive Contr4ol (n=1)	Negative Control (n=1)
Absorbance (arbitrary units)	1	0.18	0.172	0.5	0.17
	2^{-1}	0.195	0.663	0.816	0.183
	2^{-2}	0.156	0.853	0.924	0.178
	2^{-3}	0.154	0.799	0.96	0.182
	2^{-4}	0.155	0.891	0.96	0.203
	2^{-5}	0.452	0.787	0.834	0.169
	2^{-6}	0.597	0.789	0.823	0.173
	2^{-7}	0.608	0.716	0.427	0.178

The ability of two-fold serial dilutions of acetic acid and chlorhexidine digluconate to inhibit the development of biofilm generated by *P. aeruginosa* was evaluated.

Table 8. A table to show the effects of two-fold serially diluted concentrations of acetic acid and chlorhexidine digluconate on the development of biofilm produced by *P. aeruginosa* following the application and solubilisation of crystal violet dye. CHX = chlorhexidine digluconate. Absorbance values were recorded at 570 nanometres (nm). All values represented in this table are averages.

Sample	Dilution	*P. aeruginosa* & CHX (n=3)	*P. aeruginosa* & Acetic Acid (n=3)	Positive Control (n=2)	Negative Control (n=2)
Absorbance (arbitrary units)	1	3.5 (±0)	0.21 (±0.008)	0.829	0.204
	2^{-1}	3.5 (±0)	0.206 (±0.02)	0.806	0.182
	2^{-2}	3.5 (±0)	0.194 (±0.008)	0.846	0.174
	2^{-3}	3.167 (±0.3)	0.182 (±0.007)	0.852	0.18
	2^{-4}	3.111 (±0.3)	0.385 (±0.03)	0.77	0.201
	2^{-5}	2.882 (±0.6)	0.63 (±0.1)	1.17	0.224

The ability of ten-fold serial dilutions of chlorhexidine digluconate to inhibit the development of biofilm generated by *S. aureus* was evaluated. Ten-fold serial dilutions of chlorhexidine digluconate were used to further distinguish the effects of high and low concentrations of chlorhexidine digluconate on the development of biofilm by *S. aureus*.

Table 9. A table to show the effects of ten-fold serially diluted concentrations of chlorhexidine digluconate on the development of biofilm produced by *S. aureus* following the application and solubilisation of crystal violet dye. CHX = chlorhexidine digluconate. Absorbance values were recorded at 570 nanometres (nm). All values represented in this table are averages.

Sample	Dilution	*S. aureus* & CHX (n=2)	Positive Control (n=5)	Negative Control (n=2)	CHX & Tryptone Soya broth (n=1)
Absorbance (arbitrary units)	1	3.5	0.470 (±0.09)	0.146	3.5
	10^{-1}	2.183	0.771 (±0.1)	0.201	2.732
	10^{-2}	0.219	1.154 (±0.1)	0.123	0.242
	10^{-3}	0.148	1.417 (±0.2)	0.13	0.125
	10^{-4}	0.518	1.228 (±0.3)	0.121	0.102
	10^{-5}	3.107	0.868 (±0.2)	0.135	0.116

MTT Cytotoxicity Assay

The cytotoxicity of two-fold serially diluted concentrations of chlorhexidine digluconate, acetic acid, and both quaternary ammonium compounds were evaluated against A549 cells. The quantity of formazan produced by viable cells was recorded as an absorbance value at 540 nanometres (nm). In addition, a reference wavelength of 690 nm was used to eliminate background signal interference, such as those generated by precipitates, cellular debris, and scratches on the wells.

Table 10. A table to show the cytotoxic effects of two-fold serially diluted concentrations of acetic acid, chlorhexidine digluconate (CHX), benzyl-C12-14-alkyldimethylammonium chlorides (BAC), and didecyldimethylammonium chloride (DDAC) on A549 cells. The top set of values were recorded at 540 nanometres (nm). The bottom set of values represent the background signal values, recorded at 690 nanometres. All values represented in this table are averages.

Sample	Dilution	A549 & Acetic Acid (n=2)	A549 & CHX (n=2)	A549 & BAC (n=2)	A549 & DDAC (n=2)	Positive Control (n=2)	Negative Control (n=2)
Absorbance (arbitrary units)	1	0.101 0.083	1.165 0.914	0.258 0.167	0.453 0.304	0.371 0.078	0.077 0.066
	2^{-1}	0.243 0.091	0.691 0.464	0.107 0.093	0.108 0.09	0.321 0.089	0.097 0.085
	2^{-2}	0.129 0.104	0.566 0.382	0.143 0.117	0.111 0.095	0.323 0.103	0.104 0.086
	2^{-3}	0.306 0111	0.484 0.34	0.148 0.113	0.136 0.114	0.352 0.122	0.129 0.117
	2^{-4}	0.301 0.119	0.169 0.139	0.18 0.141	0.166 0.147	0.342 0.13	0.118 0.109
	2^{-5}	0.323 0.132	0.186 0.159	0.168 0.151	0.159 0.138	0.385 0.136	0.147 0.128
	2^{-6}	0.332 0.155	0.224 0.184	0.211 0.162	0.185 0.158	0.418 0.162	0.153 0.125
	2^{-7}	0.489 0.155	0.446 0.17	0.502 0.177	0.452 0.169	0.483 0.163	0.165 0.144

The cytotoxicity of ten-fold serially diluted concentrations of silver ions, ranging from 0.0074 mol/L to 0.011 mol/L, and acetic acid were evaluated against A549 cells.

Table 11. A table to show the cytotoxic effects of ten-fold serially diluted concentrations of acetic acid and silver ions on A549 cells. The bottom set of values represent the background signal values, recorded at 690 nm. All values represented in this table are averages. All values represented in this table are averages.

Sample	Dilution	A549 & Silver Ion Solution (n=3)	A549 & Acetic Acid (n=2)	Positive Control (n=2)	Negative Control (n=2)
Absorbance (arbitrary units)	1	0.821(±0.2) 0.821(±0.2)	0.104 0.083	0.337 0.081	0.11 0.072
	10^{-1}	0.278(±0.05) 0.225(±0.04)	0.29 0.1	0.395 0.089	0.122 0.083
	10^{-2}	0.141(±0.02) 0.105(±0.006)	0.353 0.109	0.421 0.097	0.119 0.083
	10^{-3}	0.166(±0.006) 0.119(±0.007)	0.395 0.13	0.425 0.121	0.148 0.117
	10^{-4}	0.292(±0.03) 0.131(±0.001)	0.365 0.142	0.417 0.124	0.135 0.105
	10^{-5}	0.313(±0.02) 0.137(±0.01)	0.336 0.147	0.394 0.127	0.161 0.122
	10^{-6}	0.320(±0.02) 0.157(±0.006)	0.422 0.178	0.434 0.162	0.175 0.139
	10^{-7}	0.353(±0.009) 0.157(±0.004)	0.397 0.179	0.415 0.159	0.196 0.158

Discussion

The antimicrobial activity of a certified disinfectant hand rub formula and its individual formula constituents, including the principal antimicrobial agent chlorhexidine digluconate, were evaluated using a selection of different biological assays. In addition, the cytotoxic properties of the aforementioned antimicrobial agents against a mammalian cell line were evaluated, in parallel, to the antimicrobial assays to provide a comprehensive understanding of the overall efficiency of the hand rub formula and its individual constituents. Although it is fundamental for antimicrobial formulae to exhibit potent activity against pathogenic microbes and ultimately inhibit the spread of disease-causing bacteria, it is equally important for antimicrobial formulae to not exhibit significant cytotoxicity. Furthermore, the concept of biocompatibility is derived from combining these two aspects of antimicrobial formulae. As described by Kramer & Müller (2008), in order to accurately evaluate the suitability of novel antimicrobial agents or formulations, both the antimicrobial activity and the potential cytotoxic effects must be assessed using *in vitro* methods in order to generate biocompatible antimicrobial agents.

Figure 1. distinctly demonstrates the skin concentrates potent antimicrobial activity against *S. aureus* over a 7-hour incubation period. In the presence of the skin concentrate, the growth of *S. aureus* was inhibited immediately. Furthermore, the growth of *S. aureus* in the presence of the skin concentrate exhibited no revival during the 7-hour incubation period, however an extended incubation period could have provided more conclusive data. In addition, this method solely provides data associated with the bacteriostatic properties of the skin concentrate. With regards to the bactericidal action of the skin concentrate, a more decisive biological assay would be required.

Table 1. illustrates that all three strains exhibited identical responses when treated with two-fold serially diluted concentrations of chlorhexidine digluconate. All three strains display an OD_{600} of <0.1 when treated with the 2^{-7} dilution; this is indicative of bacterial growth inhibition as these values align with the negative control values. Additionally, *E. coli* and *P. aeruginosa* display optical density values lower than 0.1 for the prior dilution at 2^{-6}. However, the slightly more elevated absorbance value for *S. aureus*, 0.162, could equate to contamination of one of the repeats. Wells situated on the edge of 96-well plates were prone to contamination from a variety of sources, such as from the equipment being used or from the surrounding atmosphere. Additionally, an inadequate number of serial dilutions were used in order to obtain the minimum inhibitory concentration (MIC), as the lowest dilution of chlorhexidine digluconate generated no turbidity for all three strains. Moreover, all values recorded prior to the 2^{-5} dilution would seemingly indicate extensive growth of bacteria in the treated wells, however this would be a false assumption as these elevated optical density values are a by-product of a reaction between higher concentrations of chlorhexidine digluconate and Mueller-Hinton broth. Furthermore, a precipitation reaction can be assumed due to the fact that the absorbance values decrease with decreasing concentrations of chlorhexidine digluconate, thereby indicating that reduced concentrations of chlorhexidine digluconate are able to inhibit bacterial growth without the generation of an undesired by-product obscuring the effects.

Table 2. distinguishes the effects between high and low concentrations of chlorhexidine digluconate on all three strains of bacteria with minimal inference from an undesired by-product. This was achievable by way of utilising ten-fold serial dilutions. As observed previously, all three strains responded identically to chlorhexidine digluconate. All three strains display an OD_{600} of <0.1 when treated with both the 10^{-2} and 10^{-3} dilutions; the slightly elevated

value for *E. coli*, 0.126 at the 10^{-3} dilution, represents an exception due to possible contamination of one of the repeat wells. Furthermore, these values align with the negative control values and ultimately indicate bacterial growth inhibition. As bacterial growth was observed by all strains when treated with the 10^{-4} dilution, OD_{600} of >1, it can be assumed that the 10^{-3} dilution represents the MIC. However, it must be established that ten-fold serial dilutions are not as accurate as two-fold serial dilutions in determining the MIC, due to the fact that the range between each ten-fold serial dilution is far too extensive. Furthermore, dilutions succeeding the 10^{-3} dilution yield elevated bacterial growth for each strain, more so in the case of *E. coli* and *P. aeruginosa*, both displaying OD_{600} values of >1. In addition, the value of 1.083 at the 10^{-4} dilution for *S. aureus* represents an anomaly as the subsequent dilutions yield considerably lower absorbance values; OD_{600} of 0.481-0.604. This could be attributed to a form of contamination affecting one of the side wells of the 96-well plate. The results illustrating the effects of chlorhexidine digluconate on *P. aeruginosa* sufficiently portray the activity spectrum associated with decreasing concentrations of chlorhexidine digluconate. For example, the first two dilutions display elevated absorbance values associated with the generation of an undesired by-product between higher concentrations of chlorhexidine digluconate and biological growth medium. Both the 10^{-2} and 10^{-3} dilutions display bacterial growth inhibition, whereas the subsequent dilutions display gradually increasing bacterial densities associated with decreasing concentrations of antimicrobial compound.

Table 3. illustrates the activity of chlorhexidine digluconate and the skin concentrate against Gram-negative *E. coli* and Table 4. illustrates the activity of the aforementioned compounds activity against Gram-positive *S. aureus.* The skin concentrate exhibited similar activity against both strains, with inhibition of growth observed at both the 10^{-2} and 10^{-3} dilutions; far below the concentration it is used at. Interestingly, *S. aureus* did not demonstrate a similar revival in

growth compared with *E. coli* at dilution values below the MIC, with *S. aureus* displaying OD_{600} values ranging between 0.451-0.757 for both test compounds. Bacterial growth associated with *S. aureus* at sub-MIC concentrations of both compounds seemed constrained, albeit marginally increasing. This growth behaviour was observed in Table 2. A possible explanation could be the extra outer membrane associated with Gram-negative bacteria, since *P. aeruginosa* displayed elevated bacterial growth at sub-MIC concentrations in Table 2. In addition, a column with exclusively chlorhexidine digluconate and growth medium was included, in both Table 3. and 4., to highlight the sensitivity Mueller-Hinton broth had for higher concentrations of chlorhexidine digluconate, thereby generating a cloudy precipitate. Furthermore, with regards to elevated absorbance value associated with *E. coli* treated with 1 % (v/v) chlorhexidine digluconate, 0.823, bacterial inhibition could be attributed to the by-product of the undesired reaction and not solely chlorhexidine digluconate.

Table 5. is a much clearer representation of the antimicrobial effect of two-fold serial dilutions of chlorhexidine digluconate against *S. aureus* and *E. coli*. Inhibition of both strains is clearly indicated at 0.01 % (v/v) and all subsequent dilutions up to 2^{-5}, with OD_{600} values at <0.1. Therefore, the 2^{-5} dilution can be designated the MIC. Subsequent dilutions for both strains indicate a revival in growth similar to the growth patterns observed in Table 2. and 4. The lack of undesired background signal interference is attributed to the much lower initial concentration, 0.01 % (v/v), which undoubtedly demonstrates chlorhexidine digluconates potent antimicrobial activity.

The biofilm assay represented in Table 6. distinctly demonstrates the problematic nature of using crystal violet dye to evaluate biofilm inhibitory data. It must be firmly established that optical density values can be somewhat misleading with regards to the biofilm inhibition assay

due to the staining potency of crystal violet dye. Additionally, due to the abundance of experimental aspects associated with the crystal violet assay, several errors could have been made that ultimately reduced the amount of biofilm recovered. Visually, it was achievable to distinguish the effects of high and low concentrations of test compound against biofilm development by changes in the colour intensity of the resolubilised crystal violet dye; a more intense crystal violet colour indicated an increased number of adhered microorganisms to the well. However, spectrophotometric data could be very misrepresentative with regards to a test compounds ability to inhibit biofilm development. Table 6. highlights the high affinity crystal violet had for the by-product of the reaction between chlorhexidine digluconate and growth medium. In general, the positive controls demonstrated the biofilm-producing properties of *S. aureus*, with an OD_{570} range of 0.268-0.458. Furthermore, hydrogen peroxide did not react readily with the growth medium and demonstrated biofilm-inhibitory properties across all concentrations used; OD_{570} values ranging from 0.201-0.218, which align with the negative control values. It is difficult to extract conclusive information regarding the biofilm inhibitory properties of chlorhexidine digluconate, as the strong association between chlorhexidine digluconate and the growth medium obscured any beneficial observations.

Table 7. displays results obtained from assessing the inhibitory properties of two broad spectrum antimicrobials, hydrogen peroxide and acetic acid, against biofilms associated with *P. aeruginosa*. As they did not react readily with growth medium, the assay produced yielding results which clearly represented their activity against biofilm formation. Acetic acid demonstrates inhibitory properties at 0.25 % (v/v), although subsequent dilutions do not exhibit inhibitory properties. Hydrogen peroxide demonstrates similar inhibitory properties, as it displays inhibitory properties at the 2^{-4} dilution, with an accompanying absorbance value of

0.155. In addition, the positive control values are satisfactory, with an OD_{570} range of 0.427-0.96, therefore indicative of affluent biofilm development.

Table 8. further demonstrates the common occurrence of misleading results when utilising the crystal violet assay to assess biofilm inhibitory properties. All absorbance values associated with the biofilm inhibitory properties of chlorhexidine digluconate lie within a range of 2.882-3.5, thereby making it unfeasible to extract any decisive information. However, It could be assumed that chlorhexidine digluconate does demonstrate inhibitory properties against biofilms associated with *P. aeruginosa* by analysing the effects of chlorhexidine digluconate, at concentrations far below 2 % (v/v), against planktonic cells associated with *P. aeruginosa* in Table. 2. However, this assumption would have to be suitably assessed before any certain claims are made.

Table 9. further distinguishes the effects between high and low concentrations of chlorhexidine digluconate by using ten-fold serial dilutions. In addition, a control column with exclusively test compound and growth medium was included to highlight that low concentrations of chlorhexidine digluconate did not readily chemically react with growth medium. Furthermore, ten-fold serial dilutions of chlorhexidine digluconate allowed conclusive data to be obtained, since both 10^{-2} and 10^{-3} dilutions yielded OD_{570} values that are indicative of biofilm inhibition; 0.219 and 0.148 respectively. Furthermore, the slightly higher value for the 10^{-2} dilution could be attributed to a minor reaction between chlorhexidine digluconate and the growth medium. Both sets of control values are satisfactory, as they align with the nature of their corresponding control type. All negative control values fall between 0.121 and 0.201. The positive control values are indicative of unhindered, affluent biofilm development; displaying an OD_{570} range of 0.470-1.417. Closer assessment of tables 4. & 9., concerning *S. aureus,* seemingly indicate

that inhibition of planktonic cell growth and inhibition of biofilm development occur at similar concentrations of chlorhexidine digluconate. While this similarity does make sense as reduced cell biomass in the well would yield reduced cellular adherence to the well; it does not provide insight into the effect of chlorhexidine digluconate on pre-existing biofilms. It would have been more prudent to employ a far superior biofilm inhibitory assay which does not require the application of crystal violet dye. For example, Ceri et al. (1999) described a biofilm susceptibility assay using a Calgary Biofilm Device (CBD), which does not require the application of crystal violet dye. In brief, the assay involves cultivating biofilms on specialised pegs on a lid and subsequently exposing the biofilms to an antimicrobial compound. Following exposure to an antimicrobial compound, the pegs are placed in recovery media. Following exposure to recovery media, a new lid is placed in the recovery media and the amount of viable biofilm recovered on the pegs is ascertained. Furthermore, due to time limitations and the cost of CBDs, the assay was never attempted.

Cytotoxicity data was collected by employing the tetrazolium-based MTT assay. While generally more of an accurate assay with regards to the initial starting cell number, the MTT assay was laborious and often temperamental due to microbial contamination. In addition, microbial contamination of a plate would render the entire assay redundant, whereas microbial contamination of a tissue culture flask would make it necessary to restart cell cultivation. Furthermore, the immortalised A549 cell line was used since it was an accurate representation of a mitotic cell line and its immortalised nature encouraged rapid cultivation.

Table 10. compares the cytotoxic effects of acetic acid, chlorhexidine digluconate, BAC 50, and BTC 101E. Acetic acid displayed low levels of toxicity towards A549 compared with the other test compounds, with increased viability present at the 2^{-3} dilution. The absorbance value

of 0.243 at the 2^{-1} dilution can be disregarded due to fact that the subsequent dilution displayed a reduction in viability. Furthermore, the elevated absorbance value associated with the 2^{-1} dilution could be attributed to microbial contamination. In addition, the aforementioned absorbance value could be attributed to an experimental error, such as the application of an incorrect dilution or lack of attentiveness which resulted in no test compound being applied. Interestingly, compared with its ability to inhibit biofilm development, acetic acid displayed more potent behaviour towards A549. More conclusive data could be obtained by directly assessing acetic acid against pre-existing biofilms. Two-fold serial dilutions of chlorhexidine digluconate, BAC 50, and BTC 101E all display similar toxicity levels against A549. As observed in the antimicrobial studies, higher concentrations of chlorhexidine digluconate react with the mammalian cell growth media to generate elevated absorbance values up to the 2^{-3} dilution; this is followed by a reduction in absorbance, 0.169 and 0.186, which is suggestive of a reduction in viability. Viable cells were present at the 2^{-7} dilution for all three compounds, however toxicity was observed as low as the 2^{-6} dilution; OD_{540} of 0.224 in the case of chlorhexidine digluconate which indicates minimal increase in viability. For chlorhexidine digluconate, this would equate to a concentration of 0.008 % (v/v) and 0.02 % (v/v) respectively. Regarding its effects on *E. coli* and *S. aureus*, growth inhibition was observed as low as the 2^{-7} dilution; equating to a concentration of 0.02 % (v/v). While these results would appear favourable, the minimum bactericidal concentration (MBC) would surpass this concentration, thereby indicating that chlorhexidine digluconate would be deployed in a formula at a concentration greater than the MBC. Both quaternary ammonium compounds demonstrated elevated toxicity against A549, with significant viability, OD_{540} of 0.502 and 0.452, demonstrated at the 2^{-7} dilution for both compounds.

Table 11. illustrates that a concentrated solution of silver ions displayed cytotoxic behaviour at the 10^{-3} dilution; OD_{540} of 0.166 which aligns with the negative control values. All subsequent dilution values indicate a revival in viability; 0.292, 0.313, 0.320, and 0.353. The toxic properties of silver ions are even more distinct when the toxic properties of acetic acid are included, with viability observed at the 10^{-1} dilution. However, quantitative assessment of the silver ion solution revealed it to be very saturated, thus the concentration could not be determined accurately. Furthermore, an extensively saturated silver ion solution would undoubtedly exert cytotoxic activity against a 96-well plate of adherent mammalian cells swathed in growth medium.

The results obtained throughout the course of the research are conclusive with regards to the antimicrobial potency of the skin concentrate formula and its principal antimicrobial constituent, chlorhexidine digluconate. All the assays used to analyse antimicrobial activity yielded convincing data that is indicative of potent antimicrobial activity. However, to yield more quantitative data with regards to remaining viable bacterial cells, a quantitative suspension test could have been employed. On occasion, it was difficult to derive reliable data when chlorhexidine digluconate was concerned as it commonly reacted with growth medium. In order to combat the problem concerning chlorhexidine digluconates tendency to react with liquid growth medium and ultimately hinder the detection of bacterial growth, it might have been more beneficial to employ the agar dilution method. The agar dilution method involves assessing an antimicrobial compounds capabilities on a solid agar surface, thus an undesired reaction between chlorhexidine digluconate and the agar medium might not have occurred. Regrettably, the agar dilution method was never attempted. In addition, biofilm inhibitory data was unyielding in certain aspects due to the problematic nature of crystal violet dye and the laborious methodology associated with the assay. Silver ions in solution displayed

antimicrobial activity; coinciding with scientific literature concerning the antimicrobial activity of silver. However, the method employed to generate silver ions was very rudimentary, and as a consequence, a heavily saturated solution of silver ions was produced. Furthermore, while ionic silver is recognised for its antimicrobial activity, the antimicrobial efficacy of silver nanoparticles represents a more recent area of interest. Although results and experimental procedures are not presented here, silver nanoparticle generation was attempted using a method outlined by Mir-Simon et al. (2015). However, despite the presence of silver nanoparticles being confirmed by ultraviolet-visible spectroscopy (UV-Vis), detailed characterisation could not be achieved at the University of Salford, thus antimicrobial evaluation was not attempted. Minimum bactericidal concentration (MBC) data was scarce owing to the fact that results were either incomprehensible due to contamination, or there was no outcome whatsoever to derive conclusions from. No outcome could be attributed to the requirement of a neutralising agent to neutralise the effects of the antimicrobial agent while being transferred to the appropriate agar to establish viability. Regrettably, time limitations and other aspects of the research prevented further investigation into the MBC.

Regardless of the potent antimicrobial activity of chlorhexidine digluconate, cytotoxicity data confirmed the toxic effects of chlorhexidine digluconate against mammalian cells. In addition, silver ions demonstrated cytotoxicity towards mammalian cells. However, particular aspects of the cytotoxic studies were unyielding due to the fact that the mammalian growth medium frequently reacted with all test compounds at higher concentrations. In addition, a marginal difference could occasionally be detected between both control values; this could be attributed to an experimental error that produced an insufficient amount of cells being plated per well. Whereas the mammalian cell line used was a lung carcinoma cell line, it still was a mitotic cell line of human origin. However, more conclusive data could be harvested from cytotoxic

evaluation against fibroblasts and keratinocytes. With regards to cytotoxicity testing, more data with regards to structural damage would have generated a wider understanding of the toxic effects of the antimicrobial agents. While not particularly within the realms of an MSc (By Research) project, structural analysis using transmission electron microscopy (TEM) following treatment in order to explore the various signalling pathways that could be associated with antimicrobial-induced mammalian cell death using a fluorescent dye, such as Fluo-3 AM, would have yielded far more extensive data.

The comparison between antimicrobial activity and cytotoxicity of antimicrobial compounds represents a very significant area of research due to the widespread application of antimicrobial compounds in different settings. Since topical antimicrobial compounds are applied to the skin habitually, it is important that toxic side effects are at an absolute minimum. The concept of biocompatibility is especially crucial in the medical setting, as there are an abundance of debilitated persons, thus creating an opportunistic environment for bacteria to flourish. In order to imitate *in vivo* scenarios as accurately as possible, an assortment of *in vitro* assays have been developed and enhanced in recent years. While not all of those assays have been reported here in an experimental sense, the ones that have been attempted provide an invaluable insight into the antimicrobial and cytotoxic activity of antimicrobial compounds.

In terms of results achieved over the course of the research, it was not possible to include all the data that was collected because of the quantity, and on occasion, the incomprehensible nature of them. The generation of unintelligible data was commonplace, since a lot of techniques performed over the course of the research required a substantial amount of practice to achieve a satisfactory level of self-confidence. Though assays demand a great deal of practice to achieve clear results, important intermediate steps, such as bacterial inoculations,

often require the same level of persistent practice. The next step of this research could be the habitual application of skin concentrate on a random collection of individuals to monitor its effect over a premeditated time period, using alcohol-containing formulae to serve as controls. Dyer, Gerenraich & Wadhams (1998) performed a study evaluating the immediate and persistent antimicrobial properties associated with rinse-free hand sanitisers on hands that had been purposefully contaminated with a particular species of bacteria. In brief, the aforementioned study involved contaminating hands with bacteria and subsequently applying the appropriate antimicrobial formulation. Following a standardised washing protocol, the glove juice sampling method was employed in order to extract the initial bacterial sample; this was achieved using a collection fluid. Furthermore, collection samples were then plated on the appropriate agar to obtain accurate colony counts. In addition, to ensure straightforward analysis of the effectiveness of an antimicrobial formulation, Dyer et al. (1998) used *Serratia marcescens* in the study which grows in red-coloured colonies. *In vivo* evaluations offer more reliable data, however lack of available time and ethical matters prevented any work being done in this area.

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