SUSCEPTIBILITY PATTERNS AND TOLERANCE MECHANISMS OF *Staphylococcus epidermidis* BIOFILMS TO PHYSICO-CHEMICAL STRESS EXPOSURE

BY

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DECLARATION

I declare that this is my original work and has never been presented for award of a degree in any University.

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DEDICATION

I dedicate this thesis to my wife, Milicent Awuor, my sister Irene and my dear parents, Joseph and Mary.

ABSTRACT

Bacterial biofilms remain a major public health burden. *Staphylococcus epidermidis* biofilm is the predominant cause of biofilm-associated infections. Kisumu county has a high circulation of antibiotic resistance genes, which is attributable to *S. epidermidis* biofilm, necessitating effective *S. epidermidis* biofilm control. Given the high tendency of bacteria to develop resistance to antibiotics, *S. epidermidis* biofilm control using physico-chemical disinfection is a suitable approach. In Kisumu county, heat (60°C), 1.72 M sodium chloride (NaCl), 0.178 M sodium hypochlorite (NaOCl) and 1.77 M hydrogen peroxide (H_2O_2) are the commonly used disinfectants. Studies on susceptibility of bacterial biofilms to disinfectants have focused on structurally or metabolically unique bacterial species; hence, offer limited insights on general biofilm disinfection. Despite *S. epidermidis* being a model and the most clinically relevant biofilm, its susceptibility patterns to the disinfectants remain undocumented. Mechanisms, including reduced diffusion through biofilm matrix, physiological heterogeneity within biofilm or persister cells are linked with high biofilm tolerance against antimicrobials. However, these mechanisms only provide partial explanations for biofilm's tolerance against fewer antibiotics, but not physico-chemical stresses, necessitating exploration of conclusive tolerance mechanisms. Although studies have implicated extracellular DNA (eDNA) and alternative sigma factor B (σ^B) in planktonic cells' tolerance against stressors, their contribution in biofilm's (*S. epidermidis* included) tolerance against physico-chemical stress exposure remain unknown. Hence, the susceptibility patterns, eDNA release and σ^B activity of *S. epidermidis* biofilm in response to physico-chemical stress exposure were evaluated. One *S. epidermidis* isolate per skin swab of sixty-two Kisumu county residents was used to generate a pair of biofilm and planktonic cultures. A post-test study design was adopted. The pairs were exposed to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 30 and 60 min for susceptibility determination using standard plating. Further, the pairs were exposed to optimal physico-chemical stresses (50°C, 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2) for 60 min for eDNA and σ^B activity quantification using qubit fluorometry and quantitative real-time PCR respectively. Statistical differences between groups were determined by *t*-tests using GraphPad Prism software. Significantly fewer *S. epidermidis* biofilms were killed upon exposure to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 than the planktonic cells ($p < 0.0001$). Unlike NaCl, biofilms exposed to 50°C, 5 mM NaOCl or 50 µM H_2O_2 exhibited significantly higher eDNA yields and σ^B activity than planktonic cells ($p < 0.05$). These findings demonstrated that *S. epidermidis* biofilm was more tolerant to the disinfectants, and that eDNA and σ^B activities contributed to its tolerance against the disinfectants. Collectively, the findings could inform on development of efficient disinfection approaches against *S. epidermidis* biofilm by targeting eDNA and/or σ^B; hence, reducing the burden and spread of antimicrobial tolerance.

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LIST OF ABBREVIATIONS AND ACRONYMS

agr	Accessory gene regulator
ANOVA	Analysis of variance
asp23	Alkaline shock protein 23
ATCC	American type culture collection
Atl	Autolysin
ATP	Adenosine triphosphate
CFU	Colony-forming unit
Ct	Cycle threshold
dsDNA	Double-stranded Deoxyribonucleic acid
ECM	Extracellular matrix
eDNA	Extracellular Deoxyribonucleic acid
h	Hour
H_2O_2	Hydrogen peroxide
HS	High sensitivity
KCRH	Kisumu County Referral Hospital
L	Litre
M	Molar
min	Minute
mL	Millilitre
mM	Millimolar
MOH	Ministry of Health
mRNA	Messenger Ribonucleic acid
MSA	Mannitol salt agar
NACC	National AIDs Control Council
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
OD	Optical density
PI	Propidium iodide
PMA	Propidium monoazide
RPA	Ribonuclease protein assay
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-qPCR	Quantitative real-time polymerase chain reaction
SEM	Standard error of the mean
TM	Tube method
TSB	Tryptic soy broth
USA	United States of America
UV	Ultra violet
VBNC	Viable but non-culturable
WHO	World Health Organization
µL	Microliter
σ^B	Alternative sigma factor B

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CHAPTER ONE

INTRODUCTION

1.1. Background

A bacterial population exists either as planktonic (free-floating cells) or as a biofilm (Jamal *et al*., 2015). Bacterial biofilm is a community of surface-attached bacterial cells embedded in a self-produced extracellular matrix (ECM) (Paytubi *et al*., 2017) composed of polysaccharides, proteins, water, lipids and nucleic acids (Jamal *et al*., 2015). Bacterial biofilms are ubiquitous (Tan *et al*., 2014) and account for over sixty-five percent of human infections (de la Fuente-Núñez *et al*., 2013). Biofilm formation is a survival strategy for several bacterial species against adverse conditions (Busscher & van der Mei, 2012). Bacterial biofilms provide a reservoir for pathogenic bacteria, hence are a major public health threat (Paytubi *et al*., 2017).

Staphylococcus epidermidis is a Gram-positive coagulase negative bacterium commonly linked with infections of medical implant device e.g. catheters, intrauterine devices, joint prostheses etc. (Otto, 2009; Joo & Otto, 2012), which are treated by removal of the infected device and subsequent replacement, causing an increase in morbidity and cost (Fey & Olson, 2010). Further, *S. epidermidis* biofilm is highly resistant to antibiotics and host immunity (Fey & Olson, 2010). Annually, *S. epidermidis*-related nosocomial infections account for a significant number of deaths (World Health Organization (WHO), 2014). The *S. epidermidis* biofilm is a model of bacterial biofilms (Decker *et al*., 2015). Further, *S. epidermidis* biofilm is a reservoir of antibiotic resistance and horizontal transfer genes, as well as conjugative and/or mobilizable plasmids, hence key in dissemination of antibiotic resistance genes among bacterial pathogens (Fey & Olson, 2010; Águila-Arcos *et al*., 2017). Thus, *S. epidermidis* biofilm is an important target in the control of the spread of antimicrobial tolerance genes.

Kisumu county has a high prevalence of pathogens in its soils, surface water and stored water (Baker *et al*., 2018; Barnes *et al*., 2018). Kisumu county, therefore, suffers a high burden of communicable bacterial infections, such as diarrhoea, tuberculosis and typhoid (Odongo *et al*., 2017), which are associted with biofilm-forming bacteia (Tan *et al*., 2014). Further, Kisumu county has a high variety of antibiotic resistance genes and multi-drug resistant isolates (Taitt *et al*., 2017). Being a hyper-endemic HIV area, Kisumu county has a high number of immunocompromised persons (National AIDs Control Council (NACC), 2016), who facilitate both evolution and rapid spread of resistant pathogens in the community (Kariuki & Dougan, 2014). The usage of antibiotics in the hospitals within Kisumu is high, increasing development of resistance phenotypes (Okoth *et al*., 2018). Since *S. epidermidis* biofilm is ubiquitous and harbours antimicrobial tolerance genes and horizontal gene transfer elements (Águila-Arcos *et al*., 2017), it is key in dissemination of antimicrobial tolerance in Kisumu county and many places.

Prevention of acquisition, spread and establishment of biofilm-forming bacteria, such as *S. epidermidis* (Peeters *et al*., 2008a), in domestic and healthcare settings where bacterial biofilms are most frequently encountered (Garrett *et al*., 2008; Francolini *et al*., 2010), is imperative. Due to the relatively high proclivity of bacteria to develop resistance to antibiotics (Hammer *et al*., 2012), control of *S. epidermidis* biofilm using effective physico-chemical disinfection procedures (Peeters *et al*., 2008a) may be a suitable approach. This requires a better understanding of the susceptibility patterns and mechanism(s) of tolerance of *S. epidermidis* biofilm to physico-chemical disinfectants. The following physico-chemical disinfectants are commonly utilized for point-of-use disinfection of food, water and/or medical equipment in Kisumu county and in many places: heat (60°C) (Sobsey, 2002), 1.72 M (10%) sodium chloride (NaCl) (Smith & Stratton, 2007), 0.178 M (1.2%) sodium

hypochlorite (NaOCl) (Blum *et al*., 2014) or 1.77 M (6%) hydrogen peroxide (H_2O_2) (Ministry of Health (MOH), 2007; Linley *et al*., 2012).

The susceptibility patterns of bacterial species to physico-chemical stresses, such as heat, NaCl, NaOCl and H_2O_2, have been reported previously for different bacterial species. For instance, the susceptibilities of biofilm forms of *Mycoplasma bovis* (McAuliffe *et al*., 2006), *Vibrio cholerae* O1 (Wai *et al*., 1998) and *Salmonella enterica* (Scher *et al*., 2005) to heat and/or NaCl exposures have been reported. Further, the susceptibilities of biofilm forms of *Lactobacillus plantarum* (Kubota *et al*., 2009), *Burkholderia cepacia*, *Pseudomonas aeruginosa* (Behnke *et al*., 2011; Elkins *et al*., 1999; Peeters *et al*., 2008a), *Mycobacterium avium*, *Mycobacterium intracellulare* (Steed & Falkinham III, 2006), *Escherichia coli* (Zhang *et al*., 2007) and *Klebsiella pneumoniae* (Stewart *et al*., 2001) to NaOCl and/or H_2O_2 exposures have been reported. However, the previous studies reported on *Mycoplasma*, *Mycobacterium* and *Salmonella* species, which are structurally or metabolically different from most bacterial species (Zogaj *et al*., 2001; Brooks *et al*., 2007). Further, the previous studies tested heat, NaCl, NaOCl or H_2O_2 concentrations that were either higher or lower than the concentrations used for point-of-use disinfection of pathogens in domestic and healthcare settings. Thus, the previous findings are only relevant to the respective bacterium and may not inform on general bacterial biofilm disinfection. Although *S. epidermidis* biofilm is model bacterial biofilm, the most clinically relevant bacterial biofilm (Otto, 2009; Decker *et al*., 2015) and a major disseminator of antimicrobial tolerance genes among bacterial pathogens (Fey & Olson, 2010; Aguila-Arcos *et al*., 2017), its susceptibility patterns to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 remain undocumented. This information is necessary in improving the physico-chemical disinfection guidelines to better control *S. epidermidis* biofilm, and by extension reduce the antimicrobial tolerance burden.

Multiple mechanisms have been proposed to explain the high tolerance of bacterial biofilms against antimicrobials. These include, reduced diffusion of antimicrobials through the ECM (Joo & Otto, 2012), neutralization of the antimicrobials by the ECM components (de la Fuente-Núñez *et al*., 2013), the physiological heterogeneity provided by the three-dimensional biofilm structure (Acker *et al*., 2014), higher expression of specific protective molecules (Joo & Otto, 2012), or the presence of highly resistant subpopulation of biofilm cells (persisters) (de la Fuente-Núñez *et al*., 2013). However, these mechanisms not only provide a partial explanation for the increased tolerance of bacterial biofilms against few antibiotics, such as ciprofloxacin, tetracycline and β-lactams, but also apply to a limited bacterial biofilm species (Joo & Otto, 2012; Hall & Mah, 2017). The mechanisms underlying the tolerance of *S. epidermidis* biofilm against heat, NaCl, NaOCl or H_2O_2 exposure are unknown. Thus, studies exploring the potential mechanisms underlying tolerance of bacterial biofilm against antimicrobials are needed to develop more potent bacterial biofilm eradication strategies.

Emerging line of evidence have shown that extracellular DNA (eDNA) of genomic origin is involved in the tolerance of microbial biofilms against stressors. For instance, two studies showed that eDNA is involved in the tolerance of *S. epidermidis* biofilm against vancomycin (Kaplan *et al*., 2011; Doroshenko *et al*., 2014). Moreover, Kaplan *et al*. (2012) showed the role of eDNA in *Staphylococcus aureus* planktonic cells' tolerance against β-lactam antibiotics. Further, Hathroubi *et al*. (2015) linked eDNA to *Actinobacillus pleuropneumoniae* biofilm's tolerance against penicillin G. The eDNA was also linked to *Candida albicans* (fungal) biofilm's tolerance against H_2O_2 exposure (Pemmaraju *et al*., 2016). The previous studies focused on the role of eDNA in the tolerance against conventional antibiotics, such as vancomycin, β-lactams and penicillin G. Further, only one of the previous studies reported on the role of eDNA in the tolerance against H_2O_2, but in a fungal biofilm. The potential role of

eDNA in the tolerance of bacterial biofilms against heat, NaCl, NaOCl or H_2O_2 exposure remains unknown. Therefore, understanding the role of eDNA in the tolerance of bacterial biofilms, using *S. epidermidis* biofilm, which is a model bacterial biofilm (Decker *et al*., 2015), major disseminator of antimicrobial tolerance genes (Águila-Arcos *et al*., 2017) and the most medically relevant bacterial biofilm (Otto, 2009), is necessary. This could inform on the design of more potent disinfection approaches against *S. epidermidis* biofilm and other bacterial biofilms; hence, reducing the burden and spread of antimicrobial tolerance genes among pathogenic bacteria.

Alternative sigma factor B (σ^B), a sub-unit of RNA polymerase (Paharik & Horswill, 2016), has been implicated in the tolerance of some bacterial species against stressors. For instance, σ^B has been implicated in the tolerance of *S. aureus* (Chan *et al*., 1998; Cebráin *et al*., 2009), *Bacillus cereus* (Schaik *et al*., 2004), *Bacillus subtilis* (Voelker *et al*., 1999) and *Listeria monocytogenes* (Becker *et al*., 1998; Abram *et al*., 2008) against heat and/or H_2O_2 exposure. In addition, studies have linked σ^B to the tolerance of bacteria to antibiotics e.g. vancomycin, tetracycline and β-lactams (Chen *et al*., 2011; Poole, 2012). The previous studies only focused on the planktonic forms of the bacterial species hence may not inform on the bacterial biofilm's response against heat, NaCl, NaOCl or H_2O_2-exposure. Although bacterial biofilms are a major public health burden (Paharik & Horswill, 2016), the role of σ^B in their tolerance against physico-chemical stress exposure remains unexplored. Thus, understanding the role of σ^B in the tolerance of bacterial biofilms using *S. epidermidis* biofilm, which is a model bacterial biofilm (Decker *et al*., 2015), key disseminator of antimicrobial tolerance genes (Águila-Arcos *et al*., 2017) and the most medically relevant bacterial biofilm (Otto, 2009), is necessary. This information may improve on the control of *S. epidermidis* biofilm and other

bacterial biofilms and by extension antimicrobial tolerance burden by targeting a single stress regulator, σ^B.

In *S. epidermidis*, a gene encoding an alkaline shock protein 23 (*asp23*), is transcribed from at least two different σ^B-dependent promoters and is expressed as a direct function of σ^B activity making it a good marker for σ^B activity (Knobloch *et al*., 2004; Mitchell *et al*., 2013).

To better understand the susceptibility patterns and the mechanisms underlying the tolerance of *S. epidermidis* biofilm against physico-chemical stress exposure, the susceptibility patterns, eDNA yield and *asp23* expression of *S. epidermidis* biofilm and planktonic cells (utilized as control samples) in response to heat, NaCl, NaOCl or H_2O_2 exposure were compared.

1.2. Statement of the problem

Kisumu county has a high burden of infections associated with multi-drug resistant biofilm-forming bacteria. Moreover, there is high circulation of antibiotic-resistant genes and isolates in Kisumu county. Thus, there is need to control the burden and spread of antimicrobial tolerance in Kisumu county. As a reservoir of antimicrobial-tolerance genes and horizontal gene transfer elements, *S. epidermidis* biofilm is a key disseminator of antimicrobial tolerance genes among bacterial pathogens in Kisumu county hence, is an important target in the fight against antimicrobial tolerance. Due to the high proclivity of bacteria to develop resistance to antibiotics, *S. epidermidis* biofilm control using effective physico-chemical disinfection may be a suitable approach, necessitating a better understanding of the susceptibility and mechanism of tolerance of *S. epidermidis* biofilm against physico-chemical disinfection. However, the susceptibility patterns and the underlying mechanisms of tolerance of *S. epidermidis* biofilm against heat, NaCl, NaOCl or H_2O_2 used for point-of-use disinfection of food, drinking water and/or medical equipment in Kisumu county are unknown. Thus, this study determined the susceptibility patterns and the activities of eDNA and σ^B as tolerance

mechanisms of *S. epidermidis* biofilm against heat, NaCl, NaOCl or H_2O_2 exposure. The findings could inform on the design of more potent disinfection strategies against *S. epidermidis* biofilm; hence, reducing the burden and spread of antimicrobial tolerance genes in Kisumu county and many places.

1.3. Study objectives

1.3.1. General objective

To determine the susceptibility patterns and tolerance mechanisms of *S. epidermidis* biofilm and planktonic cells to physico-chemical stress exposure.

1.3.2. Specific objectives

1. To determine the susceptibility patterns of *S. epidermidis* biofilm and planktonic cells to physico-chemical stress (heat, NaCl, NaOCl or H_2O_2) exposure.
2. To determine the relative eDNA release by *S. epidermidis* biofilm and planktonic cells in response to physico-chemical stress (heat, NaCl, NaOCl or H_2O_2) exposure.
3. To determine the relative σ^B activity in *S. epidermidis* biofilm and planktonic cells in response to physico-chemical stress (heat, NaCl, NaOCl or H_2O_2) exposure.

1.3.3. Null hypotheses

1. There is no significant difference in the susceptibility patterns of *S. epidermidis* biofilm and planktonic cells to physico-chemical stress (heat, NaCl, NaOCl or H_2O_2) exposure.
2. There is no significant difference between the relative eDNA release by *S. epidermidis* biofilm and planktonic cells in response to physico-chemical stress (heat, NaCl, NaOCl or H_2O_2) exposure.
3. There is no significant difference between the relative σ^B activity in *S. epidermidis* biofilm and planktonic cells in response to physico-chemical stress (heat, NaCl, NaOCl or H_2O_2) exposure.

1.4. Justification of the study

Bacterial biofilms are a public health menace. Bacterial biofilms are ubiquitous in virtually all environments with higher prevalence in domestic and healthcare settings (Garrett *et al*., 2008; Francolini *et al*., 2010). The *S. epidermidis* biofilm is highly resistant to antibiotics and host immune effectors (Fey & Olson, 2010). Moreover, *S. epidermidis* biofilm is the predominant cause of recurrent and relapsing infections hence cause significant burden in human healthcare systems (Fey & Olson, 2010; Paharik & Horswill, 2016). Treatment of *S. epidermidis* biofilm-mediated infections typically involves removal and replacement of the infected device, causing an increase in morbidity and cost (Fey & Olson, 2010). Further, *S. epidermidis* biofilm infection is predominantly associated with nosocomial infections that affect seven and ten out of one hundred hospitalized patients in the developed and developing countries respectively (Francolini & Donelli, 2010; WHO, 2014). Annually, *S. epidermidis* biofilm-associated nosocomial infections account for thousands of deaths in the developed and developing countries (WHO, 2014). Further, *S. epidermidis* biofilm is a major disseminator of antimicrobial-tolerance genes among bacterial pathogens in domestic and healthcare settings (Fey & Olson, 2010; Águila-Arcos *et al*., 2017). Considering the public health threat posed by *S. epidermidis* biofilm, effective point-of-use disinfection of *S. epidermidis* biofilm is necessary. Thus, the present study sought to understand the susceptibility patterns and the mechanisms employed by *S. epidermidis* biofilm against heat, NaCl, NaOCl or H_2O_2 used for point-of-use disinfection in domestic and healthcare systems. These findings could inform on the development of more potent eradication approaches against *S. epidermidis* biofilm leading to a reduction of the *S. epidermidis* biofilm-associated problems and the spread of antimicrobial-tolerance genes among bacterial pathogens.

1.5. Significance of the study

First, the finding that *S. epidermidis* biofilm was more tolerant to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 used for point-of-use disinfection in Kisumu county should prompt the public health policy makers to review and improve the current disinfection guidelines to target the bacterial biofilm growth mode. Secondly, the findings that eDNA and σ^B might be involved in the tolerance of *S. epidermidis* biofilm against heat, NaCl, NaOCl or H_2O_2 exposure advances the understanding on bacterial biofilm tolerance mechanisms. Thus, eDNA and σ^B could be explored as promising targets for the development of effective anti-*S. epidermidis* biofilm approaches by the molecular biologists and pharmaceutical companies.

1.6. Limitations of the study

The present study has two potential limitations. First, the susceptibility of *S. epidermidis* biofilm and planktonic cells were determined by the standard plate count method. However, the standard plating method has a narrow optimal countable colony range of 25-400 depending on the aliquot dilution factor and plate size (Ben-David & Davidson, 2014). The computation of log reductions from such narrow ranges of colony forming-units (CFU) results in values not exceeding 2.6. This implies that the low log reduction values obtained in the present study might be partly attributed to the narrow colony counting range. Second, due to resource constraints and the multiplicity of physico-chemical agents used in the present study, a relatively small but informative samples of *S. epidermidis* biofilm and planktonic were used for analysis of eDNA release and σ^B activity.

CHAPTER TWO

LITERATURE REVIEW

2.1. The *S. epidermidis* and bacterial biofilm formation, growth and development

Bacteria from the genus *Staphylococcus* encompass a diverse group of Gram-positive commensals that colonize mammals on the skin or mucous membranes (Paharik & Horswill, 2016). The genus *Staphylococcus* comprises forty-seven species: eight of which are coagulase-positive or coagulase-variable, thirty-eight of which are coagulase-negative and one that has both a coagulase-negative and a coagulase-positive sub-species (Becker *et al*., 2014). Within the coagulase-negative staphylococci, *S. epidermidis* is the most frequent cause of medical device-related infections, and is able to infect virtually any medical implant (Otto, 2009; Freitas *et al*., 2014). The *S. epidermidis* has a high rate of infection because of its prevalence in the normal skin flora and ability to colonize many human body surfaces, such as the anterior nares, axillae inguinal and perineal areas (Becker *et al*., 2014; Paharik & Horswill, 2016). Majority of the frequently isolated *S. epidermidis* are of sequence type 2 that exhibit *in vitro* biofilm-formation and belongs to the clonal complex 2 class (Otto, 2009). The *S. epidermidis* is the predominant cause of infections that affect seven and ten out of one hundred patients hospitalized in developed and developing countries respectively (Francolini & Donelli, 2010; WHO, 2014). The biofilm-forming capability of *S. epidermidis* is its main virulence factor (Fey & Olson, 2010). The *S. epidermidis* biofilm is a reservoir of antibiotic tolerance and horizontal transfer genes, as well as conjugative and/or mobilizable plasmids, suggesting its key role in dissemination of antibiotic resistance and tolerance determinants among bacterial pathogens (Fey & Olson, 2010; Águila-Arcos *et al*., 2017). Therefore, *S. epidermidis* biofilm is an important target in the control of the spread of antimicrobial tolerance.

A switch to the bacterial biofilm growth mode is associated with enhanced regulation of gene expression levels resulting in temporal adaptations (Garrett *et al*., 2008). Bacterial biofilm cells communicate via quorum sensing (Irie & Parsek, 2008). Bacterial biofilms produce ECM variably composed of proteins, nucleic acids, polysaccharides and water (Jamal *et al*., 2015). Bacterial biofilm formation involves at least three stages. First, initial attachment of cells to abiotic or biotic surface aided by bacterial adhesins. Second, proliferation and maturation mediated by cell-cell adhesion. Third, detachment mediated by ECM-degrading enzymes originating from the bacteria or environment (Joo & Otto, 2012; Paharik & Horswill, 2016). Microcolony formation is considered to lie between attachment and maturation, but the differences between microcolony and mature biofilm are not well defined (Paharik & Horswill, 2016). Moormeier *et al*. (2014) showed that attachment and early accumulation were succeeded by dispersal of a portion of the bacterial cells, leaving behind small foci of biofilm growth. These foci then mature into a bacterial biofilm with the characteristic tower structures. Further, the early dispersal phase referred to as “exodus” was independent of the accessory gene regulator (*agr*) system, a peptide quorum-sensing system present in all the staphylococci. However, the exodus phase was specifically modulated by the *sae*-regulated nucleases (Figure 1).

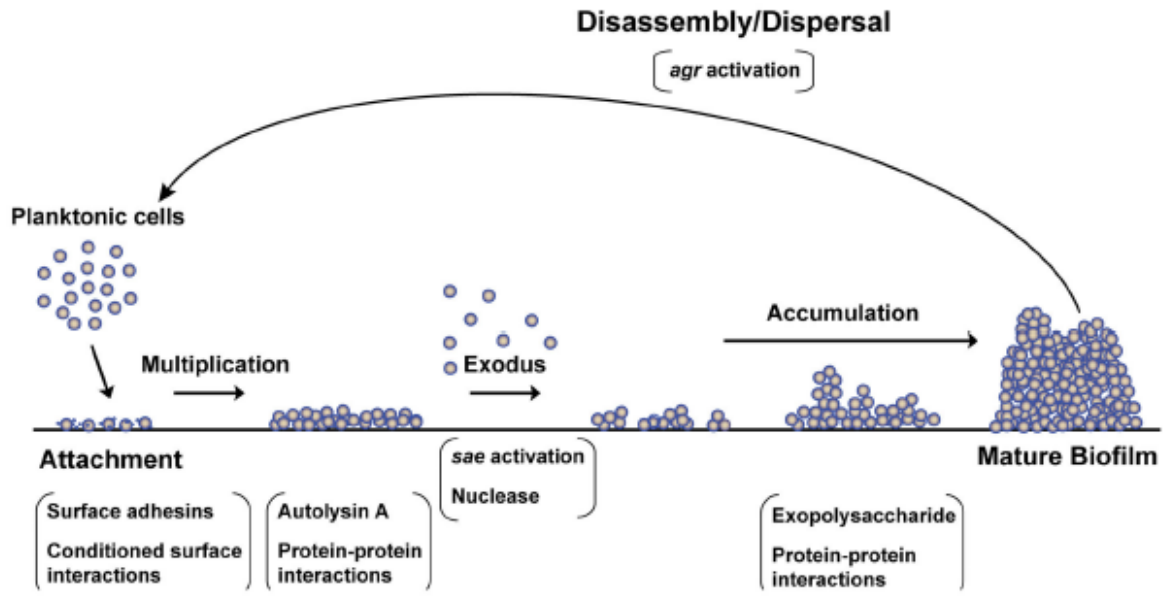

Figure 1. Bacterial biofilm development stages. After attachment, bacterial cells form a lawn of growth, which undergoes an exodus period leaving several small foci of cells. The exodus phase is mediated by the *Sae*RS system via nuclease activity. Then, foci of cells develop into a characteristic mature biofilm structure. Dispersal is mediated by the *agr* system via secreted enzymes and phenol-soluble modulins (Adapted from Paharik & Horswill, 2016).

2.2. Antimicrobial tolerance in Kisumu county and *S. epidermidis* biofilm

Kisumu county has a high prevalence of pathogens in its soils and surface water (Baker *et al*., 2018). Kisumu county, therefore, suffers a high burden of communicable bacterial infections, such as diarrhoea, tuberculosis, typhoid (Odongo *et al*., 2017), which are associated with bacterial biofilm-formers (Tan *et al*., 2014). Further, Kisumu county has a high variety of resistance genes, large number of isolates harbour five or more of the resistance genes and has a high prevalence of multi-drug resistant phenotypes (Taitt *et al*., 2017). Being a hyper-endemic HIV area (NACC, 2016), Kisumu county has a high number of immunocompromised people, who facilitate both evolution of resistant pathogens and their rapid spread in the community (Kariuki & Dougan, 2014). There is high use of antibiotics in the hospitals within Kisumu enhancing the development of resistance phenotypes (Okoth *et al*., 2018). The *S. epidermidis* biofilm is responsible for dissemination of antimicrobial tolerance genes among bacterial pathogens (Fey & Olson, 2010; Aguila-Arcos *et al*., 2017);

hence, is an important target in the fight against antimicrobial tolerance in many areas of the world, including Kisumu county.

2.3. Point-of-use physico-chemical disinfectants in domestic and human healthcare settings

Point-of-use treatment of drinking water mainly involves several methods aimed at destroying all harmful organisms. Boiling is the most effective water disinfection method irrespective of water turbidity (Sobsey & Leland, 2001). Water is brought to a ‘’rolling boil’’ for 1-3 min depending on the altitude (Kayaga & Reed, 2011). However, heating water to pasteurization temperatures (generally 55-60°C) for periods of minutes (min) to tens of min will destroy most waterborne pathogens of concern (Sobsey, 2002). However, boiling or heating water is energy consuming and changes the taste of water (Kayaga & Reed, 2011). Solar disinfection using ultra violet (UV) rays from the sun or modern UV lamps is also used in water disinfection (Sobsey, 2002; Kayaga & Reed, 2011). However, the particulates and turbidity can interfere with or reduce microbial inactivation efficiency. The UV lamps require electricity and must be replaced periodically hence expensive (Sobsey *et al*., 2002). Due to the challenges of boiling and UV disinfections, chemical disinfection using chlorine compounds are used (Kayaga & Reed, 2011). A dilute (1.2%) NaOCl solution for point-of-use water treatment (waterguard) is recommended in most resource-limited settings in Kenya (including Kisumu county) (Blum *et al*., 2014).

Effective disinfection is essential for ensuring medical equipment do not transmit infectious pathogens to patients (Rutala & Weber, 2004). The following disinfectants have been used for medical equipment disinfection: alcohols, chlorine and chlorine compounds, formaldehyde, glutaraldehyde, *ortho*-phthalaldehyde, H_2O_2, iodophors, peracetic acid, phenolics and quaternary ammonium compounds (Rutala & Weber, 2004; Rutala *et al*., 2008). The

concentration of each of the above disinfectant is dependent on where it is being used (Rutala & Weber, 2004). Further, with exception of H_2O_2 and chlorine compounds, most of these products have adverse side effects hence are not commonly used (Rutala & Weber, 2004; Rutala *et al*., 2008; Linley *et al*., 2012).

Several methods have been used to disinfect foods or food contact surfaces. Disinfectants of food contact surfaces contain chlorine compounds, peroxide and peroxyacid mixtures, carboxylic acids, quaternary ammonium compounds, acid anionic or iodine compounds (Gaulin *et al*., 2011). The food is disinfected/preserved using methods, such as canning, drying, application of sugar, pickling, smoking and salting. Of these methods, salting using 1.72 M NaCl is commonly used in most domestic settings for food preservation (Smith & Stratton, 2007).

In summary, the following are the physico-chemical disinfectants commonly used for point-of-use disinfection of water, food and/ or medical equipment in domestic and human healthcare settings in Kisumu county and other places: heat (60°C) (Sobsey, 2002), 1.72 M NaCl (Smith & Stratton, 2007), 0.178 M NaOCl (Blum *et al*., 2014) or 1.77 M H_2O_2 (MOH, 2007; Linley *et al*., 2012).

2.4. Susceptibility patterns of bacterial biofilms to physico-chemical stress exposure

2.4.1. Assessment of the previous studies on susceptibility patterns of microbial biofilm and planktonic cells to physico-chemical stress exposure

The comparison of the susceptibility patterns of biofilm and planktonic forms of bacterial species to various physico-chemical stress exposures have been reported. For instance, McAuliffe *et al*. (2006), reported that *M. bovis* biofilm subjected to 50°C for 40 min were more tolerant than the planktonic forms. However, in the study, *M. bovis* cells were exposed to 50°C, which is below 60°C recommended for pathogen elimination (Sobsey, 2002).

Further, unlike many bacterial species, *M. bovis* is cell wall-less (Brooks *et al*., 2007). Thus, the findings on *M. bovis* may not inform on the response of other bacterial biofilms to heat exposure and have limited application in general bacterial biofilm control. Another study showed that *S. enterica* serovar Typhimurium biofilm growing at the air-liquid interface were more tolerant to exposures to NaOCl concentrations ranging from 50 to 250 parts per million and heat (60°C and 70°C) than the planktonic cells (Scher *et al*., 2005). However, Scher and colleagues focused on the application of NaOCl on industrial settings (Scher *et al*., 2005) hence used NaOCl concentrations higher than 0.178 M NaOCl used in treatment of drinking water (Blum *et al*., 2014). Furthermore, unlike most bacterial species, *S. enterica* serovar Typhimurium overproduce protective cellulosic polymer (Zogaj *et al*. (2001); hence, the findings may not inform on the general bacterial biofilm control. Further, *Cryptococcus neoformans* biofilm cells were found to be more tolerant to 47°C exposure for 30 min than the planktonic forms (Martinez & Casadevall, 2007). However, *C. neoformans* is a fungal pathogen; hence, may not inform on bacterial biofilm response to heat exposure. Moreover, the temperature (47°C) used is below 60°C recommended for pathogen elimination (Sobsey, 2002).

A study reported that *V. cholerae* O1 biofilm cells were more tolerant to 2.5 molar (M) NaCl and 20 mM H_2O_2 exposures than the planktonic forms (Wai *et al*., 1998). However, the 2.5 M NaCl and 20 mM H_2O_2 used were respectively, far above and below 1.72 M NaCl and 1.77 M H_2O_2 recommended for routine pathogen disinfection (Smith & Stratton, 2007; Linley *et al*., 2012). Further, the previous study used *V. cholerae* O1, which is highly adapted to saline environments relative to most bacterial pathogens (Filho *et al*., 2011). Thus, the report by Wai *et al*. (1998) may not inform on the general bacterial biofilms response to NaCl and H_2O_2 exposure.

A report by Kubota *et al*. (2009), demonstrated that *L*. *plantarum* subsp. *plantarum* biofilm cells exposed to 10-275 parts per million of NaOCl for 30 min were more tolerant than the planktonic forms. However, the concentrations of NaOCl used were high and are mostly used in industrial settings (Scher *et al*., 2005), but not in domestic and human healthcare settings. Behnke *et al*. (2011) also reported that a co-culture of *B. cepacia* and *P. aeruginosa* biofilms was more tolerant to chlorine exposure than the planktonic forms. However, multi-species bacterial biofilms are generally more resistant than mono-species bacterial biofilms (Giaouris *et al*., 2015) thus; the report on co-culture of *B. cepacia* and *P. aeruginosa* biofilms may not inform on susceptibility of individual bacterial species biofilms. Steed and Falkinham III (2006) also reported that biofilm forms of *M*. *avium* and *M*. *intracellulare* exposed to 1 µg of chlorine/mL for 6 hours (h) were more tolerant than the planktonic forms. Unlike *S. epidermidis* and most bacterial species, mycobacteria have mycolic acid-rich membranes that enhance tolerance (Brooks *et al*., 2007; Abdallah *et al*., 2014) hence may not inform on general bacterial biofilms response to chlorine compounds. Stewart *et al*. (2001) reported that biofilm forms of *P*. *aeruginosa* or *K*. *pneumoniae* cells exposed to 1000 mg/L alkaline hypochlorite for 1 h were more resistant than the corresponding planktonic forms. Bacterial biofilms are known to mature within 24-48 h (Pintens *et al*., 2008); however, in the study reported by Stewart and colleagues, the *P*. *aeruginosa* and *K*. *pneumoniae* cells were overgrown (6 days) and accumulated high proteins and carbohydrates, which might have affected susceptibilities of the bacterial species to hypochlorites. Further, Peeters *et al*. (2008a) showed that *B. cenocepacia* biofilm cells were more tolerant to H_2O_2 (0.3-3%) and NaOCl (0.05-0.3%) than the planktonic cells. However, the H_2O_2 and NaOCl concentrations tested were lower than the 0.178 M NaOCl or 1.77 M H_2O_2 routinely used for point-of-use disinfection of drinking water (Linley *et al*., 2012; Blum *et al*., 2014) hence may not inform

on bacterial biofilm response to the oxidative stresses. Further, Elkins *et al*. (1999) showed that biofilm cells of *P*. *aeruginosa* exposed to 50 mM H_2O_2 stress for 1 h were more tolerant than the planktonic cells. Similarly, Zhang *et al*. (2007) showed that *E*. *coli* biofilm cells exposed to 20 mM H_2O_2 for 15 min were more tolerant than the planktonic cells. However, the two studies used 20 and 50 mM H_2O_2 that are below the recommended 1.77 M H_2O_2 (Linley *et al*., 2012) concentrations hence may not inform on bacterial biofilm disinfection efficiency.

Taken together, the susceptibilities of various species of bacterial biofilms to physico-chemical stress exposures have been reported. However, the previous studies reported on *Mycoplasmas*, *Mycobacteria* and *Salmonella* species, which are structurally or metabolically different from most bacterial species (Zogaj *et al*., 2001; Brooks *et al*., 2007). Further, the previous studies tested NaCl, NaOCl or H_2O_2 concentrations that were either higher or lower than the concentrations used for point-of-use disinfection of pathogens. Thus, the findings are only relevant to the respective bacterium and may not inform on general bacterial biofilm disinfection. However, despite *S. epidermidis* biofilm being a model bacterial biofilm and the most medically relevant bacterial biofilm (Otto, 2009; Decker *et al*., 2015), its susceptibility patterns to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 routinely used for disinfection in domestic and healthcare settings remain undocumented. This may inform on the effective control of diverse bacterial biofilms using physico-chemical disinfectants.

2.4.2. Overview of techniques for assessing susceptibilities of bacterial biofilms to stressors

Fluorescence-based methods, quantitative (q)-PCR, spectrophotometry, flow cytometry and plating have been used to quantify bacterial biofilm cells in susceptibility studies.

Fluorescence-based methods combined with automatic counting software are more precise, reliable and are unaffected by the user-to-user interpretation variations (Freitas *et al*., 2014). However, the techniques utilize SYTO 9 stain that is expensive and does not properly stain Gram-negative bacteria (Stiefel *et al*., 2016). In addition, the method uses propidium iodide (PI) that stains eDNA thus may overestimate the bacterial biofilm cell counts (Peeters *et al*., 2008b).

Spectrophotometry technique is used because of the simplicity of the protocol and easy optical visualization (Stiefel *et al*., 2016). However, the technique has low reproducibility, sensitivity and specificity (Pantanella *et al*., 2013). Moreover, ethanol used in the protocol does not extract the dye uniformly resulting in significant variations of the bacterial biofilm counts between and within experiments (Pitts *et al*., 2003).

A combination of qPCR together with an intercalating agent, propidium monoazide (PMA) has been used to quantify oral multi-species biofilms (Álvarez *et al*., 2013). In this technique, the PMA selectively penetrates damaged cell membranes and binds to their double-stranded (ds) DNA. The q-PCR-PMA does not overestimate cell counts (Nocker *et al*., 2007). However, the technique requires expensive PCR reagents, equipment and highly skilled personnel (França *et al*., 2012). Furthermore, complex sample preparation, primer design, optimization and interpretation of results limit its application (Pantanella *et al*., 2013).

Flow cytometry techniques utilize a combination of dyes e.g. SYTO 9 that is membrane-permeable and thus stains live and dead cells or membrane-impermeable dyes e.g. PI that stain DNA of damaged cells (Khan *et al*., 2010). Flow cytometry may help distinguish between viable but non-culturable (VBNC) cells and viable bacterial cells and produce rapid results (Khan *et al*., 2010). However, the use of PI may lead to overestimation of bacterial biofilm cells count since the dye stains both intracellular DNA and eDNA. Moreover, it is

difficult to separate bacterial biofilm cells cluster into individual cells making application of flow cytometry in bacterial biofilm quantification difficult (Ambriz-Aviña *et al*., 2014). In addition, flow cytometry requires expensive equipment and SYTO 9 dye. The technique also requires highly skilled personnel (Ambriz-Aviña *et al*., 2014).

Standard plating method is widely used because it is highly sensitive, reliable, inexpensive and readily available in most laboratories (Pan *et al*., 2014). However, the method only quantifies viable bacteria and has a narrow optimal countable colony range of 25-400 depending on the aliquot dilution factor and plate size (Ben-David & Davidson, 2014). Moreover, standard plating method cannot detect injured cells hence may underestimate bacterial cell count (Simões *et al*., 2005). Despite the shortcomings, the standard plating method is suitable for estimating the susceptibility differences between bacterial biofilm and planktonic cells (Simões *et al*., 2010).

2.5. Overview of tolerance mechanisms of bacterial biofilms against antimicrobial exposure

Generally, bacterial biofilms are highly tolerant to antimicrobials compared to their analogous planktonic forms (Paharik & Horswill, 2016). Multiple mechanisms have been proposed to explain the higher tolerance of bacterial biofilms against antimicrobials as described below.

First, the biofilm matrix may provide a diffusion barrier against (or reacts with) antimicrobial agents (reaction-diffusion inhibition) (Joo & Otto, 2012; Simões & Simões, 2013). The decreased diffusion ensures that the bacterial cells are initially exposed to a low concentration of the antimicrobial and may have time to mount a defensive response (Acker *et al*., 2014). Second, the physiological heterogeneity within the three-dimensional biofilm structure (Hall & Mah, 2017). The physiological heterogeneity creates differences in gene expression, metabolic activity and phenotype, including antimicrobial tolerance, of cells located in

different geographical areas of a biofilm (Hall & Mah, 2017). Heterogeneity arises due to the gradient of oxygen and other nutrients within the biofilm (Acker *et al*., 2014; Hall & Mah, 2017). Third, a phenomenon that contributes significantly to antibiotic tolerance in biofilms is persistence, a property of the persister cells (extremely resistant subpopulation of biofilm cells), which are more numerous in biofilms than in the planktonic populations (de la Fuente-Núñez *et al*., 2013; Hall & Mah, 2017). Persister cells can withstand the presence of stressors, likely due to transcriptional programming (de la Fuente-Núñez *et al*., 2013). Four, expression of specific protective molecules may be higher in the biofilm mode of growth, and antibiotics may also directly enhance the expression of protective mechanisms, such as overproduction of neutralizing enzymes that degrade or inactivate antibiotics (Jamal *et al*., 2015) or genes that confer antimicrobial tolerance (de la Fuente-Núñez *et al*., 2013; Hall & Mah, 2017). These and other miscellaneous mechanisms are shown in Figure 2 below.

The above tolerance mechanisms only provide partial explanation for the increased bacterial biofilm tolerance and are limited to few conventional antibiotics e.g. ciprofloxacin (Joo & Otto, 2012; Hall & Mah, 2017). Thus, there is need for exploration of more conclusive mechanisms underlying the tolerance of bacterial biofilms against physico-chemical agents, such as heat, NaCl, NaOCl and H_2O_2, to be able to design more effective bacterial biofilm eradication approaches.

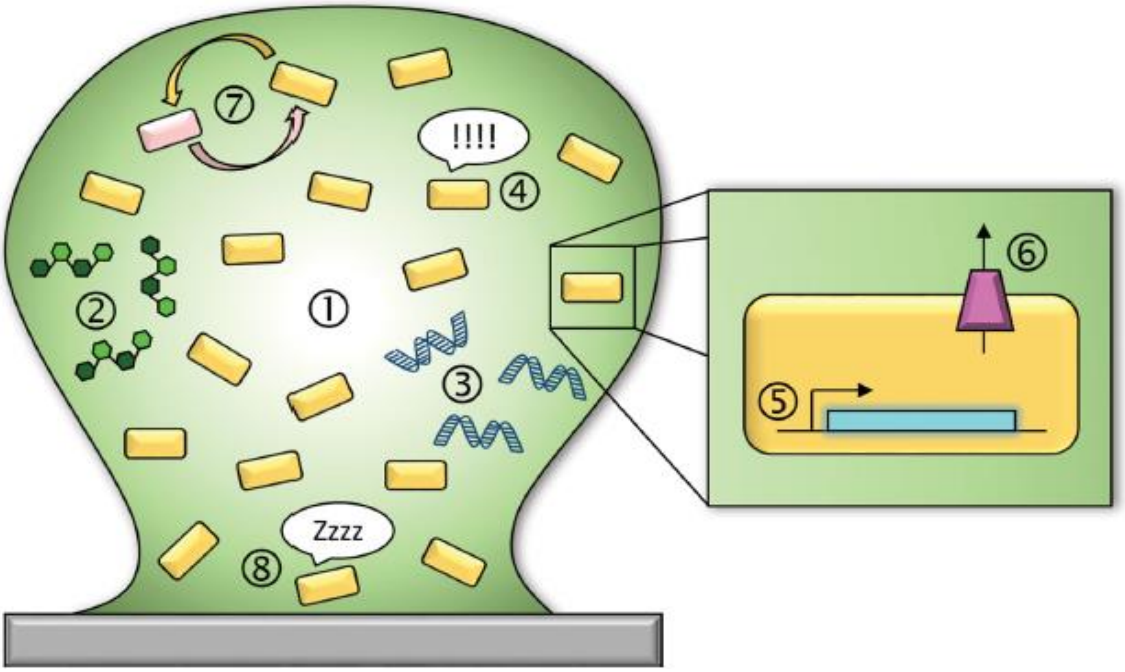

Figure 2. Overview of the major bacterial biofilm tolerance mechanisms against antimicrobials. Biofilm cells (yellow rectangles) are embedded in a mushroom-shaped matrix (shown in green). The biofilm is attached to a surface (grey rectangle). Pictorial representations of the tolerance mechanisms are numbered as follows: (1) nutrient gradient (shown here as a colour-intensity gradient) with less nutrient availability in the core of the biofilm, (2) matrix exopolysaccharides, (3) eDNA, (4) stress responses (oxidative stress response, etc.), (5) discrete genetic determinants that are specifically expressed in biofilms and whose gene products reduce biofilm susceptibility via mechanisms, (6) multidrug efflux pumps, (7) intercellular interactions (horizontal gene transfer, etc.) and (8) persister cells (Adapted from Hall & Mah, 2017).

2.6. The eDNA

2.6.1. The eDNA and assessment of previous studies on eDNA in microbial biofilms tolerance against antimicrobial exposure

Staphylococcal biofilm formation can be polysaccharide intracellular adhesin-dependent or proteins/eDNA-dependent (McCarthy *et al*., 2015). The eDNA is involved in bacterial biofilm adhesion and maintenance of structural integrity (Song *et al*., 2016). The eDNA is either actively secreted or released from bacterial cells by the following mechanisms: autolysis, necrosis, apoptosis and bacterial secretion systems via DNA-containing membrane vesicles (Vorkapic *et al*., 2016). The release of eDNA is largely regulated by autolysin (*atl*) genes (Houston *et al*., 2011). In autolysis, a subpopulation of bacterial biofilm cells are lysed,

releasing DNA (Xu & Kreth, 2013). The eDNA is a major source of substrate for horizontal gene transfer to competent bacterial biofilm cells (Okshevsky & Meyer, 2015). The eDNA can confer antibiotic resistance by binding directly to cationic antibiotics (Jones *et al*., 2013). The eDNA can also indirectly confer resistance by inducing expression of resistance genes (Mulcahy *et al*., 2008; Johnson *et al*., 2013) (Figure 3). The eDNA is essential in bacterial biofilm colonization, virulence and pathogenesis (Zatorska *et al*., 2017).

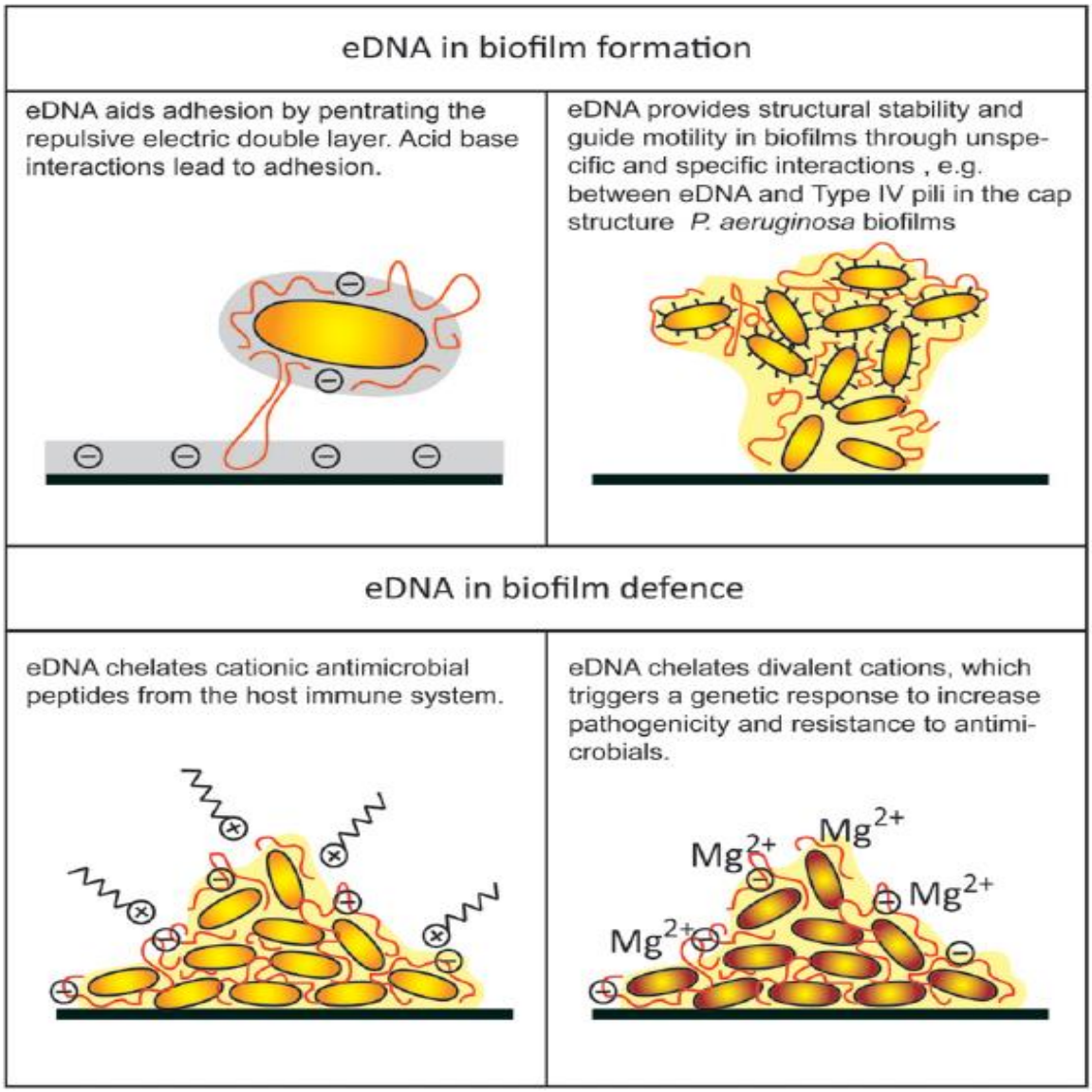

Figure 3. Schematic presentation of the roles of eDNA in bacterial biofilm formation and tolerance to antimicrobials and host immunity (Adapted from Okshevsky & Meyer, 2015).

Few studies have evaluated the role of eDNA in bacterial and fungal cells tolerance against various physico-chemical stress exposures. For instance, a report showed that *C. albicans* biofilm exposed to 2 M NaCl did not produce eDNA richer ECM than the untreated controls. Conversely, 5 mM H_2O_2 exposure led to increased eDNA release into the *C. albicans*' ECM than the untreated controls (Pemmaraju *et al*., 2016). However, *C. albicans* is a fungus hence; the findings may not inform on bacterial biofilms tolerance against NaCl or H_2O_2 exposures. Itzek *et al*. (2011) also reported that *Streptococcus gordonii* cells exposed to 1 mM or 2 mM H_2O_2 for 5 h released more eDNA than the untreated controls. However, Itzek and colleagues only reported the response of planktonic forms of *S. gordonii* to H_2O_2 exposure hence might not shed light on the bacterial biofilm forms.

The release of eDNA by bacterial and fungal cells in response to conventional antibiotics exposure has been reported. To begin with, two studies reported increased eDNA production by *S. epidermidis* biofilm cells exposed to a sub-minimum inhibitory concentration of vancomycin (Kaplan *et al*., 2011; Doroshenko *et al*., 2014). A study by Kaplan *et al*. (2012) also demonstrated that sub-minimum inhibitory concentration of β-lactam antibiotics induced eDNA release by *S. aureus* strains. However, the above studies utilized planktonic forms of *S. aureus* and/or used antibiotic hence may not inform on the biofilm response against physico-chemical stress exposure. Further, Hathroubi *et al*. (2015) showed that sub-MIC of penicillin G enhanced release of eDNA in the ECM of *A. pleuropneumoniae* biofilms. However, Hathroubi and co-workers evaluated the response to an antibiotic, but not physico-chemical stress agent. Rajendran *et al*. (2013) also showed that antifungals, such as amphotericin B and caspofungin, enhanced eDNA release in *Aspergillus fumigatus* biofilms than the untreated controls. However, fungal biofilms are structurally and metabolically different from bacterial

cells (Brooks *et al*., 2007); hence, may not inform on the bacterial biofilm response to stressors.

Overall, the previous studies have mainly focused on bacterial biofilms' tolerance against antibiotics and antifungals. Only two studies have examined the role of eDNA in the tolerance against NaCl and/or H_2O_2 exposure. Of the two studies, one was on a fungus *C. albicans* biofilm and the other on planktonic forms of *S. gordonii* hence may not inform on the role of eDNA in bacterial biofilms' tolerance against NaCl or H_2O_2 exposure. The role of eDNA in the tolerance of *S. epidermidis* biofilm or any other bacterial biofilm against physico-chemical stress exposure remains unknown. Thus, there is need to understand the role of eDNA on bacterial biofilms' tolerance against physico-chemical stress exposure using a model bacterial biofilm organism i.e. *S. epidermidis* (Decker *et al*., 2015), which is also a major disseminator of antimicrobial tolerance genes among bacterial pathogens (Águila-Arcos *et al*., 2017). This could inform on the development of more potent eradication approaches against *S. epidermidis* biofilm and other bacterial biofilms; hence, reducing the spread of antimicrobial tolerance genes among bacterial pathogens.

2.6.2. Overview of techniques for quantifying eDNA

Quantification of DNA can be conducted using UV spectroscopy, quantitative real-time PCR (RT-qPCR), fluorometry methods (Oslon & Morrow, 2012), digital PCR and phosphorus analysis (Brennan *et al*., 2009).

In UV spectroscopy, absorption of UV light at a wavelength of 260 nm is measured and the values obtained ranging from 0.1 to 1.0 OD are converted into ng/µL of dsDNA using a conversion factor of 50 ng/µL for 1 OD unit (Holden *et al*., 2009). Microvolume UV spectroscopy instruments, such as NanoDrop spectrophotometers are available. NanoDrop spectrophotometers are faster, user-friendly, require small sample volumes and are non-

destructive (Holden *et al*., 2009; Rothrock, 2011). However, the major limitation of UV spectroscopy and NanoDrop spectrophotometers is that they measure maximal absorbance of nucleic acids; thus, they do not discriminate between dsDNA, single stranded ssDNA, RNA and nucleotides (Nakayama *et al*., 2016).

In RT-qPCR technique, target sequence copy number concentration is measured and then equated to DNA concentration based on genome target sequence copy number and mass (Hospodsky *et al*., 2010). The RT-qPCR specifically quantifies intact and accessible target DNA and not total DNA (Oslon & Morrow, 2012). However, RT-qPCR requires the use of a reference standard that usually introduces uncertainties (Griffiths *et al*., 2011). Moreover, RT-qPCR requires expensive reagents and thermal cyclers (França *et al*., 2012).

In fluorometry methods, fluorescence emission from fluorescently labeled single-stranded DNA or dsDNA is used to estimate DNA concentration (Holden *et al*., 2009). Faith (2008) identified two key advantages of fluorometry equipment such as qubit fluorometers. First, the qubit fluorometers quantify the concentration of a specific molecule of interest i.e. intact dsDNA, RNA or proteins. Secondly, the qubit fluorometers generate accurate and precise data even with highly diluted samples like eDNA in the supernatant.

The digital PCR and phosphorus analysis are rarely used in eDNA quantification. This is majorly because digital PCR and phosphorus analysis require highly specialized equipment and high DNA threshold (500 μg) respectively (Brennan *et al*., 2009).

2.7. The σ^B

2.7.1. The σ^B and assessment of previous studies on σ^B in bacterial cells tolerance against antimicrobial exposure

The RNA polymerase is comprised of a dissociable subunit termed sigma (σ) factor that binds to the core subunits (ββ'α2ω) to form a "holoenzyme" (Paget, 2015). The σ subunit recruits the core RNA polymerase to recognize promoters with specific DNA sequences (Tripathi *et al*., 2014). There are two σ factors in Gram-positive bacteria like *Staphylococcus* species. First, sigma factor A (σ^A) required for housekeeping functions like cellular growth and reproduction. Second, σ^B that mediates specialized functions, such as differentiation, biofilm formation, stress response, pathogenesis (Guldimann *et al*., 2016) and virulence (Nadon *et al*., 2002). The σ^B regulon of *Staphylococcus* species encompasses approximately 200 genes involved in general stress response (Guldimann *et al*., 2016). Tolerance against antibiotics, such as tetracycline, gentamicin, β-lactam and vancomycin, is also linked with σ^B activity (Poole, 2012). Moreover, σ^B is implicated in biofilm formation of *Bacillus*, *Listeria* and *Staphylococcus* species (Savage *et al*., 2013).

The role of σ^B in the tolerance of bacteria against physico-chemical stress exposure has been reported. However, these studies were only limited to planktonic forms of different bacterial species. For example, a study reported that heat shock from 30-42°C and 0.43 M NaCl exposure upregulated σ^B expression in *B. cereus*. Further, a marginal σ^B expression was observed upon exposure of *B. cereus* to 50 μM H_2O_2 (Schaik *et al*., 2004). Voelker *et al*. (1999) also reported that σ^B mutants of *B. subtilis* exhibited 50-100-fold reduced ability to survive 54°C or 1.72 M NaCl exposure. Becker *et al*. (1998) also observed that osmotic upshift increased σ^B activity in *L. monocytogenes*. Further, *S. aureus* σ^B mutants were more sensitive to exposure to 54°C for 10 min and 7.5 mM H_2O_2 compared to the wild type.

Conversely, 1 M NaCl exposure resulted in marginal σ^B activity in the *S. aureus* cells (Chan *et al*., 1998). Abram *et al*. (2008) also showed that σ^B mutant of *L. monocytogenes* was more susceptible to 1.75 M NaCl exposure than the wild type. Cebráin *et al*. (2009) showed that σ^B mutants of *S. aureus* were significantly more susceptible to 58°C and 100 mmol/L H_2O_2 exposure than the wild types.

Although σ^B activity in response to NaOCl stress has not been directly reported, a study showed increased σ^B activity in *L. monocytogenes* biofilm exposed to lethal concentrations of benzalkonium chloride (van der Veen & Abee, 2010) than the planktonic cells. However, benzalkonium chloride and NaOCl have different modes of action. Whereas benzalkonium chloride targets bacterial cytoplasmic membranes (van der Veen & Abee, 2010), NaOCl targets multiple bacterial metabolic processes e.g. adenosine triphosphate (ATP) and DNA synthesis (Rutala & Weber, 2008). Thus, the previous study on benzalkonium chloride may not inform on bacterial biofilm's response against NaOCl exposure.

The above studies only focused on the roles of σ^B in planktonic forms of different bacterial species. However, despite bacterial biofilms being a major public health burden (Paharik & Horswill, 2016); the role of σ^B in their tolerance against physico-chemical stress exposure is yet to be understood. Therefore, understanding the role of σ^B in the tolerance of bacterial biofilms using *S. epidermidis* biofilm, which is a model bacterial biofilm (Decker *et al*., 2015), key disseminator of antimicrobial tolerance genes (Águila-Arcos *et al*., 2017) and the most medically relevant bacterial biofilm (Otto, 2009), is necessary. This information may improve on the fight against bacterial biofilms and antimicrobial tolerance burden.

In *S. epidermidis*, σ^B is an operon comprising a cluster of four genes, namely, *rsbU, rsbV, rsbW* and *sigma B* (Knobloch *et al*., 2004). The gene cluster of the σ^B operon has a σ^A-dependent promoter upstream of *rsbU* and a σ^B-dependent promoter upstream of *rsbV,*

therefore, *sigma B* gene transcription is detectable even in the absence of σ^B activity, making *sigma B* gene an unreliable target for σ^B activity (Knobloch *et al*., 2004). In contrast, *asp23* gene is transcribed from at least two different σ^B-dependent promoters (Knobloch *et al*., 2004). Furthermore, *asp23* is expressed as a direct function of σ^B activity (Mitchell *et al*., 2013) making it a good marker for σ^B activity in *S. epidermidis* (Knobloch *et al*., 2004; Pintens *et al*., 2008).

2.7.2. Overview of techniques for quantifying gene expression

Specific messenger (m) RNA in a sample can be quantified using northern blot analysis, dots/slots analysis, ribonuclease protection assays (RPA) and RT-qPCR (Roth, 2002).

Northern blot analysis has remained a common method for mRNA quantification and detection despite the emergence of superior methods such as dots/slots analysis and RPAs (Perdew *et al*., 2007). This is attributable to the fact that northern blot procedure is straightforward, inexpensive and utilizes common equipment and supplies present in most basic molecular biology laboratories (Roth, 2002; Perdew *et al*., 2007). However, Perdew *et al*. (2007) delineated three cons of northern blot analysis as follows. First, the procedure is prone to ribonuclease contamination that may compromise the quality of data obtained. Second, northern blotting is less sensitive and may not be suitable for rare genes. Last, northern blotting requires a large difference between samples (5- to 10-fold) to be significant.

Dot/slot blot analysis is analogous to the northern blot in most ways except that it has a higher throughput (Roth, 2002). The main advantage of the dot/slot blot is that many samples can be run simultaneously. Moreover, the bands to be analyzed are uniform hence easy to quantify. In addition, the RNA can be of slightly lower quality and still give a detectable signal. However, the sensitivity of dot/slot blot is slightly higher than that of northern blotting but less than that for RPA or RT-qPCR (Perdew *et al*., 2007).

The RPA is a highly sensitive method for the detection and quantitation of specific RNAs in a complex mixture of total cellular RNA. Indeed, more sensitive than the northern blotting (Roth, 2002). However, unlike northern blotting and slot/dot blot, RPA can detect low-abundance genes. In addition, RPA is the method of choice for the simultaneous detection of several RNA species (Perdew *et al*., 2007).

In RT-qPCR, the mRNA is first converted to double-stranded molecule using the enzyme reverse transcriptase (Roth, 2002). Although RT-qPCR is preferred for gene expression analysis, it requires expensive reagents and equipment (França *et al*., 2012) and the procedure is prone to contamination resulting in tube-to-tube variability (Perdew *et al*., 2007). Nevertheless, RT-qPCR has several pros, including, small sample volumes used, detection of small differences in gene expression and analysis of many genes in a large number of samples (Perdew *et al*., 2007). Moreover, RT-qPCR is superbly sensitive, robust and amenable to high-throughput gene expression analysis (Smith & Osborn, 2009). Thus, RT-qPCR is a gold standard method for mRNA quantification (Smith & Osborn, 2009; França *et al*., 2012).

Technically, bacterial RNA is difficult to study due to its short half-life (Atshan *et al*., 2012) and the complicated isolation procedure involved (Stead *et al*., 2012). The RNA isolation methods include enzymatic lysis, sonication, bead beating, cesium chloride precipitation and treatment with guanidine isothiocyanate, phenol and sodium dodecyl sulfate to inhibit RNases (Sung *et al*., 2003). However, all these RNA extraction methods are time-consuming, laborious, and costly and yield small mRNA quantities (Sung *et al*., 2003). Consequently, several commercial RNA extraction kits have been developed (Atshan *et al*., 2012). However, most of the commercial kits are not designed for and do not work well with bacterial biofilm cultures (Atshan *et al*., 2012; França *et al*., 2012). To overcome this challenge, customized RNA isolation protocols such as simple phenol method that are time saving, minimizes DNA

contamination, yields good quality and quantity bacterial biofilm RNA have been developed (Atshan *et al*., 2012) to be used independently or in combination with the commercial RNA extraction kit (Atshan *et al*., 2012; Stead *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study area

The study targeted the residents of Kisumu county, which is located within longitudes 33° 20'E and 35° 20'E and latitudes 0° 20'South and 0° 50'South (Appendix 1). Kisumu County has a population of 1,107,755 (NACC, 2016). Majority of Kisumu county residents (59.9%) seek outpatient services in public health hospitals (MOH, 2014). Kisumu County Referral Hospital (KCRH) is one of the leading referral health facilities in Kisumu county hence gives a representative picture of the county. Further, KCRH is located within the town centre, near main bus park, hence is convenient for many outpatients. To ensure that the samples were representative of Kisumu county and not KCRH, samples from outpatients who had not visited KCRH or any hospital in the preceding three months were collected in November and December 2015.

3.2. Study design

3.2.1. Research design

Study participants were recruited using a systematic random sampling technique for skin swab collection. Matched pair of biofilm and planktonic cells was generated from a single *S. epidermidis* isolate obtained from skin swab of each study participant. Susceptibility patterns of the matched pairs of *S. epidermidis* biofilm and planktonic cells challenged with 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 30 and 60 min were compared. Further, eDNA release and σ^B activity analyses were performed for the pairs of *S. epidermidis* biofilm and planktonic cells exposed to optimal physico-chemical concentrations determined in section 3.5.1 i.e. 50°C, 0.8 M NaCl, 5 mM NaOCl and 50 μM H_2O_2 for 60 min. The *S. epidermidis* biofilm and planktonic cells unexposed to heat, NaCl, NaOCl or H_2O_2 served as

controls. In this study, analogous planktonic cells were used as controls samples. For gene expression analysis, 16S rRNA gene was used as reference for normalization of expression levels of the target gene (*asp23*).

3.2.2. Sample size determination

The desired sample size was determined using Cochran (1963) formula. In Kenya, the overall prevalence of nosocomial (mostly *S. epidermidis* biofilm-related) infections is 4.4% (Ndegwa, 2015).

$$n_0 = \frac{Z^2 pq}{e^2}$$

Where:

n_0 = sample size
Z = standard normal deviate at 95% confidence level (1.96).
p = estimated proportion of biofilm-related infections (0.044).
q = 1 – p (0.956).
e = desired level of precision (0.05).

$$n_0 = \frac{(1.96)^2(0.044)(0.956)}{(0.05)^2} = 64.6$$

Ten percent (6.46) of the calculated sample size was added to accommodate any errors. Therefore, skin swabs from seventy-one outpatients were collected. However, nine of the *S. epidermidis* isolates were found to be non-biofilm forming strains (section 3.3.2) hence could not be included in the study. This reduced the desired sample size by two. However, even with the reduction, the power was still within the acceptable limits as determined by the resource equation method described elsewhere (Charan & Biswas, 2013).

3.2.3. Sampling procedure

Systematic random sampling technique is suitable for sampling outpatients attending a health clinic, where it is not possible to predict in advance, who will be attending (Degu & Tessema, 2005). Thus, in the present study, participants were recruited using systematic random sampling technique as described elsewhere (Degu & Tessema, 2005). Briefly, at the period of

sample collection, KCRH received an average of one hundred and thirty outpatients daily (daily population) from 8.00 am to 5.00 pm. About ten outpatients (daily sample size) were recruited daily for sample collection. To obtain the sampling interval (k^{th}), the daily sample size was divided by the daily population (10/130 = 1/13). Thus, every 13^{th} outpatient was selected as follows. Each day the first participant was chosen by blindly picking one out of thirteen bottle tops numbered one to thirteen. Every 13^{th} outpatient enrolled at the KCRH registry and who met the inclusion criteria below was recruited.

3.2.4. Inclusion criteria

Based on the responses from the questionnaire (Appendix 5), only volunteers who met the following criteria were recruited into the study:

1. A resident from any of the Kisumu sub-counties who had not left the county for at least three months.
2. A person who had not visited KCRH or any other hospital in the preceding three months.
3. Adult aged ≥18-65 years, able to read English or Luo and make informed consent or a child (≥5-17 years) accompanied by a guardian able to make informed consent.
4. A person who had not used antibacterial drugs and/or soaps in the preceding three months.

3.2.5. Exclusion criteria

A volunteer was not eligible for recruitment to the study if:

1. She/he had an underlying skin infection.
2. She/he was immuno-compromised.

3.2.6. Ethical considerations

Use of samples from human participants and all experimental protocols were reviewed and approved by Maseno University Ethics Review Committee (Reference number: MSU/DRPI/MUERC/000187/15) (Appendix 3). The participants were briefed on the aims

and procedures of the study in a private room from where consent was sought and questionnaire administered orally. The participants were also informed that their participation was voluntary. Moreover, the study participant's data were kept in a computer with a password only known by the principal investigator. Further, the participants were assured that the samples would only be utilized for the purposes of the study (See details on appendix 4). In addition, permission to recruit outpatients at the KCRH into the study was granted by the KCRH management. Written informed consents were obtained from all the study participants for sample collection and further analysis.

3.3. Sample collection and processing

3.3.1. Skin swabbing and *S. epidermidis* isolation procedure

Swabbing and isolation of *S. epidermidis* were conducted as described previously (Kloos & MusselWhite, 1975). Briefly, the arm joint of the non-dominating arm of the participant was rubbed vigorously with rotation over approximately 8 cm^2 for 15 seconds using a sterile cotton wool applicator moistened with sterile 0.9% NaCl (Unilab Limited, Nairobi, Kenya). Immediately, the swab was applied on mannitol salt agar (MSA; HiMedia Laboratories Pvt. Limited, Nashik, India) plates by rubbing with rotation over the entire surface and then incubated aerobically at 37°C for 24 h. Control plates were prepared to assure sterility of the cotton wool applicators, 0.9% NaCl and the MSA medium. Identification of *S. epidermidis* was based on colour on the MSA (colourless to pink colonies with no colour change to the MSA) and other tests namely, Gram staining, catalase, coagulase and novobiocin sensitivity and grown on tryptic soy agar (TSA; HiMedia Laboratories Pvt. Limited, Mumbai, India) at 37°C overnight. The *S. aureus* American Type Culture Collection (ATCC) 29213 was used as a reference control strain because it is a good biofilm former of mature biofilms within 24 h (Coraça-Huber *et al*., 2012).

3.3.2. Detection of biofilm-forming ability of the *S. epidermidis* isolates

Biofilm forming ability of *S. epidermidis* isolates were assessed by the tube method (TM) biofilm assay as previously described (Divya & Vyshnavi, 2015). Briefly, 10 mL of TSB (Sigma Aldrich Chemie GmbH, Steinheim, Germany) supplemented with 1% (weight/volume) of glucose (Unilab Limited, Nairobi, Kenya) was inoculated with 100 µL of *S. epidermidis* suspension and incubated at 37°C for 24 h. Tube contents were discarded, washed with 0.9% NaCl (Unilab Limited, Nairobi, Kenya) and dried. Dried tubes were stained with 0.1% crystal violet (Unilab Limited, Nairobi, Kenya) solution. Excess stain was removed and then rinsed with deionized water before drying in an inverted position. A visible film lining the wall and bottom of the tube was indicative of biofilm formation. The *S. epidermidis* suspensions without the film or forming a film only at the liquid-air interface were considered non-biofilm-formers. The procedure was performed in duplicate. Of the seventy-one *S. epidermidis* suspensions, sixty-two exhibited biofilm-forming ability. The rest were non-biofilm-forming strains; hence, were not included in the preceding procedures.

3.3.3. *In vitro* formation of biofilm and planktonic cultures

A pair of *S. epidermidis* biofilm and planktonic cultures was generated as previously described (França *et al*., 2012) with few modifications on the volumes. Briefly, a single colony, from a TSA plate, was inoculated into 2 mL tryptic soy broth (TSB; Sigma Aldrich Chemie GmbH, Steinheim, Germany) and incubated in GallenKamp incubator shaker (Caterpillar test and laboratory equipment, Cleveland, USA) at 37°C with shaking at 120 rpm for 18 h (overnight). To form planktonic culture, 100 µL of overnight bacterial suspension at ~1×10^9 colony-forming units (CFU)/mL concentration (which was prepared by adjusting the optical density (at 600 nm) of the overnight culture to 0.1 or 0.5) was inoculated into 10 mL of TSB (Sigma Aldrich Chemie GmbH, Steinheim, Germany) in a conical polystyrene tube

and incubated at 37°C with shaking at 120 rpm for 18 h. After incubation, the bacterial cells in suspension were centrifuged at 10,000 rpm, 4°C for 10 min. To form biofilm culture, 100 µL of the overnight bacterial suspension at ~1 × 10^9 CFU/mL was inoculated into two conical polystyrene tubes containing 10 mL of TSB supplemented with 1% glucose (Unilab Limited, Nairobi, Kenya), to enhance biofilm formation, and incubated in GallenKamp incubator shaker (Caterpillar test and laboratory equipment, Cleveland, USA) at 37°C with shaking at 120 rpm for 24 h. After incubation, the spent medium in one of the tubes was discarded and the biofilm was rinsed twice with 200 µL of 0.9% NaCl (Unilab Limited, Nairobi, Kenya). Biofilm formation was qualitatively assessed by the TM biofilm assay as described in the preceding section. In case of strong biofilm formation, the spent medium in the parallel second tube was carefully removed, and the biofilm was washed twice with 200 µL of 0.9% NaCl. One mL of 0.9% NaCl was added to the tube and vortexed for 2 min to detach the biofilm cells. The detached biofilm cell suspension was centrifuged at 10,000 rpm, 4°C for 10 min. The biofilm and planktonic cell pellets were suspended in 0.9% NaCl and the densities were adjusted to ~1 × 10^9 CFU/mL.

3.4. Susceptibility patterns of *S. epidermidis* biofilm and planktonic cells to heat, NaCl, NaOCl or H_2O_2-exposure

3.4.1. Exposure of *S. epidermidis* biofilm and planktonic cells to heat, NaCl, NaOCl or H_2O_2

The effectiveness of 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 exposure against biofilm and planktonic cultures were determined as previously described (Stewart *et al*., 2001). Briefly, 1 mL of *S. epidermidis* biofilm or planktonic suspension diluted to an OD_{600} of 0.5 (~1 × 10^9 CFU/mL) was added to 9 mL of 1.72 M NaCl (Unilab Limited, Nairobi, Kenya), 0.178 M NaOCl (Supersleek, Nairobi, Kenya) or 1.77 M H_2O_2 (RFCL Limited, New

Delhi, India) and vortexed for 2 min. For 60°C exposure, 1 mL of ~1 × 10^9 CFU/mL of *S. epidermidis* biofilm or planktonic suspension was added to 9 mL of sterile distilled water and placed in a waterbath model JSWB-11(T) (JS Research Inc, Gongju-city, Korea) at 60°C. At 0, 30 and 60 min of exposure, 1 mL was sampled for CFUs enumeration. To neutralize the NaOCl and H_2O_2-exposed cultures, 0.1% sodium thiosulphate (Unilab Limited, Nairobi, Kenya) was placed in the first dilution tube. For NaCl-exposed cultures, sterile distilled water was used instead of sodium thiosulphate. For 60°C-exposed samples, sterile water at 4°C was placed in the first dilution tube to lower the temperature. For each sample, three repeat experiments were conducted.

3.4.2. Enumeration and normalization of CFUs of *S. epidermidis* biofilm and planktonic cells

The biofilm and planktonic cultures sampled at 0, 30 and 60 min of 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 exposure were enumerated as previously described (Abdallah *et al*., 2014). Briefly, 1 mL biofilm or planktonic cells sampled at 0, 30 and 60 min were serially diluted 8-fold. Then, 100 µL of the 10^{-8} dilution was plated in duplicate on Nutrient agar (HiMedia Laboratories Pvt. Limited, Mumbai, India) and incubated for 20 h at 37°C. The CFUs were counted using Colony Counter SC6 plus (Bibby Scientific Limited, Staffordshire, United Kingdom) and converted into CFU/mL. Then, the CFU/mL was normalized into log reduction of CFU/mL as follows. A log reduction is defined as the negative $\log_{10}$ of the quotient of CFU after treatment and before treatment [$-\log_{10}(CFU_{(after\ treatment)}/CFU_{(before\ treatment)})$] (Stewart *et al*., 2001). A log reduction value is directly proportional to the difference between CFUs after and before treatment.

3.5. Quantification of the effects of heat, NaCl, NaOCl or H_2O_2-exposure on eDNA release by *S. epidermidis* biofilm and planktonic cells

According to Rodrigues *et al*. (2011), bacterial biofilms exhibiting high tolerance to disinfectants should be selected for analyses of the tolerance mechanisms. The magnitude of the log reduction of CFU/mL is directly proportional to tolerance (Stewart *et al*., 2001). Hence, *S. epidermidis* biofilm samples that showed high tolerance (i.e. the *S. epidermidis* biofilm samples with smaller log reduction of CFU/mL values from section 3.4.2 were selected) to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 and the corresponding planktonic samples were selected for eDNA quantification (n = 12) analysis.

3.5.1. Determination of the optimal heat, NaCl, NaOCl or H_2O_2 for analysis of eDNA release and σ^B activity in *S. epidermidis* biofilm and planktonic cells

Optimal condition (i.e. inducing considerable stress to *S. epidermidis* cells without severe growth inhibition) of heat, NaCl, NaOCl or H_2O_2 was determined as described by Munn *et al*. (2008) with modifications on the volumes. Briefly, 150 µL of ~1×10^9 CFU/mL of the pooled *S. epidermidis* biofilm or planktonic culture (prepared by mixing equivalent amount i.e. 150 µL of ~1×10^9 CFU/mL of *S. epidermidis* biofilm or planktonic cells drawn from six random biofilm or corresponding planktonic samples) was inoculated into 1.5 mL of increasing concentrations of NaCl (0-1.8 M), NaOCl (0-10 mM) or H_2O_2 (0-100 µM) and exposed for 60 min. For heat exposure, tubes containing 1.5 mL of sterile distilled water were inoculated with 150 µL of ~1×10^9 CFU/mL of the pooled *S. epidermidis* biofilm or planktonic culture and exposed to increasing temperatures of 0-55°C in a water bath model JSWB-11(T) (JS Research Inc, Gongju-city, Korea) for 60 min. The NaOCl and H_2O_2-exposed cultures were neutralized by 200 µL of 0.1% sodium thiosulphate (Unilab Limited, Nairobi, Kenya). Whereas, NaCl and heat-exposed cultures were neutralized by 200 µL of sterile distilled

water. Planktonic cells were collected by centrifuging the bacteria in suspension at 9,000 rpm for 8 min. Planktonic cells were collected by centrifuging the bacteria in suspension at 9,000 rpm for 8 min. For biofilm cells, the bacteria in suspension were discarded and the biofilm was gently rinsed once with 200 µL of 0.9% NaCl. One mL of 0.9% NaCl was added to the biofilm, vortexed for 2 min then centrifuged at 9,000 rpm for 8 min. The biofilm or planktonic cell pellets were suspended in 1 mL of sterile distilled water and CFUs enumerated using Colony Counter SC6 plus (Bibby Scientific Limited, Staffordshire, United Kingdom). At each temperature/concentration of the physico-chemical disinfectant, three independent experiments were performed with three technical replicates. At each temperature/concentration of heat, NaCl, NaOCl or H_2O_2, three independent experiments were performed with three technical repeats. The following temperature/concentrations of heat, NaCl, NaOCl or H_2O_2 were found to be optimal for analyses of the tolerance mechanisms: 50°C, 0.8 M NaCl, 5 mM NaOCl and 50 µM H_2O_2 (growth reduced by almost 2-fold with reference to the highest CFU value) (Appendix 2).

3.5.2. Exposure of *S. epidermidis* biofilm and planktonic cells to optimal heat, NaCl, NaOCl or H_2O_2 for eDNA quantification

The *S. epidermidis* biofilm (n = 12) and planktonic (n = 12) samples were exposed to 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2 as previously described (Linnes *et al*., 2013) with few modifications on the volumes. Briefly, 200 µL of ~1 × 10^9 CFU/mL of biofilm or planktonic cells were inoculated into 1100 µL of TSB adjusted to 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2, vortexed for 2 min and incubated at 37°C with shaking at 80 rpm for 60 min. For 50°C-exposure, 200 µL of ~1 × 10^9 CFU/mL of biofilm or planktonic cells were inoculated into 1100 µL of TSB and transferred to a water bath at 50°C for 60 min. The effects of the physico-chemical agents were neutralized as described in section 3.4.1.

Untreated controls were set up by inoculating 200 µL of ~1 × 10^9 CFU/mL of biofilm or planktonic cells into 1100 µL of TSB and incubated at 37°C for 60 min. A similar set up incubated at 25°C for 60 min served as control for heat exposure. For eDNA quantification, the eDNA was obtained from the supernatant as described in section 3.5.3. For gene expression analyses, the biofilm or planktonic cells were collected as described in the preceding section, suspended in 0.9% NaCl, adjusted to ~1 × 10^9 CFU/mL and immediately transferred into an equal volume of a 1:1 mixture of ice-cold acetone and ethanol, then kept at −80°C for at least 20 min or until further use.

3.5.3. Isolation of eDNA

To minimize variations associated with DNA precipitation, eDNA was obtained directly from the supernatant (Itzek *et al*., 2011). The eDNA released by the 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2-exposed *S. epidermidis* biofilm (n = 12) and planktonic (n = 12) samples and their unexposed controls were obtained from the supernatant as described previously (Kaplan *et al*., 2012) with few modifications on the centrifugation speed. Briefly, the 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2-exposed culture or the untreated control was centrifuged at 20,000 rpm at 4°C for 20 min. Then, 1 mL of the supernatant was pipetted into 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and centrifuged at 13,000 rpm for 3 min. Finally, 30 µL of the supernatant was suspended in 100 µL of TE buffer.

3.5.4. Quantification of eDNA

The eDNA in the supernatant was quantified using Qubit™ dsDNA high sensitivity (HS) assay kit (Invitrogen, Paisley, United Kingdom) and Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA) following the manufacturers' instructions. Briefly, Qubit working solution was prepared by diluting 1 µL of Qubit™ dsDNA HS reagent (Molecular Probes Inc., Willow Creek Road Eugene, Oregon) with 199 µL of Qubit™ dsDNA HS buffer

(Invitrogen, Paisley, United Kingdom) in a plastic tube. Then, 2 µL of the supernatant was added to 198 µL of the working solution in a plastic tube, vortexed for 3 seconds and incubated at 25°C for 2 min. The tube was loaded into a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA) to quantify eDNA in ng/µL. For each sample, three repeat measurements of eDNA quantity were performed. For each sample, the percentage change in eDNA yield was expressed by $100 \times [\{eDNA_{(exposed\ cells)} - eDNA_{(unexposed\ control)}\}/eDNA_{(unexposed\ control)}]$ was computed for *S. epidermidis* biofilm and planktonic cultures.

3.6. Quantification of the effects of heat, NaCl, NaOCl or H_2O_2-exposure on σ^B activity in *S. epidermidis* biofilm and planktonic cells

For σ^B activity measurements, *S. epidermidis* biofilm (n = 10) and the corresponding planktonic (n = 10) samples were selected as described in section 3.5. The *S. epidermidis* biofilm and planktonic samples were exposed to 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2 as described in section 3.5.2.

3.6.1. Isolation of RNA

Total RNA was isolated from the *S. epidermidis* biofilm (n = 10) and planktonic (n = 10) samples exposed to 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2 and their respective unexposed controls using a protocol described previously (Atshan *et al*., 2012). This protocol combines a simple phenol lysis of bacterial cells along with RNA isolation and purification using RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, the frozen *S. epidermidis* biofilm or planktonic cells were thawed on ice, centrifuged at 8,000 rpm for 3 min at 4°C and suspended in nuclease-free water. Then, 100 µL of ~1×10^9 CFU/mL of *S. epidermidis* biofilm or planktonic cells were vortexed vigorously for 3 min and immediately added to 100 µL of a 1:1 mixture of acid phenol and chloroform. The tube was vortexed for 1 min and incubated at 70°C for 30 min. During the 30 min incubation, vortexing was done for 1 min

after every 5 min. After 30 min, the tube was centrifuged at 12,000 rpm for 10 min. Then, 700 µL of lysis buffer (Qiagen, Hilden, Germany) was added to 100 µL of the aqueous phase. Subsequent steps were conducted following the RNeasy Mini Kit manufacturer's instructions. DNase treatment of RNA was performed using DNase I (New England Biolabs, Ipswich, England) following the manufacturer's instructions. The purity and concentration of RNA were determined using a NanoDrop 1000™ (Thermo Scientific, Waltham, USA). The RNA samples with an OD_{280}/OD_{260} ratio between 1.8 and 2.2 were used for complementary DNA (cDNA) synthesis.

3.6.2. The cDNA synthesis

To ensure equivalent amounts of RNA were converted into cDNA, dilutions of RNA in RNase-free water were performed to a final concentration of 2 µg of RNA. The cDNA was synthesized using the ProtoScript® First Strand cDNA Synthesis kit (New England Biolabs, Ipswich, England) following the manufacturer's instructions. Each reaction contained 4 µL of RNA template, 2 µL of $d(T)_{23}$ VN (50 µM) and 2 µL of nuclease-free water. The RNA was denatured for 5 min at 70°C. Then, 10 µL of M-MuLV reaction mix and 2 µL of M-MuLV enzyme mix were added making a final reaction volume of 20 µL. The complete reaction mix was incubated in a thermomixer (Eppendorf G, Hamburg, Germany) for 1 h at 42°C and 5 min at 80°C. The reaction mix was diluted with 30 µL of nuclease-free water and stored at -20°C until further use.

3.6.3. The RT-qPCR

The RT-qPCR was performed on a Rotor-Gene Q real-time thermal cycler (Qiagen, Hilden, Germany) as previously described (Gomes *et al*., 2011) with few modifications. Primers (Inqaba Biotechnical, Pretoria, South Africa) specific for 16S rRNA (reference gene) and *asp23* (target gene) of *S. epidermidis* (Table 1) were used. The 16S rRNA has been used as a

reference gene when studying the physico-chemical agents used in this study (Rodrigues *et al*., 2011). Each 20 µL of RT-qPCR reaction mixture contained 10 µL of Lunar Universal qPCR mastermix (New England Biolabs, Ipswich, England), 0.5 µL each of the forward and reverse primers, 7 µL of nuclease-free water and 2 µL of cDNA template. The thermal cycling conditions were as follows: 1 min at 95°C, followed by 45 cycles of 15 seconds at 95°C, 30 sec at 60°C (acquisition). A melt curve was included at the end of each run, with readings from 60-95°C, every 1°C for 10 min to confirm that only the desired products were amplified. A control lacking the M-MuLV enzyme mix was included in each reaction.

Table 1. Oligonucleotide primer sequences used for RT-qPCR amplification.

Target gene	Set	Sequence (5' - 3')	Reference
S. epidermidis			
asp23	Forward	CAGCAGCTTGTTTTTCTCCA	Pintens *et al*. (2008)
	Reverse	CATGAAAGGTGGCTTCACAG	
16S rRNA	Forward	GGGCTACACACGTGCTACAA	França *et al*. (2012)
	Reverse	GTACAAGACCCGGGAACGTA	
***S. aureus* ATCC 29213**			
asp23	Forward	TCGCTGCACGTGAAGTTAAA	Mitchell *et al*. (2010)
	Reverse	CAGCAGCTTGTTTTTCACCA	
16S rRNA	Forward	GTAGGTGGCAAGCGTTATCC	Karmakar *et al*. (2016)
	Reverse	CGCACATCAGCGTCAG	

3.6.4. The *asp23* expression analysis

The RT-qPCR data were analyzed using the Rotor-Gene software version 2.1.0.9 (Qiagen, Hilden, Germany). The *asp23* expression level was determined using the Efficiency$^{\Delta Ct}$ method, where, ΔCt = Ct (reference gene) - Ct (target gene) (França *et al*., 2011) for 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2-exposed and unexposed *S. epidermidis* biofilm and planktonic cells. The efficiency of the qPCR reaction was determined (with all primer sets) using several dilutions of cDNA. All primers had an efficiency of approximately 100% hence; the real efficiency i.e. 2 was substituted in the Efficiency$^{\Delta Ct}$ formula. For each RNA isolation, three repeats of *asp23* expression measurements were performed. For each sample, the

percentage change in *asp23* expression was expressed by 100 × [{*asp23* expression$_{(\text{exposed cells})}$ – *asp23* expression$_{(\text{unexposed cells})}$}⁄*asp23* expression$_{(\text{unexposed cells})}$].

3.7. Statistical analysis

Statistical analyses and graphs were performed with GraphPad Prism version 5.03 (GraphPad Software Inc., California, USA). Normality of the data was verified using D'Agostino and Pearson omnibus K2 test. Normally and non-normally distributed data were presented as mean ± standard error of the mean (SEM) and median (25^{th} and 75^{th} percentiles) respectively. Depending on data normality, differences in susceptibility patterns, eDNA release or *asp23* expression between *S. epidermidis* biofilm and planktonic cells exposed to heat, NaCl, NaOCl or H_2O_2 were analyzed by a two-tailed paired *t*-test or Wilcoxon-matched pairs signed rank test. Similarly, differences in eDNA or *asp23* expression between heat, NaCl, NaOCl and H_2O_2-exposed and unexposed controls were determined by two-tailed paired *t*-test or Wilcoxon-matched pairs signed rank test. Finally, comparisons of the effectiveness of heat, NaCl, NaOCl and H_2O_2 against *S. epidermidis* biofilm or planktonic cells were performed using repeated measures one-way analysis of variance (ANOVA) with Tukey's post-hoc. Differences with a *p* value less than 0.05 were considered statistically significant.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Susceptibility patterns of *S. epidermidis* biofilm and planktonic cells to heat, NaCl, NaOCl or H_2O_2-exposure

One of the specific aims of the present study was to determine the susceptibility patterns of *S. epidermidis* biofilm and planktonic cells to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2.

4.1.1. The *S. epidermidis* biofilm cells were less susceptible to heat exposure than the planktonic cells

The log reduction of CFU/mL of *S. epidermidis* biofilm cells (0.1102 ± 0.0063) exposed to 60°C for 30 min was significantly lower than for the planktonic cells (0.3416 ± 0.0068) (Figure 4A; Paired *t*-test, n = 62, $p < 0.0001$). Similarly, the log reduction of CFU/mL of *S. epidermidis* biofilm cells (0.3315 ± 0.0091) subjected to 60°C for 60 min was significantly lower than for planktonic cells (0.5960 ± 0.0099) (Figure 4A; Paired *t*-test, n = 62, $p < 0.0001$). These findings indicated that 60°C exposure was less effective against *S. epidermidis* biofilm cells than the corresponding planktonic cells. The present findings are in agreement with previous reports on different bacterial species, such as cell wall less *M. bovis* (McAuliffe *et al*., 2006), cellulosic polymer-overproducing *S. enterica* (Scher *et al*., 2005) and a fungus *C. neoformans* (Martinez & Casadevall, 2007), which may not inform on general bacterial biofilms' response against heat exposure. The increased heat exposure tolerance of *S. epidermidis* biofilm cells compared to the planktonic cells could probably be explained in two ways. First, since bacterial biofilms overexpress heat stress-related genes (Coenye, 2010) resulting in production of heat shock proteins that consume excess energy in form of ATP (Liu *et al*., 2015), it is probable that the *S. epidermidis* biofilm cells overexpressed heat stress-

related genes to protect them against the effects of heat exposure. Second, an increase in temperature switches the staphylococcal biofilm cells fatty acid profile such that the anteiso-C19 fatty acids known to have high melting point rises, leading to decreased membrane fluidity (Abdallah *et al*., 2014) hence the increased tolerance against heat exposure.

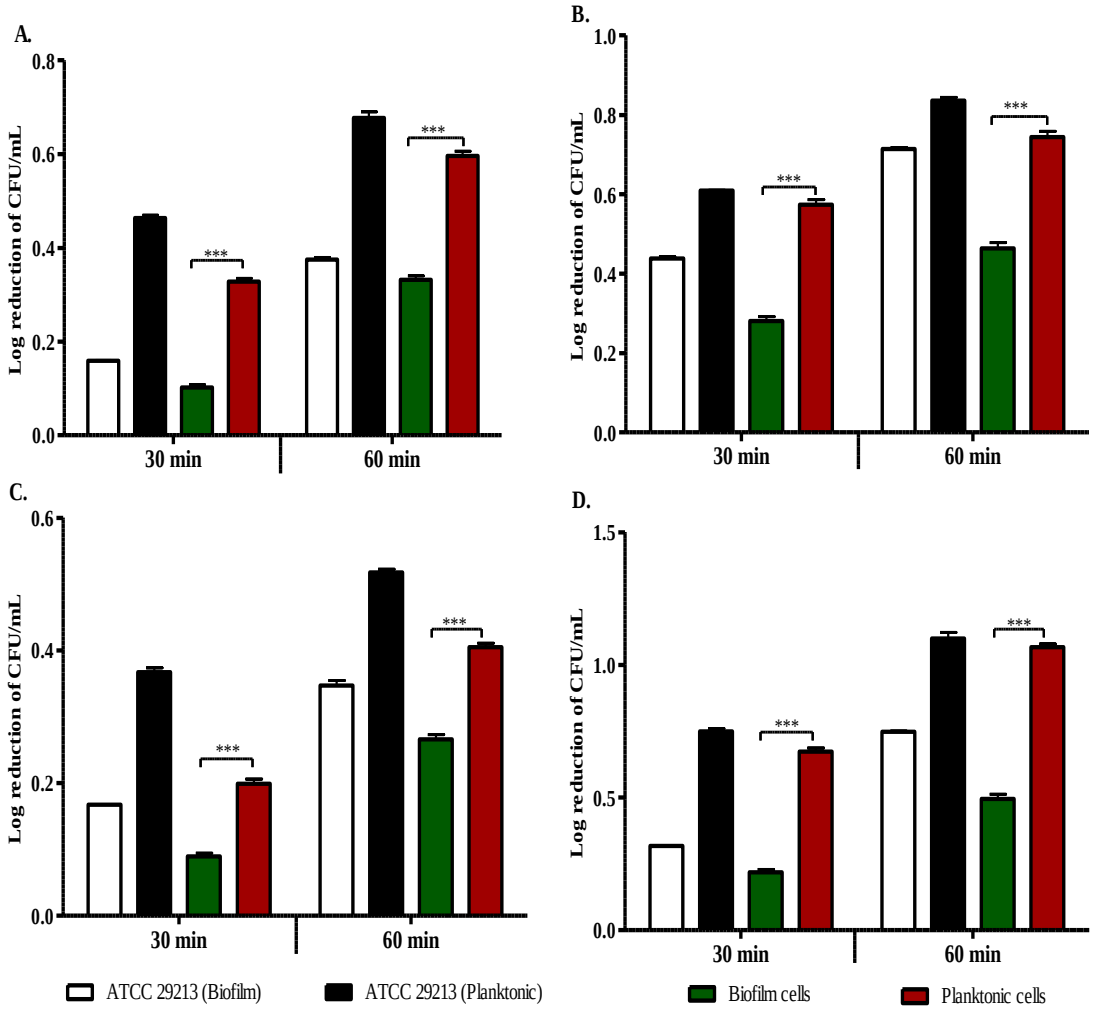

Figure 4. Susceptibility patterns of *S. epidermidis* biofilm and planktonic cells to physico-chemical stress exposure. The log reduction of CFU/mL of pairs (n = 62) of *S. epidermidis* biofilm and planktonic samples exposed to (A) 60°C, (B), 1.72 M NaCl, (C) 0.178 M NaOCl or (D) 1.77 M H_2O_2 for 30 or 60 min. For each sample, log reduction of CFU/mL measurements were performed in triplicate. Bars represent the mean ± SEM. Statistical significance between *S. epidermidis* biofilm and planktonic cultures were determined by paired *t*-test (***, $p < 0.0001$).

4.1.2. The *S. epidermidis* biofilm cells were more tolerant to NaCl exposure than the planktonic cells

When subjected to 1.72 M NaCl for 30 min, *S. epidermidis* biofilm cells exhibited a significantly lower log reduction of CFU/mL (0.2806 ± 0.0113) than the planktonic cells (0.5738 ± 0.0130) (Figure 4B; Paired *t*-test, n = 62, $p < 0.0001$). Similarly, treatment with 1.72 M NaCl for 60 min yielded a similar pattern of log reduction of CFU/mL for *S. epidermidis* biofilm (0.4639 ± 0.0143) and planktonic cells (0.7441 ± 0.0149) (Figure 4B; Paired *t*-test, n = 62, $p < 0.0001$). These results implied that 1.72 M NaCl was less effective against *S. epidermidis* biofilm cells than the planktonic cells. The findings of the present study are consistent with a previous report on *V. cholerae* O1 (Wai *et al*., 1998). However, the previous study used *V. cholerae*, a bacterium highly adapted to high salinity (Filho *et al*., 2011) and utilized 2.5 M NaCl and not 1.72 M NaCl routinely used for bacterial disinfection (Smith & Stratton, 2007). The observed increase in the tolerance of *S. epidermidis* biofilm cells could possibly be related to previous studies showing that biofilms enhance exopolysaccharides and protein production to form a water-retaining layer around biofilm cells thus protecting them from dehydration (Qurashi & Sabri, 2012; Pemmaraju *et al*., 2016). An alternative explanation could be linked to a previous observation that osmotic stress exposure enhances quorum sensing in bacterial biofilms (Cai *et al*., 2013), which confers tolerance against osmotic stress exposure (García-Contreras *et al*., 2014).

4.1.3. NaOCl exposure was more effective against *S. epidermidis* planktonic than biofilm cells

The *S. epidermidis* biofilm cells exhibited significantly lower log reduction of CFU/mL (0.0896 ± 0.0050) when exposed to 0.178 M NaOCl for 30 min than the planktonic cells (0.1991 ± 0.0071) (Figure 4C; Paired *t*-test, n = 62, $p < 0.0001$). A similar pattern of log

reduction of CFU/mL emerged upon exposure of *S. epidermidis* biofilm (0.2656 ± 0.0074) and planktonic cells (0.4043 ± 0.0067) to 0.178 M NaOCl for 60 min (Figure 4C; Paired *t*-test, n = 62, $p < 0.0001$). These results implied that *S. epidermidis* biofilm cells were more tolerant against 0.178 M NaOCl than the analogous planktonic cells. The present findings are in agreement with previous reports on different bacterial species that were either overproducing protective cellulosic polymer (Scher *et al*., 2005), were subjected to higher (Kubota *et al*., 2009) or lower NaOCl concentrations (Peeters *et al*., 2008a), were overgrown for six days (Stewart *et al*., 2001) or had protective mycolic acid-rich membranes (Steed & Falkinham III, 2006). Thus, the previous reports may not inform on the general bacterial biofilm response to NaOCl exposure. The observed increased tolerance of *S. epidermidis* biofilm cells could be due to the reaction of NaOCl with the ECM components and/or the slow diffusion across ECM barrier (Stewart *et al*., 2001) thus reducing the effect of NaOCl on the inner *S. epidermidis* biofilm cells.

4.1.4. H_2O_2 exposure was less effective against *S. epidermidis* biofilm than the planktonic cells

The *S. epidermidis* biofilm cells exposed to 1.77 M H_2O_2 for 30 min had a significantly lower log reduction of CFU/mL (0.2186 ± 0.0110) than the planktonic cells (0.6728 ± 0.0128) (Figure 4D; Paired *t*-test, n = 62, $p < 0.0001$). A similar pattern was observed for *S. epidermidis* biofilm (0.4944 ± 0.0182) and planktonic cells (1.067 ± 0.0125) (Figure 4D; Paired *t*-test, n = 62, $p < 0.0001$) exposed to 1.77 M H_2O_2 for 60 min. These findings indicated that 1.77 M H_2O_2 is more effective against *S. epidermidis* planktonic cells than the corresponding biofilm cells. The current findings concur with previous reports on different bacterial species (Wai *et al*., 1998; Peeters *et al*., 2008a). However, the previous studies used lower H_2O_2 concentrations hence might not present a clear picture of *S. epidermidis* biofilm's

response against H_2O_2 exposure. The observed higher tolerance of *S. epidermidis* biofilms could probably be due to the neutralization of H_2O_2 by the ECM components and/or overproduction of catalase enzymes that decompose the H_2O_2 (Peeters *et al*., 2008a) thereby reducing its effects on the inner *S. epidermidis* biofilm cells.

Generally, a disinfectant resulting in a log reduction unit above three (99.9% bacterial reduction) is considered effective against a bacterial biofilm (Rodrigues *et al*., 2011). However, the *S. epidermidis* biofilm exhibited low log reduction units below three when subjected to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2. This implied that 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 were ineffective against the *S. epidermidis* biofilm hence creating a public healthcare concern. However, the observed low log reduction units could partly be due to the narrow CFU counting range of the plating method (Ben-David & Davidson, 2014) employed in the present study for enumeration of the *S. epidermidis* CFUs.

4.1.5. Comparison of the effectiveness of heat, NaCl, NaOCl or H_2O_2-exposure against *S. epidermidis* biofilm or planktonic cells

The present study also compared the effectiveness of 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 against *S. epidermidis* biofilm or planktonic cells. The log reductions of CFU/mL of *S. epidermidis* biofilm cells differed significantly when exposed to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 30 or 60 min (Repeated measures one-way ANOVA, n = 62, $p < 0.0001$). At 30 min exposure, Tukey's post hoc showed that the log reduction of CFU/mL was significantly highest for 1.72 M NaCl followed by 1.77 M H_2O_2, 60°C and 0.178 M NaOCl in that order ($p < 0.0001$; Figure 5A). At 60 min exposure, Tukeys post hoc showed that the log reduction of CFU/mL was significantly highest for 1.77 M H_2O_2, followed by 1.72 M NaCl, 60°C and 0.178 M NaOCl in that order ($p < 0.0001$; Figure 5A).

The results indicated that the susceptibilities of *S. epidermidis* biofilm cells exposed to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 30 min were not dependent on the diffusion rate (molecular weight) (NaCl > H_2O_2 > heat > NaOCl). On the contrary, susceptibilities of *S. epidermidis* biofilm cells subjected to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 60 min were dependent on the diffusion rate (H_2O_2 > NaCl > heat > NaOCl).

The log reductions of CFU/mL of *S. epidermidis* planktonic cells differed significantly when exposed to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 30 or 60 min (Repeated measures one-way ANOVA, n = 62, $p < 0.0001$). At 30 or 60 min exposure, Tukey's post hoc revealed that the log reduction of CFU/mL of both *S. epidermidis* planktonic cells were significantly highest for 1.77 M H_2O_2, followed by 1.72 M NaCl, 60°C and 0.178 M NaOCl in that order ($p < 0.0001$; Figure 5B). These results implied that the susceptibilities of *S. epidermidis* planktonic cells exposed to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 30 or 60 min were dependent on the diffusion rate (H_2O_2 > NaCl > heat > NaOCl).

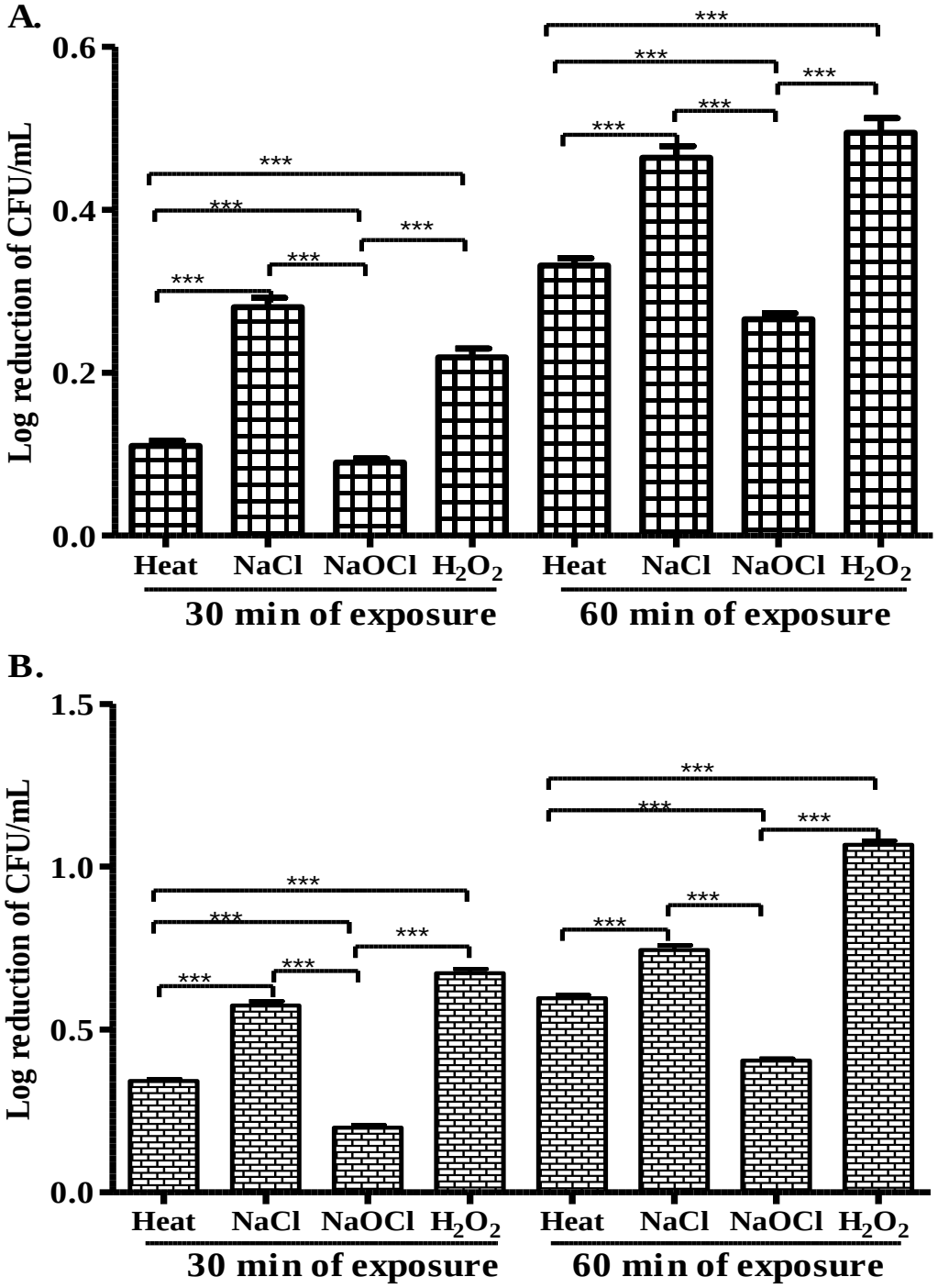

Figure 5. Comparison of the effectiveness of physico-chemical stress exposure against *S. epidermidis* biofilm and planktonic cells. The log reduction of CFU/mL for *S. epidermidis* (n = 62) biofilm (A) and planktonic (B) samples exposed to 60°C, 1.72 M NaCl, 0.178 M NaOCl and 1.77 M H_2O_2 for 30 and 60 min. For each sample, log reduction of CFU/mL measurements were performed in triplicate. Bars represent the mean ± SEM. Statistical comparisons were performed by repeated measures one-way ANOVA with Tukey's post-hoc (***, $p < 0.0001$).

Bacterial biofilms' tolerance against antimicrobials is mostly attributed to the agent's reaction with and/or slow diffusion across the ECM i.e. the reaction-diffusion inhibition mechanism (Stewart *et al*., 2001; Simões & Simões, 2013). The observation that susceptibility pattern of *S. epidermidis* biofilm cells exposed to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 60 min depended on the diffusion rate (molecular weight) (H_2O_2 > NaCl > heat > NaOCl) appear to support the reaction-diffusion inhibition mechanism. Considering previous report showing that NaOCl (with largest molecular weight) diffuses across the ECM in 50 min (Stewart *et al*., 2001), all the other agents should have crossed the ECM within the 60 min of exposure and killed an equivalent number of *S. epidermidis* biofilm and planktonic cells. Taking into account the observation that more *S. epidermidis* planktonic cells were killed compared to the corresponding biofilm cells at 60 min of exposure and that susceptibilities of biofilm cells at 30 min of exposure did not correspond to the diffusion rate, suggested that the reaction-diffusion inhibition mechanism does not fully account for the observed increased tolerance of *S. epidermidis* biofilm against 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2. This lends credence to the existence of complementary mechanism(s) of tolerance against heat, NaCl, NaOCl or H_2O_2 exposure in *S. epidermidis* biofilm, such as eDNA release (Okshevsky & Meyer, 2015) and upregulation of biofilm-specific protective molecules (de la Fuente-Núñez *et al*., 2013).

4.2. Effects of heat, NaCl, NaOCl or H_2O_2-exposure on eDNA release by *S. epidermidis* biofilm and planktonic cells

The results presented above suggested that the reaction-diffusion inhibition mechanism did not fully account for the relatively increased *S. epidermidis* biofilm cells tolerance against the physico-chemical stress exposure. Therefore, the second specific aim of the present study was

to evaluate the eDNA release as a potential mechanism underlying the tolerance of *S. epidermidis* biofilm against 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 μM H_2O_2 exposure

4.2.1. Heat exposure enhanced eDNA release by *S. epidermidis* biofilm cells, but not by planktonic cells

The eDNA yield by *S. epidermidis* biofilm (*Median* = 81.18%) and planktonic cells (*Median* = 20.82%) exposed to 50°C for 60 min were not significantly different (Figure 6A; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.4697$). Further, the 50°C-exposed *S. epidermidis* biofilm cells released significantly increased eDNA than the unexposed controls (Table 2; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.0098$). However, the eDNA released by the 50°C-exposed *S. epidermidis* planktonic cells and the respective unexposed controls (25°C) were not statistically different (Table 2; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.7910$). Taken together, the findings implied that 50°C exposure enhanced eDNA release by *S. epidermidis* biofilm cells, but not by planktonic cells. The observation that unlike the planktonic forms, the biofilm forms of *S. epidermidis* subjected to 50°C released significantly increased eDNA than the unexposed controls suggested a role of eDNA in the tolerance of *S. epidermidis* biofilm against heat exposure.

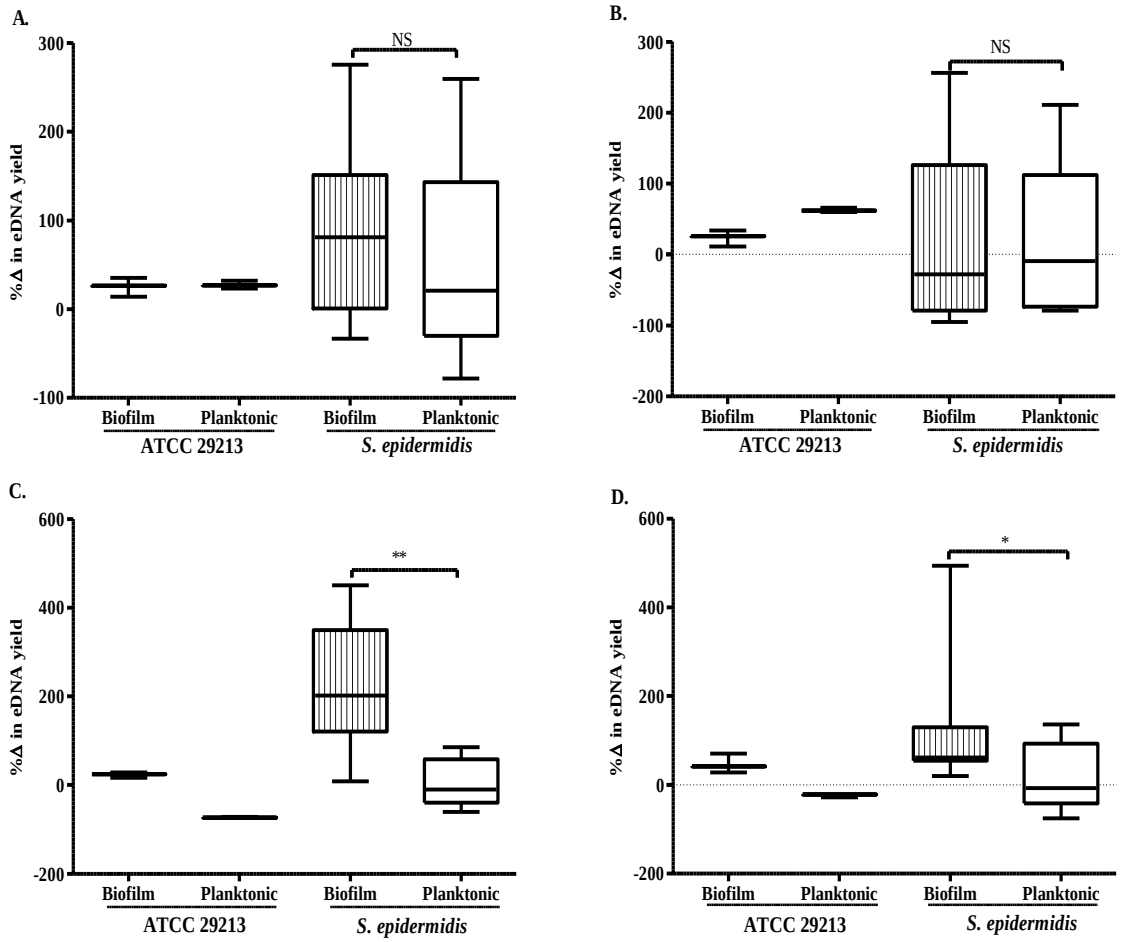

Figure 6. Effect of physico-chemical stress exposure on eDNA release by *S. epidermidis* biofilm and planktonic cells. The percentage change in eDNA yield by pairs (n = 12) of *S. epidermidis* biofilm and planktonic samples exposed to (A) 50°C, (B) 0.8 M NaCl, (C) 5 mM NaOCl or (D) 50 μM H_2O_2 for 60 min. The obtained results for biofilm or planktonic cells are presented as the percentage change in eDNA yield calculated in relation to the unexposed controls. The horizontal line across the box is the median percentage change in eDNA yield, the lower and upper ends of the box are the 25th and 75th percentiles. The whiskers are the minimum and maximum percentage changes in eDNA yield. For each sample, three repeat eDNA quantity measurements were performed. Statistical significance between *S. epidermidis* biofilm and planktonic cultures were determined by Wilcoxon matched-pairs signed rank test (NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$).

Table 2. Effect of physico-chemical stress exposure on eDNA release by *S. epidermidis* biofilm and planktonic cells.

Type of cell	Mean ± SEM of eDNA yield in ng/µL by cells exposed to optimal physico-chemical stresses			
	Heat	**NaCl**	**NaOCl**	**H_2O_2**
Biofilm				
Untreated	0.110 ± 0.019	0.482 ± 0.159	0.089 ± 0.021	0.231 ± 0.056
controls	0.221 ± 0.058	0.285 ± 0.095	0.292 ± 0.072	0.451 ± 0.095
Treated cells	***p* = 0.0098**	*p* = 0.3271	***p* = 0.0005**	***p* = 0.0005**
Planktonic	0.405 ± 0.101	0.242 ± 0.067	0.526 ± 0.128	0.137 ± 0.031
Untreated	0.394 ± 0.071	0.219 ± 0.089	0.536 ± 0.123	0.112 ± 0.015
controls	*p* = 0.7910	*p* = 0.6672	*p* = 0.9097	*p* = 0.7910
Treated cells				

The *S. epidermidis* biofilm (n = 12) and planktonic (n = 12) samples were exposed to heat 25°C (controls) and 50°C or grown in TSB with or without 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2 for 60 min for eDNA analysis. For each sample, triplicate measurements of eDNA yield were performed. Values represent the mean ± SEM. Boldface represent a statistically significant difference between physico-chemical stress-exposed and unexposed *S. epidermidis* biofilm or planktonic cells as determined by Wilcoxon matched-pairs signed rank test ($p < 0.05$).

4.2.2. NaCl exposure did not affect eDNA release by *S. epidermidis* cells

The eDNA release by *S. epidermidis* biofilm (*Median* = -27.94%) and planktonic cells (*Median* = -9.07%) exposed to 0.8 M NaCl for 60 min were not significantly different (Figure 6B; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.8501$). Further analysis showed that 0.8 M NaCl-exposed *S. epidermidis* biofilm cells yielded less eDNA than the unexposed controls although not statistically different (Table 2; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.3271$). Unexpectedly, the NaCl stress treated *S. epidermidis* biofilms released less eDNA than the untreated controls (Table 2; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.6672$). This unexpected observation could be due to NaCl-induced exopolysaccharide production in the ECM (Qurashi & Sabri, 2012; Pemmaraju *et al*., 2016) which might have formed strong bond with the eDNA (Song *et al*., 2016) rendering eDNA largely inaccessible for quantification. Taken together, the finding implied that 0.8 M NaCl exposure had no effect on eDNA release by *S. epidermidis* biofilm and planktonic cells. The

present finding concurs with a previous report on *C. albicans* biofilm (Pemmaraju *et al*., 2016), which is a fungus hence may not inform on bacterial biofilms response to NaCl exposure. The observation that there was no significant eDNA release by *S. epidermidis* biofilm and planktonic cells exposed to 0.8 M NaCl suggested that eDNA is not involved in the tolerance against NaCl exposure. In support of this interpretation, a study showed that *autolysin* (*atl*) gene, which is often associated with eDNA release is not affected by osmotic stresses (Houston *et al*., 2011).

4.2.3. NaOCl or H_2O_2-exposure enhanced eDNA release by *S. epidermidis* biofilm, but not by planktonic cells

The *S. epidermidis* biofilms subjected to 5 mM NaOCl for 60 min exhibited significantly increased eDNA yield (*Median* = 202.30%) than the planktonic cells (*Median* = -9.67%) (Figure 6C; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.0015$). Further, the 5 mM NaOCl-treated *S. epidermidis* biofilm cells yielded significantly more eDNA than the unexposed controls (Table 2; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.0005$). On the contrary, the eDNA yield by the 5 mM NaOCl-exposed *S. epidermidis* planktonic cells and the untreated controls was not statistically different (Table 2; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.9097$).

When subjected to 50 μM H_2O_2 for 60 min, *S. epidermidis* biofilms (*Median* = 61.32%) stimulated significantly increased eDNA release than the planktonic cells (*Median* = -7.24%) (Figure 6D; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.0210$). Further analyses revealed that 50 μM H_2O_2-exposed *S. epidermidis* biofilm cells produced significantly more eDNA than the unexposed controls (Table 2; Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.0005$). In contrast, the eDNA yield between 50 μM H_2O_2-exposed *S. epidermidis* planktonic cells and the unexposed controls was not significantly different (Table 2;

Wilcoxon matched-pairs signed rank test, n = 12, $p = 0.7910$). Taken together, these findings indicated that 5 mM NaOCl or 50 μM H_2O_2 exposure enhanced eDNA release by *S. epidermidis* biofilm cells, but not by planktonic cells. The finding concurs with previous reports on *Streptococcus gordonii* (Itzek *et al*., 2011; Xu & Kreth, 2013) and *C. albicans* biofilm (Pemmaraju *et al*., 2016). However, the previous reports only focused on the planktonic forms of *S. gordonii* and a fungus *C. albicans* hence may not inform on eDNA release by bacterial biofilms in response to H_2O_2.

Taken together, NaOCl or H_2O_2 exposure damage genomic DNA triggering eDNA release by a subpopulation of bacterial cells (Itzek *et al*., 2011). A possible explanation for the observed higher eDNA release by the biofilm cells could be related to the extracellular DNases released alongside eDNA in the following ways. First, unlike the planktonic cells, bacterial biofilm cells form small-protected pockets (Doroshenko *et al*., 2014) that could be protecting most eDNA from DNases degradation. Second, bacterial biofilms eDNA is mostly bound to the ECM components (Song *et al*., 2016) hence may not be easily accessible to the DNases. Third, bacterial biofilms produce relatively fewer DNases than the planktonic cells (Tang *et al*., 2013) thus minimizing the eDNA degradation. Fourth, bacterial biofilms induce release of proteolytic exoenzymes that inactivate the DNases locally (Whitchurch *et al*., 2002). Taken together, the explanations above suggest that eDNA and DNases release by bacterial biofilm cells are highly regulated processes. This implied that bacterial planktonic cells majorly release eDNA to be degraded for nutrients whereas bacterial biofilm cells induce eDNA release both as a nutrient source and for tolerance against NaOCl or H_2O_2 exposure.

Although DNase appears to provide a more plausible explanation for the increased eDNA release by *S. epidermidis* biofilm exposed to NaOCl or H_2O_2, the presence of DNase was not quantitatively measured. Further studies with DNase (+) controls and treatment groups may

be necessary to confirm the direct link between eDNA and bacterial biofilm tolerance against heat, NaOCl or H_2O_2 exposures.

4.3. Effect of heat, NaCl, NaOCl or H_2O_2-exposure on σ^B activity in *S. epidermidis* biofilm and planktonic cells

The third specific aim of the present study was to evaluate the expression of *asp23* in *S. epidermidis* biofilm and planktonic cells in response to 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 μM H_2O_2 exposure.

4.3.1. The activity of σ^B was elevated to higher extent in *S. epidermidis* biofilm exposed to heat than in planktonic cells

The *S. epidermidis* biofilm cells (45767 ± 7237%) exposed to 50°C had a significantly higher *asp23* expression compared to the planktonic cells (25165 ± 4392%) (Figure 7A; Paired *t*-test, n = 10, $p = 0.0259$). Further, *S. epidermidis* biofilm cells exposed to 50°C showed increased *asp23* expressions than those exposed to 25°C (Table 3; Paired *t*-test, n = 10, $p < 0.0001$). Similarly, *S. epidermidis* planktonic cells exposed to 50°C exhibited significantly increased *asp23* expression levels than those exposed to 25°C (Table 4; Paired *t*-test, n = 10, $p < 0.0001$). These results showed that σ^B activity is enhanced in the *S. epidermidis* biofilm and planktonic cells in response to 50°C exposure, but with significantly higher activities in the biofilm cells. The present observation on planktonic cells is in agreement with previous reports on different *Bacillus* species (Voelker *et al*., 1999 Schaik *et al*., 2004). A possible explanation for the increased σ^B activity levels in *S. epidermidis* biofilm and planktonic cells in response to 50°C exposure may be an increase in heat-dependent metabolic activities regulated by σ^B, such as ATP synthesis, but not necessarily as a heat tolerance strategy. Alternatively, the observed higher σ^B activity in *S. epidermidis* biofilm suggested that tolerance of the biofilm against heat-exposure might be dependent on σ^B activity.

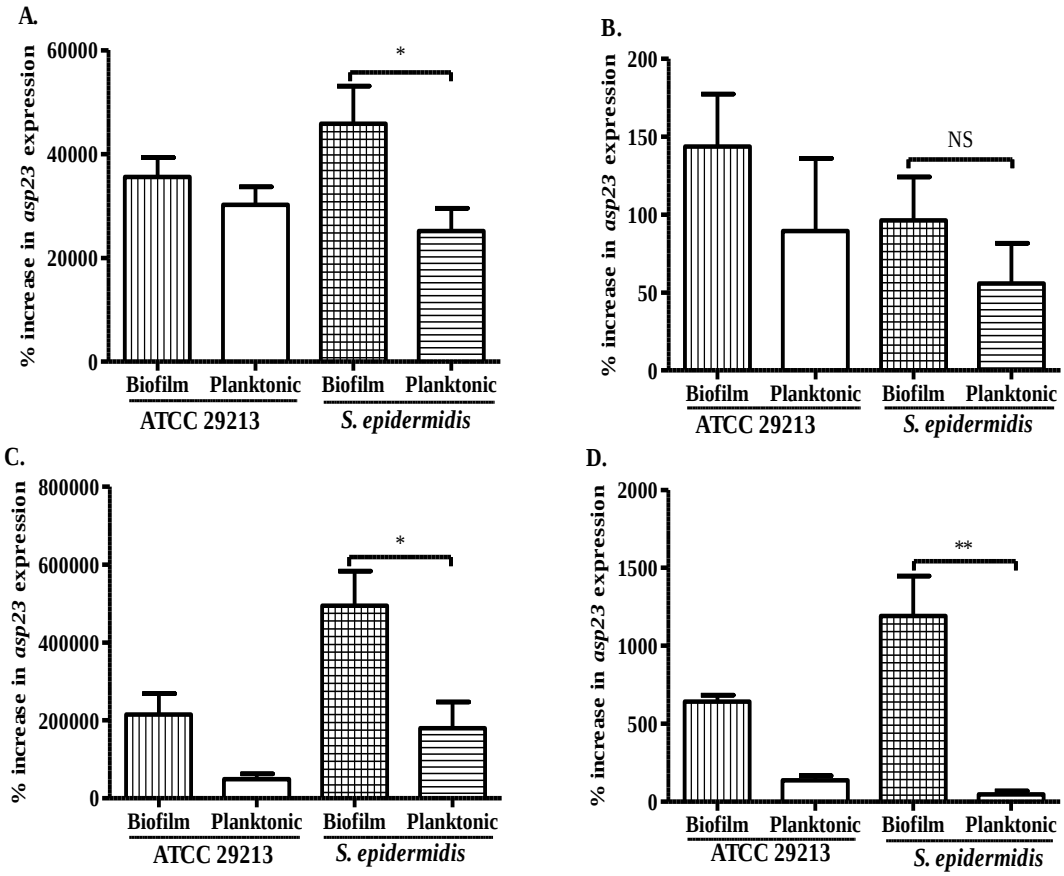

Figure 7. Effects of physico-chemical stress exposure on *asp23* expression in *S. epidermidis* biofilm and planktonic cells. The *S. epidermidis* biofilm (n = 10) and planktonic (n = 10) samples were exposed to (A) 50°C, (B) 0.8 M NaCl, (C) 5 mM NaOCl or (D) 50 µM H_2O_2 for 60 min for *asp23* expression analysis. The obtained results for biofilm or planktonic cells are presented as the percent increase in *asp23* expression levels calculated in relation to the unexposed controls. For each sample, three repeat gene expression measurements were performed. Bars represent the mean ± SEM. Statistical significance between the *S. epidermidis* biofilm and planktonic cells was evaluated using two-tailed paired *t*-test (*, $p < 0.05$; NS, $p > 0.05$; **, $p < 0.01$).

Table 3. Effect of physico-chemical stress exposure on *asp23* expressions in *S. epidermidis* biofilm and planktonic cells.

Type of cell	Mean ± SEM of *asp23* expressions in cells exposed to optimal physico-chemical stresses			
	Heat	**NaCl**	**NaOCl**	**H_2O_2**
Biofilm				
Unexposed	0.0178 ± 0.0021	0.0062 ± 0.0012	0.2859 ± 0.0051	0.0078 ± 0.0010
Exposed	7.478 ± 0.9350	0.0127 ± 0.0032	112.1 ± 16.72	0.0869 ± 0.0161
	***p* < 0.0001**	***p* = 0.0203**	***p* < 0.0001**	***p* = 0.0002**
Planktonic				
Unexposed	0.0003 ± 0.0000	0.0052 ± 0.0011	0.0425 ± 0.0085	0.0141 ± 0.0019
Exposed	4.751 ± 0.6449	0.0081 ± 0.0028	56.47 ± 16.37	0.0220 ± 0.0055
	***p* < 0.0001**	*p* = 0.1831	***p* = 0.0073**	*p* = 0.0725

The *S. epidermidis* biofilm (n = 10) and planktonic (n = 10) samples were exposed to heat 25°C (controls) and 50°C or grown in TSB with or without 0.8 M NaCl, 5 mM NaOCl or 50 µM H_2O_2 for 60 min for gene expression analysis. For each sample, three repeats of gene expression measurements were performed. Boldface represent a statistically significant difference between physico-chemical stress-exposed and unexposed *S. epidermidis* biofilm or planktonic cells as determined by paired *t*-test ($p < 0.05$).

4.3.2. NaCl exposure enhanced σ^B activity in *S. epidermidis* biofilm, but not in planktonic cells

The increase in *asp23* expression levels observed in the *S. epidermidis* biofilm (96.31 ± 27.82%) and planktonic cells (55.68 ± 26.03%) treated with 0.8 M NaCl were not statistically different (Figure 7B; Paired *t*-test, n = 10, $p = 0.4029$). Further, the biofilms exposed to 0.8 M NaCl exhibited increased *asp23* expressions than the unexposed controls (Table 3; Paired *t*-test, n = 10, $p = 0.0203$). On the contrary, the *asp23* expression in planktonic cells exposed to 0.8 M NaCl and the unexposed controls was not significantly different (Table 3; Paired *t*-test, n = 10, $p = 0.1831$). These findings implied that 0.8 M NaCl exposure enhanced σ^B activity in the *S. epidermidis* biofilm cells, but not in the planktonic cells. Contradicting observations have been reported for planktonic forms of different bacterial species (Becker *et al*., 1998; Chan *et al*., 1998; Schaik *et al*., 2004; Abram *et al*., 2008). Considering that NaCl-exposure response regulatory mechanisms in different bacterial species may not follow common patterns (Sihto *et al*., 2015), the discrepancy between the present and previous outcomes

could be attributed to the different regulatory patterns in planktonic cells of *S. epidermidis* and the other bacterial species. Since σ^B is critical in cell wall envelope homeostasis (Guldimann *et al*., 2016), it is probable that the 0.8 M NaCl concentration tested had a little effect on *S. epidermidis* cell wall homeostasis, resulting in the observed low σ^B activity levels. The present observation that σ^B activity was significantly higher in the 0.8 M NaCl-exposed *S. epidermidis* biofilms than the untreated controls suggested that σ^B might be involved in the tolerance of *S. epidermidis* biofilm against NaCl exposure.

4.3.3. The σ^B activity was upregulated more in NaOCl-exposed *S. epidermidis* biofilm than in planktonic cells

The *asp23* expression levels in *S. epidermidis* biofilm cells (494925 ± 87896%) subjected to 5 mM NaOCl were significantly higher than that of the planktonic cells (180444 ± 67507%) (Figure 7C; Paired *t*-test, n = 10, $p = 0.0109$). Moreover, biofilms treated with 5 mM NaOCl exhibited significantly increased *asp23* expressions than the unexposed controls (Table 3; Paired *t*-test, n = 10, $p < 0.0001$). Similarly, planktonic cells exposed to 5 mM NaOCl had significantly higher *asp23* expression than the unexposed controls (Table 3; Paired *t*-test, n = 10, $p = 0.0073$). These results suggested that exposure to 5 mM NaOCl upregulates σ^B activity in both *S. epidermidis* biofilm and planktonic cells, but with significantly higher activities in the biofilm cells. The present observation is in agreement with a previous report on *L. monocytogenes* exposed to a chlorine-based disinfectant, benzalkonium chloride (van der Veen & Abee, 2010). Of note, benzalkonium chloride and NaOCl have different modes of action (Rutala *et al*., 2008; van der Veen & Abee, 2010), therefore, the previous reports may not inform on σ^B activity in *S. epidermidis* biofilm and planktonic cells exposed to NaOCl. A possible explanation for the enhanced σ^B activity in the *S. epidermidis* biofilm and planktonic cells in response to 5 mM NaOCl exposure is an increase in the σ^B-dependent metabolic

activities affected by NaOCl exposure, such as DNA synthesis (O'Byrne & Karatzas, 2008; Rutala *et al*., 2008). The observed significantly higher σ^B activity levels in biofilm cells suggested that tolerance of the *S. epidermidis* biofilm against NaOCl-exposure might be dependent on σ^B activity.

4.3.4. H_2O_2 exposure enhanced σ^B activity in *S. epidermidis* biofilm, but not in planktonic cells

The *S. epidermidis* biofilm cells (1191 ± 256.6%) exhibited a significantly higher *asp23* expression compared to the planktonic cells (45.31 ± 22.15) in response to 50 μM H_2O_2 exposure (Figure 7D; Paired *t*-test, n = 10, $p = 0.0020$). Furthermore, *S. epidermidis* biofilms subjected to 50 μM H_2O_2 exhibited significantly increased *asp23* expressions than the unexposed controls (Table 3; Paired *t*-test, n = 10, $p = 0.0002$). Conversely, the *asp23* expression in planktonic cells exposed to 50 μM H_2O_2 and the unexposed controls was not statistically different (Table 3; Paired *t*-test, n = 10, $p = 0.0725$). Taken together, these data revealed that 50 μM H_2O_2 exposure enhanced σ^B activity in *S. epidermidis* biofilm cells, but not in the planktonic cells. The present observation on planktonic cells contradicts previous reports, showing that σ^B activity is significantly enhanced in H_2O_2-treated cells than the untreated controls (Chan *et al*., 1998; Schaik *et al*., 2004). This contradiction suggests that the regulatory mechanism(s) controlling H_2O_2 exposure in *S. epidermidis* planktonic cells is different from other bacterial species. A possible explanation for the observed marginal σ^B activity levels in the present and previous studies is that σ^B activity is H_2O_2 concentration-dependent. The observed significantly higher σ^B activity levels in biofilm cells exposed to 50 μM H_2O_2 suggested that σ^B activity might be having a significant contribution in the *S. epidermidis* biofilm's tolerance against H_2O_2 exposure.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1. Summary

In summary, significantly fewer *S. epidermidis* biofilm cells were killed upon exposure to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2 for 30 or 60 min than the planktonic cells. The eDNA released by the 50°C-exposed *S. epidermidis* biofilm and planktonic cells was not statistically different. However, the 50°C-exposed *S. epidermidis* biofilm cells released significantly higher eDNA than the unexposed controls. Further, there was no significant difference in eDNA released by 0.8 M NaCl-exposed *S. epidermidis* biofilm and planktonic cells. In stark contrast, 5 mM NaOCl or 50 μM H_2O_2-treated *S. epidermidis* biofilm cells released significantly higher eDNA than the planktonic cells. Further, *S. epidermidis* biofilm cells exposed to 50°C, 5 mM NaOCl or 50 μM H_2O_2 exhibited significantly higher σ^B activity levels than the planktonic cells. Conversely, no significant difference in σ^B activity levels were detected between *S. epidermidis* biofilm and planktonic cells exposed to 0.8 M NaCl. On the contrary, *S. epidermidis* biofilm cells exposed to 0.8 M NaCl showed significantly higher σ^B activity than the unexposed controls.

5.2. Conclusions and implications

This is the first study reporting on the susceptibility patterns, eDNA release and σ^B activities of *S. epidermidis* biofilm in response to heat, NaCl, NaOCl or H_2O_2 exposure. The following conclusions and implications may be drawn from the results presented.

1. The *S. epidermidis* biofilm was more tolerant to 60°C, 1.72 M NaCl, 0.178 M NaOCl or 1.77 M H_2O_2-exposure. Thus, there is need to review and improve the current point-of-use physico-chemical disinfection guidelines to effectively target the bacterial biofilm growth forms.

2. The *S. epidermidis* biofilm significantly enhanced eDNA release in response to 50°C, 5 mM NaOCl or 50 μM H_2O_2-exposure, suggesting a role of eDNA in the biofilm's tolerance against heat, NaOCl or H_2O_2-exposure. Therefore, eDNA may be a potential target for novel anti-biofilm approaches.
3. The *S. epidermidis* biofilm significantly increased σ^B activity in response to 50°C, 0.8 M NaCl, 5 mM NaOCl or 50 μM H_2O_2-exposure, suggesting the importance of σ^B activity in the biofilm's tolerance against heat, NaCl, NaOCl or H_2O_2-exposure. Thus, σ^B may be a promising target for novel anti-staphylococcal biofilm strategies.

5.3. Recommendations

5.3.1. Recommendations for the present study

Based on the analyses of the findings and the potential limitations of the present study, the following recommendations may be drawn:

1. The present study used standard plating method, which may not detect injured or VBNC cells leading to underestimation of bacterial cell count. There is need to use techniques, such as flow cytometry or next generation sequencing, which can detect injured and VBNC cells to study the susceptibility patterns of *S. epidermidis* biofilm to heat, NaCl, NaOCl or H_2O_2 exposure.
2. The present study did not quantify the release of DNases. There is need for inclusion of DNase (+) controls and treatment groups to confirm the direct link between eDNA and the tolerance of *S. epidermidis* biofilm against heat, NaCl, NaOCl or H_2O_2 exposure.
3. The present study did not incorporate σ^B mutants or promoter reporters. There is need for inclusion of the mutants or promoter reporters to reach a more definite conclusion on the dependence of *S. epidermidis* biofilm on σ^B activity for survival against heat, NaCl, NaOCl or H_2O_2 exposure.

5.3.2. Suggestions for future study

1. Multi-species biofilms present a greater challenge than the mono-species biofilms (Giaouris *et al*., 2015). Since *S. epidermidis* interacts with a plethora of skin microbes, it is necessary to understand at the molecular level, the effects of such interactions on the susceptibility of *S. epidermidis* biofilm to heat, NaCl, NaOCl or H_2O_2 exposure.
2. Since eDNA is potentially required in the tolerance of *S. epidermidis* biofilm against heat, NaOCl or H_2O_2 exposure, there is need to understand the molecular and biophysical mechanisms through which eDNA promote *S. epidermidis* biofilm's tolerance against heat, NaOCl or H_2O_2 exposure.
3. Given that σ^B regulates several genes, there is need for differential profiling of the σ^B-regulated genes to identify the gene involved in the tolerance of *S. epidermidis* biofilm against heat, NaCl, NaOCl or H_2O_2 exposure.

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APPENDICES

Appendix 1. Physical map of the study area

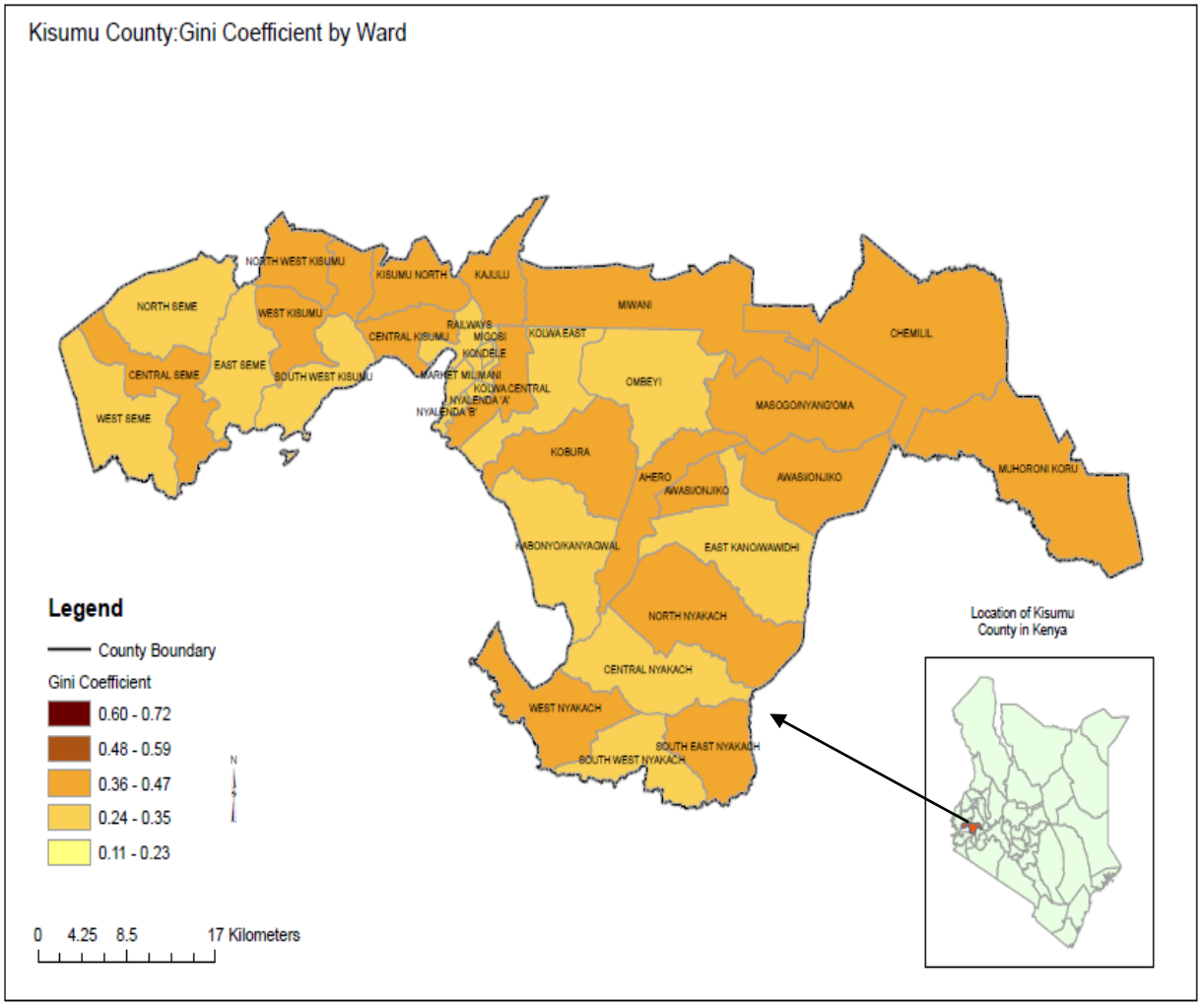


Figure 8. Physical map of the study area (Adapted from Kenya National Bureau of Statistics (KNBS), 2013).

Appendix 2. Curves for determination of the optimal heat, NaCl, NaOCl or H_2O_2 for analysis of the tolerance mechanisms of *S. epidermidis* cells

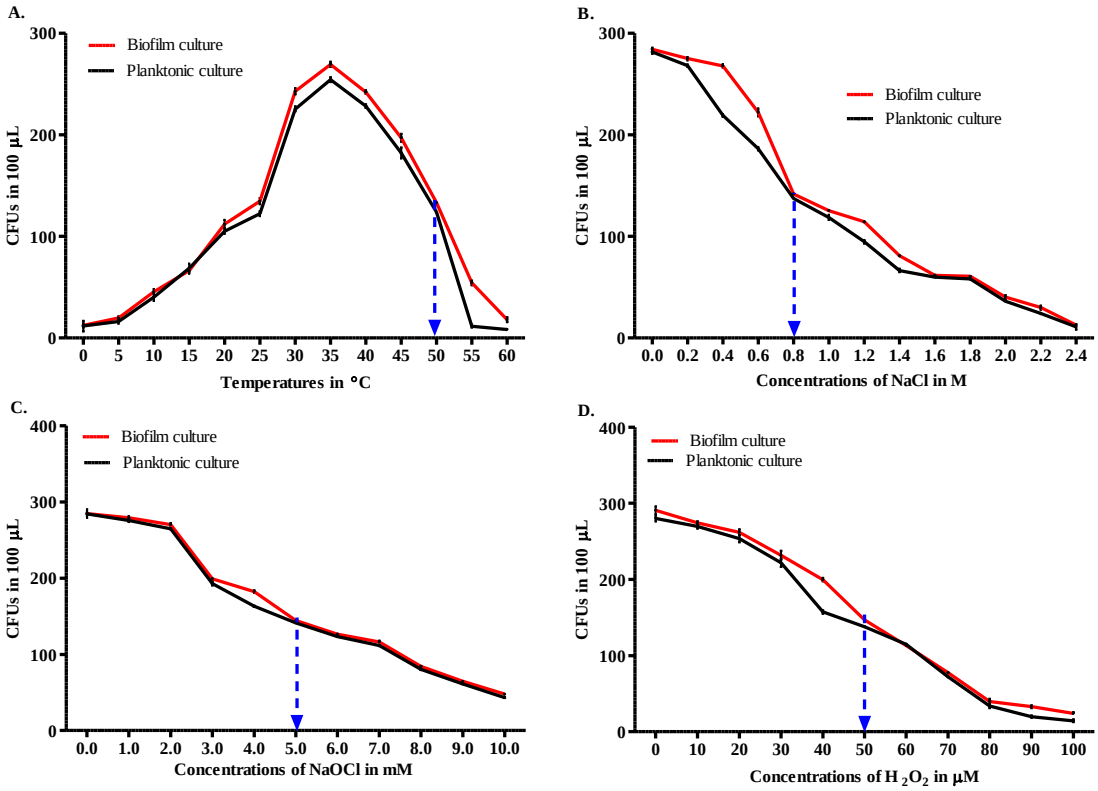
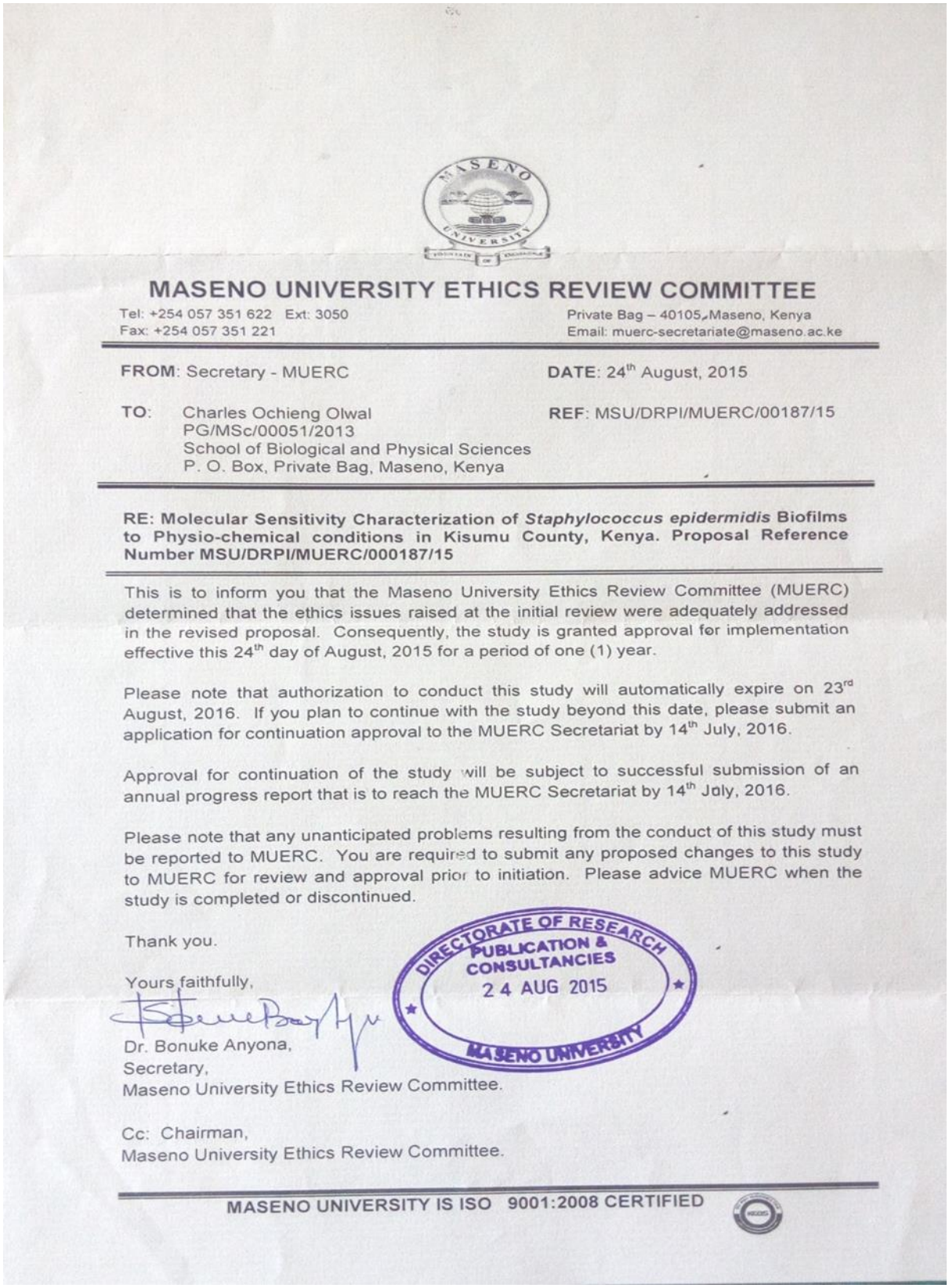


Figure 9. The growth of *S. epidermidis* biofilm and planktonic cells exposed to increasing physico-chemical stress concentrations. A pooled *S. epidermidis* biofilm or planktonic culture (prepared by mixing equivalent numbers of the biofilm or planktonic cells drawn from six different samples) was subjected to increasing temperatures/concentrations of heat (A), NaCl (B), NaOCl (C) or H_2O_2 (D) for 60 min. At each temperature/concentration, *S. epidermidis* planktonic cells were enumerated on TSA plates. The plots at each temperature/concentration depict the mean ± standard deviation of three independent experiments with three technical replicates. The blue dotted arrow represents the temperature or concentration at which *S. epidermidis* biofilm and planktonic cells were considerably stressed (growth reduced by almost 2-fold with reference to the highest CFU value).

Appendix 3. Ethical clearance letter



MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
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Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 24th August, 2015

TO: Charles Ochieng Olwal
PG/MSc/00051/2013
School of Biological and Physical Sciences
P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/00187/15

RE: Molecular Sensitivity Characterization of *Staphylococcus epidermidis* Biofilms to Physio-chemical conditions in Kisumu County, Kenya. Proposal Reference Number MSU/DRPI/MUERC/000187/15

This is to inform you that the Maseno University Ethics Review Committee (MUERC) determined that the ethics issues raised at the initial review were adequately addressed in the revised proposal. Consequently, the study is granted approval for implementation effective this 24th day of August, 2015 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 23rd August, 2016. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 14th July, 2016.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 14th July, 2016.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

Yours faithfully,

DIRECTORATE OF RESEARCH PUBLICATION & CONSULTANCIES 24 AUG 2015 MASENO UNIVERSITY

Dr. Bonuke Anyona,
Secretary,
Maseno University Ethics Review Committee.

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

Appendix 4. Sample consent form

Consent to Participate in Research

Title

Molecular Sensitivity Characterization of *Staphylococcus epidermidis* Biofilms to Physico-chemical stress agents in Kisumu County, Kenya.

Introduction

My name is **Olwal Charles Ochieng'**. I am a graduate student at Maseno University, Kenya, working with my faculty supervisors, **Dr. Paul Oyieng'** and **Dr. Daniel Ochiel** of Zoology department. We are planning to conduct a research study, which we invite you to take part in.

You are being invited to participate in this study because you are a resident of Kisumu County where the study is based.

Purpose

The purpose of this study is to investigate the susceptibility of biofilms to the physico-chemical conditions commonly used for pathogen eradication in Kenya. The findings of the study will inform public health policies on biofilm eradication from water, food and medical devices. About seventy-one (71) participants will take part in this study.

Procedures: If you agree to be in this study, you will be asked to do the following:

- Identify your non-dominating arm.
- A lab technician will swab your arm joint with sterile cotton wool applicator moistened with normal saline.
- It will then be applied directly on mannitol salt agar and incubated overnight before being safely transported to Maseno University lab for further analysis.
- This will be done in Kisumu district hospital laboratory and it will take at most 10 min of your time.

Benefits: There is no direct benefit to you anticipated from participating in this study. However, it is hoped that the information gained from the study will help in controlling and eliminating the infections associated with biofilms in the country and other parts of the world.

Risks/Discomforts: This study poses no known risk to you.

Confidentiality: Your study data will be handled confidentially. If results of this study are published or presented, individual names and other personally identifiable information will not be used. To minimize the risks to confidentiality, we will restrict access to study records.

Retaining research records: When the research is completed, I may save the samples for use in future research done by others or myself. I will retain this study information for up to five years after the study is over. The same measures described above will be taken to protect confidentiality of this study data.

Ownership of specimens: If you consent to giving sample as part of this study, the sample will become the property of Maseno University. The samples and the DNA they contain may be used in this research and in other research, and may be shared with other organizations. The specimens could lead to discoveries or inventions that may be of value to Maseno University or to other organizations. Other investigators intending to use the samples will have to be given written permission from MUERC through the investigator.

Compensation: You will not be paid for taking part in this study.

Participation in research is voluntary: You have the right to decline to participate or to withdraw at any point in this study without penalty.

Questions/complaints: If you have any questions or concerns about this study, you may contact Charles Olwal at **0710937793** or email (**olwalc@yahoo.com**). In case of any questions about your rights or in case of adverse effect/event you can contact; **Maseno University Ethics Review Committee (MUERC) Secretariat** on Tel. no.: **057 351221** or write to them through P.O. Box Private Bag, Maseno or email at **muercsecretariat@maseno.ac.ke**.

Consent statement:

I have read the comments above and agree to participate in this research under the terms outlined herein. I understand that if I have any questions or concerns regarding this project I can contact the investigator at the above location or the Maseno University Ethics Review secretariat (Please read this section loudly for taping as proof of consent). You have been given a copy of this consent form to keep.

Participant's signature_____Date _____

OR

Parent/guardian's signature_____Date _____

Appendix 5. Sample oral questionnaire for recruitment of study participants

I am **Charles O. Olwal**, a graduate student from Maseno University. I am currently collecting skin swab samples for my master's project titled ''Molecular Sensitivity Characterization of *Staphylococcus epidermidis* Biofilms to Physico-chemical stress agents in Kisumu County, Kenya''. I would like to ask you a few questions to verify your eligibility for recruitment into this study. Thank you.

Q1. Which county do you come from?

…….

Q2. Which sub-county or constituency do you come from? .....

Q3. Have you ever visited Kisumu county referral hospital or any other hospital?

A. YES (B) NO

Q4. If yes, when was the last time you visited a hospital?

A. 1 month ago

B. 2 months ago

C. More than 3 months ago

Q5. Have you ever visited any other county?

A. YES (B) NO

Q6. If YES, when did you come back to Kisumu County?

A. 1 month ago

B. 2 months ago

C. More than 3 months ago

Q7. How old are you?

A. Less than 18years

B. 18-65years

C. Above 65years

Q8. If less than 18 years, were you accompanied to the hospital by your parent or guardian?

A. YES (B). NO

Q9. Which soap do you normally use for bathing? ……..

Q10. For how long have you used the soap?

A. Less than 3months

B. More than 3months

C. I can't remember

Q11. Have you ever had any bacterial infection?

A. YES (B) NO

Q12. If yes, did you take any medicine for the bacterial infection?

A. YES (B) NO

Q13. If YES, when is the last time you took medicine for the bacterial infection?

A. Less than 3months

B. More than 3months

C. I can't remember

Q14. Do you have any skin infection?

A. YES

B. NO

C. I prefer not to answer

Q15. Are you immuno-compromised?

A. YES

B. NO

C. I prefer not to answer