Understanding the resistance and virulence mechanisms of *Staphylococcus epidermidis* triggered during skin disinfection, blood production and storage

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ABSTRACT

Bacterial contamination of platelet concentrates (PCs) represents the highest post-transfusion infectious risk. The skin flora bacterium *Staphylococcus epidermidis* has been reported to be the predominant aerobic contaminant of PCs. The Ramirez' group has shown that *S. epidermidis* can form surface-attached bacterial aggregates known as biofilms, and can outcompete other coagulase-negative staphylococci, such as *Staphylococcus capitis,* in PCs. The ability of *S. epidermidis* to form biofilms has been linked to increased pathogenicity and missed detection during PC screening with an automated culture system (BacT/ALERT). This thesis aimed at investigating the proliferative advantage and resistance mechanisms displayed by *S. epidermidis* in the PC milieu. Furthermore, in an effort to enhance PC safety for transfusion patients, I studied the anti-biofilm properties of essential oils and antimicrobial peptides (AMPs).

My studies aimed at improving PC safety by focussing on both the point of introduction of bacterial contaminants (blood collection), and the stage at which bacterial contaminants can form biofilms and proliferate (PC storage). *S. epidermidis* can be found in the skin of blood donors as biofilms, which are resistant to the blood donor skin disinfectant currently used by Canadian Blood Services, chlorhexidine-gluconate and isopropyl alcohol (CHG-IPA). Here, several plant-extracted essential oils were evaluated for their ability to enhance the anti-biofilm activity of CHG-IPA. Data revealed that the *Lavandula multifida* oil and its main component (linalool) greatly enhanced the activity of CHG-IPA against *S. epidermidis* biofilms. Furthermore, the ability of a combination of three synthetic AMPs to inhibit *S. epidermidis* biofilm formation during PC storage was assessed These results showed that the combination of AMPs could inhibit biofilm formation but was ineffective against pre-formed *S. epidermidis* biofilms.

The accumulation associated protein (Aap) encoded by the *aap* gene, found in most *S. epidermidis* strains and absent in *S. capitis,* plays a role in biofilm formation. When *S. epidermidis aap* is transformed into *S. capitis*, this bacterium displayed increased biofilm formation and proliferated to higher

concentrations compared to untransformed *S. capitis* and to a *S. epidermidis aap* deletion mutant. Based on these results, *aap* appears to play a role in providing *S. epidermidis* a proliferative advantage in PCs by enhancing biofilm formation. Lastly, the GraRS system and SepA were studied for their role in *S. epidermidis* resistance to platelet-derived AMPs using the synthetic AMP PD4 as a model molecule. Results indicate that the GraS mechanism is involved in resistance towards PD4. The work presented in my thesis provides further insights into why *S. epidermidis* has a proliferative advantage in the PC storage environment and allows for the proposal of alternative methods to enhance PC safety for transfusion patients.

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LIST OF ABBREVIATIONS

µm: Micrometer

µM: Micromolar

Aap: Accumulation-associated protein

ACD: Acid citrate dextrose

ACD-A: Adenine, Citrate Dextrose-Formula A

ADP: Adenosine Diphosphate

AMPs: Antimicrobial Peptide

ATCC: American type culture collection

AtlE: Autolysin E

Bap: Biofilm-associated protein

Ca^{2+}: Calcium

CD62P: P-selectin

CDI: Coefficient of drug interaction

CFU: Colony forming unit

CHG: Chlorhexidine-gluconate

CO_2: Carbon Dioxide

CoNS: Coagulase-negative staphylococci

CPD: Citrate phosphate dextrose

CPDA: Citrate phosphate dextrose adenine-1

CTAP-3: Connective Tissue Activating Peptide 3

eDNA: extracellular Deoxyribonucleic Acid

Embp: Extracellular matrix binding protein

EOs: Essential oils

FP-B: Fibrinopeptide B

GC-MS: Chromatography-mass spectrometry

GlcNAc: N-acetylglucosamine

GP: Glycoprotein

GraRS: Antimicrobial peptide sensor system

GRAS: Generally regarded as safe

H: Hour

HBD-3: Human beta defensin-3

HLA: Human leukocyte antigen

IPA: Isopropyl alcohol

LB: Luria-Bertani broth

LBA: Luria-Bertani agar

Min: Minute

mM: Millimolar

MSCRAMM: Microbial surface components recognizing adhesive matrix molecules

NetCAD: Network centre for applied development

O_2: Oxygen

OD: Optical density

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PCs: Platelet concentrates

PD4: Platelet-derived peptide

PF4: Platelet Factor 4

PGA: Poly-γ-DL-glutamic acid

PIA: Polysaccharide intercellular adhesin

Pls: Plasmin-sensitive

PLTs: Platelets

PRT: Pathogen reduction technologies

RBCs: Red blood cell concentrates

SAGM: Saline adenine glucose mannitol

SCID: Severe combined immunodeficiency

SDs: Standard deviations

TSA: Trypticase soy agar

TSB: Trypticase soy broth

TSBg: Trypticase soy broth supplemented with glucose

Tβ-4: Thymosin Beta 4

UV light: Ultraviolet light

Vol: Volume

vWF: Von Willebrand factor

WBCs: White blood cells

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CHAPTER 1.

General Introduction

1.1 TRANSFUSION MEDICINE

In 1818, the first human-to-human blood transfusion was reported by Dr. James Blundell in Medico-Chirurgical Society of London; however, the use of transfusions for patient treatment was limited in the 19th century (Greenwalt, 1997). The discovery of the four main blood groups in the early 20th century by Dr. Landsteiner [groups A, B, and C (more commonly known as group O)], and Drs. Decastello and Sturli (group AB) opened new doors in the field of transfusion medicine (McCullough, 2012).

The clotting of blood posed a serious obstacle in the implementation of blood transfusions in the 19th century, therefore various medical devices were developed to facilitate direct donor to recipient transfusions. The disadvantages of direct donor to recipient transfusion included having a donor readily available and occurrence of blood-borne diseases such as syphilis, which required improvements in blood storage to enhance blood availability and safety (McCullough, 2012). In 1915, several groups described sodium citrate as the first anticoagulant to be used in blood transfusions (Greenwalt, 1997; Lewisohn, 1924; Weil, 1915). Since then, new additive solutions have been reported to avoid coagulation and improve the shelf life and quality of blood products. Notable examples include Acid Citrate Dextrose (ACD) additive solution developed by Loutit and Mollison in 1943 (Loutit and Mollison, 1943), and Citrate Phosphate Dextrose (CPD) and Citrate Phosphate Dextrose Adenine-1 (CPDA-1) additive solutions developed by Gibson and colleagues in 1957 (Gibson et al., 1957).

Transfusion medicine underwent dramatic advances during World Wars I and II. For example, Captain Oswald Robertson performed 22 transfusions of whole blood (stored at cold temperatures for up to 26 days with an additive solution containing sodium citrate) during World War I (Chandler et al., 2012), and during World War II, the Rh blood group system was discovered (The American National Red Cross, 2002). The first blood bank was established in Barcelona in response to the growing need for blood during the Spanish Civil War (McCullough, 2012). Whole blood was initially stored in glass containers until 1960s when sterile plastic storage bags were introduced (McCullough, 2012; Gullbring, 1964). The

plastic bags allow the separation of one whole blood into several blood components such as red blood cell concentrates (RBCs), plasma, and platelet concentrates (PCs).

1.2 BLOOD COMPONENTS MANUFACTURED AT CANADIAN BLOOD SERVICES

Canadian Blood Services manufactures several blood products including RBCs, plasma, and PCs for treatment of patients in need. Each year Canadian Blood Services collects approximately 900,000 units of whole blood and approximately 30,000 apheresis donations, and manufactures more than a million of RBC, plasma and PC units (Canadian Blood Services, 2007).

Whole blood is collected in containers containing CPD. RBCs are separated by centrifugation and filtration from leukocytes, plasma, and platelets. The RBCs are suspended in saline adenine glucose mannitol (SAGM), and stored at 1-6^{o}C for a maximum of 42 days to treat anemic patients (Canadian Blood Services, 2017a).

Plasma products produced at Canadian Blood Services are used to treat patients with a wide variety of coagulation factor and plasma protein deficiencies. Consequently, these plasma products are required to have varying compositions of these factors. The plasma products produced at Canadian Blood Services include apheresis fresh frozen plasma (frozen within eight hours of collection time), frozen plasma (frozen within 24 hours of collection time), cryosupernatant plasma, and cryoprecipitate. These products are manufactured using different combinations of centrifugation and filtrations and stored for a maximum of one year at -18^{o}C or colder (Canadian Blood Services, 2017a).

PCs are used to treat patients with bleeding disorders due to platelet dysfunction or deficiency. PC manufacturing processes are described below (Canadian Blood Services, 2017a).

1.3 PLATELET CONCENTRATES

1.3.1 Platelets

Platelets are anucleated cells that are two to five micrometers (μm) in diameter. They are discoid-shaped, granulated, and mainly responsible for hemostasis. Platelets are derived from the fragmentation of megakaryocytes, a giant bone marrow progenitor cell. The normal platelet count in healthy individuals

ranges from 150× 10^9/ L to 400 × 10^9/ L, and the lifespan of circulating platelets is approximately seven to ten days (Harrison and Briggs, 2013; White, 2013).

Platelets play a key role in the hemostatic response to vascular injury. In general, during vascular injury circulating platelets are exposed to collagen and the collagen-von Willebrand factor (vWF) complex which activate platelets via platelet surface receptors such as α2β1 and glycoprotein (GP) Ib-IX. This results in the alteration of the platelet shape from discoid to a sticky irregular shape, which allows plug formation at the site of vascular injury. In addition, activated platelets recruit and activate other circulating platelets by releasing adenosine diphosphate (ADP), thromboxane A2, and calcium (Ca^{2+}), thereby enhancing plug formation. The platelet aggregation that follows can be regulated by several factors including the platelet surface glycoprotein (GP) IIb-IIIa and the cell adhesion molecule CD62P (P-selectin) (Brass et al., 2013).

1.3.1.1 Platelets and the Immune System

Platelets are not only essential for haemostasis, but they also play a role in innate and adaptive host defences against infection during vascular injury. Upon platelet activation, platelets release intracellular granules that are expressed on the platelet membrane surface and other granules are released into the host circulation. The granules in the host circulation contain chemokines and cytokines to enhance the recruitment and activation of neutrophils and macrophages to the site of infection (Yeaman, 2014; Semple et al., 2011). Upon activation, platelets also release alpha granules that possess antimicrobial activity as a defense mechanism against bacterial infection as described in the next section (Yeaman, 2014; Jenssen et al., 2006; Tang et al., 2002).

1.3.1.2 Platelet-Derived Antimicrobial Peptides

A variety of human cells including cells of the immune system such as neutrophils and platelets produce antimicrobial peptides (AMPs). Generally, AMPs are cationic, amphipathic molecules that are approximately 12 to 100 amino acids in length, and with a chemical structure that allows them to cross and disrupt the negatively charged bacterial cell membrane (Bahar and Ren, 2013; Jenssen et al., 2006). Activated platelets release antimicrobial effector molecules including platelet factor 4 (PF4), connective

tissue activating peptide 3 (CTAP-3), fibrinopeptide B (FP-B), and thymosin Beta-4 (Tβ-4) that have been shown to have bactericidal effects against *Staphylococcus aureus* and *Escherichia coli*. Interestingly, PF4 and CTAP-3 demonstrated synergistic bactericidal effect against *E. coli* (Tang et al., 2002). Since AMPs exhibit broad-spectrum activity against bacteria, and can work synergistically with antibacterial drugs, there has been a lot of interest in investigating the role of AMPs as a new generation of bactericidal agents (Sierra et al., 2017; Różalski et al., 2013; Jenssen et al., 2006).

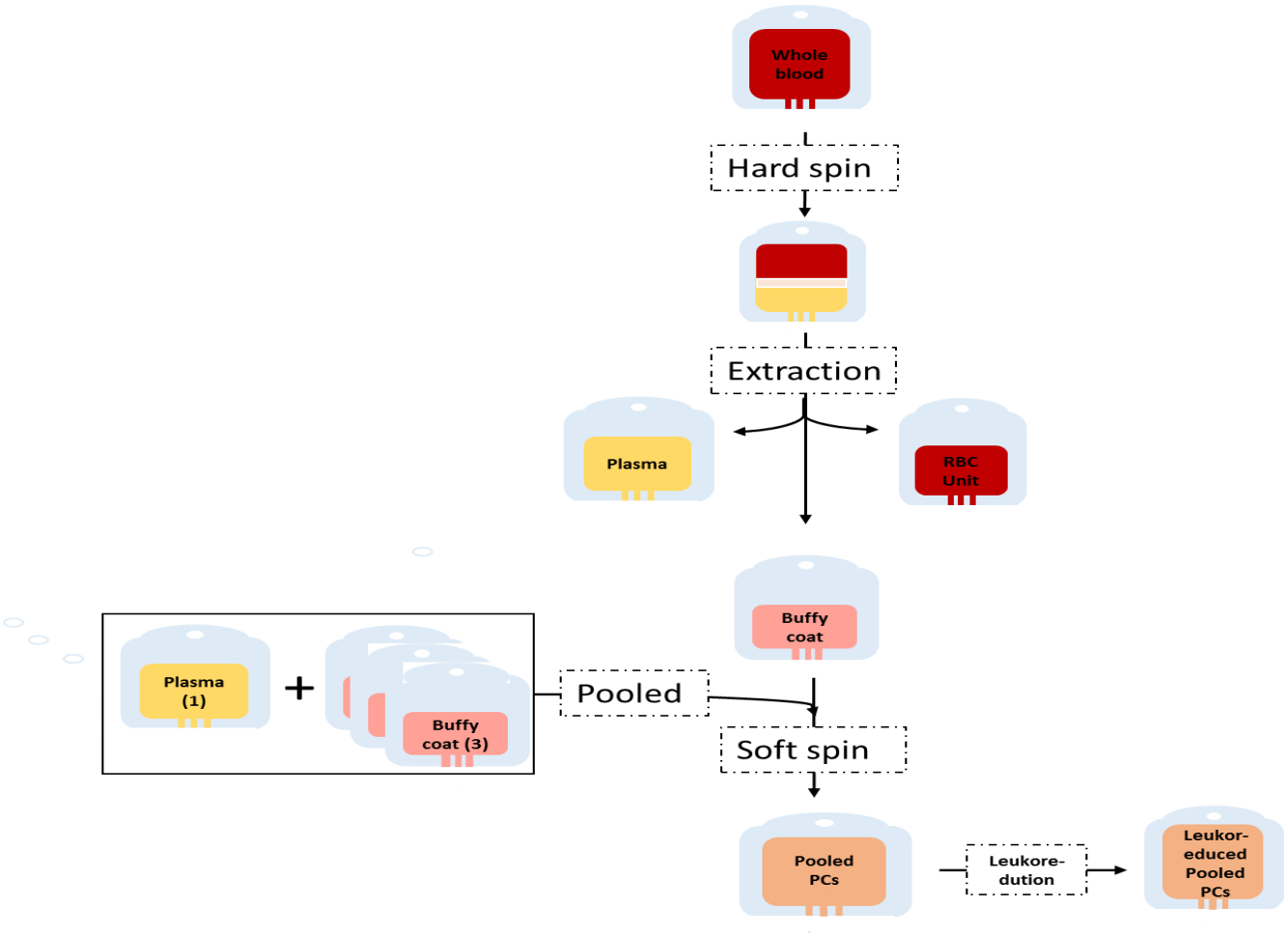
1.3.2 PC Production

PCs are manufactured at Canadian Blood Services using two approaches: the buffy coat method and the apheresis method (Fig. 1.1). In the buffy coat production method, four whole blood (ABO-matched) donations collected in CPD are spun to separate platelets and leukocytes (buffy coat) from RBCs and plasma. The buffy coat components are then extracted, and the four buffy coat units are pooled together and suspended in one of the autologous plasma units. Any residual leukocytes are removed from the pooled platelet unit via filtration in a process called leukoreduction and the final product is a PC pool. In the apheresis method of PC production, a single donor's blood is collected in a closed sterile system and processed through an apheresis (centrifugation) machine where PCs and plasma are separated and the rest of the blood components are returned into the donor's bloodstream (Canadian Blood Services, 2017a; Canadian Blood Services, 2017b). Apheresis PCs are mixed with ACD-A (adenine, citrate dextrose-formula A) anticoagulant solution, and ideally used to treat patients who have become alloimmunized as a result of recurrent PC transfusions and thus require apheresis PCs that are human leukocyte antigen (HLA) matched to avoid immune rejection of this product (Canadian Blood Services, 2016a; Vamvakas, 2009).

1.3.3 PCs for Therapeutic Use

In 1960, the introduction of plastic storage bags that allowed for the separation of PCs from the rest of the blood components dramatically enhanced transfusion therapy in medical practice. Shortly after, Hersh

(A) Buffy coat method



(B) Apheresis method

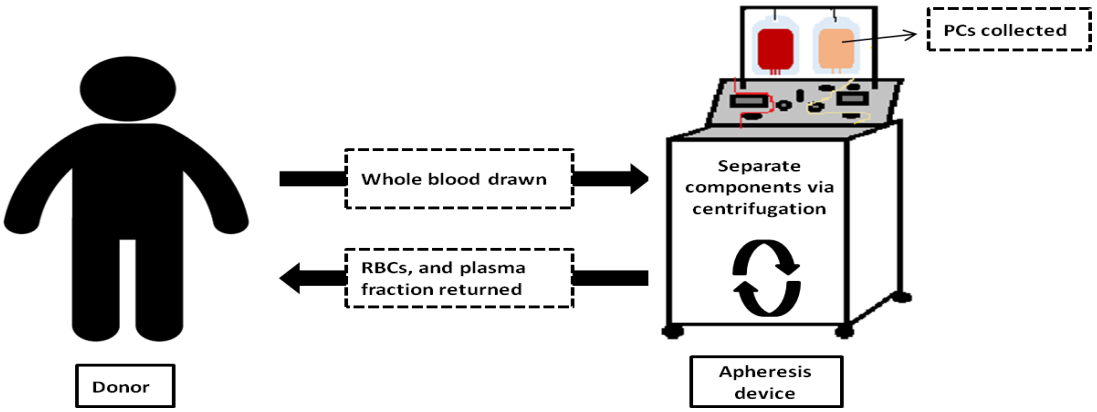


Figure 1.1. Production methods of PCs. Diagrams represent (A) buffy coat method and (B) apheresis method.

and coworkers demonstrated that PC transfusion could successfully treat acute leukemia-induced hemorrhage (Hersh et al., 1965; Stroncek and Rebulla, 2007). Currently, PC transfusions are used to treat patients with bleeding disorders caused by decreased or dysfunctional platelets (Canadian Blood Services, 2017a). The transfusion of PCs has become an important part in the treatment of haematological malignancies (such as acute leukemia), solid tumor chemotherapy, and marrow failure (Stroncek and Rebulla, 2007).

1.3.4 Platelet Storage Lesion

PCs are stored in gas-permeable plastic containers with an additive solution containing dextrose at a concentration of approximately 25 g/L, at 20-24^{o}C under constant gentle agitation for up to 7 days (Canadian Blood Services, 2017a; Canadian Blood Services, 2017b). The shelf life of PCs is restricted by the storage related deterioration of platelet function and quality known as platelet storage lesion (Rijkers et al., 2017). This PC deteriorating process can be monitored *in vitro* by assessing platelet counts, shape, size, pH, O_2 consumption, glucose consumption, lactate production, CO_2 production, hypotonic shock response, and increased expression of platelet activation indicator CD62P (P-selectin) surface expression (Perrotta et al., 2013).

1.4 BACTERIAL CONTAMINATION OF PCs

In modern transfusion medicine, bacterial contamination of PCs is a major concern in developed countries. It is the predominant cause of infectious transfusion reactions with sporadic cases of fatalities that are reported worldwide (Horth et al., 2018; Thyer et al., 2018; Ramirez-Arcos et al., 2017; Food and Drug Administration, 2016; Kou et al., 2015; Palavecino et al., 2012). Septic transfusion reactions owing to bacterial contamination have been reported to occur at a rate of approximately 1/100,000 transfused PC units (Ramirez-Arcos et al., 2017; Walther-Wenke and Schmidt, 2011). These rates have been suggested to be an under estimation since not all reactions can be retroactively correlated to a transfusion event

(Benjamin et al., 2017; Walther-Wenke et al., 2010; Eder et al., 2009). Of all blood products, PCs are the most susceptible to bacterial contamination due to their storage conditions, which offer bacteria an ideal environment for proliferation (Canadian Blood Services, 2017b; Ramirez-Arcos and Goldman, 2012).

1.4.1 Strategies used to prevent bacterial contamination in PCs

At Canadian Blood Services, several strategies have been implemented to prevent and/or reduce the bacterial contamination of blood products. The first strategy involves assessing blood donors' general health by filling a mandatory questionnaire and measuring donors' body temperature on the day of donation to prevent transfusion-transmissible diseases (Canadian Blood Services, 2016b; Ramirez-Arcos and Goldman, 2012). The next approach is the implementation of blood donor skin disinfection at the venipuncture site to reduce the contamination of blood products by skin microflora. Diversion pouches were implemented in 2003; these divert the first 30-40 mL of donated blood, which is deemed most likely to contain bacterial contaminants that are acquired locally from the donor's skin (Jenkins et al., 2011). At Canadian Blood Services, 100% of PC products are tested for bacterial contamination using the automated bacterial detection system BacT/ALERT which has been demonstrated to have a high detection sensitivity of 1-10 colony forming units (CFU)/mL of bacteria per PC unit (Jenkins et al., 2011; Benjamin and McDonald, 2014). In August 2017, Canadian Blood Services implemented a new testing algorithm that allowed for the extension of shelf life of PCs from five to seven days, thereby improving PC safety (Ramirez-Arcos et al., 2018). Various changes associated with the new testing algorithm include delayed testing at ≥ 36 hours post collection; inoculation of aerobic and anaerobic culture bottles; and post-sampling quarantine at ≥ 6 hours. Testing algorithms used by Canadian Blood Services for 5-day PCs (March 2004 to August 2017) and 7-day PCs (since August 2017) are described in Table 1.1. Although PC screening for bacterial contamination has enhanced PC safety, septic transfusion reactions still occur as outlined in Table 1.2.

Table 1.1. PCs testing algorithms for bacterial contamination implemented at Canadian Blood Services (Ramirez-Arcos et al., 2018)

Condition	**March 2004 – August 2017**	**Since August 2017**
PC Shelf life	Five days	Seven days
Sampling time post-collection	24-30 h	≥ 36 h
Volume	8-10mL	16-20mL
BacT/ALERT culture bottles	Aerobic	Aerobic and anaerobic
Hold on tested PCs before release into inventory	None	≥ 6 h

Table 1.2. Septic transfusion reactions documented at Canadian Blood Services

	January 2010 to December 2016	**August 2017 to July 2018**
Number of PC products tested (buffy coat pools and apheresis units)	788,725	97,588
Number of septic transfusion reactions	6	1
Implicated Bacteria	• Coagulase-negative staphylococci (n = 3) * • *S. aureus* (n = 3)	• *S. epidermidis*
Reference	Ramirez-Arcos et al., 2017	Personal communication Dr. S. Ramirez-Arcos

* One case of *S. epidermidis* septic reaction led to fatality (Kou et al., 2015)

1.4.1.1 Donor Skin Disinfection

Skin flora bacteria have been implicated as the predominant contaminants of PCs; thus, optimal disinfection of the antecubital area before venipuncture is an crucial step to reduce PC contamination rates (Ramirez-Arcos and Goldman, 2012). Before implementing any new skin disinfectant, blood centers consider factors including acceptability by phlebotomists, availability in the market, and suitability for blood donors (Story-Roller and Weinstein, 2016; Washer et al., 2013; McDonald, 2011; Barenfanger et al., 2004). It is also important to consider the skin disinfectant type, concentration, time of contact on skin, and mode of action (e.g., scrub, swab) since these factors influence the efficacy of skin disinfection (Ramirez-Arcos and Goldman, 2012; McDonald et al., 2010). Consequently, countries including Canada, the United States, and the United Kingdom have implemented a one-step skin disinfection method, the ChloraPrep™ kit (Ramirez-Arcos and Goldman, 2010; Benjamin et al., 2011; McDonald et al., 2010). ChloraPrep is a swab stick containing 3 mL of 2% chlorhexidine-gluconate (CHG) and 70% isopropyl alcohol (IPA) (Ramirez-Arcos and Goldman, 2010). This combination of disinfectants (CHG-IPA) with a 30-second application time reduces but does not eliminate the rate of PC contamination (Ramirez-Arcos and Goldman, 2010; Benjamin et al., 2011; McDonald et al., 2010). Several studies have documented that essential oils have antibacterial activity and high penetration through skin (Hendry et al., 2009; Karpanen et al., 2010; Karpanen et al., 2008a), which could potentially be used to enhance the efficacy of CHG-IPA as described in chapter two (Alabdullatif et al., 2017).

1.4.1.2 Bacterial detection methods

There are several detection methods for bacterial contamination in PCs that are generally divided into culture and rapid methods as shown in Table 1.3. Culture methods require incubation of contaminant bacteria for at least 24 hours and usually possess high sensitivity (Table 1.3). Rapid methods are usually performed prior to transfusion, and only require a few hours but have lower sensitivity than culture methods (Table 1.3) (Störmer and Vollmer, 2014). One or more of the detection methods are used by several countries to enhance blood product safety, and interestingly the BacT/ALERT system

Table 1.3. Bacterial detection methods

Methods	Manufacturer	Principle of detection	Comments	Reference
Culture methods				
1. BacT/ALERT	BioMerieux	Special culture bottles are used to detect bacterial growth by increasing CO_2 production, which changes the color of sensor at the bottom of bottle	• Sample volume 4-10mL • Sensitivity 1-10 CFU/mL	Benjamin and McDonald, 2014; Störmer and Vollmer, 2014
2. BACTEC	BD Biosciences	Special culture bottles are used to detect bacterial growth by fluorescence which corresponds to the amount of CO_2 production	• Sample volume 4-10mL • Sensitivity 1-10 CFU/mL	Savini et al., 2009; Störmer and Vollmer, 2014
3. VersaTrek	Trek Diagnostics	Special culture bottles with pressure changes detector in the headspace used to monitor bacterial growth by gas consumption and production	• Sample volume 4 mL • Sensitivity 10-20 CFU/mL	Nanua et al., 2009; Störmer and Vollmer, 2014
4. pH measurement	Not applicable	Bacterial growth detection measured by rate of pH change	• Sample volume 15 mL • Sensitivity 10^3-10^6 CFU/mL	Barker et al., 2010
5. Haemonetics (eBDS)	Haemonetics	Bacterial growth detection measured by electric changes due to oxygen consumption	• Sample volume 2-3 mL • Sensitivity 1-10 CFU/mL	McDonald et al., 2005
6. Microcalorimetry	TA Instruments	Bacterial metabolism during growth measured by heat flow over time	• Sample volume ~1mL • Sensitivity 1-10 CFU/mL	Trampuz et al., 2007
Rapid methods				
1. BactiFlow	BioMerieux	Flow cytometer is used to detect bacteria based on non-fluorescent fluorochrome cleavage by intercellular esterase in viable cells	• Sample volume 1mL • Sensitivity 300-500 CFU/mL • The time to result is 1 hour	Dreier et al., 2009; Vollmer et al., 2013; Störmer and Vollmer, 2014

2. Pan Genera Detection (PGD)	Verax Biomedical Inc.	Lateral flow immunoprecipitation used to detect bacterial cell wall antigens (lipopolysaccharide or lipoteichoic acid)	• Sample volume 0.5mL • Sensitivity 10^4-10^6 CFU/mL • The time to result is 1.5 hour	Vollmer et al., 2010; Störmer and Vollmer, 2014
3. BacTx	Immunetics	Colorimetric assay used to detect bacterial peptidoglycan	• Sample volume 1mL • Sensitivity 10^3-10^6 CFU/mL • The time to result is 1 hour	Palavecino et al., 2010; Ramirez-Arcos et al., 2014
4. Nucleic acid test (NAT)	Not applicable	Detect bacteria by amplification of nucleic acids (e.g., 16S rDNA)	• Sample volume 1mL • Sensitivity 12-155 CFU/mL • The time to result is 4 hours	Sireis et al., 2011

is the most commonly used in blood banks worldwide to detect bacterial contamination (Elantamilan et al., 2017; Esmaili et al., 2017; Das and Baruah; 2016; Störmer and Vollmer, 2014; McDonald, 2013). The BacT/ALERT system is an automated culture method that is used at least 24 hours post donation. Briefly, PC samples are inoculated into aerobic and/or anaerobic culture bottles containing 40 mL of media that enhances bacterial growth. The inoculated bottles are placed into the BacT/ALERT system and incubated at 35-37^{o}C for up to 7 days. The colorimetric sensor at the bottom of the bottles changes color from green to yellow when pH decreases because of CO_2 production by growing bacteria. This change in colour is detected by the BacT/ALERT system and flagged as positive for the existence of bacteria (McDonald et al., 2017; Thorpe et al., 1990).

Even though the BacT/ALERT system has a high sensitivity (1-10 CFU/mL), missed detections of bacterial contamination of PCs has been reported (Food and Drug Administration, 2016; Kou et al., 2015; Benjamin and McDonald, 2014). Missed detections could be related to very low initial bacterial concentrations in the PC unit, slow bacterial growth, and bacterial ability to form surface-attached cell aggregates embedded within a self-produced matrix known as biofilms (McDonald, 2013; Murphy et al. 2008; Ali et al., 2014; Greco-Stewart et al., 2012). It has been estimated that the initial concentration of bacteria in contaminated PC units is approximately 1-100 CFU/PC unit, which could cause false-negative screening results due to sampling error even with delayed sampling (Ramirez-Arcos et al., 2018; McDonald et al., 2017; McDonald, 2013).

1.4.1.3 Pathogen Reduction Technologies

Several countries have implemented pathogen inactivation to prevent transfusion of contaminated PCs. There are three pathogen reduction technologies (PRT) that can be used to treat PCs. Two of these PRT, Intercept™ and Mirasol®, are available in the market while the third technology, THERAFLEX UV-Platelets, is currently used in clinical trials (Devine and Schubert, 2016). Intercept (Cerus, California, USA) uses UV light (320-400 nm) in combination with the nucleic acid photosensitizer amotosalen which causes a covalent crosslink of any nucleic acids where amotosalen is bound, thereby preventing replication of pathogens. Mirasol (Terumo BCT, Colorado, USA) utilizes UV light (280-360 nm) in

combination with the nucleic acid photosensitizer riboflavin (vitamin B2) which interacts with the nucleic acid causing oxidation of guanine, thereby preventing replication of infectious agents. On the other hand, THERAFLEX (Macopharma, Tourcoing, France) uses UV light (254 nm) to prevent nucleic acid replication by forming pyrimidine dimers in the absence of any photosensitizers (Devine and Schubert, 2016). Apart from their ability to reduce bacterial contamination in PCs, PRT also minimize the risk of graft-versus-host disease since they inactivate white blood cells. Though PRT hold great promise, these technologies have shown to have adverse effects on platelet quality indicated by increased glucose metabolism, and increase CD62P expression on the platelet surface, which is known as an indicator of platelet activation (Reikvam et al., 2010; Devine and Schubert, 2016). Furthermore, they have been reported to be ineffective at eradicating the safety risk caused by bacterial spores and non-enveloped viruses (Schlenke, 2014; Devine and Schubert, 2016). Nevertheless, there have been no reports of septic transfusion reactions in countries where PRT have been implemented (Jutzi et al., 2018). In the United States, the Intercept system has been implemented at the University of Colorado Hospital in 2016 with no transfusion-transmitted bacterial infections reported up to date (Neff, 2016). Intercept is the only PRT approved for use in PC products in Canada (Cerus 2018); however, this technology has not been implemented by any of the two Canadian blood suppliers Héma-Québec or Canadian Blood Services.

1.4.2 Bacteria implicated in PC contamination

A wide range of Gram-negative and Gram-positive bacteria can proliferate in PC products, and they can reach clinical dangerous levels during PC storage (Ramirez-Arcos et al., 2017; Canellini et al., 2010). Contamination of PCs with Gram-negative bacteria (e.g., *E. coli*, *Serratia marcescens*, and *Klebsiella pneumoniae*) is less common, but contamination with this group of bacteria is most likely to cause septic reactions and fatality. By contrast, Gram-positive skin flora bacteria [coagulase-negative staphylococci (CoNS) and *Propionibacterium acnes*] are the predominant PC contaminants (Ramirez-Arcos et al., 2017; Canellini et al., 2010; Müller et al., 2015; Girgis et al., 2014; Zhu et al., 2009; Kuenhnert et al., 2001).

The coagulase-negative *Saphylococcus epidermidis* is the most commonly isolated aerobic bacterial contaminant of PCs (Ramirez-Arcos et al., 2017; Müller et al., 2015; Dickson et al., 2013). This

can be attributed to the fact that *S. epidermidis* is a predominant colonizer of the skin and can be found specifically in the antecubital area from where it is thought to be introduced into PC products by venipuncture during phlebotomy (Ramirez-Arcos and Goldman, 2012; Grice and Segre, 2011; Karpanen et al., 2010; Kloos and Musselwhite, 1975; Otto, 2009).

1.4.2.1 Staphylococcus epidermidis

S. epidermidis is a commensal resident of the normal human skin flora. There are several mechanisms that provide *S. epidermidis* with the ability to survive in human skin. Human skin cells produce the AMP human beta defensin-3 (HBD-3) but the *S. epidermidis* displays resistance to the bactericidal action of HBD-3. This bacterium possesses the antimicrobial peptide sensor (APS) system, also known as GraRS, which increases the positive charge of the bacterial cell membrane and teichoic acids, and exports AMPs, including HBD-3, out of the cells (Li et al., 2007a). In addition, *S. epidermidis* secretes the SepA protease that can degrade anionic AMPs like the sweat gland-produced dermcidin (Lai et al., 2007). *S. epidermidis* also secretes compounds like poly-γ-DL-glutamic acid (PGA) and phenol-soluble modulin toxins to resist phagocytosis, lyse neutrophils, and withstand environments with high salt concentrations (Cheung et al., 2010; Kocianova et al., 2005).

Although *S. epidermidis* is considered to be harmless, this bacterium is an opportunistic nosocomial pathogen responsible for most infections associated with indwelling medical devices, mainly due to its ability to form biofilms (Schaeffer, 2015; Otto, 2009; Rohde 2007; Costerton, 2007).

1.4.2.1.1 Staphylococcus epidermidis biofilm formation

The pathogenicity of *S. epidermidis* has been linked to its ability to form biofilms (Otto, 2009). The biofilm environment increases resistance to antibiotics by reducing antibiotics penetration (Singh et al., 2010), among other mechanism (Shapiro et al., 2011; Otto, 2009). *S. epidermidis* biofilms have been demonstrated to be able to evade eradication by the immune system with reports demonstrating that *S. epidermidis* biofilms display heightened protection from neutrophil-dependent killing compared to free-floating bacteria (Vuong et al., 2004; Kocianova et al., 2005; Kristian et al., 2008).

The process of biofilm formation can be divided into four steps: attachment, accumulation, maturation, and dispersal (Fig. 1.2). Primary attachment of *S. epidermidis* to biotic and abiotic surfaces can be mediated by several surface proteins including autolysin E (AtlE) and microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Otto, 2009). Attachment is followed by accumulation (cell-cell adhesion and proliferation) which is an essential step for biofilm formation and has been shown to be mediated by polysaccharides (such as the polysaccharide intercellular adhesin; PIA), proteins [including the accumulation-associated protein (Aap), the extracellular matrix binding protein (Embp), the biofilm-associated protein (Bap)], and/or extracellular DNA (eDNA) (Schaeffer et al., 2015; Büttner et al., 2015; Hodgson et al., 2014; Qin et al., 2007; Rohde et al., 2007; Tormo et al., 2005). As the biofilm matures, single cells or cell aggregates start to detach from the biofilm as a result of mechanical stress or quorum sensing signalling (Otto, 2009).

S. epidermidis production of PIA is essential for *in vitro* and *in vivo* biofilm formation in some strains (Otto, 2009; Zhang et al., 2003). PIA-dependent biofilm formation is driven by the enzymes encoded by the intercellular adhesin operon (*icaADBC*) and regulated by the IcaR protein. IcaA and IcaD produce a chain of N-acetylglucosamine (GlcNAc) polymers which constitute the backbone of PIA (Otto, 2009; Gerke et al., 1998). IcaC has been suggested to be responsible for the export of the polymer chains, while IcaB appears to direct the de-acetylation of the N-acetylglucosamine chain increasing the positive charge in PIA to enhance attachment to the negatively charged bacterial cell surface (Otto, 2009; Vuong et al., 2004).

Although PIA production is commonly associated to a biofilm-positive phenotype, PIA-independent biofilm formation by *S. epidermidis* strains associated with clinical infections has been reported (Rohde et al., 2007). Approximately 33% of *S. epidermidis* clinical isolates have been documented to be incapable of producing PIA (Schaeffer et al., 2015; Fey and Olson, 2010). Furthermore, the environment in which bacteria exist could influence whether biofilms can be formed, as well as the nature of the biofilm structure itself.

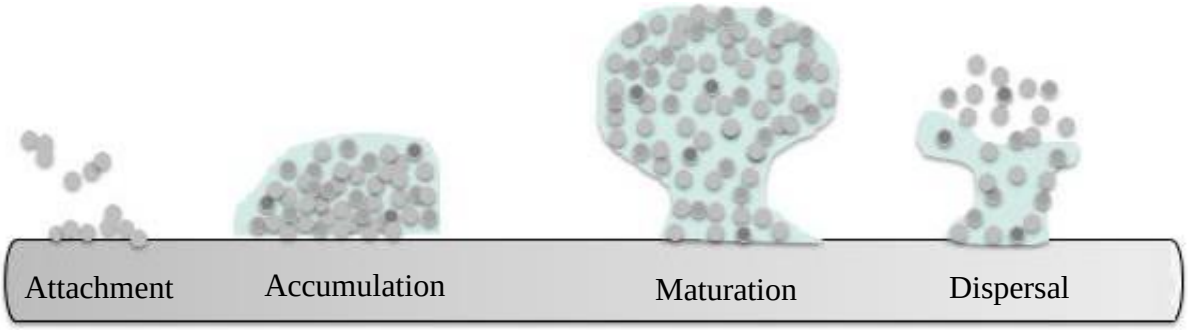


Figure 1.2. Biofilm formation process steps. (1) Irreversible attachment to biotic and abiotic surfaces (2) cell to cell adhesion causing accumulation and the production of the extracellular matrix (light green) (3) maturation (4) dispersal of single cell or cell aggregates.

Interestingly, the Ramirez's laboratory documented that *S. epidermidis ica* negative strains that were unable to form biofilms in laboratory media could form biofilms in PCs (Ali et al., 2014; Hodgson et al., 2014). In addition, the structure of the biofilm matrix of *S. epidermidis* is modified when grown in PCs, exhibiting protein-based biofilm matrix instead of polysaccharide-based biofilm matrix in laboratory media (Loza Correa et al., 2017). This suggests that there are other mechanisms involved in biofilm formation during PC storage.

Recent reports have focused on Aap as a contributor to PIA-independent *S. epidermidis* biofilm formation (Schaeffer et al., 2015; Macintosh et al., 2009; Rohde et al., 2005). Aap is a cell wall-anchored protein (LPXTG) composed of two domains (A and B) that can be found in 90% of *S. epidermidis* isolates (Schaeffer et al., 2015; Rohde et al., 2007). It has been documented that the A domain plays an essential role in bacterial adhesion to plastic surfaces and epithelial cells. The cleavage of the A domain by proteases (e.g., SepA and granulocyte proteases) allow the liberated B domain to enhance cell-cell accumulation, thus promoting biofilm formation (Schaeffer et al., 2015).

1.5 HYPOTHESES

S. epidermidis biofilm resistance to the bactericidal action of blood donor skin disinfectants can be overcome by a synergistic action of currently used disinfectants and plant-extracted natural oils. Furthermore, biofilm-associated Aap protein and the expression of resistance mechanisms to AMPs confer an advantage to *S. epidermidis* for growth in PCs.

1.6 OBJECTIVES

Objective 1: To evaluate the anti-biofilm synergistic activity of skin disinfectants and plant-extracted natural oils

The use of blood donor skin disinfectants, 2% chlorhexidine-gluconate (CHG) and 70% isopropyl alcohol (IPA), is a crucial step to prevent/reduce bacterial contamination of blood products with skin flora bacteria such as *S. epidermidis* (Taha et al., 2014; Benjamin et al., 2011; McDonald, 2011; Ramirez-Arcos and Goldman, 2010). It has been demonstrated *S. epidermidis* can exist in the skin as biofilms, and *S. epidermidis* biofilms

display resistance to CHG-IPA (Costerton, 2007; Taha et al., 2014). In addition, it has been shown that essential oils synergistically increase the antibacterial activity of CHG (Hendry et al., 2009; Karpanen et al., 2008a). Therefore, this study was undertaken to test several plant-extracted essential oils in combination with CHG or CHG-IPA for their ability to eradicate *S. epidermidis* biofilms.

Objective 2: To evaluate the contribution of Aap to the proliferative advantage of *S. epidermidis* in PCs

Blood centres worldwide have reported that *S. epidermidis* is the predominant aerobic contaminant of PCs while other staphylococci, such as *S. capitis*, are involved with lower contamination rates (Müller et al., 2015; Girgis et al., 2014; Zhu et al., 2009; Kuehnert et al., 2001). Furthermore, under experimental conditions, it has been shown that *S. epidermidis* outcompetes *S. capitis* during PC storage (Taha et al., 2014). The Accumulation Associated Protein (Aap), encoded by the *aap* gene, is implicated in biofilm formation in *S. epidermidis* and is absent in *S. capitis* (Schaeffer et al., 2015; Cameron et al., 2015; Rohde et al., 2005). In this study, the role of *S. epidermidis aap* in enhancing biofilm formation in PCs and conferring an advantage to skin flora bacteria for proliferation in this environment was investigated.

Objective 3: To evaluate the anti-biofilm activity of synthetic AMPs

A combination of three synthetic AMPs, the platelet-derived peptide (PD4) and two arginine-tryptophan repeats (RW3 and RW4), could serve as bactericidal agents during PC storage. This combination of AMPs has a bactericidal effect against *S. epidermidis* free-floating (non-biofilm) cells in PCs (Mohan et al., 2014). In this study, the ability of the PD4, RW3, and RW4 combination to inhibit *S. epidermidis* biofilm formation and to eradicate mature biofilms was evaluated.

Objective 4: To investigate mechanisms of AMP resistance by *S. epidermidis* in PCs

The antimicrobial peptide sensor (GraRS) system and the SepA protease represent two mechanisms of *S. epidermidis* resistance toward AMPs found in the skin (Li et al., 2007a; Lai et al., 2007). This study investigated whether the predominant proliferation of *S. epidermidis* in PCs could be attributed to resistance to platelet-derived AMP by either of these two mechanisms.

CHAPTER 2.

Enhancing Blood Donor Skin Disinfection Using Natural Oils

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Running title: Essential oils improve skin disinfection

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2.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

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M. Alabdullatif, I. Boujezza and Dr. S. Ramirez-Arcos conceived the study and designed the experiments described herein. Dr. M. Mekni, Dr. A. Landoulsi and Dr. M. Taha provided guidance on the experimental design. M. Alabdullatif conducted the anti-biofilm and GC-MS assays and analyzed the results. I. Boujezza conducted the essential oils extraction and rabbit irritation assay. D. Kumaran assisted with data interpretation. M. Alabdullatif and Dr. Q. Yi performed statistical analyses. M. Alabdullatif, D. Kumaran and Dr. S. Ramirez-Arcos wrote the manuscript.

2.2 ABSTRACT

Effective donor skin disinfection is essential in preventing bacterial contamination of blood components with skin flora bacteria like *Staphylococcus epidermidis*. Cell aggregates of *S. epidermidis* (biofilms) are found on the skin and are resistant to the commonly used donor skin disinfectants chlorhexidine-gluconate and isopropyl alcohol. It has been demonstrated that essential oils synergistically enhance the antibacterial activity of chlorhexidine-gluconate. The objective of this study was to test plant-extracted essential oils in combination with chlorhexidine-gluconate or chlorhexidine-gluconate plus isopropyl alcohol for their ability to eliminate *S. epidermidis* biofilms. The composition of oils extracted from *Artemisia herba-alba, Lavandula multifida, Origanum marjoram, Rosmarinus officinalis*, and *Thymus capitatus* was analyzed using gas chromatography-mass spectrometry. A rabbit model was used to assess skin irritation caused by the oils. In addition, the anti-biofilm activity of the oils used alone or in combination with chlorhexidine-gluconate or chlorhexidine-gluconate plus isopropyl alcohol was tested against *S. epidermidis* biofilms. Essential oil concentrations 10%, 20%, and 30% were chosen for anti-biofilm assays, because skin irritation was observed at concentrations greater than 30%. All oils except for *O. marjoram* had anti-biofilm activity at these three concentrations. *L. multifida* synergistically enhanced the anti-biofilm activity of chlorhexidine-gluconate and resulted in the highest anti-biofilm activity observed when combined with chlorhexidine-gluconate plus isopropyl alcohol. Gas chromatography-mass spectrometry revealed that the main component contributing to the activity of *L. multifida* oil was a natural terpene alcohol called linalool. The anti-biofilm activity of chlorhexidine-gluconate plus isopropyl alcohol can be greatly enhanced by *L. multifida* oil or linalool. Therefore, these components could potentially be used to improve blood donor skin disinfection.

2.3 INTRODUCTION

In modern transfusion medicine, bacterial contamination of platelet concentrates (PCs) is implicated as the primary transfusion-associated infectious risk (Corash, 2011). PCs are prepared in an additive solution that

provides neutral pH and a dextrose concentration of approximately 25 g/L. In addition, PCs are stored under agitation at 22 ± 2°C. Together, these conditions offer contaminating bacteria an optimal environment for proliferation (Canadian Blood Services, 2015; Ramirez-Arcos and Goldman, 2012). Blood centers in the United States, Canada, Germany, China, and New Zealand have documented that coagulase-negative *Staphylococcus epidermidis* is the most commonly isolated aerobic contaminant of PCs (Müller et al., 2015; Dickson and Dinesh, 2013; Zhu et al., 2009; Jacobs et al., 2001). *S. epidermidis* is a commensal inhabitant of human skin that favors moist niches like the antecubital area and is thought to be introduced into blood products by venipuncture at the time of collection (Ramirez-Arcos and Goldman, 2012; Karpanen et al., 2010; Grice and Segre, 2011). Several strategies have been implemented to overcome bacterial contamination of PCs and include: donor screening to detect potential symptoms of infection, donor arm skin disinfection before venipuncture, the diversion of the first 30 to 40 mL of the donated blood, and the screening of PCs for bacterial contamination (Korte and Marcelis, 2014; Ramirez-Arcos and Goldman, 2012). Despite these efforts, bacterial contamination of PCs and missed detections continue to occur, which in some cases have led to fatal *S. epidermidis*-associated transfusion reactions (Kou et al., 2015; US Food and Drug Adminstration, 2015).

Chlorhexidine is a cationic disinfectant with broad-spectrum activity, which has contributed to the increased efficacy of donor skin disinfection over the past decade (Milstone et al., 2008; Mangram et al., 1999). Canadian Blood Services has implemented the use of a one-step ChloraPrep kit, which contains 2% chlorhexidine-gluconate (CHG) and 70% isopropyl alcohol (IPA). This combination of disinfectants (CHG-IPA) has been shown to decrease PC contamination rates, but it has not been able to eliminate the risk (Ramirez-Arcos and Goldman, 2012; Benjamin et al., 2011; Milstone et al., 2008). We previously demonstrated that *S. epidermidis* biofilms (cell aggregates embedded within a self-produced matrix) exhibit a heightened resistance to the currently used disinfectant, in contrast to their planktonic (free-floating cells) counterparts, which are susceptible to elimination by CHG-IPA (Taha et al., 2014).

Essential oils (EOs) have been documented by various studies as having antibacterial properties (Lakehal et al., 2016; Douhri et al., 2014; Chedia et al., 2013; Wang et al., 2012; Vàgi et al., 2005). This activity

has mostly been attributed to oxygenated terpenoid compounds (e.g., phenolic terpenes and alcohols) found in EOs, such as linalool, eucalyptol (1,8-cineole), and thymol. In addition, it has been shown that EOs enhance the penetration of CHG through human skin and augment antibacterial activity through synergistic interactions with CHG (Bassolé et al., 2012; Karpanen et al., 2010; Karpanen et al., 2008a; Bassolé et al., 2010; Hendry et al., 2009). The aim of this study was to test several plant-derived EOs in combination with the one-step disinfectant (CHG-IPA) for their ability to eliminate *S. epidermidis* biofilms.

2.4 MATERIALS AND METHODS

I. Bacterial strains

Biofilm-positive *S. epidermidis* Hamburg 9142 strain isolated from a blood culture (Hodgson et al., 2014; Mack et al., 1992), was stored in brain heart infusion broth (Becton, Dickinson) containing 15% glycerol (volume/volume) at -80°C until required.

II. EO isolation and analysis

EOs were extracted in Tunisia using hydrodistillation from the aerial parts of five native plants, namely: *Artemisia herba-alba*, *Rosmarinus officinalis*, *Lavandula multifida*, *Origanum marjoram*, and *Thymus capitatus*. The qualitative and quantitative compositions of the extracted EOs were analyzed using gas chromatography-mass spectrometry (GC-MS), which was performed at the John L. Holmes Mass Spectrometry Facility at the University of Ottawa on an Agilent 7820A gas chromatograph using a 5975 series mass selective detector with a Stabilwax column (Restek) (30 m, 0.25-mm internal diameter, and 0.25-µm film thickness). Helium was used as the carrier gas with a constant flow rate of 1.8 mL per minute. The oven temperature ramp ran from 40 to 220°C at 10°C per minute, and then remained at 220°C for 2 minutes. The GC-MS detector remained off for the first 3 minutes to avoid the solvent peak (ethyl acetate). A 1-µL sample of the extracted oil was diluted in 40 µL ethyl acetate, of which a 1-µL aliquot was injected into the GC-MS, and the Wiley 275 database was used to detect and quantify the EO components.

III. Reagents

All working stocks of EOs, linalool, and disinfectants were prepared in an 8% (volume/volume) Tween-80 solution (Sigma-Aldrich). Each of the five EOs was diluted to a concentration ranging from 10 to 50% (volume/volume) (Syed and Peh, 2014; Chamdit and Siripermpool, 2012; Hendry et al., 2012), linalool (97%; Sigma-Aldrich) was diluted to a concentration of 4.5% (volume/volume), and aqueous CHG 20% (weight/volume; Sigma-Aldrich) and IPA 100% (volume/volume; Fisher Scientific) were diluted to obtain 2% (volume/volume) and 70% (volume/volume) solutions, respectively (Taha et al., 2014; Hendry et al., 2012).

The neutralizing solution used in this study was composed of 0.5% (weight/volume) lecithin (Fischer Scientific) and 4% (volume/volume) Tween-20 (Fisher Scientific) in distilled water and was prepared according to established procedures (Taha et al., 2014; Kampf et al., 2005). The toxicity and efficacy of the neutralizer were validated according to protocols prescribed in the American Society for Testing and Materials (ASTM E1054-08) (E35 Committee, American Society for Testing and Materials International, 2008).

IV. Rabbit irritation assay

The assay was performed according to recommendations prescribed by the US Environmental Protection Agency (Office of Prevention, Pesticides, and Toxic Substances [OPPTS] 870.2500) (US Environmental Protection Administration, 1998). Three rabbits, each weighing approximately 1.5 kg, were used in the assay for each oil tested. The day before the experiment, the rabbits were anesthetized with 1 mL/kg urethane 66% (weight/volume), and a 5 × 8 cm area on the dorsal side was shaved and cleaned with water. The next day, the area was cleaned with ultra-pure water and a pen was used to delimit two circular spots 3 cm apart. One spot served as the nonirritant control where 10 μL of the Tween-80 solution was administered, and the other spot served as the test site where 10 μL of the oil in Tween-80 solution was applied. Signs of irritation were assessed at the 30-second, 10-minute, 1-hour, and 4-hour time points.

V. Anti-biofilm activity of EOs, linalool, and disinfectants

Anti-biofilm activity assays were performed as previously described by Taha and colleagues with some modifications (Taha et al., 2014). Briefly, *S. epidermidis* Hamburg 9142 was streaked on Trypticase Soy Agar plates (Difco, BD Diagnostics) and grown overnight at 37°C. Overnight cultures were prepared by inoculating one to three colonies in 3 mL of Müeller Hinton broth (Difco, BD Diagnostics) and incubating the suspension for 17 or 18 hours at 37°C with agitation at 260 revolutions per minute. Overnight cultures were adjusted to an optical density of 600 nm (OD_{600}) = 0.1, corresponding to approximately 1.0 x 10^7 colony-forming units per milliliter (CFU/mL) of *S. epidermidis* in Müeller Hinton broth. Each well of a 96-well plate (Corning Inc.) was inoculated with 0.1 mL of the adjusted culture and incubated at 37°C for 24 hours to allow biofilm formation. After biofilm formation, the planktonic cells were removed, and each well was gently washed three times with 0.1 mL of 0.9% saline, pH 7.4.

Each of the five EOs at three concentrations (10%, 20%, and 30%) or 4.5% linalool were tested either individually, or in combination with CHG, or in combination with CHG-IPA against *S. epidermidis* biofilms. The biofilms were exposed to 0.1 mL of treatment for 30 seconds to mimic skin-disinfection practices (Taha et al., 2014), after which 0.2 mL of the neutralizing solution was added for 5 minutes. The neutralizing solution was removed, and each well was gently washed three times with 0.2 mL of 0.9% saline. Fresh 0.9% saline (0.2 mL) was added to wells, and biofilm cells were dislodged from the 96-well plate by sonication at 60Hz for 30 minutes using an ultrasonic cleaner (Model 5510; Branson Ultrasonics Corp.), as previously optimized (Greco-Stewart et al., 2012). Each suspension was serially diluted in 0.9% saline and plated on Trypticase Soy Agar. The plates were incubated overnight at 37°C, and the colonies were enumerated.

VI. Analysis of drug interaction

To determine the nature of the interactions between the extracted oil and CHG, the coefficient of drug interaction (CDI) was calculated as follows: CDI = AB / (A × B), where AB stands for the ratio of the bacterial counts (CFU/mL) obtained for the combination treatment (EO + CHG) group to the bacterial counts (CFU/mL) obtained for the control group, and A (EO) or B (CHG) is the ratio of the counts obtained for the single

treatment group to the counts obtained for the control group. Depending on the CDI value, the interaction was assigned as either antagonistic (CDI >1), additive (CDI=1), or synergistic (CDI <1) (Zhao et al., 2014; Chou and Talalay, 1984). Synergistic interactions are generally characterized by the effect of a combination treatment being greater than the sum of the effects of the individual treatments, whereas additive interactions are characterized by the effect of a combination treatment being equal to the sum of the effects of the individual treatments; and, finally, an antagonistic interaction is characterized by the effect of the combination treatment being less than sum of the effects of the individual treatments (Yap et al., 2014; Chou and Talalay, 1984).

VII. Statistical analyses

A log-10 transformation was performed on all viable counts obtained. Log-reduction was expressed as the log difference in the viable cell counts in treated cultures versus untreated cultures (controls). A mixed-model analysis was performed using Statistical Analysis System software (SAS Institute, Inc.) for multiple comparisons between treatments. For the adjustment of multiple comparisons, Tukey's method was applied, and p values less than 0.05 were considered significant. Each treatment was performed at least three independent times with two replicates per repetition.

2.5 RESULTS

I. High concentrations of EOs cause skin irritation in the rabbit model

If irritation was observed at any of the time points tested, then it was deemed to have a positive reaction (+). If no irritation was observed after 4 hours of exposure, then the reaction was deemed to be negative (-). The control solution did not produce any signs of irritation on the rabbit skin. Furthermore, no positive reactions were observed for any of the EOs tested up to a concentration of 30%, with the exception of *T. capitatus*, which caused signs of irritation at 30% in one rabbit. Higher concentrations of all EOs (40% and 50%) caused skin irritation (Table 2.1). Consequently, the 10%, 20%, and 30% concentrations of the EOs were chosen for the anti-biofilm assays.

Table 2.1. Rabbit irritation scores for the five extracted EOs

Plant	Extract oil concentration							
	50%	40%	30%	25%	20%	15%	12.5%	10%
T. capitatus	+++	+++	--+	---	---	---	---	---
O. marjoram	+++	--+	---	---	---	---	---	---
L. multifida	+++	---	---	---	---	---	---	---
R. officinalis	+++	++-	---	---	---	---	---	---
A. herba-alba	+++	++-	---	---	---	---	---	---

+, irritation; -, no irritation (n = 3).

II. EOs demonstrate anti-biofilm activity

Five plant-extracted EOs suspended in 8% Tween-80 solution were tested in this study for their anti-biofilm activity. The 8% Tween-80 solution (control) did not give rise to any significant difference in viable counts when exposed to preformed biofilms for 30 seconds compared with distilled water. Furthermore, the control solution did not affect the anti-biofilm properties of either CHG or CHG-IPA (data not shown). With the exception of *R. officinalis* and *A. herba-alba*, which displayed similar anti-biofilm activity ($p = 0.614$), all other EOs differed significantly from one another in their ability to reduce viable biofilm counts ($p < 0.05$) (Table 2.2). *O. marjoram* did not exhibit anti-biofilm activity at any tested concentration ($p > 0.05$); however, the other four EOs showed significant anti-biofilm activity ($p < 0.05$) (Table 2.2). According to the observed log-reduction (CFU/ mL), *L. multifida* had the strongest effect (reduction: 1.319, 1.594 and 1.599 for the 10%, 20%, and 30% groups, respectively), followed by *T. capitatus* (reduction: 1.168, 1.259, and 1.422, respectively), and *R. officinalis* (reduction: 0.598, 0.709, and 0.794, respectively). *A. herba-alba* gave rise to significant log reductions of 0.472 and 0.563 only at higher concentrations of 20% and 30%, respectively (Table 2.2).

III. *L. multifida* synergistically enhances the effect of CHG

CHG significantly reduced bacterial concentrations by approximately 2 logs compared to the 8% Tween-80 solution control ($p < 0.0001$) (Fig. 2.1). When the five EOs were tested in conjunction with CHG, a significant increase in anti-biofilm activity was observed compared with the reductions observed for the EOs alone ($p < 0.0001$). Interestingly, the combination of *T. capitatus* or *L. multifida* with CHG resulted in enhanced anti-biofilm activity compared with the reductions observed for CHG alone ($p < 0.001$) (Table 2.2). To determine the nature of the interactions between the EOs and CHG, the CDI was calculated. It is noteworthy that only *L. multifida* exhibited a synergistic effect with CHG, whereas the other four EOs had an antagonistic effect (Table 2.2).

Table 2.2. Log reduction of *S. epidermidis* (CFU/mL) by EOs when used individually or in combination with 2% CHG

EO concentration, %	CFU/mL Log reduction ± SD* EO	p-value †	CFU/mL Log reduction ± SD* EO plus CHG	p-value ‡	CDI	Result
A. herba-alba						
Control, 0%	0.000 ± 0.000	.	2.220 ± 0.211	.	.	.
10%	0.198 ± 0.288	0.356	2.259 ± 0.141	0.999	1.445	Anta
20%	0.472 ± 0.141	0.029	2.410 ± 0.138	0.722	2.055	Anta
30%	0.563 ± 0.163	0.027	2.453 ± 0.149	0.477	2.293	Anta
L. multifida						
Control, 0%	0.000 ± 0.000	.	1.864 ± 0.273	.	.	.
10%	1.319 ± 0.203	0.008	3.309 ± 0.193	< 0.001	0.693	Syn
20%	1.594 ± 0.161	0.003	3.529 ± 0.283	< 0.001	0.656	Syn
30%	1.599 ± 0.068	0.001	3.709 ± 0.098	< 0.001	0.491	Syn
O. marjoram						
Control, 0%	0.000 ± 0.000	.	1.957 ± 0.117	.	.	.
10%	-0.023 ± 0.260	0.891	1.802 ± 0.374	0.994	2.158	Anta
20%	0.185 ± 0.254	0.335	2.084 ± 0.065	0.998	1.163	Anta
30%	0.539 ± 0.356	0.120	2.295 ± 0.223	0.633	1.677	Anta
R. officinalis						
Control, 0%	0.000 ± 0.000	.	2.220 ± 0.386	.	.	.
10%	0.598 ± 0.101	0.009	2.402 ± 0.182	0.815	1.744	Anta
20%	0.709 ± 0.093	0.006	2.554 ± 0.097	0.149	1.576	Anta
30%	0.794 ± 0.119	0.007	2.611 ± 0.125	0.058	1.648	Anta
T. capitatus						
Control, 0%	0.000 ± 0.000	.	1.882 ± 0.105	.	.	.
10%	1.168 ± 0.124	0.004	2.583 ± 0.081	< 0.001	2.865	Anta
20%	1.259 ± 0.177	0.007	2.799 ± 0.148	< 0.001	2.167	Anta
30%	1.422 ± 0.099	0.002	2.821 ± 0.100	< 0.001	2.779	Anta

* Mean $\log_{10}$ reduction (CFU/mL) ± SD; n=3.
† The p value is shown for each EO concentration compared to controls.
‡ The p value is shown for each EO plus CHG compared to CHG alone.
SD = standard deviation; Anta = antagonistic; Syn = synergistic.

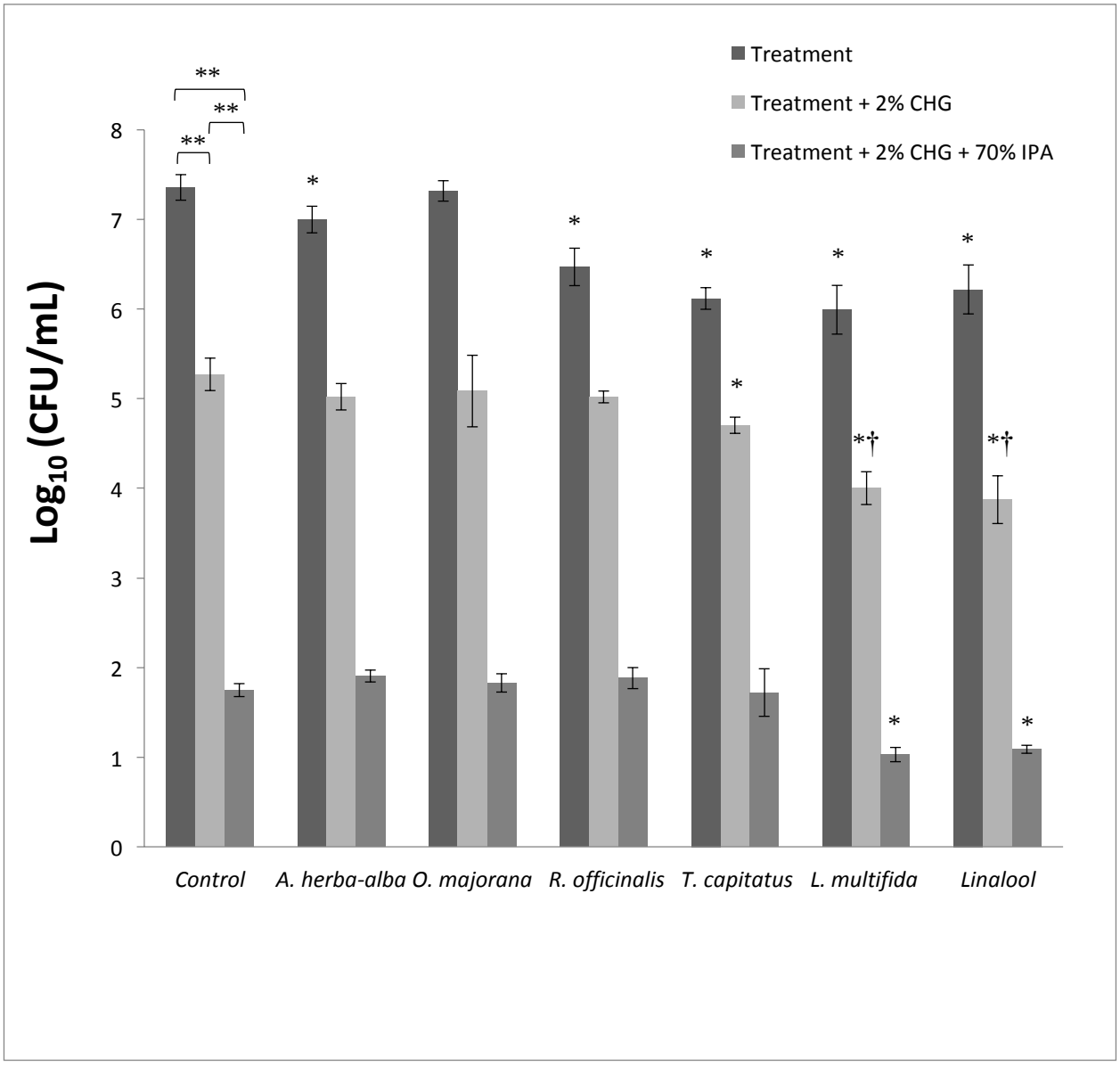

Figure 2.1. Anti-biofilm activity of 10% EOs and 4.5% linalool when used individually or in combination with either 2% CHG or with 2% CHG and 70% IPA. Three independent replicates of the EO and linalool experiements were performed (n=3). Mixed-model analysis was performed for the multiple comparisons with *$p < 0.05$ and **$p < 0.0001$. A dagger (†**)** indicates that synergy was observed in combination with CHG as assessed by the coefficient of drug interaction. Control: 8% Tween 80, Treatment = Control or EO.

IV. *L. multifida*-derived EOs contained the highest percentage of oxygenated terpenoids, consisting primarily of linalool

GC-MS analysis of the qualitative and quantitative chemical composition of the EOs revealed that *L. multifida* had the highest percentage of oxygenated terpenoids (84.17%) followed by *T. capitatus* (81.18%), whereas *O. marjoram* had the lowest oxygenated terpenoid content of the EOs tested (31.79%) (Table 2.3). The most abundant oxygenated terpenoid compounds identified in the different EOs were as follows: linalool in *L. multifida* (45%), thymol in *T. capitatus* (36%), camphor in *R. officinalis* (25%) and *A. herba-alba* (23%), and 4-terpineol in *O. marjoram* (9%).

V. *L. multifida* and linalool displayed the highest anti-biofilm activity in combination with CHG-IPA

Because no significant differences were observed in the synergy exhibited by the different concentrations of EOs when used in conjunction with CHG (Table 2.2), the lowest concentration of the oils (10%) was used to assess anti-biofilm activity. CHG-IPA resulted in a reduction of bacterial concentrations of up to 3 logs, which was significantly higher compared with the reductions observed for CHG alone ($p < 0.0001$) (Fig. 2.1). The addition of CHG-IPA to the EOs or to linalool significantly decreased bacterial concentrations compared with the oils or linalool alone ($p < 0.0001$), with bacterial reductions of up to 5.5 logs being observed. Both *L. multifida* and linalool displayed synergistic interactions with CHG and resulted in the most significant increase in anti-biofilm activity when used in combination with CHG and IPA ($p < 0.05$) (Fig. 2.1). There were no differences in anti-biofilm activities between *L. multifida* and linalool when used in conjunction with either CHG or CHG and IPA ($p > 0.05$).

2.6 DISCUSSION

Blood centres around the world continually strive to provide safe and effective blood products to patients. The disinfection of the skin at the venipuncture site is paramount in achieving this goal. In an effort to improve skin disinfection, various blood centers have implemented the use of a one-step disinfectant containing 2% CHG and 70% IPA (Korte and Marcelis, 2014; Ramirez-Arcos and Goldman, 2012). This has resulted in the reduction of

Table 2.3. Total percentage of oxygenated terpenoids and the most abundant oxygenated terpenoid in EOs

EO	Total percentage of oxygenated terpenoids, %	Most abundant oxygenated terpenoid compound (%)
A. herba-alba	49.871	Camphor (22.72)
L. multifida	84.174	Linalool (45.09)
O. marjoram	31.79	4-terpineol (8.79)
R. officinalis	54.59	Camphor (25.04)
T. capitatus	81.181	Thymol (35.92)

PC contamination rates compared with the previously used two-step povidone-iodine disinfectant process (Benjamin et al., 2011). Despite the observed reduction in bacterial contamination in PCs, the one-step disinfectant has not been able to completely eliminate this risk (Kou et al., 2015; US Food and Drug Adminstration, 2015; Ramirez-Arcos and Goldman, 2012). In nature, bacteria exist in matrix-enclosed communities called biofilms. This has been found to be true even on the skin, where *S. epidermidis* biofilms have been observed in between squamous epithelial cells up to five cells deep (10-20 µm of the outermost layer of the skin) (Karpanen et al., 2008b; Costerton, 2007). Interestingly Taha and coworkers reported that, although the combination of CHG and IPA can cause a reduction in viable *S. epidermidis* biofilms, it is not effective at eliminating them (Taha et al., 2014). Bacteria can be found in the deeper layers of the skin (up to 1500 µm) within hair follicles; however, Karpanen and colleagues demonstrated that 2% CHG can only penetrate to depths of less than 300 µm in the skin after contact for up to 30 minutes (Karpanen et al., 2008b; Selwyn and Ellis, 1972). These findings help shed a light on a potential cause of the continued bacterial contamination of PCs and other blood products.

Various studies have assessed the disinfectant properties of EOs and their ability to enhance transdermal drug delivery (Herman and Herman, 2015). Their appeal is derived in part from the ability of EOs to have anti-biofilm activity, as evidenced by the reduction of viable *S. epidermidis*, *Staphylococcus aureus*, and *Escherichia coli* biofilms by eucalyptus oil. Furthermore, it has been demonstrated that eucalyptus oils also enhance the anti-biofilm activity of chemical disinfectants like CHG (Hendry et al., 2009; Karpanen et al., 2008a). However, the use of eucalyptus oil is limited, because skin irritation caused by the use of concentrations as low as 1% has been reported (Villaplana and Romaguera, 2000).

Taken together, these findings emphasize the need to assess new disinfectants for their anti-biofilm activity, their penetrative properties, and their potential to cause allergic reactions. Here, we describe five EOs that did not cause allergic contact dermatitis in a rabbit model when concentrations below 30% were used. An analysis of the anti-biofilm properties of these individual oils revealed that all the oils tested had anti-biofilm

properties with the exception of *O. marjoram*, whereas *L. multifida* provided the most significant reduction in viable *S. epidermidis* biofilms. GC-MS data indicated that *L. multifida* had the highest composition of oxygenated terpenoids. Interestingly, Bassolé and coworkers reported that EOs with high oxygenated terpenoid content exhibited high antibacterial activity (Bassolé et al., 2010; Bassolé et al., 2012). Our results demonstrate a similar trend against *S. epidermidis* biofilms. Further assays are warranted to demonstrate the effectiveness of EOs against other biofilm-forming bacteria that can be isolated from contaminated blood components such as *Pseudomonas fluorescens* or *Serratia marcescens* (Ramirez-Arcos and Goldman, 2012).

GC-MS data also revealed that a large proportion of the oxygenated terpenoids found in *L. multifida* was composed of linalool. We were able to establish that linalool was the active component in *L. multifida*, because no significant differences in anti-biofilm activity were observed when either linalool or *L. multifida* was used under the three conditions tested. Previous studies have demonstrated that linalool has antibacterial properties and can synergistically interact with other chemical components like eugenol or carvacrol, enhancing bactericidal effects (Bassolé et al., 2010; Bassolé et al., 2012). To our knowledge, our study is the first to demonstrate that linalool can synergistically interact with disinfectants like CHG, enhancing anti-biofilm effects, in addition to its ability to enhance the anti-biofilm properties of CHG-IPA. These data give credence to the use of linalool in conjunction with CHG-IPA as a means to reduce the potential of bacterial contamination of blood products.

The US Food and Drug Administration has classified EOs and their oxygenated terpenoids as "generally regarded as safe" (or GRAS) because of their low irritancy and toxicity (Vaddi et al., 2002). Although it has been shown that exposure to linalool in the air could contribute to allergic reactions (Hagvall et al., 2008), a study investigating the subcutaneous absorption of linalool into the blood stream demonstrated that only 121 ng/mL of linalool was present in the blood stream after a 20-minute abdominal massage with approximately 0.45 g of linalool, and no adverse reactions were reported (Jäger et al., 1992). Our rabbit irritation assays demonstrated that the EOs tested in this study do not cause contact dermatitis when used at concentrations below 30%; however, it is unknown whether the combination of EOs with CHG would cause an allergic

reaction. We and others have reported that CHG can cause skin reactions in blood donors (Ramirez-Arcos and Goldman, 2010; Alaman et al., 2007); therefore, assessing the allergenic potential of the combination of linalool and CHG-IPA on the skin merits further investigation. It has been shown that linalool acts as an enhancer of transdermal penetration (Herman and Herman, 2015; Jäger et al., 1992); thus, it would be important to demonstrate that this property is maintained in a blood donation scenario when using *L. multifida* EO or linalool in combination with CHG-IPA. Our study highlights the potential use of linalool as a candidate to supplement CHG-IPA for blood donor skin disinfection because of its synergistic interactions with CHG and its ability to enhance the anti-biofilm properties of CHG-IPA.

2.7 ACKNOWLEDGMENTS

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CHAPTER 3.

Role of the Accumulation Associated Protein (Aap) on the Predominance of *Staphylococcus epidermidis* as a Platelet Contaminant

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3.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

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M. Alabdullatif and Dr. S. Ramirez-Arcos conceived the study, analyzed the data, and wrote the manuscript. Experiments were performed by M. Alabdullatif.

3.2 ABSTRACT

Staphylococcus epidermidis is a predominant contaminant of platelet concentrates (PCs), outcompeting other skin flora bacteria such as *Staphylococcus capitis.* The Accumulation Associated Protein (Aap), encoded by the *aap* gene, is involved in formation of bacterial aggregates (biofilms) in *S. epidermidis* and is absent in *S. capitis*. In this study, the role of *S. epidermidis aap* in enhancing biofilm formation and conferring an advantageous growth in PCs was investigated. Biofilm formation assays of *S. epidermidis* 1457, *S. epidermidis* 1457Δ*aap, S. capitis* 517, and *S. capitis* 517 carrying *S. epidermidis aap* (*S. capitis* 517/pRB*aap*) were performed in glucose-supplemented trypticase soy broth (TSBg) and PCs. Additionally, competition assays with paired cultures (1:1 ratio) of *S. epidermidis* and *S. capitis* strains were seeded in PCs, followed by determination of viable counts of each organism at the end of PC storage. *S. epidermidis aap* had no effect on biofilm formation in TSBg. By contrast in PCs, *S. epidermidis* 1457 showed higher biofilm formation than *S. epidermidis* 1457Δ*aap* ($P < 0.05$). Biofilm formation was also enhanced in *S. capitis* 517/pRB*aap* compared to *S. capitis* 517 ($P = 0.054$). Competition assays showed that *S. epidermidis* 1457 outcompeted *S. capitis* 517 and importantly, *S. capitis* 517/pRB*aap* outcompeted *S. capitis* 517 and *S. epidermidis* 1457Δ*aap*. This study demonstrated that *S. epidermidis aap* plays a role in biofilm formation in PCs conferring an advantageous proliferation to skin flora bacteria in this milieu. The molecular mechanisms of action of Aap merit further investigation.

3.3 INTRODUCTION

Bacterial contamination in platelet concentrates (PCs) poses a major safety risk for transfusion patients (US Food and Drug Administration, 2016; Kou et al., 2015; Ramirez-Arcos and Goldman, 2012). PCs are a therapeutic product suspended in an additive solution that provides a neutral pH and high dextrose concentration (approximately 25 g/L). Furthermore, PCs are stored in gas-permeable plastic containers under constant agitation at 22±2^{o}C for up to 7 days. These conditions offer contaminating bacteria an ideal environment for proliferation (Canadian Blood Services, 2017b; Ramirez-Arcos and Goldman, 2012). Coagulase-negative staphylococci, mostly found in human skin, are predominantly implicated in bacterial contamination of PCs

with *Staphylococcus epidermidis* as the main aerobic contaminant while other staphylococci, such as *Staphylococcus capitis*, have been implicated with lower contamination rates (Müller et al., 2015; Girgis et al., 2014; Zhu et al., 2009; Kuenhnert et al., 2001). Despite mitigation strategies to reduce the risk of septic transfusion events, fatal transfusion-associated cases due to contamination of PCs with *S. epidermidis* still occur (US Food and Drug Administration, 2016; Kou et al., 2015).

Detection of staphylococci during PC screening is challenging due to the slow growing characteristics of these organisms and their ability to adhere to the inner surface of PC plastic bags forming bacterial aggregates enclosed in a matrix known as biofilms (Ali et al., 2014; Greco-Stewart et al., 2012; Von Eiff et al. 1999). During PC storage, activated platelets secrete antimicrobial peptides (AMPs), which have antibacterial properties (Yeaman, 2014; Tang et al., 2002). We have demonstrated that *S. epidermidis* biofilms are resistant to the bactericidal action of AMPs (Alabdullatif et al., 2018) and it is thus proposed that biofilm formation during PC storage results in advantageous growth of this organism in PCs.

The staphylococcal biofilm matrix is typically composed of polysaccharides such as the polysaccharide intercellular adhesin (PIA), but it can also contain proteins (including the accumulation-associated protein; Aap), and extracellular DNA (Loza Correa et al., 2017). Rohde and coworkers demonstrated that *S. epidermidis* strains unable to produce PIA were still able to form Aap-based biofilms (Rohde et al., 2005). Aap is encoded by the *aap* gene and is a cell wall-anchored protein (LPXTG) composed of two domains (A and B). The A domain is involved in bacterial adhesion to epithelial cells and plastic surfaces. Upon cleavage of the A domain, the liberated B domain enhances cell to cell accumulation promoting biofilm formation (Paharik et al., 2017; Schaeffer et al., 2015; Macintosh et al., 2009; Rohde et al., 2005). Other staphylococci such as *S. capitis* do not carry the *aap* gene; however, some *S. capitis* isolates carry the *pls* gene encoding the plasmin-sensitive protein (Pls), which shares domain and sequence homology with *S. epidermidis* Aap (Cameron et al., 2015).

We have previously demonstrated that *S. epidermidis* outcompetes *S. capitis* when grown in PCs (Taha et al., 2014). Here we have assessed the role of *S. epidermidis aap* in enhancing biofilm formation and thereby conferring a proliferative advantage to this bacterium in PCs.

3.4 MATERIALS AND METHODS

I. Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids for this study were listed in Table 3.1. *Staphylococcus* was grown in trypticase soy broth (TSB; BD Biosciences) or TSB agar (TSA; BD Biosciences). Wild-type *S. epidermidis* 1457, *S. epidermidis* 1457Δ*aap, S. epidermidis* 1457Δ*aap/*pRB*aap*, wild-type *S. capitis* 517, and *S. capitis* 517/pRB*aap* were used for *aap* studies. *S. capitis* strains 512, 517, 521, 525, 07/2010 were used for PCR amplification of the *pls* gene. *S. epidermidis* 1457 and *S. capitis* 517, and their derivatives, were used for competition studies. *Escherichia coli* was grown in Luria-Bertani broth (LB; EMD Chemicals) or LB agar (LBA). *E. coli* DC10B was used for plasmid passage to bypass the strong restriction barrier present in *S. epidermidis* and *S. capitis* (Cui et al., 2015; Monk et al., 2012). When appropriate, antibiotics were used at concentrations of 10 µg/ml chloramphenicol (Cm; Fisher Scientific) or 10 µg/ml tetracycline (Tet; Sigma-Aldrich).

II. Polymerase chain reaction (PCR) for amplification of *S. epidermidis aap* and *S. capitis pls*

The primer pair used to amplify *S. epidermidis aap* are $aap_{for}2$ (5′-GAAGCACCGAATGTTCCAACTATC-3′) and $aap_{rev}2$ (5′-TGGTTCTGCTTTTGTTGGACCATAC-3′) (Rohde et al., 2005). *S. capitis pls* was amplified with primer pair pls_{for} (5'-TTCTGTTGTTGGTTCACCAC-3'), and pls_{rev} (5'-AAGGGAATGGCCAAGACTTC-3') designed using the sequence of *S. capitis* AYP1020 (GeneBank accession number CP007601.1). Amplifications were done in a thermal cycler PCR system (Mastercycler ep, Eppendorf, Hamburg, Germany): initial denaturation 2 minutes/94^{o}C; 30 cycles of denaturation (30 seconds/94^{o}C), annealing for 30 seconds (51^{o}C for *aap* and 49^{o}C for *pls*), and extension (60 seconds for *pls* and 180 seconds for *aap*) at 68^{o}C; final extension (5 minutes/ 68^{o}C). Reactions were performed using OneTaq DNA Polymerase (New England Biolabs) according to the manufacturer's recommended protocol. Chromosomal DNA was obtained from cell suspensions in nuclease-free water adjusted to an optical density of 600 nm = 0.1 [approximately 10^7 colony forming units (CFU)/mL]. Nuclease-free water was used as a negative control.

Table 3.1. Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Characteristics *	Reference
***S. epidermidis* strains**		
1457	Central venous catheter isolate	Li et al., 2007a
1457 *Δaap*	*aap* allelic replacement mutant, Tet^r	Schaeffer et al., 2015
1457 *Δaap*/pRB*aap*	1457 *Δaap* mutant complemented with plasmid carrying *aap*	This study
1457/pRB474	Wild type 1457 carrying vector plasmid	This study
1457 *Δaap*/pRB474	1457 *Δaap* mutant carrying the vector plasmid	This study
***S. capitis* strains**		
512	PCs contaminant isolate	Greco-Stewart et al., 2013
517	PCs contaminant isolate	Greco-Stewart et al., 2013
517/pRB*aap*	Wild type 517 carrying a plasmid containing *S. epidermidis aap*	This study
517/pRB474	Wild type 517 carrying vector plasmid	This study
521	PCs contaminant isolate	Greco-Stewart et al., 2013
525	PCs contaminant isolate	Greco-Stewart et al., 2013
CBS 07/2010	PCs contaminant isolate	Greco-Stewart et al., 2013
***E. coli* strains**		
DC10B	Used as plasmid passage to bypass the strong restriction barrier present in *S. epidermidis* and *S. capitis* (restriction negative, modification positive)	BEI Resources
Plasmids		
pRB474	shuttle vector for cloning in staphylococci and *E. coli*, and containing constitutive gene expression in staphylococci via *vegII* promoter, Cm^r	Rohde et al., 2005
pRB*aap*	Plasmid carrying *S. epidermidis aap* gene, Cm^r	Schaeffer et al., 2015

* Cm^r: chloramphenicol resistance; Tet^r: tetracycline resistance

III. Plasmid transformation

E. coli: Electroporation of pRB*aap* and pRB474 (Table 3.1) into *E. coli* DC10B was performed as follows: overnight cultures of *E. coli* were grown in 10 mL LB and then diluted with fresh LB (1:100) and incubated at 37ºC with agitation 260rpm to obtain an OD_{600} = 0.5 (final volume 40mL). The cultures were chilled in ice for 15 min, with all following steps performed in ice. Cells were harvested at 5,000 × g for 5 min at 4ºC, and pellets were resuspended in 40 mL of sterilized cold water. The centrifugation and resuspension steps were repeated once. The cells were then centrifuged and resuspended first in 20 mL, then in 2 mL, and finally in 80 µL of sterilized cold 10% glycerol. Aliquots of 40 µL were prepared and frozen at -80ºC. For electroporation, competent cells were thawed in ice, and approximately 0.02-0.05 µg of plasmid was added to competent cells followed by electroporation at 1.8 kv, 2.5 msec in a MicroPulser electroporator (Bio-Rad) at room temperature. Electroporated cells were immediately resuspended in 0.5 mL of super optimal broth supplemented with glucose (SOC; Qiagen). The culture was incubated at 37ºC for 60 min, and then 200 µL were separately plated onto two LBA plates containing 10 µg/ml chloramphenicol plates, which were incubated overnight at 37ºC.

Staphylococcus: Electroporation of *E. coli*-derived pRB*aap* and pRB474 into *S. epidermidis* 1457Δ*aap* was performed for complementation studies. Transformation of pRB*aap* into *S. capitis* 517 was performed to investigate the role of *S. epidermidis aap* on biofilm formation and proliferation while transformation of pRB474 into *S. capitis* 517 was used as a control. *S. capitis* 517 carrying *S. epidermidis aap* was named *S. capitis* 517/pRB*aap* (Table 3.1). The electroporations were performed as follows: electrocompetent cells were prepared as described by Monk and colleagues (Monk et al., 2012) with some modifications. Overnight cultures of *S. epidermidis* were grown in 10 mL TSB and then diluted with fresh TSB to an optical density of 578nm= 0.5 in a final volume of 40 mL. The cultures were incubated for 30 min at 37ºC and then chilled in ice for 10 min, with all following steps done in ice. The cells were harvested at 4,000 × g for 10 min at 4ºC and the pellets were resuspended in 40 mL of sterilized cold water. The cells were centrifuged and resuspended once again. The centrifugation and resuspension steps were repeated first in 4.7 mL, then in 1.8 mL, and finally in 235 µL of sterilized cold 10% glycerol and aliquots of 40 µL were prepared. For electroporation, a competent

cells aliquot was placed in ice for 5 min and then left at room temperature for 5 min. The cells were then centrifuged at 11337 × g for 3 min and resuspended in 40 µL of sterilized 10% glycerol and 500mM sucrose. Approximately 0.1-0.5 µg of plasmid was added to the competent cells, followed by electroporation at 2.1 kv, 2.5 msec in a MicroPulser electroporator. Cells were immediately resuspended in 0.5 mL of TSB supplemented with 500 mM sucrose. The culture was incubated at 37ºC for 60 min, and then 200 µL were separately plated onto two TSA plates containing 10 µg/ml chloramphenicol (TSA_{Cm}) then incubated overnight at 37ºC. *S. capitis* was electroporated as described above for *S. epidermidis* except that the plasmid concentration was increased to 1.5-4 µg (Cui et al., 2015).

IV. Plasmid purification and confirmation

Plasmids pRB474 or pRB*aap* were extracted from *E. coli* DC10B using a QIAGEN MiniPrep Spin Kit 2.0 (QIAGEN) using the manufacturer's protocol. After purification, the plasmids were concentrated using an ethanol precipitation method (Cui et al., 2015). pRB474 or pRB*aap* were then transformed into *S. epidermidis* 1457, *S. epidermidis* 1457Δ*aap,* or *S. capitis* 517 as described above, followed by plasmid purification as described above with the exception that staphylococcal suspensions were performed in Buffer P1 containing 0.2 mg/mL lysostaphin (Sigma-Aldrich) and incubated at 37ºC for 30 min prior to plasmid purification. Plasmid concentration was measured in a BioDrop µLITE (BioDrop) spectrophotometer and analyzed using PCR and restriction digest. A 50 µL restriction digest mixture [15 µL plasmid (60-85µg/mL), 5 µL NEBuffer, 1 µL restriction enzyme(s), completed with sterilized distilled water] was prepared and incubated at 37ºC for 75 min. Restriction enzymes (New England Biolabs) including BglI individually or in combination with BamHI or PstI were selected for pRB474 while KpnI and HindIII individually or in combination were selected for pRB*aap* to confirm plasmid identity.

V. Platelet concentrates

Whole blood units were collected from healthy volunteer donors and PC preparation (collection, sterility, and storage) was performed in accordance with Canadian Blood Services Standard Operating Procedures. PC units were manufactured using the buffy-coat method by pooling four buffy-coats and suspending the pool in one of

the four donors' plasma units. The PC pools were manufactured at the Canadian Blood Services Network Centre for Applied Development (netCAD, Vancouver). Ethical approval for this study was provided by the Canadian Blood Services Research Ethics Board.

VI. Biofilm formation assays

Staphylococcus strains were streaked on TSA plates and grown overnight at 37ºC. A few colonies were then selected to inoculate 3 mL of TSB which incubated at 37ºC with agitation at approximately 260 rpm for 24 h. Overnight cultures were inoculated in duplicate into 6-well polystyrene tissue culture plates (Corning Inc., Corning, NY) to final concentration of approximately 10^7 CFU/mL with a final volume of 3 mL of either TSB supplemented with 0.5% glucose (TSBg; Difco) or PCs. Plates containing TSBg were incubated at 37ºC for 24 h while culture plates containing PCs were incubated under platelet storage conditions (at 20-24ºC on a platform platelet agitator for 5 days) to obtain mature biofilms (Ali et al., 2014; Taha et al., 2014). A set of duplicate plates were prepared, one to be used for biofilm formation determination using a crystal violet method (Ali et al., 2014), and the second for biofilm dislodging and determination of bacterial cell counts in the biofilms (Taha et al., 2014).

Crystal violet assay: After biofilm formation, planktonic cells were removed by gently washing each well three times with 3 mL of phosphate buffered saline (PBS; pH 7.4) and stained for 30 min with 3 mL of 0.3% crystal violet (Becton, Dickinson, and Company, Sparks, MD, USA). The wells were then washed three times with PBS to remove unbound crystal violet stain. Following the washing steps, 3 mL of destaining solution (20% acetone and 80% ethanol) was added to each well and incubated with agitation (approx. 100 rpm) for 15 min to elute the bound stain. Then, 200 µL samples were taken from each well and transferred to wells of a 96-well plate (Corning Inc., Corning, NY). Absorbance readings of the bacterial cultures were subtracted from the negative controls (only TSBg or PCs with no bacteria added), which read at a wavelength 492 nm in a microplate reader (Expert Plus, ASYS Hitech GmbH, Eugendort, Austria) (Ali et al., 2014).

Viable cell determination: After biofilm formation, planktonic cells were removed by gently washing with PBS three times. Instead of adding crystal violet staining, 3 mL of PBS was added to each well and scraped to dislodge biofilms and cells were homogenized by repeated pipetting and vigorous mixing. Each suspension was serially 10-fold diluted and plated on TSA. Plates were incubated overnight at 37^{o}C and colonies were counted (Alabdullatif et al., 2018; Taha et al., 2014).

VII. Competition assays

S. epidermidis 1457, 1457Δ*aap,* and 1457Δ*aap/*pRB*aap* strains were used for competition studies with *S. capitis* 517 and *S. capitis* 517/pRB*aap.* Staphylococcal cultures were streaked on TSA plates and grown overnight at 37^{o}C. Following incubation, three colonies were selected to inoculate 10 mL of TSB, which were incubated overnight at 37^{o}C with agitation at approximately 260rpm, then supplemented with 15% glycerol and adjusted to a turbidity corresponding to OD_{600}= 0.1. The homogeneous suspensions were divided into several aliquots (1.0 mL) and stored at -80^{o}C. One random aliquot was selected of each strain for determination of bacterial concentrations, thereby these stocks with pre-determined concentration were used to inoculate PCs. Paired cultures of *S. epidermidis* and *S. capitis* at a 1:1 ratio were seeded in PCs (each strain had a final concentration of approximately 100 CFU/PC unit). After PC agitation for 20 mins, 8 to 10 mL of PCs was injected into BacT/ALERT aerobic culture bottles followed by incubation in the BacT/ALERT system to confirm bacterial survival. After 5 days of PC storage, spiked PC units were sampled for viable counts by serial dilutions and colony counts on TSA. *S. epidermidis* and *S. capitis* strains were differentiated on TSA plates as *S. epidermidis* 1457 produces white colonies while *S. capitis* 517 grows forming yellow colonies (Taha et al., 2014). Five colonies of S. *capitis* 517/pRB*aap* after day 5 incubation in PCs were sub-cultured twice in TSA_{Cm} and the presence of pRB*aap* was confirmed by PCR. For competition assays between *S. capitis* 517 and S. *capitis* 517/pRB*aap*, spiked PC units were sampled in plain TSA and TSA_{Cm} (only *S. capitis* 517/pRB*aap* can grow in the presence of chloramphenicol) and the difference in the number of colonies obtained between plain TSA and TSA_{Cm} yielded the colony counts of *S. capitis* 517.

VIII. Statistical analyses

A log-10 transformation was used for viable cell counts, and calculation of means and standard deviations (SDs) were performed using Microsoft Excel. A mixed-model analysis was performed using Statistical Analysis System software (SAS Institue, Inc.). P values less than 0.05 were considered significant. Biofilm formation assays were performed three independent times with two replicates per repetition while competition assays were performed three independent times.

3.5 RESULTS

I. PCR detection of the *aap* and *pls* genes

The presence of the *aap* gene in *S. epidermidis* and *S. capitis* strains was determined by PCR amplification. A 3,209 bp gene fragment containing *aap* was amplified from *S. epidermidis* 1457 but not from *S. epidermidis* 1457Δ*aap*. While *aap* was absent in *S. capitis* 517 and *S. capitis* 517/pRB474, the gene was detected in the transformed strain *S. capitis* 517/pRB*aap* (Fig. 3.1a). The presence of *S. capitis pls*, an *aap* homologs, was determined by PCR amplification in five *S. capitis* strains. The gene was only detected in *S. capitis* 512 (Fig. 3.1b).

II. *S. epidermidis aap* enhances biofilm formation in PCs but not in TSBg

Biofilm formation assays of *S. epidermidis* and *S. capitis* strains were performed in TSBg and PCs. Figures 3.2a and 3.2b show that *S. epidermidis aap* had no effect on biofilm formation when assays were performed in TSBg. However, biofilm formation of *S. epidermidis* 1457 in PCs was higher than biofilm formation of *S. epidermidis* 1457Δ*aap* and *S. epidermidis* 1457Δ*aap*/pRB474 ($P < 0.05$). Complementation studies of the *S. epidermidis* 1457Δ*aap* mutant with plasmid pRB*aap* restored biofilm formation (Figs 3.2c and 3.2d). Notably, *S. capitis* 517/pRB*aap* showed greater biofilm formation than *S. capitis* 517 and *S. capitis* 517/pRB474 (Figs 3.2c and 3.2d).

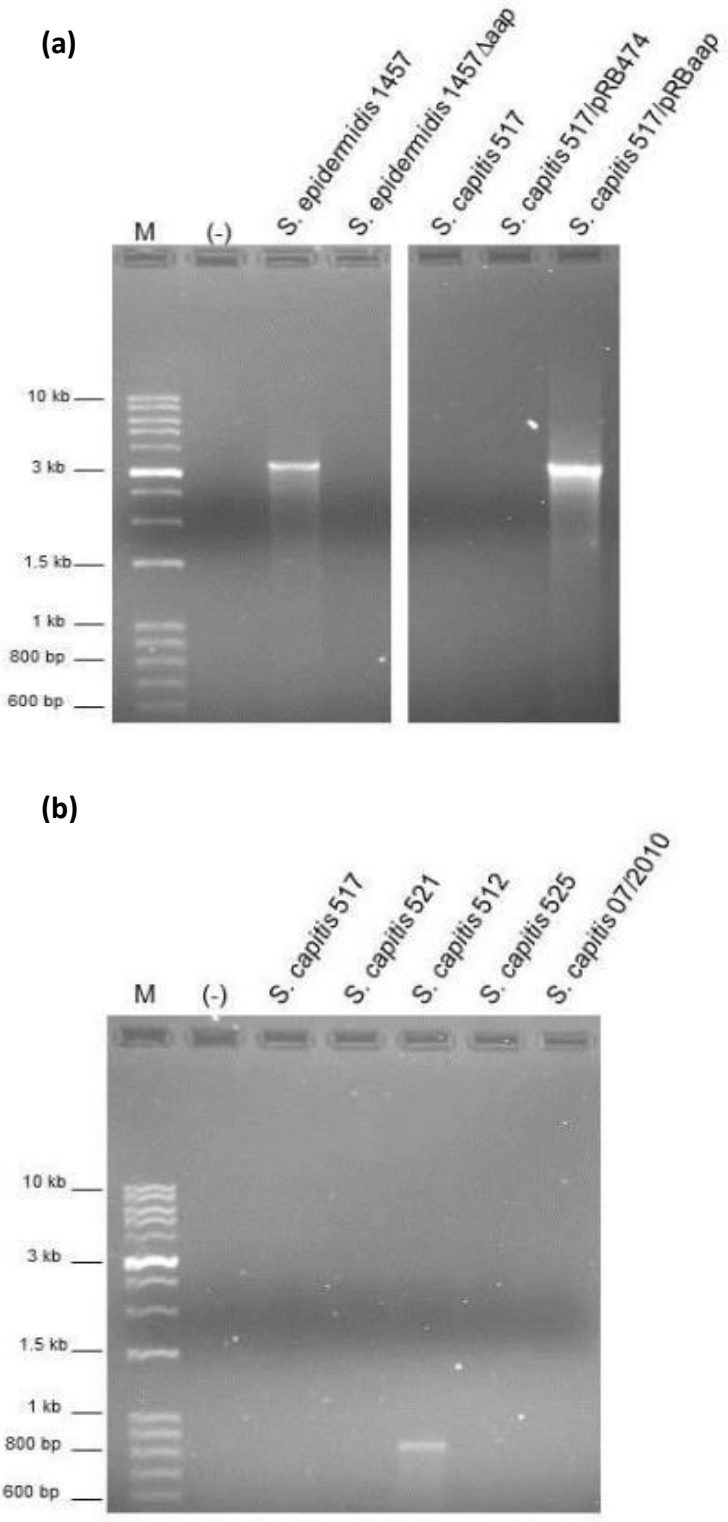

Figure 3.1. PCR detection of *S. epidermidis aap* (a) and *S. capitis pls* (b). M = molecular weight marker (1 KB Plus DNA ladder; Smobio Excelband); (-) = no DNA template (negative control). (a) The expected size of the DNA fragment containing *aap* is 3,209 bp. (b) The expected size of the DNA fragment for *pls* is 781 bp.

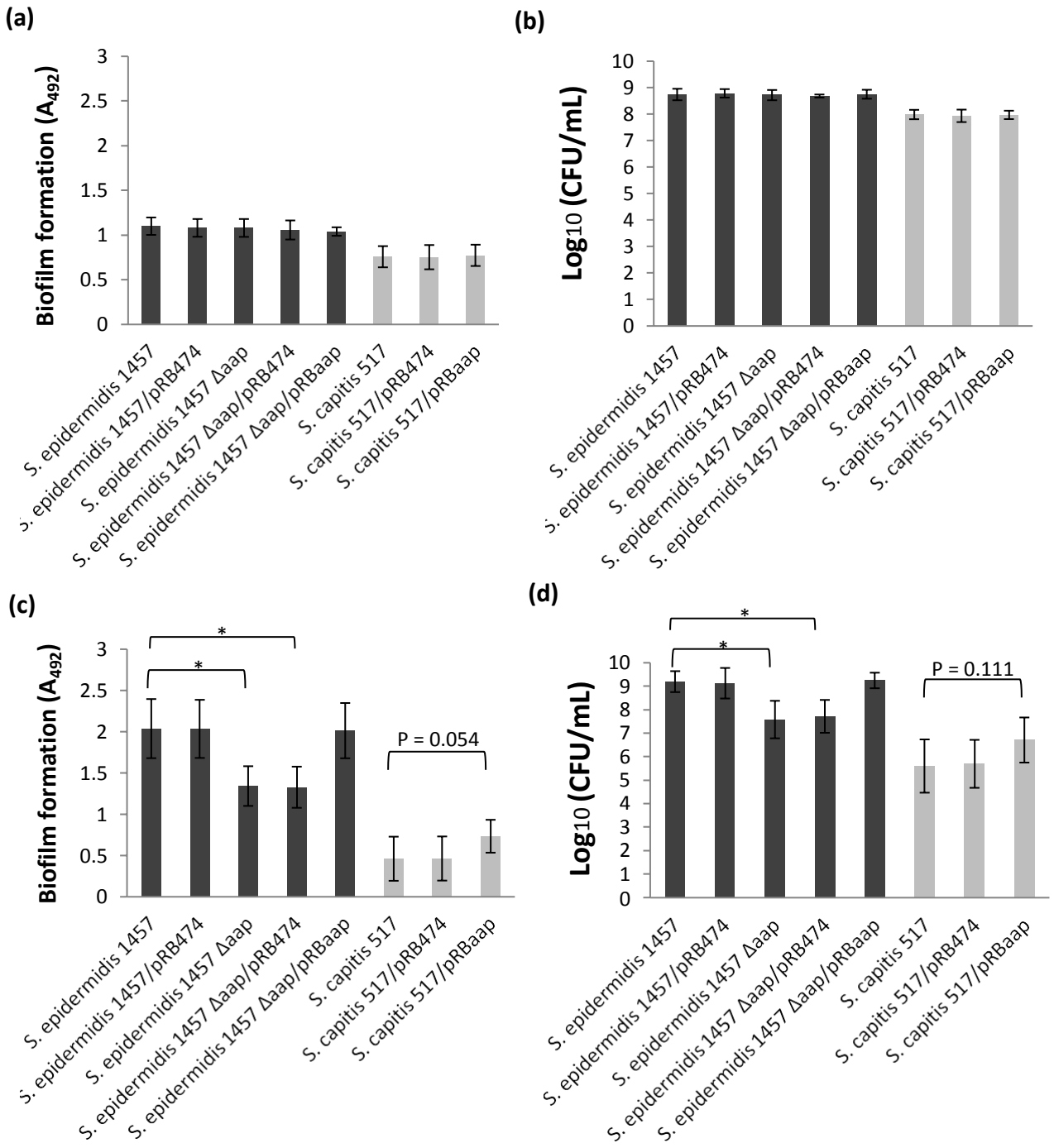

Figure 3.2. Biofilm formation of *S. epidermidis* and *S. capitis* strains in TSBg or PCs. Biofilm formation was analyzed by: **1)** semi-quantification using a crystal violet assay in TSBg cultures (a) and PCs (c), and **2)** determination of viable counts in TSBg cultures (b) and PCs (d). Black bars represented *S. epidermidis* strains while grey bars represented *S. capitis* strains. Results are presented as mean ±SD of three separate experiments with two replicates per experiment. * represents a significant difference between two strains ($p < 0.05$).

III. *S. epidermidis aap* confers proliferative advantage in PCs

Competition assays between *S. epidermidis* and *S. capitis* were performed following previously established protocols (Taha et al., 2014). *S. epidermidis* 1457 was inoculated along *S. capitis* 517 at a 1:1 ratio in PCs on day 0. After 5 days of PC storage, *S. epidermidis* 1457 reached a higher concentration than *S. capitis* 517 ($P < 0.05$) (Fig. 3.3). However, the concentration of *S. epidermidis* 1457Δ*aap* was significantly lower compared to wild-type *S. epidermidis* 1457 ($P < 0.05$) (Fig. 3.3). Importantly, *S. capitis* 517/pRB*aap* resulted in preferential proliferation when compared to *S. capitis* 517 and *S. epidermidis* 1457Δ*aap* (Fig. 3.3).

3.6 DISCUSSION

In this study we demonstrated that *S. epidermidis aap* enhanced biofilm formation and conferred an advantageous growth to *S. epidermidis* and *S. capitis* in PCs. Previously, we observed that PIA-negative *S. epidermidis* strains, with a biofilm negative phenotype, could form biofilms in PCs with enhanced pathogenicity (Ali et al., 2014; Hodgson et al., 2014). In addition, several studies have documented biofilm-positive clinical infections caused by PIA-negative *S. epidermidis*. This suggests the involvement of PIA-independent biofilm formation mechanisms (Rohde et al., 2007; Rohde et al., 2005). In a recent study, we demonstrated that the structure of the cell wall and the biofilm matrix of *S. epidermidis* is remodeled when grown in PCs, displaying a biofilm matrix of proteinaceous nature (Loza Correa et al., 2017). Protein-dependent biofilm formation may involve several proteins such as Aap, extracellular matrix binding protein (Embp), or/and biofilm-associated protein (Bap) (Büttner et al., 2015; Rohde et al., 2007; Tormo et al., 2005). Schaeffer and colleagues demonstrated that Aap was implicated in *S. epidermidis* biofilm formation, and a knockout of the *aap* gene significantly reduced *S. epidermidis* concentration compared to a wild type strain, using a rat jugular catheter model (Schaeffer et al., 2015). Since the majority (~90%) of *S. epidermidis* isolates have *aap* (Rohde et al., 2007), which is absent in *S. capitis* (Cameron et al., 2015), we aimed to investigate the role of *S. epidermidis aap* in enhancing biofilm formation and conferring an advantageous growth to staphylococci in PCs.

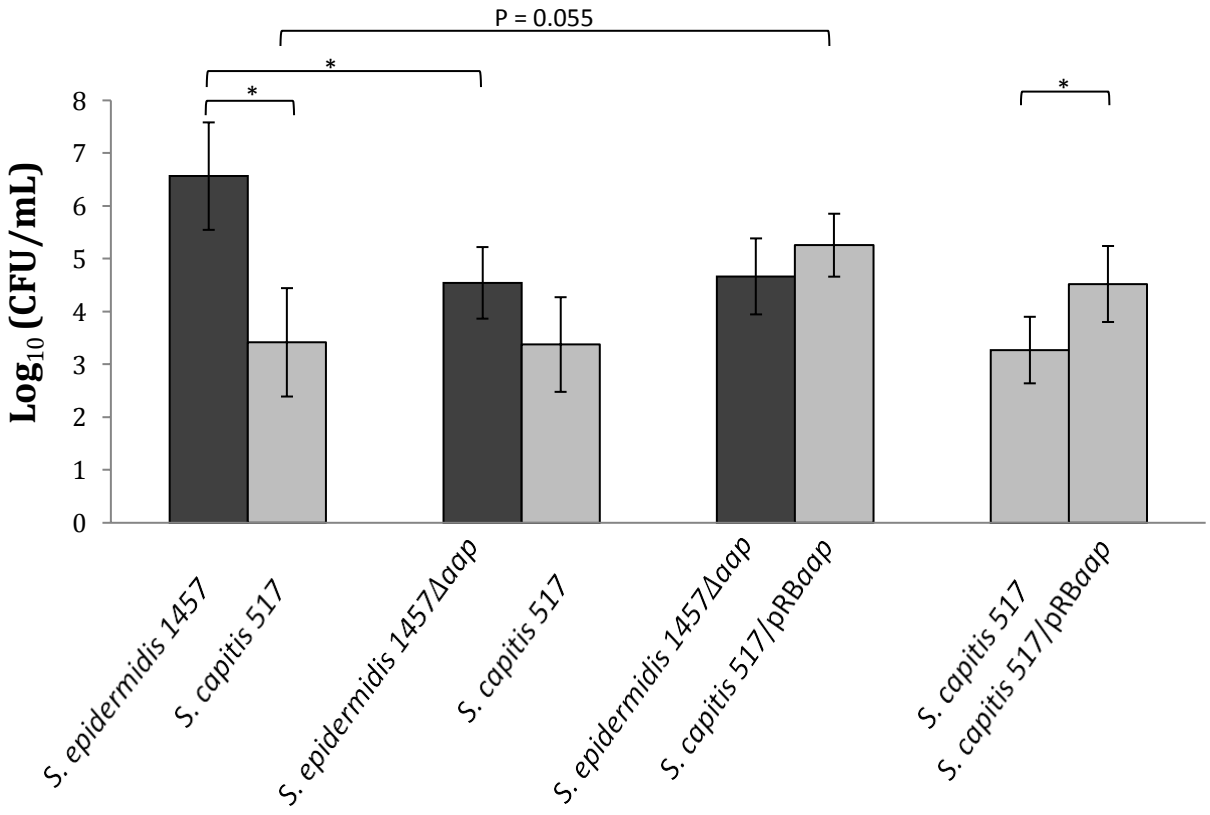

Figure 3.3. Competition assays of *S. epidermidis* and *S. capitis* strains in PCs. Paired cultures were seeded at a 1:1 ratio and viable counts were determined after 5 days of PC storage. Black bars represented *S. epidermidis* while grey bars represented *S. capitis*. Results are presented as mean ±SD of three separate experiments. * represents a significant difference between two strains ($p < 0.05$).

Our studies demonstrated that *S. epidermidis aap* had no effect on biofilm formation by *S. epidermidis* and *S. capitis* strains under laboratory storage conditions. However, a knockout of the *aap* gene significantly reduced the ability of *S. epidermidis* for biofilm formation in PCs. In addition, *S. capitis* carrying *S. epidermidis aap* displayed enhanced biofilm formation in PCs compared to native *S. capitis* confirming the role of *S. epidermidis aap* in biofilm formation during PC storage, and supporting our previous study with *S. epidermidis* forming protein-based biofilms in PCs (Loza Correa et al., 2017).

A recent skin microbiome study demonstrated that *S. epidermidis* is the predominant organism within the staphylococci group while *S. capitis* is found in skin at a lower rate (Grice and Segre, 2011). We speculate that *S. epidermidis aap* could also confer predominance of *S. epidermidis* in the skin since the A domain of Aap is known for promoting adhesion to corneocytes (Macintosh et al., 2009). Similarly, the Aap A domain has been shown to enhance attachment to plastic surfaces that could be linked to increased adhesion to PC plastic bags resulting in enhanced biofilm formation (Schaeffer et al., 2015). As cleavage of the A domain by endogenous proteases (e.g., SepA) and host-derived proteases (e.g., granulocyte proteases) promotes *S. epidermidis* biofilm formation (Paharik et al., 2017; Schaeffer et al., 2015; Rohde et al., 2005), host-derived proteases present in PCs could process Aap and enhance biofilm formation in this milieu.

We have previously demonstrated that *S. epidermidis* outcompetes *S. capitis* in PCs (Taha et al., 2014). To prove that *S. epidermidis aap* plays a role in preferential proliferation in PCs, competition assays were performed between *S. epidermidis* and *S. capitis* strains with different genetic backgrounds. Our results demonstrated that *aap* conferred an advantageous growth in PCs as the *S. epidermidis aap* knockout resulted in lower bacterial concentrations than its wild-type counterpart. Similar results were shown by Schaeffer and coworkers who reported that the knockout of *aap* gene reduced *S. epidermidis* concentration up to 3 Log_{10} using a rat catheter model (Schaeffer et al., 2015). More importantly, we demonstrated that *S. capitis* carrying *aap* reached higher concentrations compared to native *S. capitis* and the *S. epidermidis aap* mutant. We hypothesize that the presence of *aap* allows the formation of protein-based biofilms in PCs, which we have recently demonstrated to confer resistance to AMPs (Alabdullatif et al., 2018), resulting in preferential bacterial

proliferation in this environment. Whether *S. capitis pls* plays a similar role as *S. epidermidis aap* was not determined since the only strain carrying *pls, S. capitis* 512, cannot be used for competition assays as it produces white colonies which are undistinguishable from *S. epidermidis* colonies.

To our knowledge, our study is the first to investigate the role of *aap* on staphylococcal biofilm formation in PCs. Since the knockout of *aap* in *S. epidermidis* reduced but did not eliminate biofilm formation in PCs, there may be other proteins such as Embp and Bap involved in this process (Los et al., 2010; Stevens et al., 2008; Rohde et al., 2007; Tormo et al., 2005), which merits further investigation. Our results showed that *aap* enhances biofilm formation and confers an advantageous growth to *S. epidermidis* and *S. capitis* in PCs. It would be interesting to expand these experiments to include other coagulase-negative staphylococci and to investigate the molecular mechanism of action of Aap during staphylococcal proliferation in PCs.

3.7 ACKNOWLEDGMENTS

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CHAPTER 4.

Antimicrobial peptides: an effective approach to prevent bacterial biofilm formation in platelet concentrates

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Running title: Antibiofilm activity of antimicrobial peptides

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4.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

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M. Alabdullatif and Dr. S. Ramirez-Arcos conceived the study, analyzed the data, and wrote the manuscript; microbiology assays were performed by M. Alabdullatif; and Dr. C.D. Atreya contributed to the synthesis of peptides used in the study, intellectual discussion, and editing the manuscript.

4.2 ABSTRACT

The safety of platelet concentrates (PCs) is a major concern in transfusion medicine due to contamination mainly with skin Gram-positive bacteria. The predominant contaminant, *Staphylococcus epidermidis,* forms bacterial cell aggregates (biofilms) in PCs posing a safety risk for transfusion patients. Combinations of synthetic antimicrobial peptides (AMPs) have demonstrated bactericidal activity in PCs. Herein, we have evaluated the ability of a mix of AMPs to inhibit biofilm formation and/or eradicate *S. epidermidis* biofilms. Three synthetic AMPs, the platelet-derived peptide (PD4) and two arginine-tryptophan repeats (RW3 and RW4), were used for bactericidal and antibiofilm experiments in glucose-supplemented trypticase soy broth (TSBg) and PCs spiked with three biofilm-forming strains of *S. epidermidis*. Time-killing assays were performed to evaluate the bactericidal capability of the peptides. Inhibition of biofilm formation was assayed by seeding *S. epidermidis* into TSBg or PC cultures supplemented with the AMPs. Biofilm eradication assays were performed after AMP treatment of preformed biofilms with and without mechanical dislodging. Biofilms were measured using a crystal violet assay. Time-killing assays demonstrated that all *S. epidermidis* strains were eliminated after 24 hours of AMP treatment. While inhibition of biofilm formation was observed for all *S. epidermidis* strains in TSBg and PCs, the AMP treatment was only effective to reduce the bacterial load of mechanically dislodged biofilms. The combination of three synthetic AMPs (PD4-RW3-RW4) can be used to inhibit biofilm formation by *S. epidermidis* to enhance PC safety. However, further investigation is needed to improve their activity against mature *S. epidermidis* biofilms.

4.3 INTRODUCTION

Bacterial contamination of blood products represents the highest posttransfusion infectious risk (Hong et al., 2016; Ramirez-Arcos and Goldman, 2012). Platelet concentrates (PCs) are the most susceptible blood component to bacterial contamination due to their storage conditions (constant agitation at 22 ± 2^{o}C with an additive solution containing dextrose concentration of approx. 25 g/L), which provide an ideal environment for bacteria to grow (Canadian Blood Services, 2015; Ramirez-Arcos and Goldman, 2012). Blood centres worldwide have documented that skin flora bacteria are the predominant PC contaminants, with *Staphylococcus*

epidermidis being the most commonly isolated aerobic organism (Müller et al., 2015; Jacobs et al., 2011; Zhu et al., 2009). Several strategies have been implemented to reduce the risk of bacterial contamination, including donor screening questionnaire, improved skin disinfection methods, diversion of the first aliquot of the donated blood, and PC screening for bacterial contamination (De Korte et al., 2014; Ramirez-Arcos and Goldman, 2012). Unfortunately, despite these measures, transfusion-associated morbidity and mortality events due to contamination of PCs with *S. epidermidis* continue to be reported (Kou et al., 2015; US Food and Drug Administration, 2015; De Korte et al., 2014).

The ability of *S. epidermidis* to form aggregates of cells enclosed in a matrix called biofilm during PC storage contributes to missed detection of this bacterium during routine screening (Ali et al., 2014; Greco-Stewart et al., 2012). Importantly, we have reported that *S. epidermidis* is able to form biofilms in PCs, independently of its ability to form biofilms in regular media (i.e., nonbiofilm formers convert to a biofilm positive phenotype in PCs) (Ali et al., 2014). It has been shown that biofilm formation by *S. epidermidis* during PC storage results in increased pathogenicity (Hodgson et al., 2014).

Antimicrobial peptides (AMPs), 12 to 100 amino acids long, have emerged as a new generation of bactericidal agents due to their broad-spectrum activity (Huang et al., 2010; Jenssen et al., 2006). Generally, AMPs have a positive net charge that allows them to interact and penetrate the negatively charged bacterial cell membrane, which effectively increases membrane permeability and eventually causes bacterial cell death (Huang et al., 2010; Jenssen et al., 2006). AMPs are produced by a variety of cells including human cells of the immune system such as neutrophils and platelets (PLTs) (Yeaman, 2014). During PC storage, PLTs release proteins with microbicidal activity from alpha granules when they are activated as a defense mechanism against infection (Yeaman, 2014). Yeaman and colleagues (Yeaman et al., 2007) have demonstrated that a synthetic PLT-derived peptide (PD4) from the PLT microbicidal protein exhibits bactericidal effect against *Staphylococcus aureus* and *Salmonella typhimurium* (Mohan et al., 2014). In addition, short synthetic peptides containing arginine-tryptophan (RW) repeats have been shown to exhibit significant bactericidal activity in contaminated PCs (Mohan et al., 2014; Hou et al., 2010; Mohan et al.,

2010a). Importantly, PCs treated with the RW3 and RW4 peptides maintained their *in vitro* quality properties during storage for up to 7 days (Bosch-Marcé et al., 2014a).

Combination antimicrobial therapy, which uses a mixture of two AMPs or more, can synergistically enhance their bactericidal activity (Yu et al., 2016). Mohan and coworkers (Mohan et al., 2014) demonstrated that several combinations of synthetic AMPs including PD4, RW3, and RW4 could serve as bactericidal agents during PC storage. These AMPs have shown bactericidal effect on free-floating (nonbiofilm cells) of *S. epidermidis* in PCs. In this study, we have evaluated the ability of PD4, RW3, and RW4 to inhibit biofilm formation by *S. epidermidis* and to eradicate mature biofilms.

4.4 MATERIALS AND METHODS

I. Bacterial strains

S. epidermidis Hamburg 9142 isolated from a blood culture (Mack et al., 1992), *S. epidermidis* AZ-39 isolated from skin (Taha et al., 2018), and commercial *S. epidermidis* American Type Culture Collection (ATCC) 35983 purchased from ATCC, which was originally isolated from human blood, were used in this study. All strains have a biofilm-positive phenotype. While strains Hamburg 9142 and ATCC 35983 form biofilm matrices mainly composed of polysaccharides under laboratory conditions, *S. epidermidis* AZ-39 has a protein-rich biofilm matrix (Loza Correa et al., 2017; Oliveira and Cunha, 2010). All bacterial strains were stored in brain heart infusion broth (Becton, Dickinson, and Company) with 15% glycerol (vol/vol) at -80^{o}C until used.

II. PCs

Whole blood units were collected from healthy unpaid volunteer donors with informed and signed consent forms. PCs were manufactured using the buffy coat method by pooling four buffy coats and suspending the pool in one of the four donors' plasma at the Canadian Blood Services Network Centre for Applied Development (netCAD). PC collection, sterility, and storage conditions were performed in accordance with Canadian Blood Services Standard Operating Procedures. Ethical approval for this study was provided by the Canadian Blood Services Research Ethical Board.

III. Peptide synthesis

Three AMPs, PD4 (AALYKKKIIKKLLES), RW3 (RWRWRW), and RW4 (RWRWRWRW), were synthesized at the core facility of the Center for Biologics Evaluation and Research, US Food and Drug Administration, and purified by reverse phase high-performance liquid chromatography as described previously (Mohan et al., 2014; Mohan et al., 2010b). Synthetic peptides were reconstituted individually in phosphate-buffered saline (PBS), pH 7.4. A stock solution of the combination of AMPs was made to 10 mmol/L in PBS and stored at -20^{o}C until required. On the day of the assay, a 10 µmol/L final concentration of AMPs combination was prepared as it has been shown that this concentration is optimal for bactericidal assays while maintaining PC quality (Mohan et al., 2014; Bosch-Marcé et al., 2014a).

IV. Time-killing assays

S. epidermidis cultures were streaked onto trypticase soy agar (TSA; Difco, BD Biosciences) plates and grown overnight at 37^{o}C. The following day, one to three colonies were used to inoculate 3 mL of trypticase soy broth (TSB; BD Biosciences), which was incubated for 24 hours at 37^{o}C with agitation at 260 rpm. The overnight cultures were diluted in TSB supplemented with 0.5% of glucose (TSBg; Difco), and the suspension was adjusted to a concentration of approximately 10^5 colony-forming units (CFU)/mL, which was confirmed by performing serial dilutions and plating of the bacterial suspension. The assays were set up in 96-well polystyrene tissue culture plates (Corning, Inc.) with a final volume of 200 µL in each well containing 160 µL of fresh TSBg, 20 µL of the 10^5 CFU/mL bacterial suspension, and 20 µL of a 100 µmol/L combination of AMPs (PD4, RW3, RW4). The final bacterial concentration in each well was 10^4 CFU/mL while AMPs were at a final concentration of 10 µmol/L. PBS (pH 7.4) served as the negative control treatment. The culture plates were incubated at 37^{o}C for 0, 20, 60, 90, and 120 minutes and 24 hours. Suspensions of posttreatment times 0 and 20 minutes were collected, serially diluted 10-fold, and plated. The 200-µL suspensions of times 60, 90, and 120 minutes and 24 hours were directly plated without further dilutions.

V. Biofilm formation

Crystal violet assay

S. epidermidis cultures were streaked on TSA plates and grown overnight at 37ºC. After incubation, one to three colonies were used to inoculate 3 mL of TSB and the suspension was incubated for 24 hours at 37ºC with agitation at 260 rpm. The overnight cultures were diluted in TSBg and adjusted to an optical density of 600 nm = 0.1, which corresponds to approximately 10^7 CFU/mL of *S. epidermidis.* Wells of six-well polystyrene tissue culture plates (Corning, Inc.) were seeded with 3 mL of this suspension and incubated at 37ºC with no agitation for 24 hours to obtain mature biofilms. For biofilm formation in PCs, a similar approach was used but instead of preparing the inocula in TSBg, the suspensions were prepared in PCs, which were then dispensed into six-well plates and incubated under PLT storage conditions, at 22 ± 2ºC on a platform PLT agitator for 5 days to produce mature biofilms (Ali et al., 2014; Greco et al., 2007). After incubation, each well was gently washed three times with 3 mL of PBS to remove free-floating cells and bacterial aggregates (biofilms) attached to the plastic of the culture plates were stained for 30 minutes with 3 mL of 0.3% crystal violet (Becton, Dickinson, and Company). The wells were then washed three times with PBS to remove any unbound stain. Following previously established procedures, 3 mL of a destaining solution (20% acetone and 80% ethanol) was added to each well and incubated with agitation (approx. 100rpm) for 15 minutes to elute the bound crystal violet. This elution was done once; subsequently, six 0.2-mL aliquots taken from the 3-mL eluted solution were transferred to six wells of a 96-well plate, and the absorbance of each sample seeded in the well was read at a wavelength 492 nm in a microplate reader (Expert Plus, ASYS Hitech GmbH) (Ali et al., 2014; Greco et al., 2007). Absorbance of the bacterial cultures was obtained by averaging the values obtained for the six aliquots. Absorbance readings from the negative controls (wells with just TSBg or PCs) were subtracted from readings of the bacterial cultures.

Viable cell determination

To determine the viable cell counts of mature biofilms, a similar process as the one described above for the crystal violet assay was followed. Free-floating cells were discarded, and biofilms were washed with PBS three

times. Instead of crystal violet staining, 3 mL of PBS was added to each well and the plates were scraped to dislodge the biofilm cells (Taha et al., 2014). Each suspension was serially diluted 10-fold and plated on TSA. Plates were incubated overnight at 37^{o}C and colonies were enumerated.

VI. Inhibition of biofilm formation

Inhibition of biofilm formation assays were prepared in six-well culture plates with a final volume of 3 mL in each well containing: 2.4 mL of TSBg or PCs, 0.3 mL of the 10^5 CFU/mL bacterial suspension, and 0.3 mL of a 100 µmol/L combination of AMPs (PD4, RW3, RW4). The final bacterial concentration in each well was 10^4 CFU/mL while AMPs were at a final concentration of 10 µmol/L. Negative controls had PBS added to the culture instead of the AMPs mix. The samples were then incubated for biofilm formation, and a crystal violet assay and viable cell enumeration were performed as described above.

VII. Eradication of preformed biofilms

Without mechanical dislodging

Mature (preformed) *S. epidermidis* biofilms in six-well culture plates were washed with PBS and treated with 3 mL of the combination of AMPs (10 µmol/L final concentration) or with PBS (negative control). The plates were incubated at 37^{o}C for 24 hours, after which a crystal violet assay and viable cell enumeration were performed.

With mechanical dislodging

To evaluate if dislodged biofilms would be more susceptible to the bactericidal action of AMPs, mature biofilms in six-well plates were subjected to mechanical disruption before AMP treatment. In the first approach, mature biofilms were washed with PBS and partially dislodged by scraping, leaving visible aggregates, before being treated with AMPs. In the second approach, washed and scraped biofilms were homogenized by repeated pipetting and vigorous mixing (at 3200 rpm for 10 sec). Disrupted biofilm cultures treated with AMPs were incubated at 37^{o}C for 24 hours, after which a viable cell enumeration was performed.

VIII. Statistical analyses

All assays were performed three independent times with two replicates per repetition. A log transformation was performed on bacterial colony counts, and calculation of means and standard deviations (SDs) were performed with computer software (Excel, Microsoft Crop.). Statistical analyses were performed using two-tailed paired t test and p values less than 0.05 were considered significant.

4.5 RESULTS

I. A combination of the PD4-RW3-RW4 AMPs eliminates *S. epidermidis* after 120 minutes of exposure

The initial inoculum of all *S. epidermidis* strains subjected to the time-killing assays was confirmed to be approximately 10^4 CFU/mL. Viable counts of treated *S. epidermidis* gradually decreased after treatment with the combination of AMPs at 20 minutes (approx.1.5 log reduction), 60 minutes (approx. 2.5 log reduction), 90 minutes (approx. 3.7 log reduction), and 120 minutes (approx. 4 log reduction; Fig. 4.1). At 24 hours, a complete elimination of viable cells was observed for all *S. epidermidis* strains. The negative controls (cells treated with PBS) reached concentrations of more than 10^7 CFU/mL (Fig. 4.1).

II. *S. epidermidis* biofilm formation is effectively inhibited by a combination of the PD4-RW3-RW4 AMPs

Cultures of *S. epidermidis* for biofilm formation assays were performed in TSBg or PCs to which a mix of the AMPs PD4-RW3-RW4 was added. All *S. epidermidis* cultures in TSBg and PCs to which PBS was added as a negative control demonstrated biofilm formation with a final bacterial concentration of approximately 10^8 CFU/mL in TSBg and PCs (Fig. 4.2). Semiquantification of biofilm formation using a crystal violet assay demonstrated inhibition of biofilm formation in *S. epidermidis* cultures seeded in TSBg (Fig. 4.2A) and PCs (Fig. 4.2C) containing the combination of AMPs. Inhibition of biofilm formation was confirmed as no viable bacteria were recovered in the AMP-treated cultures grown in TSBg (Fig. 4.2B) or PCs (Fig. 4.2D).

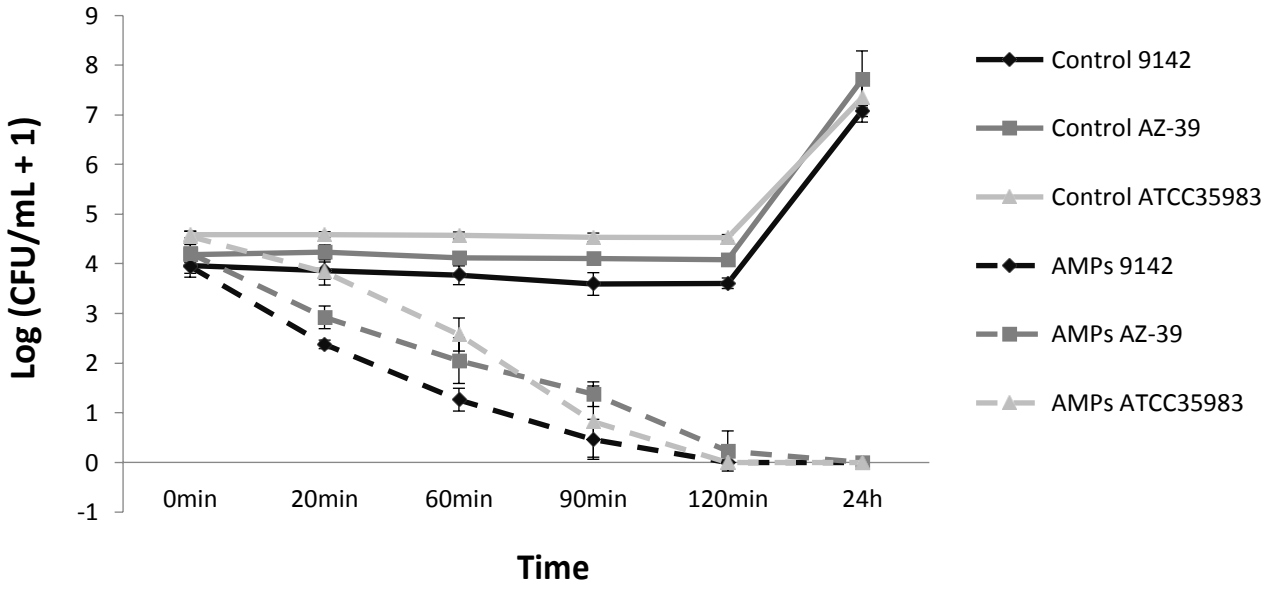

Figure 4.1. Time-killing assays. Bacterial counts were normalized (log + 1) since complete clearance of bacteria has been observed. Results are presented as mean ± SD of three separate experiments (n = 3). Control = cells treated with PBS; AMPs = cells treated with the combination of three AMPs PD4-RW3-RW4.

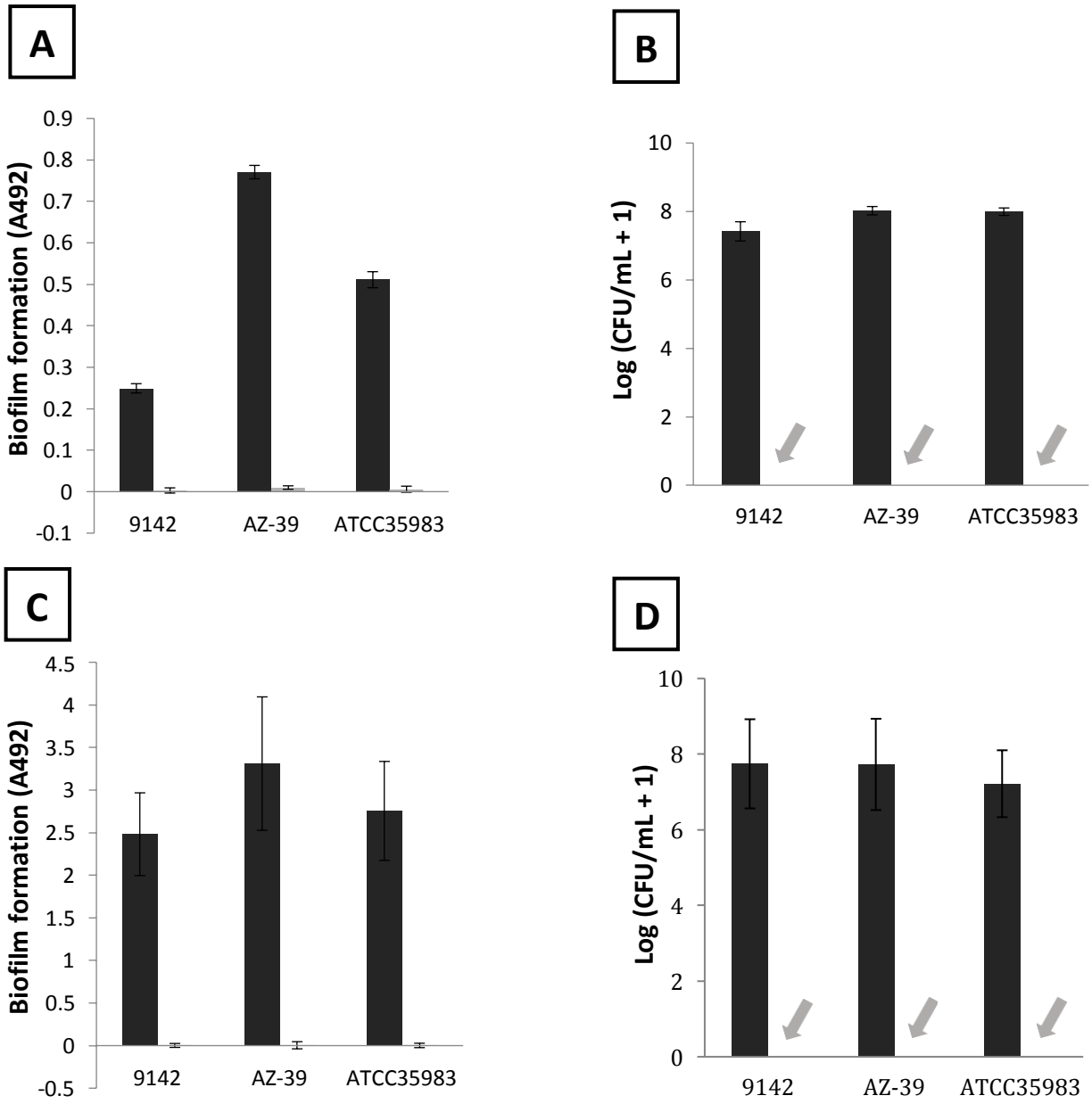

Figure 4.2. Inhibition of biofilm formation by AMPs. Biofilm formation was analyzed by: **1)** semiquantification using a crystal violet assay in TSBg cultures (A) and PCs (C), and **2)** determination of viable counts in TSBg cultures (B) and PCs (D). Viable counts were normalized (Log + 1). Black bars represent cultures treated with PBS (negative control); grey bars (indicated with an arrow) show cultures treated with AMPs. Results are presented as mean ± SD of three separate experiments.

III. Mature biofilms are only susceptible to AMP bactericidal effect when they are mechanically dislodged

As shown in Fig. 4.3, AMP treatment of preformed *S. epidermidis* biofilms did not have a bactericidal effect. However, when mature biofilms were dislodged by scraping, the AMPs combination was effective in decreasing viability of *S. epidermidis* biofilm cells grown in TSBg by approximately 1 log but not in PCs (Figs 4.4A and 4.4B, respectively). Interestingly, when scraped biofilms were homogenized by repeated pipetting and vigorous mixing, allowing for complete biofilm dislodging, AMP treatment significantly reduced ($p < 0.05$) bacterial concentrations by approximately 2.5 log in biofilm cells grown in TSBg (Fig. 4.4C) and by approximately 1.5 log in biofilm cells grown in PCs (Fig. 4.4D).

4.6 DISCUSSION

In this study, we demonstrate that a combination of synthetic AMPs is effective in inhibiting biofilm formation by three biofilm-forming strains of *S. epidermidis* in PCs. Previously, we have shown that the PC storage environment promotes biofilm formation by *S. epidermidis* isolates traditionally considered to be biofilm-negative (Ali et al., 2014). Importantly, biofilms formed in PCs have increased pathogenicity as demonstrated using nematode-killing assays (Hodgson et al., 2014). Biofilm formation by *S. epidermidis* has also been linked to missed detection during PC screening (Greco-Stewart et al., 2012; Ali et al., 2014). Moreover, *S. epidermidis* biofilms resist the killing action of skin disinfectants used on blood donors and have an antagonist effect on other skin contaminants resulting in their preferential proliferation in PCs (Taha et al., 2014). It is therefore important to implement strategies to prevent biofilm formation during PC storage or to treat preformed biofilms.

Previously Mohan and colleagues (Mohan et al., 2014) have demonstrated that the combination of synthetic AMPs used in this study (PD4-RW3-RW4) was effective in reducing the viability of common bacterial contaminants in PCs. Furthermore, Bosch-Marcè and coworkers (Bosch-Marcè et al., 2014b) have confirmed that when used individually, these AMPs do not elicit immune response in rabbits and do not significantly affect PCs recovery in a SCID mouse model.

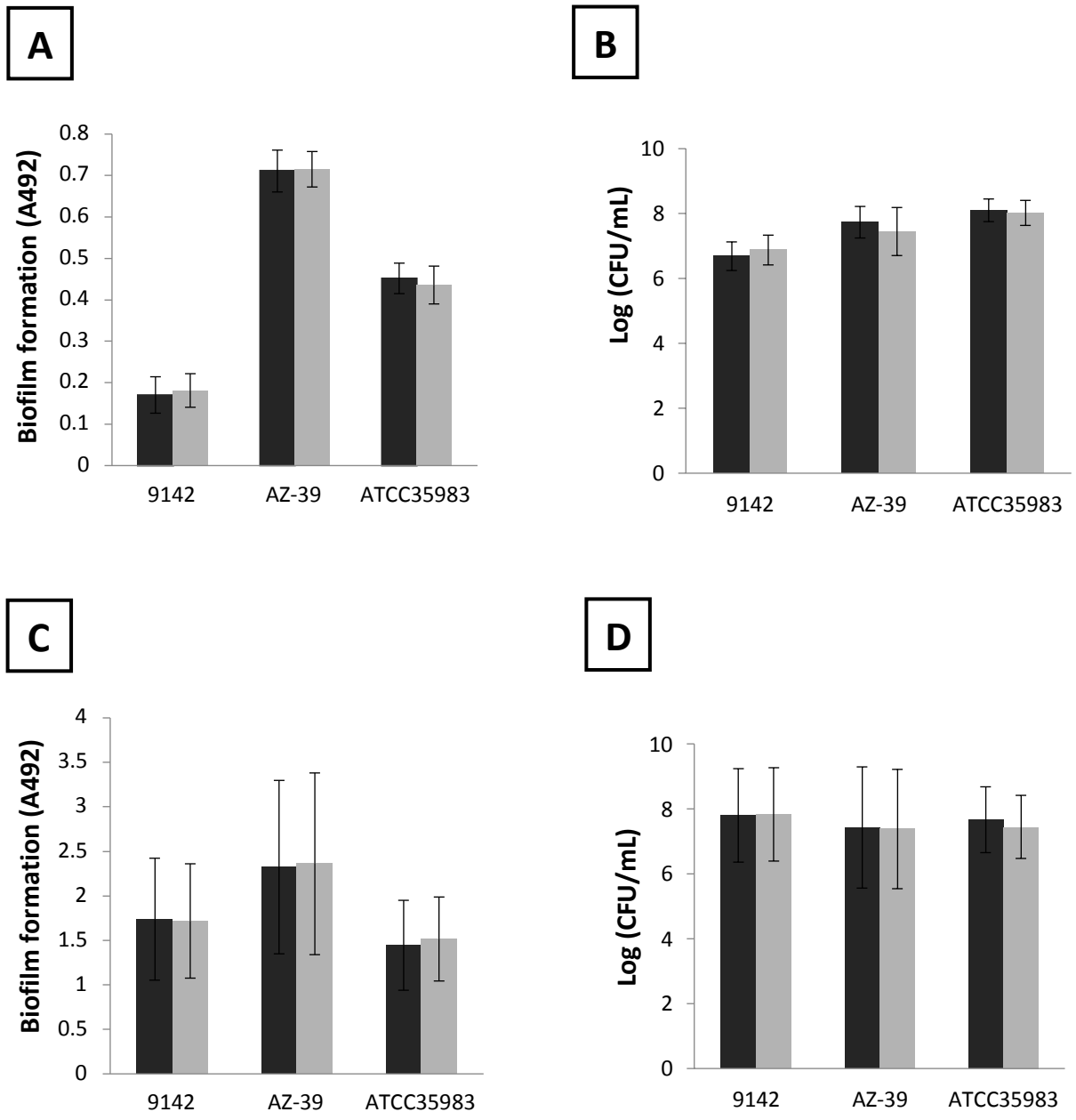

Figure 4.3. Eradication of preformed biofilms with AMPs without mechanical dislodging. Mature biofilms in TSBg (A and B) and PCs (C and D) were treated with AMPs, and residual biofilms were semiquantified using a crystal violet assay (A and C) and by determination of viable cell counts (B and D). Black bars represent cultures treated with PBS (negative control); grey bars represent AMP-treated biofilms. Results are presented as mean ± SD of three separate experiments.

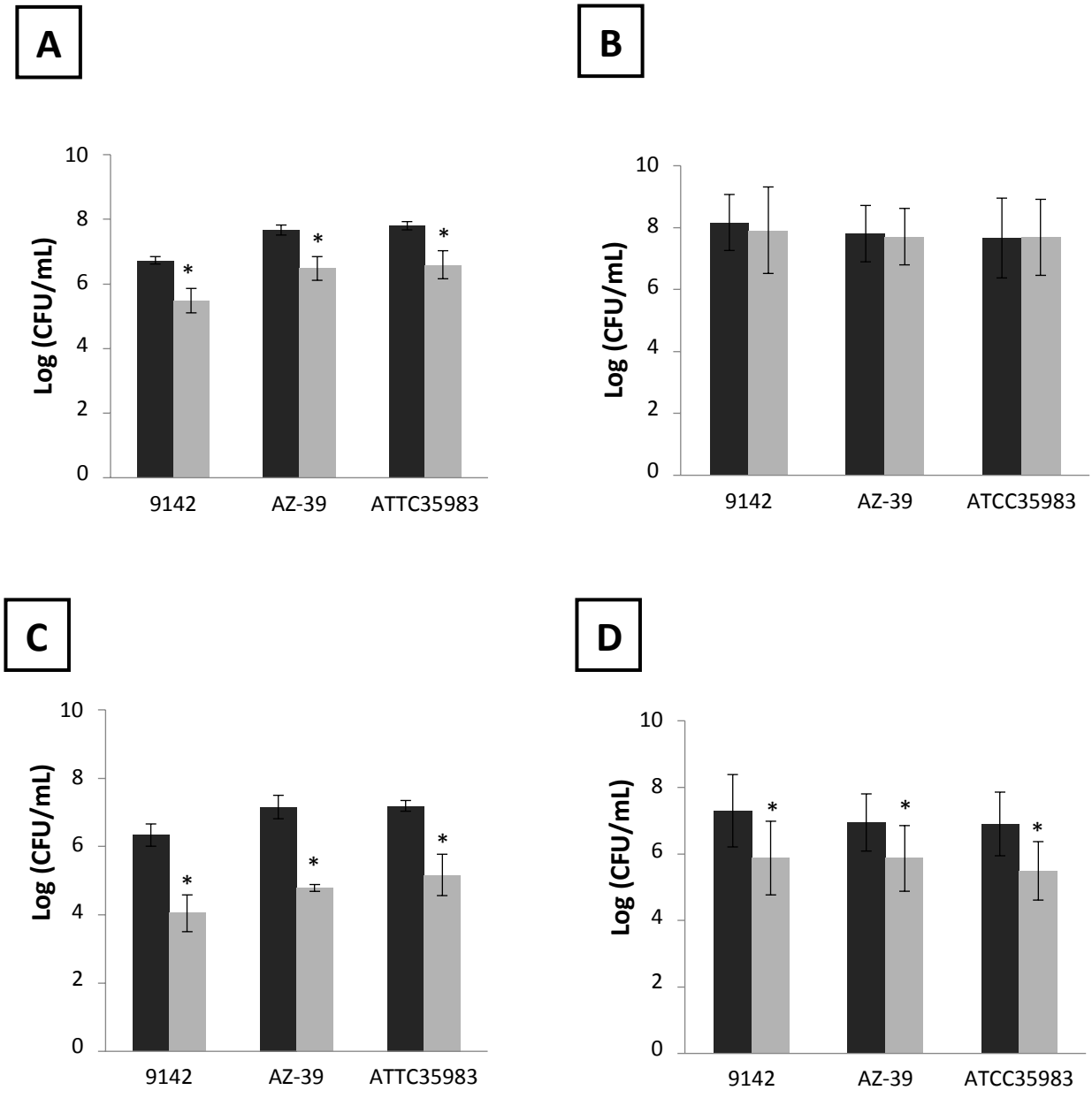

Figure 4.4. Eradication of preformed biofilms with AMPs after mechanical dislodging. Mature biofilms in TSBg (A and C) and PCs (B and D) were scraped (A and B) or scraped, pipetted, and vigorously mixed (C and D) before treatment with PBS (black bars; negative control) or with AMPs (grey bars). Viable counts were determined and results are presented as mean ± SD of three separate experiments. * Significant difference in viable counts between biofilms treated with AMPs versus the negative control ($p < 0.05$).

Our time-killing assays demonstrated that a combination of the three AMPs PD4-RW3-RW4 were effective in reducing viable counts of three *S. epidermidis* isolates by approximately 4 log after 120 minutes of AMP treatment. Similar results were shown by Mohan and coworkers (Mohan et al., 2014) who reported that synthetic AMPs had a bactericidal effect in spiked PCs with bacterial reductions greater than 3 log after 90 minutes of AMP treatment. Importantly, no differences in the action of the AMPs were observed between these isolates despite their origin and differences in biofilm-associated factors.

Herein, we aimed at evaluating the antibiofilm activity of synthetic AMPs, which have shown bactericidal effects in nonbiofilm cells. Our results demonstrated that AMPs were effective in preventing biofilm formation, which supports our observations with the time-killing assays. Although intact mature *S. epidermidis* biofilms were resistant to the bactericidal action of the AMPs combination, mechanically dislodged biofilms (i.e., scraped or vigorously mixed) showed reduced viable cell counts posttreatment with AMPs. *S. epidermidis* biofilm cells are cohesively embedded in a biofilm matrix, which can be composed of polysaccharides, such as the polysaccharide intercellular adhesion, proteins, and extracellular DNA (Loza Correa et al., 2017; Joo and Otto, 2015). It is likely that the biofilm matrix is responsible for the resistance to AMPs since biofilms are known to display resistance to treatment with antimicrobials by different mechanisms, one of which is the barrier posed by the biofilm matrix for antibiotic penetration (Joo and Otto, 2015).

We have evidence that isolates of *S. epidermidis* grown in TSBg can form biofilms coated with matrixes of different chemical compositions. While strains ATCC 35983 and 9142 have a polysaccharide intercellular adhesion-based matrix, *S. epidermidis* AZ-39 has a protein-based biofilm matrix (Loza Correa et al., 2017; Oliveira and Cunha, 2010). However, no differences in susceptibility to AMPs between the three strains was noted once the biofilms produced in TSBg cultures were mechanically dislodged. This suggests that the physical cohesiveness of mature biofilms formed in culture media could be as important as the chemical composition of the matrix for resistance against the bactericidal effect of AMPs. It is feasible that AMPs are sequestered within the biofilm matrix, a mechanism of resistance previously described by Joo and Otto (Joo and Otto, 2015).

Interestingly, it has been shown that one of the AMPs used in this study, RW4, was effective in killing *Escherichia coli* preformed biofilms in laboratory media due to RW4 capability to penetrate through the negatively charged exopolysaccharide matrix (Hou et al., 2009). The role of the biofilm matrix on AMP resistance by biofilms should be further explored.

We also observed differences in the resistance to AMPs by *S. epidermidis* dislodged biofilms grown in TSBg and PCs. Biofilms formed in PCs became susceptible to AMP treatment only when they were completely homogenized and no aggregates were present. Greco-Stewart and coworkers (Greco-Stewart et al., 2013) observed that biofilms grown in PCs are thicker than biofilms formed in TSBg; thereby, AMPs may have easier access to *S. epidermidis* cells in TSBg. Furthermore, we have recently demonstrated that PC storage induces structural changes in the *S. epidermidis* cell wall and biofilm matrix composition. The biofilm matrix of *S. epidermidis* grown in PCs is mainly of proteinaceous nature independently of the chemical composition of the biofilm matrix when the same strain is grown in TSBg (Loza Correa et al., 2017). Analysis of the cell wall of *S. epidermidis* biofilms grown in PCs revealed that the peptidoglycan has shorter muropeptides and reduced serine content compared to biofilms grown in TSBg (Loza Correa et al., 2017). Furthermore, the peptidoglycan of *S. epidermidis* biofilms is amidated and O-acetylated, two modifications that have been shown to be involved in increased resistance to AMPs in staphylococci (Sukhithasri et al., 2013).

The practical application of synthetic AMPs therefore resides on their potential use to prevent biofilm formation in PCs (i.e., bacterial aggregation and attachment to PC containers) and not to eliminate mature biofilms. Further investigation is needed to evaluate the effectiveness of the tested AMPs against a broader range of bacterial species and to test other AMP combinations. It is also acknowledged that clinical studies in humans are needed before the use of AMPs have a practical application in transfusion settings. It is nevertheless important to highlight that bacterial detection during PC screening would be enhanced by using AMPs since we have demonstrated that biofilm formation during PC storage results in missed detection with culture systems (Greco-Stewart et al., 2012; Ali et al., 2014). We have also shown that biofilms formed during PC storage have enhanced pathogenicity and could pose a higher safety risk to transfusion patients (Hodgson et

al., 2014), which would be averted by inhibiting biofilm formation with AMPs. Overall, the results presented herein provide promising evidence for the use of synthetic AMPs to prevent *S. epidermidis* biofilm formation in PCs, which would result in enhanced safety from the risk of bacterial sepsis for transfusion patients.

4.7 ACKNOWLEDGMENTS

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CHAPTER 5.

Staphylococcus epidermidis resistance mechanisms against the synthetic platelet-derived antimicrobial peptide PD4

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Running title: *S. epidermidis graS* mediates resistance to antimicrobial peptides

5.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

The manuscript "*Staphylococcus epidermidis* resistance mechanisms against the synthetic platelet-derived antimicrobial peptide PD4" will be submitted for peer-review as a short report.

M. Alabdullatif and Dr. S. Ramirez-Arcos conceived the study, analyzed the data, and wrote the manuscript; microbiology assays were performed by M. Alabdullatif; and Dr. C.D. Atreya contributed to the synthesis of platelet-derived antimicrobial peptide (PD4) used in the study.

5.2 ABSTRACT

Mechanisms of *Staphylococcus epidermidis* resistance to the bactericidal action of antimicrobial peptides (AMPs) present in the skin include the antimicrobial peptide sensor system (GraRS) and the SepA protease. In this study, we investigated whether the predominance of *S. epidermidis* in platelet concentrates (PCs) can be attributed to resistance to a platelet-derived AMP. A synthetic AMP (PD4), which was derived from platelet factor-4, was used in antibacterial assays against common PC contaminants including *Klebsiella pneumoniae*, *Escherichia coli*, *Serratia marcescens*, *Staphylococcus aureus*, and coagulase-negative staphylococci (*Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus caprae*, and *Staphylococcus saprophyticus*). PD4 reduced the concentration of *K. pneumoniae*, *E. coli*, *S. marcescens*, and *S. aureus* by approximately 1 $\log_{10}$ ($p < 0.05$) compared to negative control PBS, while coagulase-negative staphylococci displayed resistance to PD4. To investigate *S. epidermidis* resistance mechanisms toward PD4, antibacterial assays were performed against wild-type *S. epidermidis* 1457 and *S. epidermidis* 1457*graS* and *sepA* mutants. While wild-type *S. epidermidis* 1457 and *S. epidermidis* 1457Δ*sepA* mutant were resistant to PD4, the concentration of *S. epidermidis* Δ*graS* was decreased when treated with PD4 by approximately 0.5 $\log_{10}$ ($p < 0.05$) compared to *S. epidermidis* 1457. This study demonstrated that *S. epidermidis* (and likely other coagulase-negative staphylococci) displays resistance to PD4 involving GraS. Investigation of the molecular mechanisms involved in potential synergy with other resistance systems merits further attention.

5.3 INTRODUCTION

In transfusion medicine, bacterial contamination of platelet concentrates (PCs) poses the highest infectious risk in developed countries with transfusion-associated morbidity and mortality events reported recently (Ramirez-Arcos et al., 2017; Food and Drug Administration, 2016). PCs provide an ideal environment for bacterial proliferation due to their storage conditions in constant agitation at 20-24^{o}C. In addition, PCs are prepared with an additive solution containing a glucose concentration of approximately 25 g/L (Canadian Blood Services,

2017b; Ramirez-Arcos and Goldman, 2012). The skin flora bacterium *Staphylococcus epidermidis* is recognized as the predominant aerobic contaminant of PCs, and it is believed that this organism is introduced during the blood collection procedure (Ibáñez-Cervantes et al., 2017; Müller et al., 2015). During PC storage, activated platelets release antimicrobial peptides (AMPs), whose bactericidal action is overcome by *S. epidermidis* and other bacteria able to proliferate in PCs (Yeaman, 2014; Mohan et al., 2014; Tang et al., 2002), by so far unknown AMP resistance mechanisms.

There are two reported mechanisms of *S. epidermidis* resistance toward AMPs in the skin. The first mechanism utilizes an antimicrobial peptide sensor (APS) system, also known as GraRS, which confers resistance to the human AMP beta defensins-3 (HBD-3) produced by skin cells (Li et al., 2007a). GraS is responsible for AMP sensing, which in turn activates the GraR regulator. GraR controls the expression of several genes including *dltABCD* and *mprF*, resulting in an increase of the positive charge of teichoic acids in the cell wall and cell membrane, respectively. GraR also up-regulates *vraFG,* which play a role in exporting AMPs attached to the cell membrane (Li et al., 2007a). The second resistance mechanism involves the *S. epidermidis* SepA protease for AMP degradation (Lai et al., 2007).

Generally, AMPs have a cationic net charge that allows them to interact with the negatively charged bacterial cell membrane, leading to increased membrane permeability and eventually causing cell death (Huang et al., 2010; Jenssen et al., 2006). Platelet factor-4 (PF4) is the most abundant platelet antimicrobial protein with a C-terminal domain containing a net cationic charged and an α-helix structure. The synthetic platelet-derived AMP PD4 is a derivative of PF4 that is composed of the last 15 amino acid of the C-terminal domain of PF4 (Yeaman, 2007). PD4 showed high bactericidal effect against *Staphylococcus aureus* and *Salmonella typhimurium* (Yeaman, 2007). Furthermore, Mohan and colleagues have shown that PD4 displays antibacterial activity against *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Bacillus cereus*, but not *S. epidermidis* (Mohan et al., 2014). As a result, PD4 was selected in this study as a molecule model to further the investigation of *S. epidermidis* resistance towards PC AMPs.

5.4 MATERIALS AND METHODS

I. Bacterial strains and plasmids

Bacterial strains and plasmids for this study are listed in Table 5.1. Bacterial stocks were stored in brain heart infusion broth (Becton, Dickinson, and Company, Sparks, MD, USA) or trypticase soy broth (TSB; BD Biosciences) with 15% glycerol (v/v) at -80^{o}C until further use. All bacteria were grown in TSB or TSB agar (TSA; BD Biosciences) plates, and antibiotics [10 µg/ml chloramphenicol (Fisher Scientific) or 100 µg/ml ampicillin (Fisher Scientific)] were used when needed.

II. Peptide synthesis

PD4 (AALYKKKIIKKLLES) was synthesized using solid phase method at the United States Food and Drug Administration (FDA) and purified using reverse-phase high-performance liquid chromatography. Lyophilized PD4 was reconstituted in phosphate buffered saline (PBS), pH 7.4, to prepare a stock solution at a concentration of 10 mM that was stored at -20^{o}C. On the day of the assay, a 10 µM final concentration of PD4 was prepared.

III. Construction of a *S. epidermidis sepA* deletion mutant

Construction of a *S. epidermidis sepA* deletion mutant was performed following protocols described by Li and colleagues (Li et al., 2007a) with some modifications. Upstream and downstream amplicons of 965 bp and 966 bp were generated by primers sets sepA2-for1/sepA2-rev1 and sepA2-for2/sepA2-rev2, respectively (Table 5.2.), using a suspension of *S. epidermidis* 1457 wild-type in nuclease-free water as a template chromosomal DNA. Then, nested PCR was performed (to reduce non-specific binding in products) by amplifying the previous PCR products (965 bp and 966 bp) with primers sets attB1/sepA2-rev1 and sepA2-for2/attB2 (Table 5.2) to get amplicons of 1006 bp and 1007 bp, respectively. The resulting nested PCR products were used to construct a *sepA* deletion mutant. After digestion with BamHI (New England Biolabs), the two amplicons were ligated using T4 DNA ligase (New England Biolabs) at 15^{o}C overnight. The ligation mixture was combined with pKOR1 (temperature-sensitive plasmid; Table 5.1) (Bae and Schneewind, 2006) and BP Clonase mix (Invitrogen) in a 20 µL reaction volume and incubated overnight at room temperature as described by the

Table 5.1. Bacterial strains and plasmids used in this study

Bacterial strains, or plasmids	**Strain (Source/Reference)**	**Comment**
Staphylococcus epidermidis	9142 (Mack et al., 1992)	Blood culture isolate
	1457 (Li et al., 2007a)	Central venous catheter isolate
	1457 Δ*graS* (Li et al., 2007a)	*graS* allelic replacement mutant
	1457 Δ*graS*/pRB*graS* (This study)	*graS* allelic replacement mutant with plasmid carrying full length *graS*
	1457 Δ*sepA* (This study)	*sepA* allelic replacement mutant
	AZ-39 (Taha et al., 2018)	Skin isolate
	ST-10002 (Taha et al., 2017)	Contaminated PCs
	ATCC 35984 (American Type Culture Collection)	Catheter sepsis
Staphylococcus capitis	517 (Greco-Stewart et al., 2013)	Contaminated PCs
Staphylococcus caprae	BPA 07073 (Canadian Blood Sservices)	
Staphylococcus saprophyticus	BPA 07099 (Canadian Blood Sservices)	
Klebsiella pneumoniae	WHO-C245 (World Health Organization)	
Serratia marcescens	BT-10023A (Canadian Blood Sservices)	
Staphylococcus aureus	CBPA-BT-12003 (Canadian Blood Services)	
Escherichia coli	CBPA-BT-13003 (Canadian Blood Services)	
	DH5α (New England Biolabs)	Cloning strain, suitable for high efficiency transformation
	DC10B (BEI Resources)	Used as a gateway strain before transformation of plasmids into *S. epidermidis* (restriction negative, modification positive)
Plasmids	**Comment (Reference)***	
pRB474	*E. coli* and staphylococci shuttle cloning plasmid; constitutive gene expression in staphylococci via *vegII* promoter, Cm^R (Rohde et al., 2005)	
pRB*graS*	Complementation plasmid of *graS* gene, Cm^R (This study)	
pKOR1	Staphylococcus allelic exchange vector with anhydrotetracycline inducer of *secY* antisense RNA for counterselection; Ap^R and Cm^R in *E. coli*; Cm^R in *S. epidermidis* (Bae and Schneewind, 2006)	
pKO*sepA*	pKOR1 containing in-frame deletion *sepA* construct; Ap^R and Cm^S in *E. coli*; Cm^R in *S. epidermidis* (This study)	

***** Cm^R: chloramphenicol resistance; Cm^S: chloramphenicol sensitive; Ap^R: ampicillin resistance

Table 5.2. Primers used in this study

Primer	Sequences*	Reference
sepA-F	5’-CTACACGCCAACACTGTCCCATG-3’	Paharik et al., 2017
sepA-R	5’-CCGAACAAAGCAGCTTACAACACA-3’	Paharik et al., 2017
attB1	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3’	Fossum et al., 2009
attB2	5’-GGGGACCACTTTGTACAAGAAAGCTGGGT-3’	Fossum et al., 2009
sepA2-for1	5’-AAAAAGCAGGCTTTATACAGGGGCTTGTTATG-3’	This study
sepA2-rev1	5’-CAACACATggatccTTCTTCATTTTATTACCTCCT-3’	This study
sepA2-for2	5’-CTAGTCATggatccATGGGACAGTGTTGGTGTG-3’	This study
sepA2-rev2	5’-AGAAAGCTGGGTAGCTATAGACTGGGAATGTGTAA-3’	This study
sepA-Fc	5’-TGACATTTGAGCAGTTGAACAA-3’	This study
SeapsSBam	5’-GACATGGATAGTGCAATTggatccAAAGTTGGTAAAGG-3’	Li et al., 2007a
SeapsSXba	5’-CACTCCTGTCATACTTCAACCAAATTAtctagaTACGAAGAC-3’	Li et al., 2007a

*: restriction sites are underlined.

manufacturer's instructions. The BP recombination reaction resulting in pKOR1 containing the ~ 2 kb in-frame *sepA* deletion (pKO*sepA*; Table 5.1.), was transformed into *E. coli* DH5α. The *E. coli* transformants that were resistant to ampicillin and susceptible to chloramphenicol carried pKO*sepA*. The pKO*sepA* plasmid was purified using a QIAGEN MiniPrep Spin Kit 2.0 (QIAGEN). The ~ 2 kb in-frame *sepA* deletion in pKO*sepA* was verified by PCR amplification and sequencing (Stemcore Laboratories, Ottawa, ON) with primer set sepA2-for1/sepA2-rev2. Two µL (83.4 µg/mL) of purified pKO*sepA* was electroporated into *E. coli* DC10B competent cells in a 1 mm gap electroporation cuvette on ice at 1.8 kv, 2.5 msec with a MicroPulser electroporator (Bio-Rad) at room temperature, and recovered in 0.5 mL of SOC (Qiagen). Electroporated cells were then incubated at 37ºC for 1 hour, plated on TSA with ampicillin, and incubated overnight at 37ºC. Of note, pKO*sepA* transformation into *E. coli* DC10B is required to bypass the restriction barrier present in *S. epidermidis* (Monk et al., 2012). Two µL (~ 0.2 µg) of purified pKO*sepA* from *E. coli* DC10B was then electroporated into *S. epidermidis* 1457 in a 1 mm gap electroporation cuvette at 2.1 kv, 2.5 msec with a MicroPulser electroporator at room temperature, and recovered in 0.5 mL of TSB supplemented with 500mM sucrose as described by Monk and coworkers (Monk et al., 2012). Electroporated cells were incubated at 37ºC for 1 hour, plated on TSA with chloramphenicol (TSAcm), and incubated overnight at 37ºC. The *S. epidermidis* 1457 transformants with resistance to chloramphenicol were expected to contain pKO*sepA*, which was verified by PCR amplification with primer set sepA2-for1/sepA2-rev2. Allelic exchange for *S. epidermidis* 1457 containing pKO*sepA* (*S. epidermidis* 1457/pKO*sepA*) was performed as outlined by Bae and Schneewind (Bae and Schneewind, 2006). Briefly, *S. epidermidis* 1457/pKO*sepA* was inoculated into 10 mL of TSB with chloramphenicol and incubated overnight at 37ºC, to increase *S. epidermidis* 1457/pKO*sepA* concentration. Then, a 100µL of the resulting culture was streaked on TSAcm and incubated overnight at 43ºC, which is a non-permissive condition for plasmid replication and selects for allelic exchange. Large colonies were selected and inoculated into 5 mL of plain TSB then incubated overnight at 30ºC, to facilitate plasmid excision. The resulting culture was diluted and streaked on TSA with anhydrotetracycline (500 ng/mL; used as a selection for plasmid excision and loss) and incubated overnight at 37ºC. Large colonies then were streaked on plain TSA and TSAcm, and then incubated

overnight at 37ºC. *S. epidermidis* colonies that were sensitive to chloramphenicol (loss of plasmid) were subjected to colony PCR screening. Colonies which were negative for the 204 bp amplicon product (sepA-F/sepA-R; internal primers of *sepA* gene) were selected for verification of in-frame *sepA* deletion using PCR amplification and sequencing with a combination of internal and external primers.

IV. Complementation of a Δ*graS* mutant

The *graS* gene was PCR amplified, using the primers (Table 5.2) SeapsSBam/SeapsSXba listed in Li and coworkers (Li et al., 2007a). The PCR product was digested with XbaI and BamHI (New England Biolabs), and cloned into XbaI/BamHI-digested plasmid pRB474 (Table 5.1) to create pRB*graS* (Table 5.1) which was transformed into *E. coli* DH5α competent cells. Presence of *graS* in pRB*graS,* was confirmed by PCR amplification and sequencing with primer set SeapsSBam/SeapsSXba. Then, pRB*graS* was transformed into *E. coli* DC10B and then into *S. epidermidis* 1457 Δ*graS* to obtain the complemented strain *S. epidermidis* 1457 Δ*graS/*pRB*graS,* which was confirmed by PCR amplification and sequencing with primer set SeapsSBam/SeapsSXba.

V. Antibacterial assays

Several bacterial strains (Table 5.1) were tested following protocols described by Mohan and coworkers (Mohan et al., 2014) with some modifications. Fresh bacterial colonies from TSA plates were selected to inoculate 3 mL of TSB which were incubated at 37ºC with agitation at approximately at 260 rpm for 24 h. Overnight cultures were adjusted to optical density of 600 nm = 0.1 in TSB, and grown to mid-log phase. One mL of mid-log phase cultures was centrifuged at ~ 3000 × g for 15 min. The pellet was washed three times with PBS (pH = 7.4) and resuspended in PBS to give approximately 1.0×10^5 colony forming unit (CFU)/mL, which was confirmed by performing serial dilutions and plating on TSA. The assays were set up in 96-well plates (Corning Inc., Corning, NY) with a final volume of 200 µL in each well containing 160 µL of fresh PBS, 20 µL of the 10^5 CFU/mL bacterial suspension, and 20 µL of a 100 µM of PD4. The final bacterial concentration in each well was ~ 10^4 CFU/mL while PD4 was at a final concentration of 10 µM. PBS served as the negative control treatment. The

bacterial cultures with PD4 or negative control were incubated for 120 min at 37ºC, then plated on TSA to determine colony counts.

VI. Statistical analyses

The numbers of CFU were log-10 transformed for statistical comparisons, and calculation of means and standard deviation (SDs) by using Micrsoft Excel. All assays were done three independent times with two replicates per repetition. Statistical analyses were done using two-tailed paired t-test and p values less than 0.05 considered statistically significant.

5.5 RESULTS

I. Coagulase-negative staphylococci displayed resistance to PD4

The final concentration of all bacterial strains subjected to antibacterial assays was confirmed to be approximately 10^4 CFU/mL. Antibacterial assays demonstrated that PD4 exhibited bactericidal activity against *K. pneumoniae*, *E. coli*, *S. marcescens*, and *S. aureus* with a reduction of approximately 1 $\log_{10}$ ($p < 0.05$) compared to the negative control (PBS). Meanwhile, the concentration of coagulase-negative staphylococci including *S. epidermidis*, *Staphylococcus capitis*, *Staphylococcus caprae*, and *Staphylococcus saprophyticus* was not reduced displaying resistance to PD4 (Fig. 5.1).

II. *S. epidermidis graS* is involved in resistance to the bactericidal action of PD4

All *S. epidermidis* strains exhibited resistance to PD4 (Fig. 5.1). To investigate *S. epidermidis* resistance mechanisms toward PD4, antibacterial assays were performed against *S. epidermidis* 1457 wild-type, and *S. epidermidis* 1457*graS* and *sepA* mutants. The concentration of *S. epidermidis* 1457 wild-type and the *S. epidermidis*1457Δ*sepA* mutant was not reduced showing resistance to PD4 (Fig. 5.2). However, resistance to PD4 was significantly decreased in *S. epidermidis* Δ*graS* by approximately 0.5 $\log_{10}$ ($p < 0.05$) compared to negative control PBS, and restored in genetically complemented mutant strain (*S. epidermidis* Δ*graS*/pRB*graS*) (Fig. 5.2).

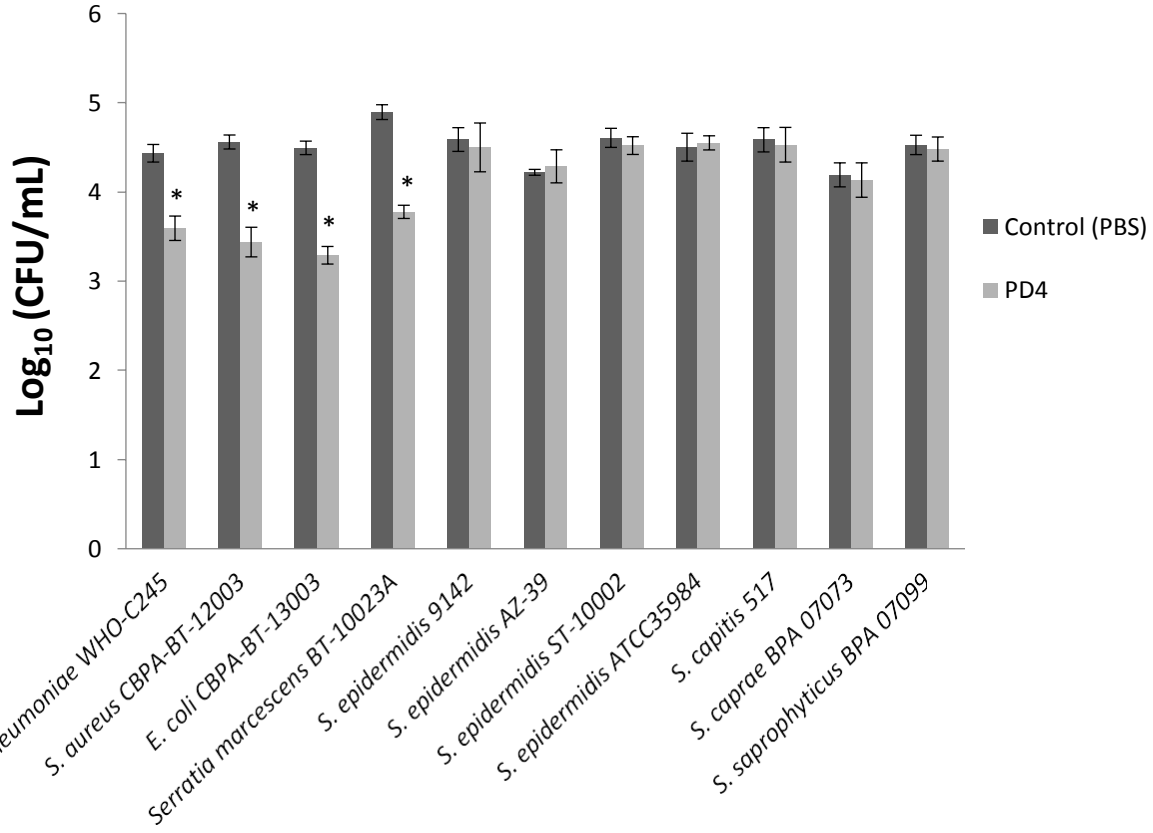

Figure 5.1. Antibacterial assays of PD4 against a common bacterial contaminants of PCs. Black bars represented cultures treated with PBS (negative control) while grey bars showed cultures treated with PD4. Results were presented as avarage ±SD of three separate experiments with two replicates per repetition. * indicates a significant difference in viable counts between cultures treated with PD4 versus the negative control ($p < 0.05$).

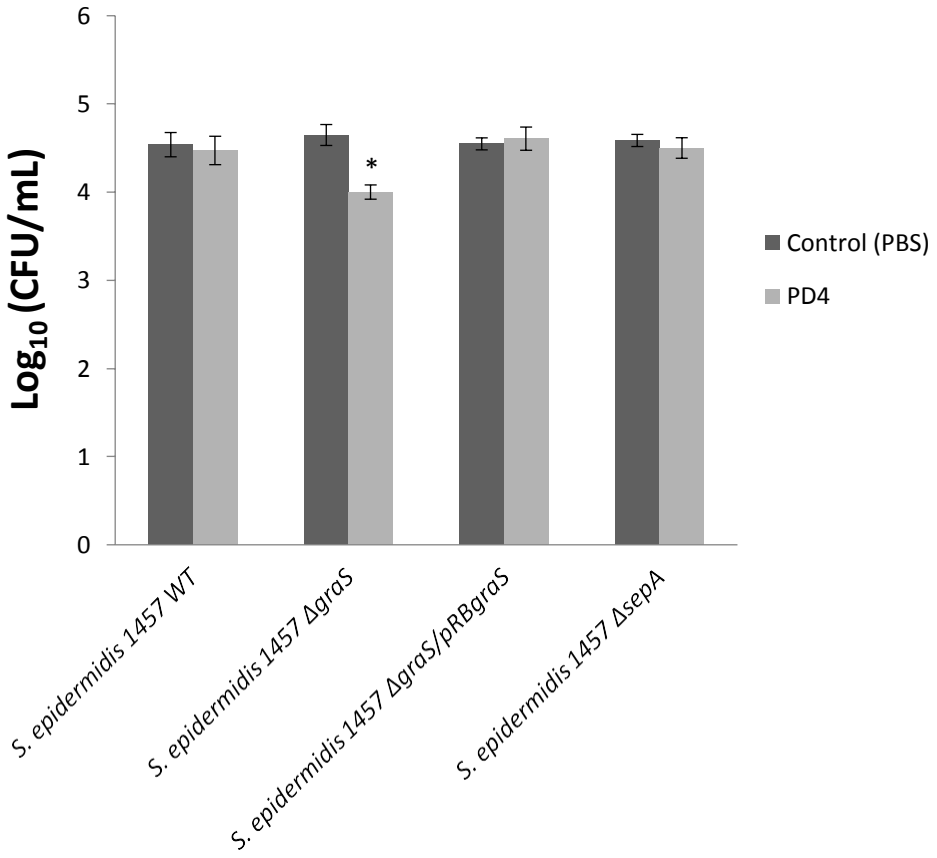

Figure 5.2. Antibacterial assays of PD4 against *S. epidermidis* 1457 wild-type and *S. epidermidis* 1457 Δ*graS* and Δ*sepA* mutants. Black bars represented cultures treated with PBS (negative control) while grey bars showed cultures treated with PD4. Results were presented as avarage ±SD of three separate experiments with two replicates per repetition. * represents a significant difference in viable counts between cultures treated with PD4 versus the negative control ($p < 0.05$).

5.6 DISCUSSION

In this study, we demonstrated that coagulase-negative staphylococci displayed resistance to the platelet-derived AMP PD4. It has previously shown that PD4 displayed antibacterial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *B. cereus*, but not *S. epidermidis* (Mohan et al., 2014). Blood centers worldwide have reported coagulase-negative staphylococci are the predominant aerobic PC contaminants with *S. epidermidis* being the species most frequently isolated (Müller et al., 2015; Girgis et al., 2014; Zhu et al., 2009; Kuenhnert et al., 2001). Even though platelets release AMPs during PC storage (Yeaman, 2014; Tang, 2002), *S. epidermidis,* and other PC contaminant bacteria are able to proliferate (Taha et al., 2014; Greco-Stewart et al., 2012) and therefore are resistant to the bactericidal action of AMP by unknown mechanisms.

Previously, we demonstrated PC storage enhances *S. epidermidis* aggregation and production of a self-matrix known as a biofilm (Ali et al., 2014; Hodgson et al., 2014), which exhibit resistance to synthetic AMPs (Alabdullatif et al., 2018). Herein, we demonstrate that *graS* is involved in *S. epidermidis* resistance to platelet-derived AMP. Cameron and colleagues demonstrated that the *graRS* gene is present in both *S. capitis* and *S. epidermidis* (Cameron et al., 2015), which could explain the resistance by the coagulase-negative staphylococci.

Our results demonstrated that *K. pneumoniae, E. coli, S. marcescens*, and *S. aureus* cultures were reduced but not eliminated when treated with PD4. Gram-negative bacteria such as *S. marcescens* use the PhoPQ two component regulatory system which reduces the net negative charge of lipopolysaccharide and thus decreases the attraction of AMPs to cell membrane (Lin et al., 2014). Meanwhile, Gram-positive bacteria such as *S. aureus* use GraRS to increase the positive charge of cell wall and cell membrane to avoid attraction of AMPs (Li et al., 2007b). In addition, Gram-negative and Gram-positive bacteria produce enzymes to degrade AMPs such as OmpT protease in *E. coli* and aureolysin protease in *S. aureus* (Band and Weiss, 2015; Lai et al., 2007). Taken together, these examples of resistance mechanisms could explain the reduction, but not the elimination of PC contaminants including *K. pneumoniae, E. coli, S. marcescens, S. aureus* (Ramirez-Arcos et al., 2017; Mark and Shauna; 2005) when treated with PD4.

The GraRS system is found in both *S. aureus* and *S. epidermidis* but the extracellular loop that detects AMPs in *S. aureus* GraS is different from the one found in *S. epidermidis*. As documented by Li and coworkers, a serine residue is present in the extracellular loop of *S. epidermidis*, instead of the proline residue found in *S. aureus*. The presence of a proline causes a bend in the extracellular loop of *S. aureus* GraS and this results in AMP selectivity. For instance, the skin AMP HBD-3 is not recognized by *S. aureus* GraRS, whereas HBD-3 is recognized by *S. epidermidis* GraRS system. As a result, *S. epidermidis* exhibits more resistance to HBD-3 compared to *S. aureus* (Li et al., 2007a; Li et al., 2007b). Similar results were found in our study where *S. epidermidis* displayed more resistance toward PD4 compared to *S. aureus*, however, further investigation is required to determine if the difference between the GraS extracellular loop between the two bacteria could play a role in PD4 resistance. Furthermore, *S. epidermidis graS* and *sepA* mutants had significantly decreased ability to survive after phagocytic interaction with human neutrophils compared to the wild-type strain (Cheung et al., 2010). These observations could be due to reduced resistance against AMPs produced by neutrophils. In order to understand whether a similar pattern is observed during PC storage, AMPs produced from platelets (from PCs) could be induced by using adenosine diphosphate (ADP) as demonstrated by Różalski and coworkers (Różalski et al., 2013). To our knowledge, this is the first report to date studying *S. epidermidis* resistance mechanism toward a synthetic platelet AMP, and a further study merits investigation.

CHAPTER 6.

Conclusions and Future Directions

Bacterial contamination of PCs represents the highest post-transfusion infectious risk (Hong et al., 2016; Ramirez-Arcos and Goldman, 2012). The skin flora bacterium *S. epidermidis* is implicated as the predominant aerobic PC contaminant (Ramirez-Arcos et al., 2017; Müller et al., 2015; Dickson and Dinesh, 2013; Zhu et al., 2009; Jacobs et al., 2001). Furthermore, severe *S. epidermidis*-associated transfusion reactions have been reported (Ramirez-Arcos et al., 2017; Food and Drug Administration, 2016; Kou et al., 2015; De Korte et al., 2014). At Canadian Blood Services, several strategies have been implemented to reduce and/or prevent bacterial contamination of PC products including donor skin disinfection, diversion of the first aliquot of donated blood, and PC screening for bacterial contamination (Ramirez-Arcos and Goldman, 2010; Jenkins et al., 2011; Ramirez-Arcos and Goldman, 2012). The ability of *S. epidermidis* to resist donor skin disinfection and escape detection during PC screening has been linked to its ability to form biofilms (Taha et al., 2014; Ali et al., 2014; Greco-Stewart et al., 2012). The Ramirez group has shown that *S. epidermidis* can form biofilms and can outcompete other coagulase-negative staphylococci in PCs (Hodgson et al., 2014; Ali et al., 2014; Taha et al., 2014). The work in this thesis was designed to understand the resistance mechanisms that provide the proliferative advantage exhibited by *S. epidermidis* in the PC environment, and proposed methods to enhance PC safety. The most important findings of this thesis are summarized in Table 6.1.

The implementation of the combination of chlorhexidine-gluconate and isopropyl alcohol for blood donor skin disinfection has resulted in the reduction of PC contamination in several countries (Ramirez-Arcos and Goldman, 2010; Benjamin et al., 2011; McDonald et al., 2010); however, the risk of transfusing contaminated PCs has not been eliminated. Bacteria are found in the deeper layers of the skin within hair follicles (Karpanen et al., 2008b; Selwyn and Ellis, 1972), and they can exist as biofilms in between epithelial cells (Costerton, 2007). Consequently, effective skin disinfection poses a challenge and is compounded by the demonstrated resistance of biofilms to skin disinfectants (Taha et al., 2014).

Essential oils (EOs) have been studied by various groups for their antibacterial properties, and they have been shown to increase the activity of commonly used skin disinfectants by enhancing their penetration into the skin and their anti biofilm activity (Bassolé et al., 2012; Karpanen et al., 2010; Karpanen et al., 2008a; Bassolé et al., 2010; Hendry et al., 2009). In this work, it has been demonstrated that EOs derived from the plant *L. multifida* and its main component linalool, an enhancer of transdermal penetration (Herman and Herman, 2015; Jäger et al., 1992), can greatly improve the anti-biofilm activity of chlorhexidine-gluconate and isopropyl alcohol. This work highlights the potential of using EOs to enhance the potency of currently used donor skin disinfectants. However, further studies aimed at investigating the allergenic potential of *L. multifida* or linalool in combination with chlorhexidine-gluconate and isopropyl alcohol, as well as the penetrative power of the same, merit consideration.

It has been documented that the formation of biofilms in PCs results in increased pathogenicity and is linked to missed detection during PC screening (Hodgson et al., 2014; Ali et al., 2014; Greco-Stewart et al., 2012). AMPs have recently gained attraction as a potential bactericidal agent due to their ability to eradicate planktonic bacterial cells in laboratory media (Mohan et al., 2014). This work showed that the combination of three synthetic AMPs (PD4-RW3-RW4) eliminated *S. epidermidis* planktonic cells in PCs and inhibited biofilm formation. Furthermore, it has been reported that these individual AMPs do not elicit an immune response or affect platelet quality or function in animal models (Bosch-Marcé et al., 2014a; Bosch-Marcè et al., 2014b). Therefore, this combination of AMPs could potentially be incorporated with anticoagulant solutions or could be used to coat PC storage bags to inhibit bacterial biofilm formation. However, further investigation would be required to evaluate the effectiveness of the combination of AMPs against a broader range of PC contaminants, and its effects on platelet quality and function.

The Ramirez group has shown that *S. epidermidis* outcompetes *S. capitis* when grown in PCs (Taha et al., 2014). This work demonstrated that *S. epidermidis aap* plays a role in enhancing biofilm formation thereby conferring a proliferative advantage to this bacterium in PCs. The enhanced ability of *S. epidermidis* to form biofilms in the PC milleu could result in increased resistance to PC-derived AMPs as was previously

demonstrated with a combination of synthetic AMPs (Alabdullatif et al., 2018). Enhanced biofilm formation could also account for the missed detection of *S. epidermidis* during routine screening (Ali et al., 2014; Greco-Stewart et al., 2012). It would be interesting to expand these experiments to include other coagulase-negative staphylococci and to investigate the molecular mechanism of action of Aap during staphylococcal proliferation in PCs.

The predominance of *S. epidermidis* as a skin flora bacterium has been related to its ability to resist the bactericidal action of AMPs present on the skin (Li et al., 2007a; Lai et al., 2007). Whether the *graS* or *sepA* contributed to the resistance to platelet-derived AMP thus leading to the predominance of *S. epidermidis* in PCs were investigated in this thesis. This study demonstrated that *graS* is involved in the resistance exhibited by *S. epidermidis* to platelet derived AMP, however since complete elimination was not observed in the *graS* knockout mutant, it is evident that other mechanisms are involved in the AMP resistance displayed by *S. epidermidis.* Therefore, investigation of the molecular mechanisms involved in potential synergy with other resistance systems merits further attention.

The studies described in this thesis were aimed at improving the safety of blood products, specifically PCs. Results indicate that the current donor skin disinfecatants could potentially be enhanced with naturally derived essential oils. Results in this thesis also highlighted the potential use of synthetic AMPs to abrogate biofilm formation during PC storage. Both approaches could have a direct and meaningful impact on the safety of transfusion products by minimizing the contamination of blood collections and inhibiting biofilm formation of bacterial contaminants during PC storage. Furthermore, an understanding of the players and resistance mechanisms involved in conferring *S. epidermidis* an advantageous growth in PC milleu could help identify potential inhibition targets. Two players involved in conferring *S. epidermidis* an advantageous growth in PC milleu, namely *app* and *graS*. These studies provide a launching point for further investigations that could elucidate the finer details associated with *S. epidermidis* proliferation and resistance mechanisms, and therefore reveal potential inhibition targets. The studies decribed in this thesis have therefore enhanced our understanding

of various aspects associated with transfusion safety and has provided insights into means by which standard practice could be changed.

Table 6.1. Major findings in this thesis

Hypotheses: *S. epidermidis* biofilm resistance to the bactericidal action of blood donor skin disinfectants can be overcome by a synergistic action of currently used disinfectants and plant-extracted natural oils. Furthermore, I postulate that the biofilm-associated Aap protein and the expression of resistance mechanisms to AMPs confer an advantage to *S. epidermidis* for growth in PCs.				
Objectives	**Conclusion**	**Index**	**Future direction**	**Chapter (Reference)**
• Did the following essential oils (or its main components) synergistically enhance the anti-biofilm activity of chlorhexidine-gluconate: - *Artemisia herba-alba*? - *Lavandula multifida* (linalool)? - *Origanum marjoram*? - *Rosmarinus officinalis*? - *Thymus capitatus*?	No Yes (Yes) No No No	Table 2.2	*L. multifida* oil or linalool could potential be used to enhance the blood skin disinfectant currently used by Canadian Blood Services	Chapter 2 (Alabdullatif et al., 2017)
• Can the anti-biofilm activity of the blood donor skin disinfectants chlorhexidine-gluconate and isopropyl alcohol be significantly enhanced by *L. multifida* oil (or linalool)?	Yes (Yes)	Fig. 2.1		

• Did *S. epidermidis aap* enhance biofilm formation of staphylococci strains in: - TSBg? - PCs? • Did *S. epidermidis aap* enhance biofilm formation and confer an advantageous growth in PCs with: - *S. epidermidis*? - *S. capitis*?	No Yes Yes Yes	Fig. 3.2 Fig. 3.3	The molecular mechanisms of action of Aap, and other proteins involved in biofilm formation in PCs merit further investigation	Chapter 3
• Did the combination of three synthetic AMPs (PD4-RW3-RW4) inhibit *S. epidermidis* biofilm formation? • Did the combination of AMPs decrease bacterial concentration of preformed *S. epidermidis* biofilm when they were: - Not mechanically dislodged? - Mechanically dislodged?	Yes No Yes	Fig. 4.2 Fig.4.3 Fig. 4.4	The combination of AMPs can be used to enhance PC safety, and further investigation is needed to improve their activity against preformed *S. epidermidis* biofilms	Chapter 4 (Alabdullatif et al., 2018)
• Did *S. epidermidis* display resistance to platelet-derived AMPs (PD4) involving: - *graS*? - *sepA*?	Yes No	Fig. 5.2	Other mechanisms of *S. epidermidis* resistance toward platelet AMPs merit further investigation	Chapter 5

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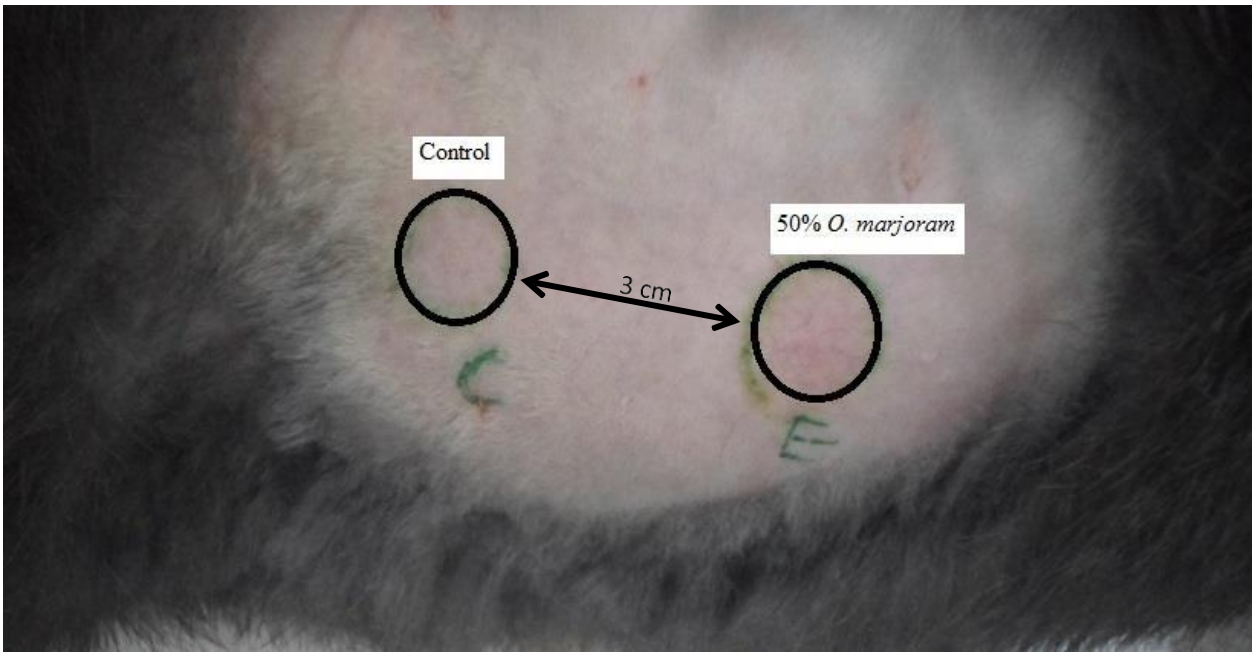
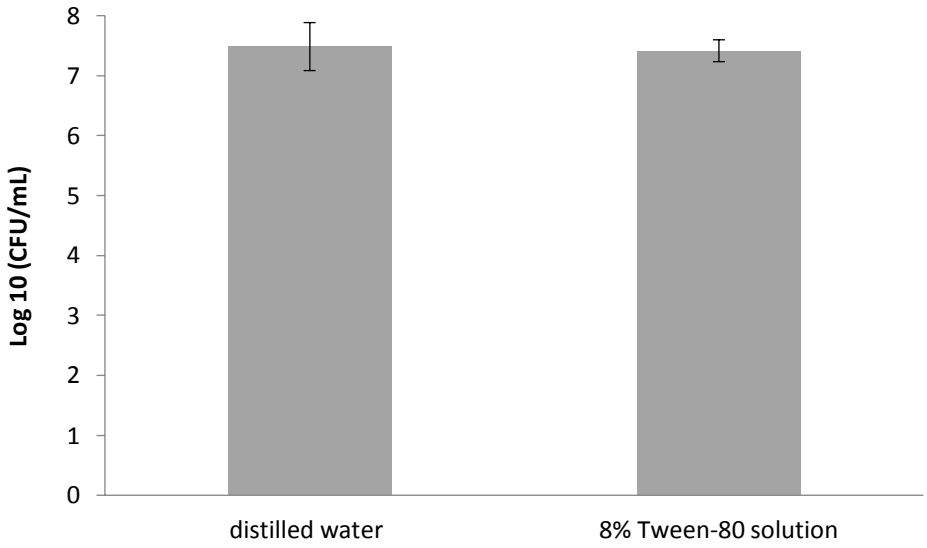
APPENDICES



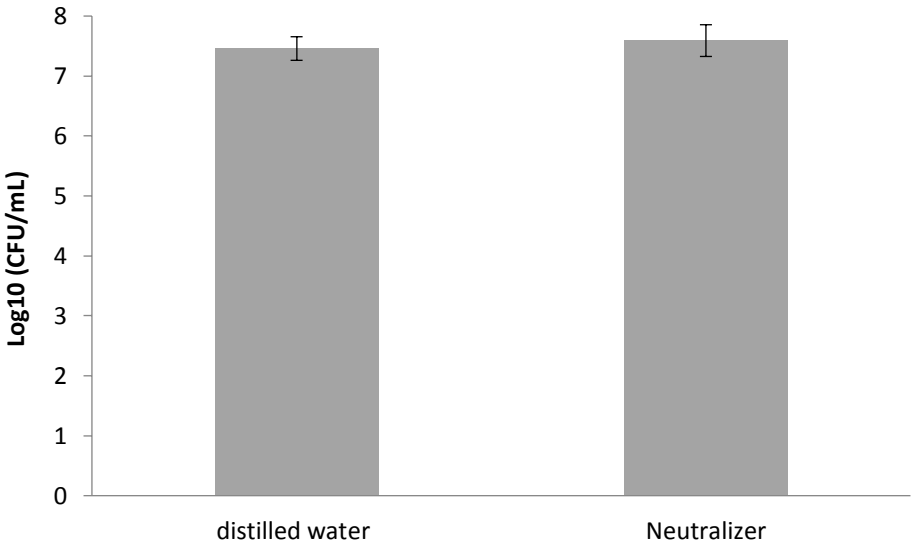
Appendix A.1. Rabbit skin irritation assay showing non-irritant control where 10 µL of the Tween-80 solution was applied while the other spot served as the test site where 10 µL of *O. marjoram* (50%) in Tween-80 solution was applied showing irritation after 10 minutes.

Appendix A.2. Oxygenated terpenoid compounds for each essential oil

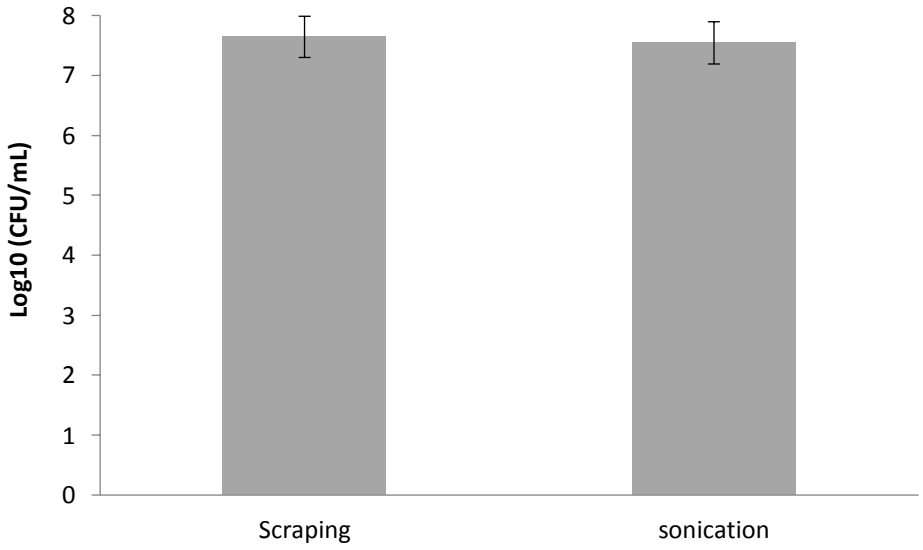
Oxygenated terpenoid compounds	*Artemisia herba-alba*	*Lavandula multifida*	*Rosmarinus officinalis*	*Origanum marjoram*	*Thymus capitatus*
1-octen-3-ol					1.491%
4-terpineol		14.769%	6.506%	8.785%	
Alpha terpineol		1.532%		7.174%	0.657%
Alpha-thujone	8.476%				
Beta-thujone	4.688%				
Borneol	4.835%		13.671%		5.305%
Camphor	22.724%	1.958%	25.038%		
Carvacrol		16.418%			31.619%
Caryophyllene oxide				1.304%	3.820%
Cis-sabinene hydrate				8.002%	0.836%
Endo-brneol		0.679%			
Eucalyptol	3.980%	3.023%	9.372%		
Eugenol					1.530%
Linalool		45.093%			
P-Cymen-8-ol		0.702%			
Thymol					35.923%
Terpinene-1-ol	1.741%			1.187%	
Pinocarvone	3.427%				
Linalyl acetate				3.416%	
Spathulenol				1.922%	
Total percent of oxygenated terpenoids	49.871%	84.174%	54.587%	31.79%	81.181%



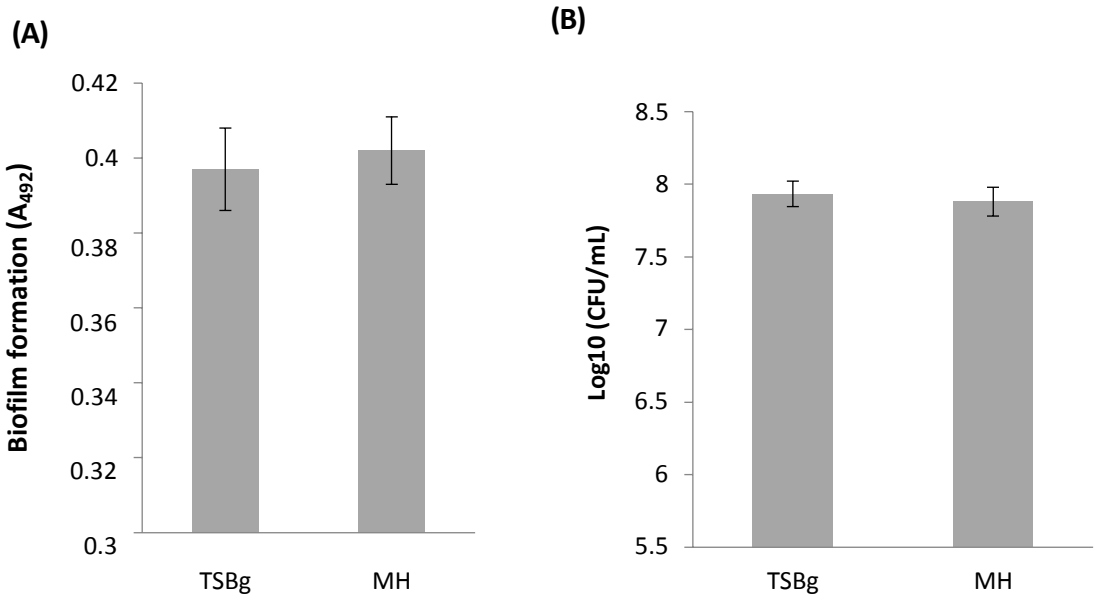
Appendix A.3. There is no significant difference ($p > 0.05$) in viable counts between the 8% Tween-80 solution (control) and distilled water when exposed to preformed *S. epidermidis* 9142 biofilms for 30 seconds. Results are presented as mean ±SD of three separate experiments with two replicates per experiment. Statistical analyses were performed using two-tailed paired t test and p value less than 0.05 was considered significant.



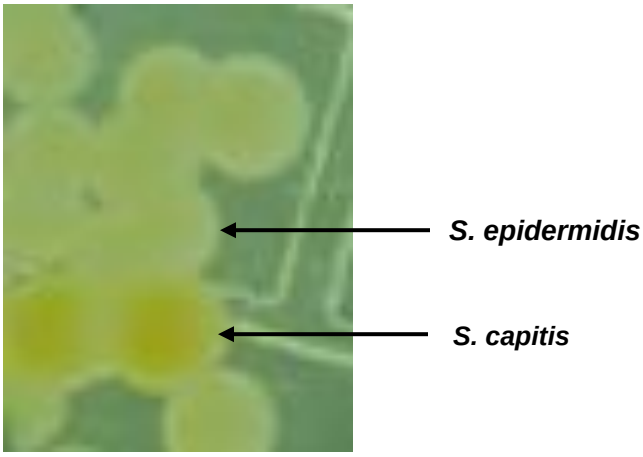
Appendix A.4. The toxicity of neutralizer was compared with distilled water when exposed to preformed *S. epidermidis* 9142 biofilms for 5 minutes. Results are presented as mean ±SD of three separate experiments with two replicates per experiment.



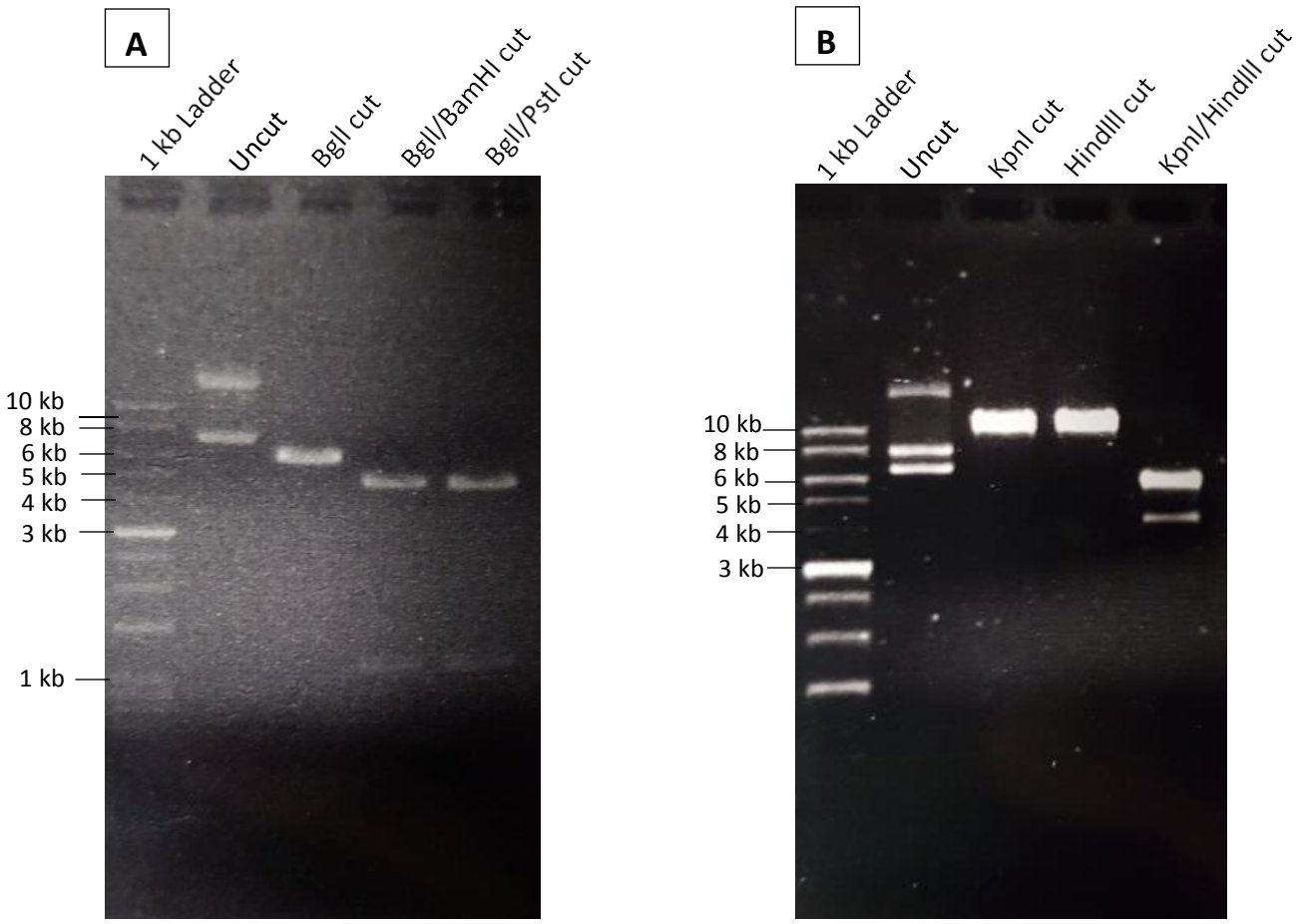
Appendix A.5. There is no significant difference ($p > 0.05$) in viable counts between scraping or sonication (at 60Hz for 30 minutes using an ultrasonic cleaner) methods to disrupt preformed *S. epidermidis* 9142 biofilms. Results are presented as mean ±SD of three separate experiments with two replicates per experiment. Statistical analyses were performed using two-tailed paired t test and p value less than 0.05 was considered significant.



Appendix A.6. Comparing *S. epidermidis* 9142 biofilm formation in TSBg and MH was evaluated using crystal violet assay (a) and viable count (b). Results are presented as mean ±SD of three separate experiments with two replicates per experiment.



Appendix B.1. *S. epidermidis* 1457 produces white colonies while *S. capitis* 517 grows forming yellow colonies on TSA plate.



Appendix B.2. Restriction digests of pRB474 (A) and pRB*aap* (B) in *E. coli* DC10B were separated at 100V for 75 min in 0.75% agarose gel. Gels were visualized under UV light using ethidium brodmide (5 µL-0.625mg/mL).

Appendix B.3. The *aap* nucleotide sequence of *S. epidermidis* 1457 (GenBank accession number KJ920749)

ATGGGCAAACGTAGACAAGGTCCTATTAATAAAAAAGTGGATTTTTTACCTAACAAATTAAACAAGTATT
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Appendix B.4. The *pls* nucleotide sequence of *S. capitis* AYP1020 (GeneBank accession number CP007601.1)

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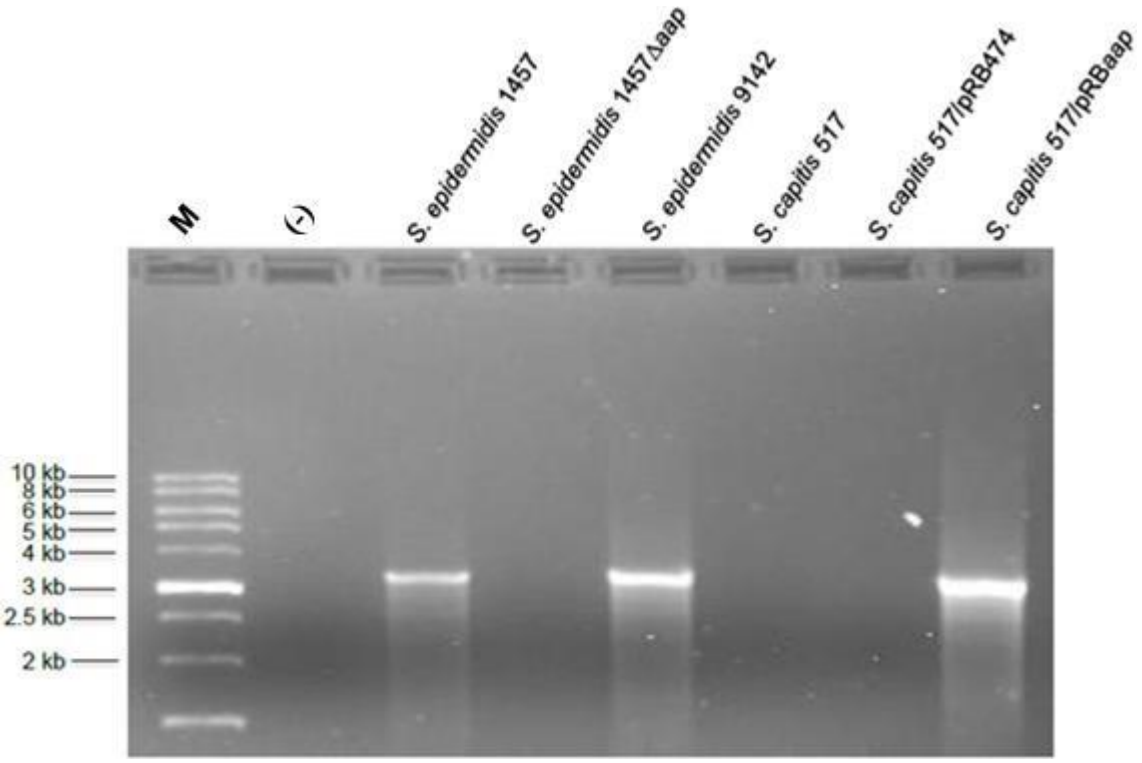
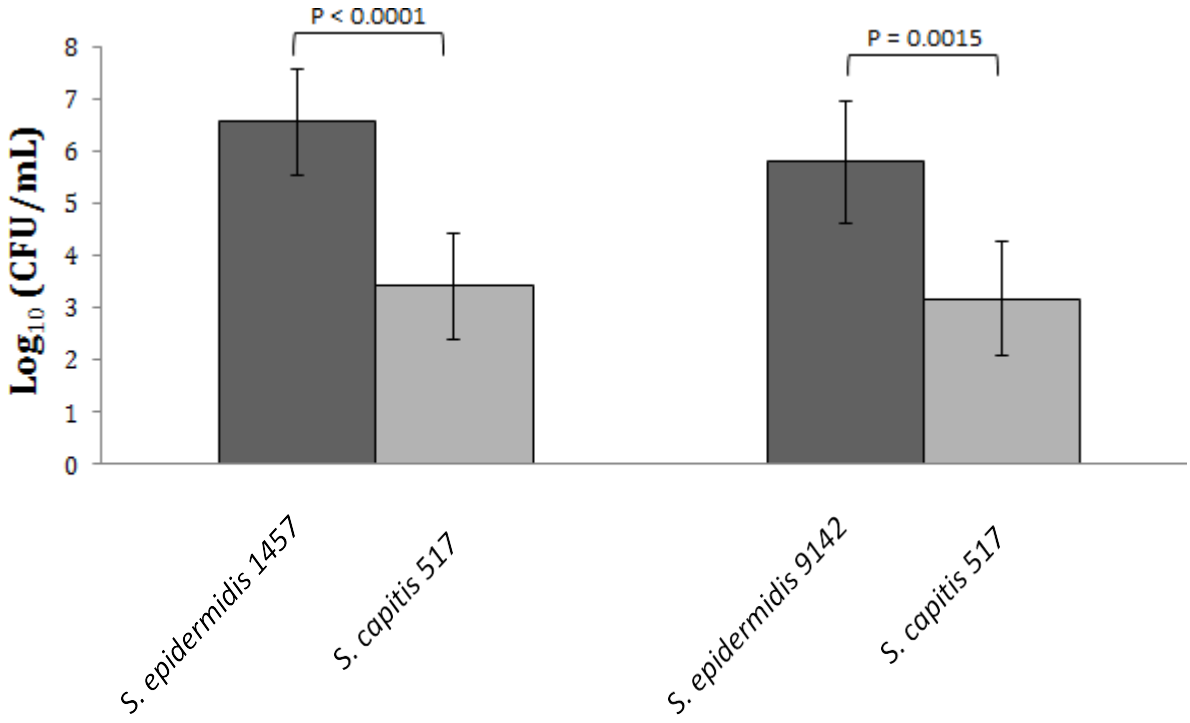
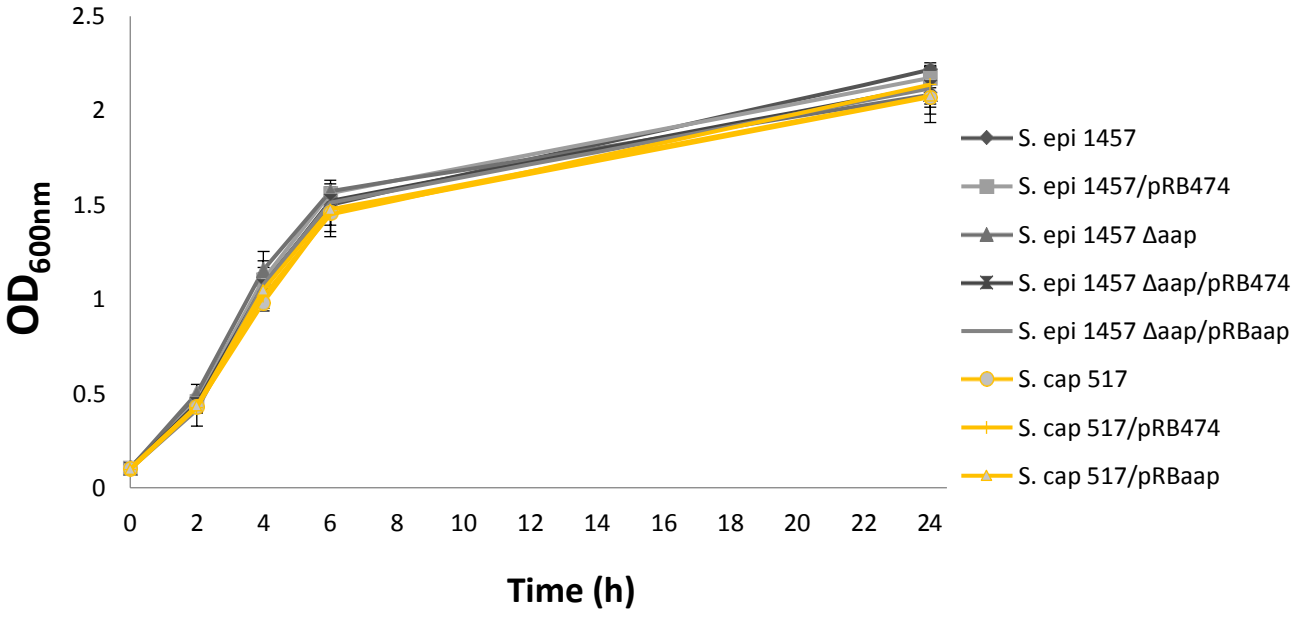


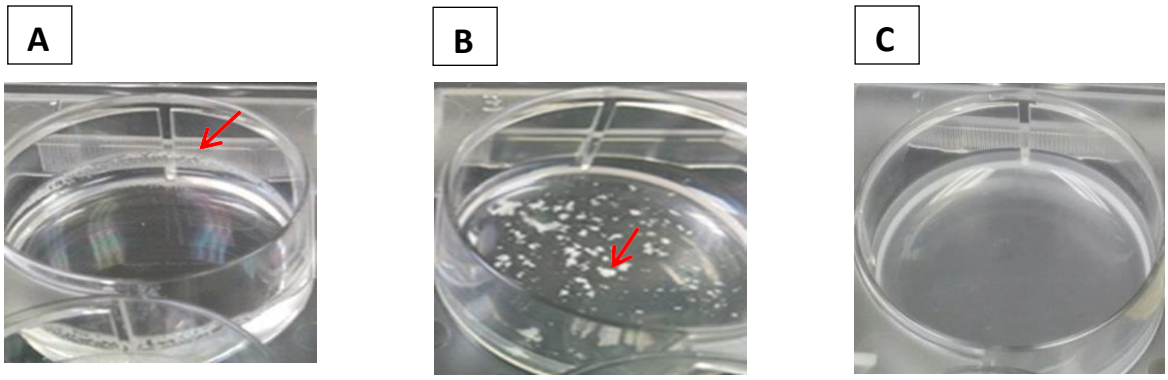
Appendix B.5. PCR detection of *S. epidermidis aap*. M = molecular weight marker (1 KB Plus DNA ladder; Smobio Excelband); (-) = no DNA template (Negative control). The expected size of the DNA fragment containing *aap* is 3,209 bp.



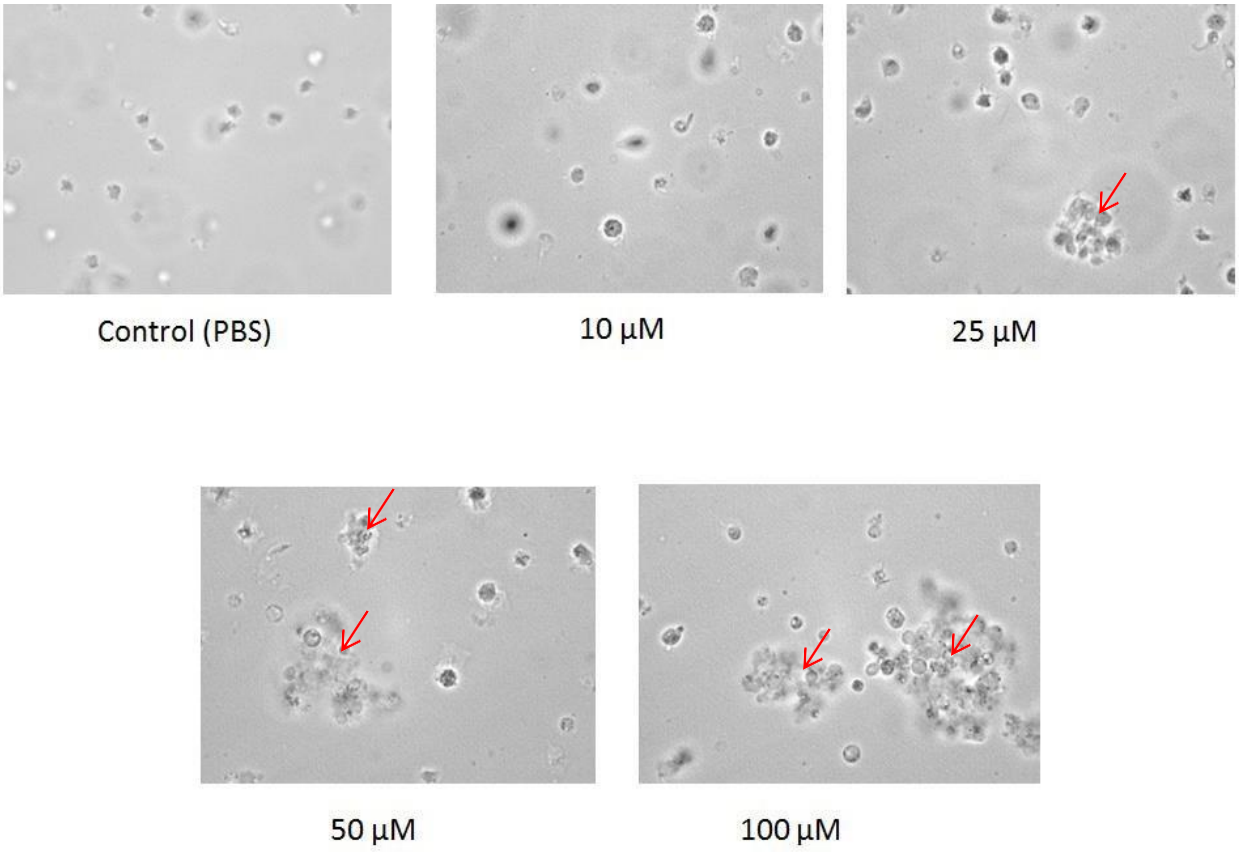
Appendix B.6. Competition assays of *S. epidermidis* and *S. capitis* strains in PCs. Paired cultures were seeded at a 1:1 ratio and viable counts were determined after 5 days of PC storage. Black bars represented *S. epidermidis* while grey bars represented *S. capitis*. Results are presented as mean ±SD of three separate experiments. Statistical analyses were performed using two-tailed paired t test.



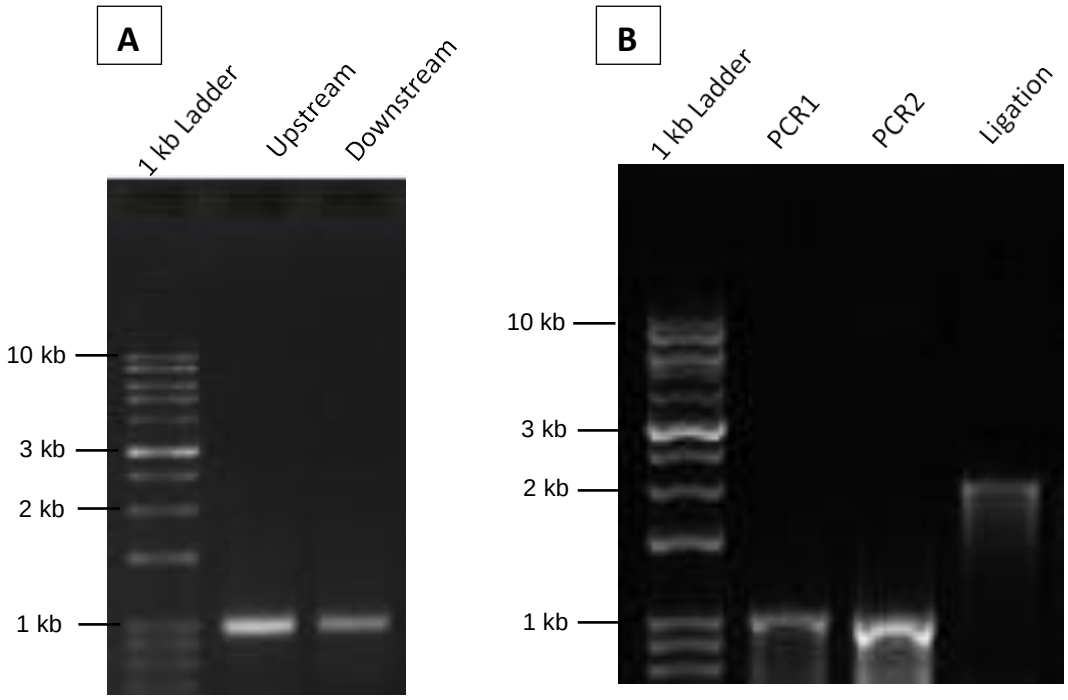
Appendix B.7. Growth curves of *S. epidermidis* and *S. capitis* strains in TSB media. Overnight cultures were adjusted to optical density (OD_{600nm}) = 0.1 in TSB, and then were incubated for 24 hours with a constant agitation at ~260 rpm at 37°C. Results are presented as mean ±SD of three separate experiments.



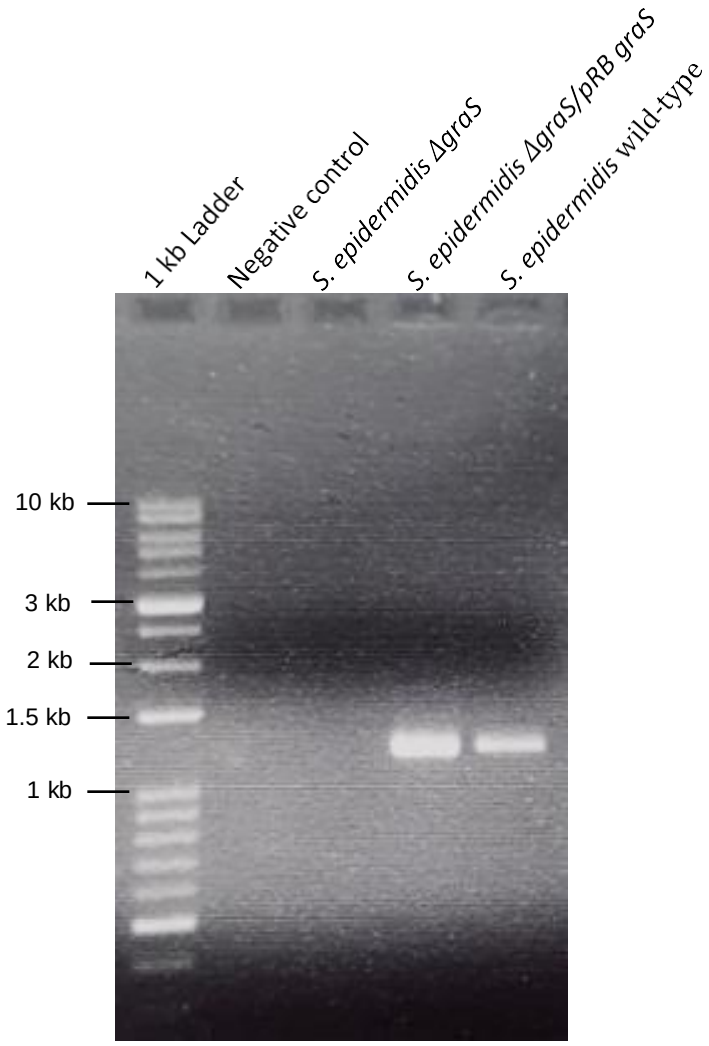
Appendix C.1. AMP treatment of preformed (mature) biofilm with no mechanical disruption (biofilm attached to cell wall; A), scraping (leaving visible aggregates; B), and scraped biofilms were homogenized by repeated pipetting and vigorous mixing (C).



Appendix C.2. AMPs combination (PD4-RW3-RW4) of 10 µM, 25 µM, 50 µM, and 100 µM concentrations effect on PCs after 5 days incubation using 100X oil-immersion microscope. Accumulation of platelets is noted with 25 µM, 50 µM, 100 µM concentrations as indicated in red arrows.



Appendix D.1. Construction of *sepA* deletion mutant of *S. epidermidis* 1457. Upstream and downstream amplicons of 965bp and 966 bp were generated by primers sets sepA2-for1/sepA2-rev1 and sepA2-for2/sepA2-rev2, respectively (A). The nested PCR was performed by amplifying the previous PCR products (965bp and 966 bp) with primer sets attB1/sepA2-rev1 and sepA2-for2/attB2 to get amplicons of 1006 bp (PCR1) and 1007 bp (PCR2), respectively (B). After digesting PCR1 and PCR2 with BamHI, the two amplicons were ligated using T4 DNA ligase (B).



Appendix D.2. The *graS* gene was PCR amplified using the primers SeapsSBam/SeapsSXba. The expected size of the DNA fragment containing *graS* is 1230 bp (~1.2 kb).

Appendix D.3. Sequencing pRB*graS* using SeapSxba primer

```
Go directly to Alignment
Multalin version 5.4.1
Copyright I.N.R.A. France 1989, 1991, 1994, 1996
Published research using this software should cite
Multiple sequence alignment with hierarchical clustering
F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890
Symbol comparison table: blosum62
Gap weight: 12
Gap length weight: 2
Consensus levels: high=90% low=50%
Consensus symbols:
 ! is anyone of IV
 $ is anyone of LM
 % is anyone of FY
 # is anyone of NDQEBZ

 MSF:   1230    Check:   0        ..
 Name: S.epidermidis  Len:  1230  Check: 7950  Weight:  1.00
 Name: Sequance       Len:  1230  Check: 5351  Weight:  1.00
 Name: Consensus      Len:  1230  Check: 3843  Weight:  0.00

//

           1                                                                                                                  130
S.epidermi CACTCCTGTC ATACTTCAAC CAAATTATCT TTTTACGAAG ACAAACTTAA TGTAGACTAT CATATCTTCT TTAATTTACA TCTTTTATTA TATCGATTAT TTATTACAAT TGTGATAATA GTAAGTAACA
  Sequance                                                    ACTTTAA TGTAGACTAT CATATCTTCT TTAATTTACA TCTTTTATTA TATCGATTAT TTATTACAAT TGTGATAATA GTAAGTAACA
 Consensus  .......... .......... .......... .......... ...AacTTAA TGTAGACTAT CATATCTTCT TTAATTTACA TCTTTTATTA TATCGATTAT TTATTACAAT TGTGATAATA GTAAGTAACA

           131                                                                                                                260
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           261                                                                                                                390
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 Consensus  TTGTTCTTTC ACGCTTTGTA CAAGGTATAA TCCCATACCA GAAGACGCAG TATCGTTGCG GTCTGTTGTA GAAGTAAATC CTCTATCAAA TATACGTGGT AAATCTCTTT TACTAATTCC ACGACCGTAG

           391                                                                                                                520
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 Consensus  TCTTTAATTT TTAAAACGAC GTGTCCTTCT ATGTTATAAC CACTTAAATT TATTGTAGAA TTATCACTAT ATTTCAAAGA GTTAGATAGA ACTTGCCTAA TCATCATACG GCACCATTTA ACATCTGTAT

           521                                                                                                                650
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  Sequance AAACCTTTTG TTCGTCTTTA AAATCTAATT CAAAACCTAT CCCTTTTGCC TGACTGATAT GTCGAGTAAC TTGTATTTCA TCTATAACCA TTCTCTTTAA TGAAATATAA TCAAAATACA TATCACGATG
 Consensus  AAACCTTTTG TTCGTCTTTA AAATCTAATT CAAAACCTAT CCCTTTTGCC TGACTGATAT GTCGAGTAAC TTGTATTTCA TCTATAACCA TTCTCTTTAA TGAAATATAA TCAAAATACA TATCACGATG

           651                                                                                                                780
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  Sequance TTGTGTTTCA AGCCTTGTTA AATATAATTG CTTATCTAAC ATCTCATTAA TACGAGACCA TTCAAATAAT AACGCTCGCT TACGCTGATC ATCATTCTCT TGATCTATCA ATAATTTCAT AGCCGTAACA
 Consensus  TTGTGTTTCA AGCCTTGTTA AATATAATTG CTTATCTAAC ATCTCATTAA TACGAGACCA TTCAAATAAT AACGCTCGCT TACGCTGATC ATCATTCTCT TGATCTATCA ATAATTTCAT AGCCGTAACA

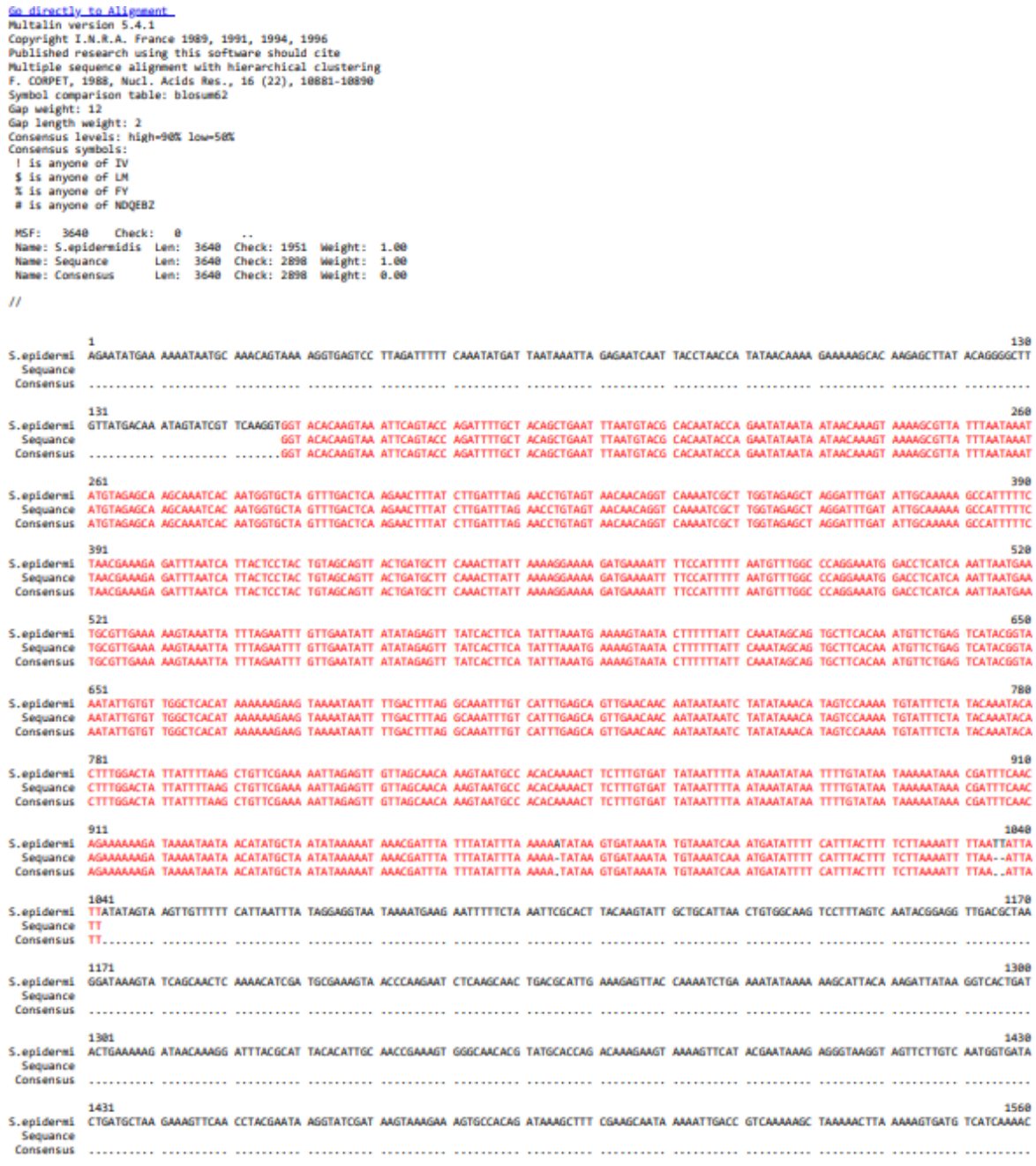
           781                                                                                                                910
S.epidermi GGTGTTTTTA TATCATGAAC AAACTCTGTA ATTGTTTGTT CATGATTTTT AATTTGTAAT TGTTGTTCGA CTACTTTCTC TTTTTGAGCG GCTATATGAC GATACAGATA ATCTATGACT TGTTGTTGAA
  Sequance GGTGTTTTTA TATCATGAAC AAACTCTGTA ATTGTTTGTT CATGATTTTT AATTTGTAAT TGTTGTTCGA CTACTTTCTC TTTTTGAGCG GCTATATGAC GATACAGATA ATCTATGACT TGTTGTTGAA
 Consensus  GGTGTTTTTA TATCATGAAC AAACTCTGTA ATTGTTTGTT CATGATTTTT AATTTGTAAT TGTTGTTCGA CTACTTTCTC TTTTTGAGCG GCTATATGAC GATACAGATA ATCTATGACT TGTTGTTGAA

           911                                                                                                               1040
S.epidermi ATGGGGTTTC TGCCAAATCT TTATGCTTAA TTTCTTCAAT TTCTTTATCT TCATAAAAAT GTTTAGATAG ACGGACTTCT TTAACAAACG TAAATAATAA GAAAAGTATA CTCAATCCTA CGTTTAATAT
  Sequance ATGGGGTTTC TGCCAAATCT TTATGCTTAA TTTCTTCAAT TTCTTTATCT TCATAAAAAT GTTTAGATAG ACGGACTTCT TTAACAAACG TAAATAATAA GAAAAGTATA CTCAATCCTA CGTTTAATAT
 Consensus  ATGGGGTTTC TGCCAAATCT TTATGCTTAA TTTCTTCAAT TTCTTTATCT TCATAAAAAT GTTTAGATAG ACGGACTTCT TTAACAAACG TAAATAATAA GAAAAGTATA CTCAATCCTA CGTTTAATAT

           1041                                                                                                              1170
S.epidermi CACTATATAA AATACACTTT CAACTGAAAT CTCATAATCT ATGTAGGCAA CACCTAATAA AATAATGTTG AGAAATAATA TCCACAATAT CCAATTAATA CGAGATTTTA TAAAAAACCA AAACCATCGA
  Sequance CACTATATAA AATACACTTT CAACTGAAAT CTCATAATCT ATGT-GGCAA CACCTAATAA AATAATGTTG AGAAATAATA TCCACAATAT CCAATTAATA CGAGATTTTA TAAAAA-CCA AAACCATCGA
 Consensus  CACTATATAA AATACACTTT CAACTGAAAT CTCATAATCT ATGT.GGCAA CACCTAATAA AATAATGTTG AGAAATAATA TCCACAATAT CCAATTAATA CGAGATTTTA TAAAAA.CCA AAACCATCGA

           1171                                                  1230
S.epidermi AAATTATTCA TGAGCTAAGT ATCCTTTACC AACTTTGGTT TCAATTGCAC TATCCATGTC
  Sequance AAATTATTCA TGAGCTAAGT ATCCTTTACC AACTTTGGAT CCCGGGGGTA C
 Consensus  AAATTATTCA TGAGCTAAGT ATCCTTTACC AACTTTGGaT cCaaggGcaa c.........
```

Appendix D.4. Sequencing *S. epidermidis* Δ*sepA* using SepA2-for1 primer



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Go directly to Alignment
Multalin version 5.4.1
Copyright I.N.R.A. France 1989, 1991, 1994, 1996
Published research using this software should cite
Multiple sequence alignment with hierarchical clustering
F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890
Symbol comparison table: blosum62
Gap weight: 12
Gap length weight: 2
Consensus levels: high=90% low=50%
Consensus symbols:
 ! is anyone of IV
 $ is anyone of LM
 % is anyone of FY
 # is anyone of NDQEBZ

 MSF:   3640    Check:   0        ..
 Name: S.epidermidis  Len:  3640  Check: 1951  Weight:  1.00
 Name: Sequance       Len:  3640  Check: 2898  Weight:  1.00
 Name: Consensus      Len:  3640  Check: 2898  Weight:  0.00

//

          1                                                                                                                  130
S.epidermi AGAATATGAA AAAATAATGC AAACAGTAAA AGGTGAGTCC TTAGATTTTT CAAATATGAT TAATAAATTA GAGAATCAAT TACCTAACCA TATAACAAAA GAAAAAGCAC AAGAGCTTAT ACAGGGGCTT
  Sequance
 Consensus .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          131                                                                                                                260
S.epidermi GTTATGACAA ATAGTATCGT TCAAGGTGGT ACACAAGTAA ATTCAGTACC AGATTTTGCT ACAGCTGAAT TTAATGTACG CACAATACCA GAATATAATA ATAACAAAGT AAAAGCGTTA TTTAATAAAT
  Sequance                        GGT ACACAAGTAA ATTCAGTACC AGATTTTGCT ACAGCTGAAT TTAATGTACG CACAATACCA GAATATAATA ATAACAAAGT AAAAGCGTTA TTTAATAAAT
 Consensus .......... .......... .......GGT ACACAAGTAA ATTCAGTACC AGATTTTGCT ACAGCTGAAT TTAATGTACG CACAATACCA GAATATAATA ATAACAAAGT AAAAGCGTTA TTTAATAAAT

          261                                                                                                                390
S.epidermi ATGTAGAGCA AGCAAATCAC AATGGTGCTA GTTTGACTCA AGAACTTTAT CTTGATTTAG AACCTGTAGT AACAACAGGT CAAAATCGCT TGGTAGAGCT AGGATTTGAT ATTGCAAAAA GCCATTTTTC
  Sequance ATGTAGAGCA AGCAAATCAC AATGGTGCTA GTTTGACTCA AGAACTTTAT CTTGATTTAG AACCTGTAGT AACAACAGGT CAAAATCGCT TGGTAGAGCT AGGATTTGAT ATTGCAAAAA GCCATTTTTC
 Consensus ATGTAGAGCA AGCAAATCAC AATGGTGCTA GTTTGACTCA AGAACTTTAT CTTGATTTAG AACCTGTAGT AACAACAGGT CAAAATCGCT TGGTAGAGCT AGGATTTGAT ATTGCAAAAA GCCATTTTTC

          391                                                                                                                520
S.epidermi TAACGAAAGA GATTTAATCA TTACTCCTAC TGTAGCAGTT ACTGATGCTT CAAACTTATT AAAAGGAAAA GATGAAAATT TTCCATTTTT AATGTTTGGC CCAGGAAATG GACCTCATCA AATTAATGAA
  Sequance TAACGAAAGA GATTTAATCA TTACTCCTAC TGTAGCAGTT ACTGATGCTT CAAACTTATT AAAAGGAAAA GATGAAAATT TTCCATTTTT AATGTTTGGC CCAGGAAATG GACCTCATCA AATTAATGAA
 Consensus TAACGAAAGA GATTTAATCA TTACTCCTAC TGTAGCAGTT ACTGATGCTT CAAACTTATT AAAAGGAAAA GATGAAAATT TTCCATTTTT AATGTTTGGC CCAGGAAATG GACCTCATCA AATTAATGAA

          521                                                                                                                650
S.epidermi TGCGTTGAAA AAGTAAATTA TTTAGAATTT GTTGAATATT ATATAGAGTT TATCACTTCA TATTTAAATG AAAAGTAATA CTTTTTTATT CAAATAGCAG TGCTTCACAA ATGTTCTGAG TCATACGGTA
  Sequance TGCGTTGAAA AAGTAAATTA TTTAGAATTT GTTGAATATT ATATAGAGTT TATCACTTCA TATTTAAATG AAAAGTAATA CTTTTTTATT CAAATAGCAG TGCTTCACAA ATGTTCTGAG TCATACGGTA
 Consensus TGCGTTGAAA AAGTAAATTA TTTAGAATTT GTTGAATATT ATATAGAGTT TATCACTTCA TATTTAAATG AAAAGTAATA CTTTTTTATT CAAATAGCAG TGCTTCACAA ATGTTCTGAG TCATACGGTA

          651                                                                                                                780
S.epidermi AATATTGTGT TGGCTCACAT AAAAAAGAAG TAAAATAATT TTGACTTTAG GCAAATTTGT CATTTGAGCA GTTGAACAAC AATAATAATC TATATAAACA TAGTCCAAAA TGTATTTCTA TACAAATACA
  Sequance AATATTGTGT TGGCTCACAT AAAAAAGAAG TAAAATAATT TTGACTTTAG GCAAATTTGT CATTTGAGCA GTTGAACAAC AATAATAATC TATATAAACA TAGTCCAAAA TGTATTTCTA TACAAATACA
 Consensus AATATTGTGT TGGCTCACAT AAAAAAGAAG TAAAATAATT TTGACTTTAG GCAAATTTGT CATTTGAGCA GTTGAACAAC AATAATAATC TATATAAACA TAGTCCAAAA TGTATTTCTA TACAAATACA

          781                                                                                                                910
S.epidermi CTTTGGACTA TTATTTTAAG CTGTTCGAAA AATTAGAGTT GTTAGCAACA AAGTAATGCC ACACAAAACT TCTTTGTGAT TATAATTTTA ATAAATATAA TTTTGTATAA TAAAAATAAA CGATTTCAAC
  Sequance CTTTGGACTA TTATTTTAAG CTGTTCGAAA AATTAGAGTT GTTAGCAACA AAGTAATGCC ACACAAAACT TCTTTGTGAT TATAATTTTA ATAAATATAA TTTTGTATAA TAAAAATAAA CGATTTCAAC
 Consensus CTTTGGACTA TTATTTTAAG CTGTTCGAAA AATTAGAGTT GTTAGCAACA AAGTAATGCC ACACAAAACT TCTTTGTGAT TATAATTTTA ATAAATATAA TTTTGTATAA TAAAAATAAA CGATTTCAAC

          911                                                                                                               1040
S.epidermi AGAAAAAAGA TAAAATAATA ACATATGCTA ATATAAAAAT AAACGATTTA TTTATATTTA AAAAATATAA GTGATAAATA TGTAAATCAA ATGATATTTT CATTTACTTT TCTTAAAATT TTAATTATTA
  Sequance AGAAAAAAGA TAAAATAATA ACATATGCTA ATATAAAAAT AAACGATTTA TTTATATTTA AAAA-TATAA GTGATAAATA TGTAAATCAA ATGATATTTT CATTTACTTT TCTTAAAATT TTAA--ATTA
 Consensus AGAAAAAAGA TAAAATAATA ACATATGCTA ATATAAAAAT AAACGATTTA TTTATATTTA AAAA.TATAA GTGATAAATA TGTAAATCAA ATGATATTTT CATTTACTTT TCTTAAAATT TTAA..ATTA

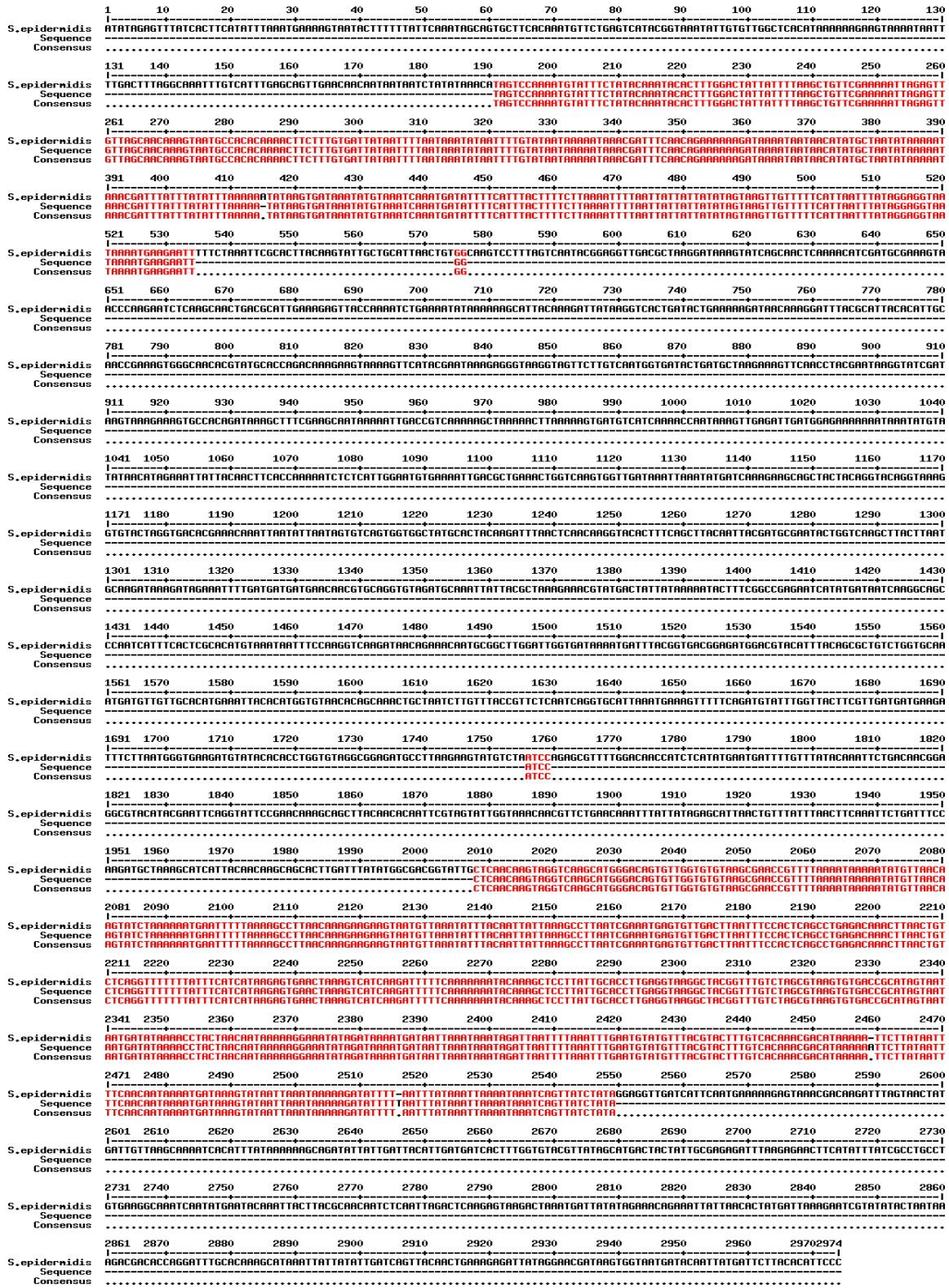
          1041                                                                                                              1170
S.epidermi TTATATAGTA AGTTGTTTTT CATTAATTTA TAGGAGGTAA TAAAATGAAG AATTTTTCTA AATTCGCACT TACAAGTATT GCTGCATTAA CTGTGGCAAG TCCTTTAGTC AATACGGAGG TTGACGCTAA
  Sequance TT
 Consensus TT........ .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          1171                                                                                                              1300
S.epidermi GGATAAAGTA TCAGCAACTC AAAACATCGA TGCGAAAGTA ACCCAAGAAT CTCAAGCAAC TGACGCATTG AAAGAGTTAC CAAAATCTGA AAATATAAAA AAGCATTACA AAGATTATAA GGTCACTGAT
  Sequance
 Consensus .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          1301                                                                                                              1430
S.epidermi ACTGAAAAAG ATAACAAAGG ATTTACGCAT TACACATTGC AACCGAAAGT GGGCAACACG TATGCACCAG ACAAAGAAGT AAAAGTTCAT ACGAATAAAG AGGGTAAGGT AGTTCTTGTC AATGGTGATA
  Sequance
 Consensus .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          1431                                                                                                              1560
S.epidermi CTGATGCTAA GAAAGTTCAA CCTACGAATA AGGTATCGAT AAGTAAAGAA AGTGCCACAG ATAAAGCTTT CGAAGCAATA AAAATTGACC GTCAAAAAGC TAAAAACTTA AAAAGTGATG TCATCAAAAC
  Sequance
 Consensus .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
```

Appendix D.5. Sequencing *S. epidermidis* Δ*sepA* using sepA-Fc primer



Appendix D.6. The *graS* nucleotide sequence of *S. epidermidis* 1457 (GeneBank accession number ARG67332.1)

TTAAAATGACAATCTTGTCACCTTAGACATGCGCTCAATGATTTCATTTTGTTGTGGGAAAATGAAATAA
AACGTTGTTCCTTTCCCCACTATTGAATCAACTTTAACTTCAATCCCAAGTTGTTCTTTCACGCTTTGTA
CAAGGTATAATCCCATACCAGAAGACGCAGTATCGTTGCGGTCTGTTGTAGAAGTAAATCCTCTATCAAA
TATACGTGGTAAATCTCTTTTACTAATTCCACGACCGTAGTCTTTAATTTTTAAAACGACGTGTCCTTCT
ATGTTATAACCACTTAAATTTATTGTAGAATTATCACTATATTTCAAAGAGTTAGATAGAACTTGCCTAA
TCATCATACGGCACCATTTAACATCTGTATAAACCTTTTGTTCGTCTTTAAAATCTAATTCAAAACCTAT
CCCTTTTGCCTGACTGATATGTCGAGTAACTTGTATTTCATCTATAACCATTCTCTTTAATGAAATATAA
TCAAAATACATATCACGATGTTGTGTTTCAAGCCTTGTTAAATATAATTGCTTATCTAACATCTCATTAA
TACGAGACCATTCAAATAATAACGCTCGCTTACGCTGATCATCATTCTCTTGATCTATCAATAATTTCAT
AGCCGTAACAGGTGTTTTTATATCATGAACAAACTCTGTAATTGTTTGTTCATGATTTTTAATTTGTAAT
TGTTGTTCGACTACTTTCTCTTTTTGAGCGGCTATATGACGATACAGATAATCTATGACTTGTTGTTGAA
ATGGGGTTTCTGCCAAATCTTTATGCTTAATTTCTTCAATTTCTTTATCTTCATAAAAATGTTTAGATAG
ACGGACTTCTTTAACAAACGTAAATAATAAGAAAAGTATACTCAATCCTACGTTTAATATCACTATATAA
AATACACTTTCAACTGAAATCTCATAATCTATGTAGGCAACACCTAATAAAATAATGTTGAGAAATAATA
TCCACAATATCCAATTAATACGAGATTTTATAAAAAACCAAAACCATCGAAAATTATTCAT

Appendix D.7. The *sepA* nucleotide sequence of *S. epidermidis* 1457 (GeneBank accession number ARG65652.1)

ATGAAGAATTTTTCTAAATTCGCACTTACAAGTATTGCTGCATTAACTGTGGCAAGTCCTTTAGTCAATA
CGGAGGTTGACGCTAAGGATAAAGTATCAGCAACTCAAAACATCGATGCGAAAGTAACCCAAGAATCTCA
AGCAACTGACGCATTGAAAGAGTTACCAAAATCTGAAAATATAAAAAAGCATTACAAAGATTATAAGGTC
ACTGATACTGAAAAAGATAACAAAGGATTTACGCATTACACATTGCAACCGAAAGTGGGCAACACGTATG
CACCAGACAAAGAAGTAAAAGTTCATACGAATAAAGAGGGTAAGGTAGTTCTTGTCAATGGTGATACTGA
TGCTAAGAAAGTTCAACCTACGAATAAGGTATCGATAAGTAAAGAAAGTGCCACAGATAAAGCTTTCGAA
GCAATAAAAATTGACCGTCAAAAAGCTAAAAACTTAAAAAGTGATGTCATCAAAACCAATAAAGTTGAGA
TTGATGGAGAAAAAAATAAATATGTATATAACATAGAAATTATTACAACTTCACCAAAAATCTCTCATTG
GAATGTGAAAATTGACGCTGAAACTGGTCAAGTGGTTGATAAATTAAATATGATCAAAGAAGCAGCTACT
ACAGGTACAGGTAAAGGTGTACTAGGTGACACGAAACAAATTAATATTAATAGTGTCAGTGGTGGCTATG
CACTACAAGATTTAACTCAACAAGGTACACTTTCAGCTTACAATTACGATGCGAATACTGGTCAAGCTTA
CTTAATGCAAGATAAAGATAGAAATTTTGATGATGATGAACAACGTGCAGGTGTAGATGCAAATTATTAC
GCTAAAGAAACGTATGACTATTATAAAAATACTTTCGGCCGAGAATCATATGATAATCAAGGCAGCCCAA
TCATTTCACTCGCACATGTAAATAATTTCCAAGGTCAAGATAACAGAAACAATGCGGCTTGGATTGGTGA
TAAAATGATTTACGGTGACGGAGATGGACGTACATTTACAGCGCTGTCTGGTGCAAATGATGTTGTTGCA
CATGAAATTACACATGGTGTAACACAGCAAACTGCTAATCTTGTTTACCGTTCTCAATCAGGTGCATTAA
ATGAAAGTTTTTCAGATGTATTTGGTTACTTCGTTGATGATGAAGATTTCTTAATGGGTGAAGATGTATA
CACACCTGGTGTAGGCGGAGATGCCTTAAGAAGTATGTCTAATCCAGAGCGTTTTGGACAACCATCTCAT
ATGAATGATTTTGTTTATACAAATTCTGACAACGGAGGCGTACATACGAATTCAGGTATTCCGAACAAAG
CAGCTTACAACACAATTCGTAGTATTGGTAAACAACGTTCTGAACAAATTTATTATAGAGCATTAACTGT
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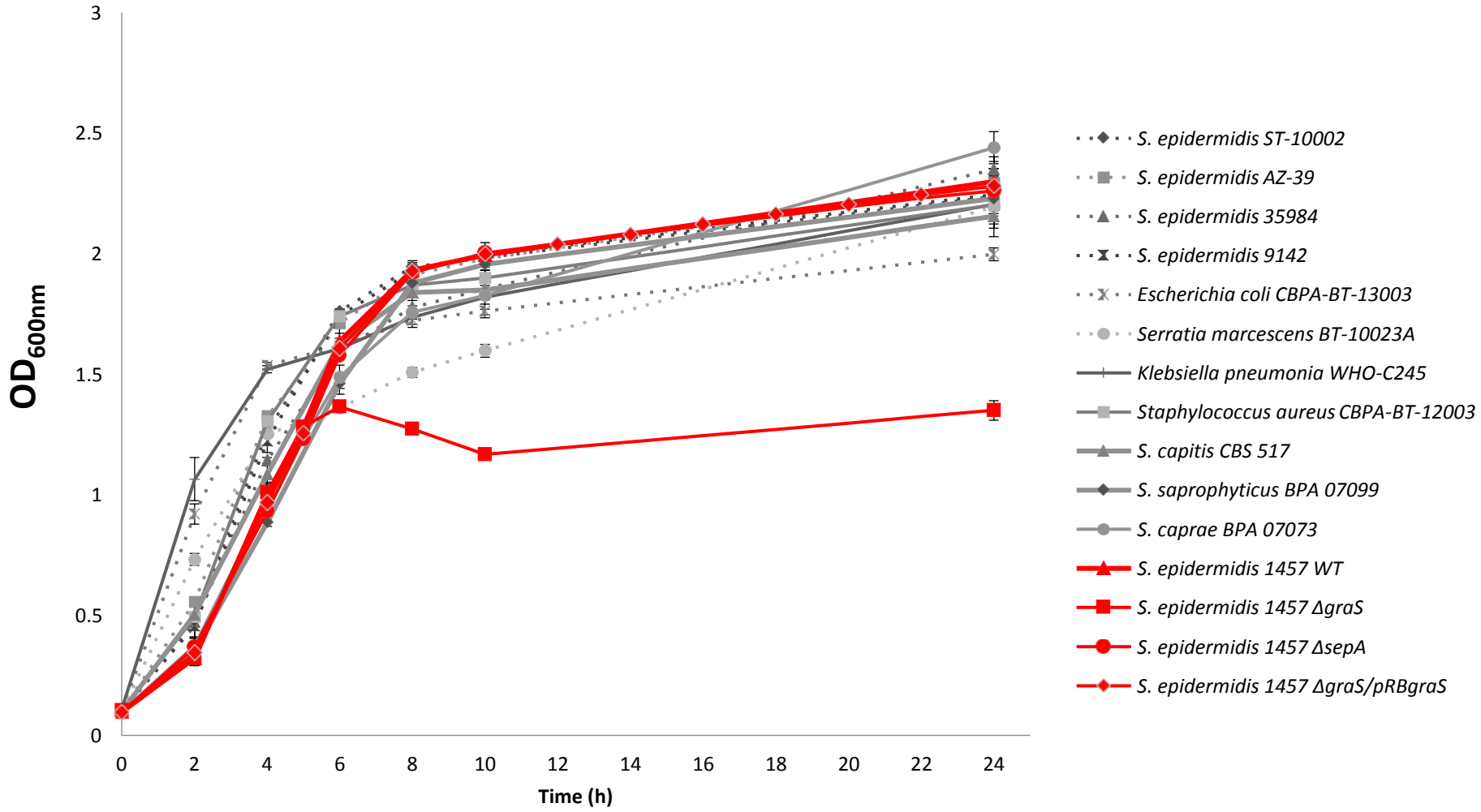
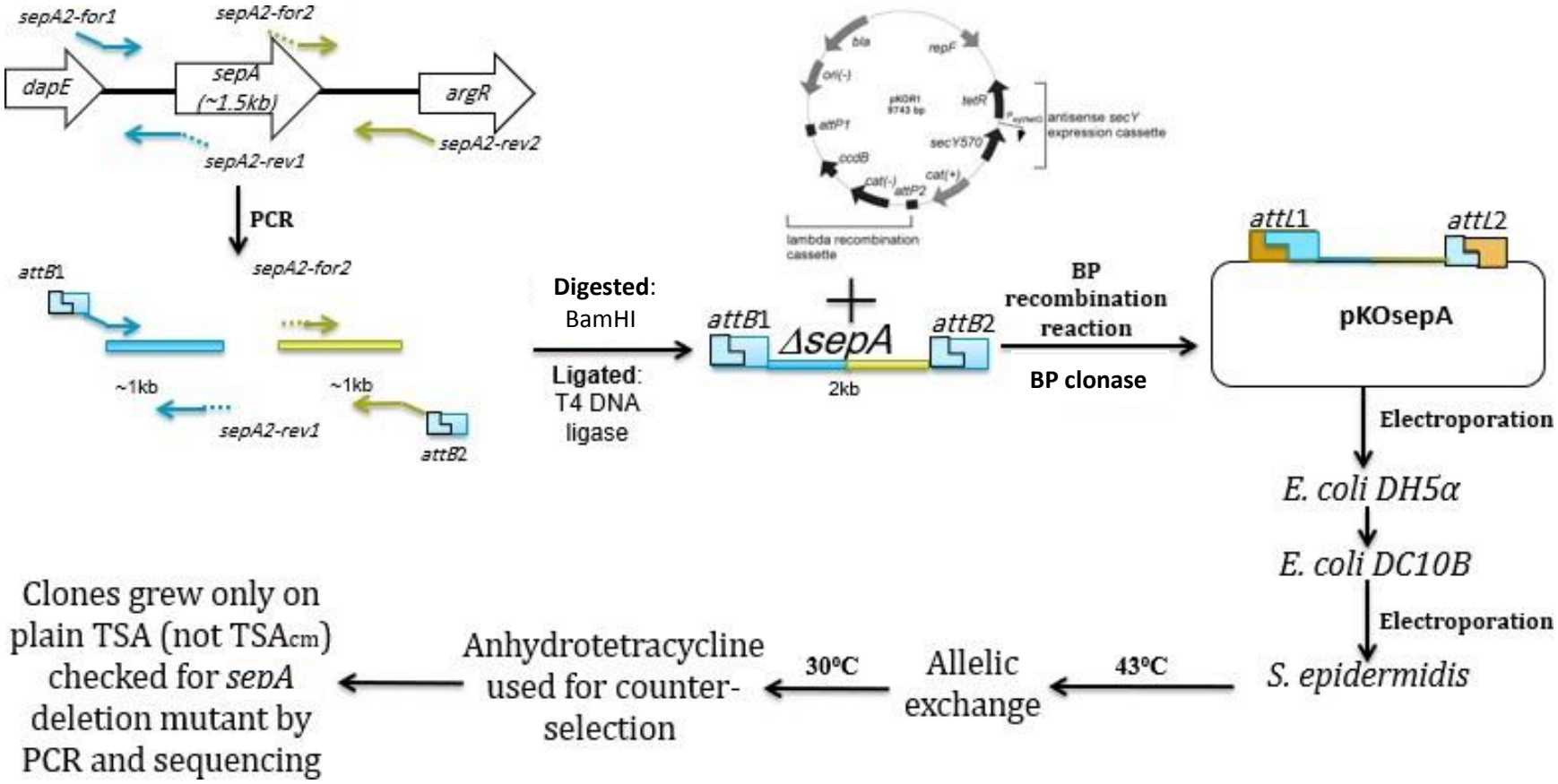


Appendix D.8. Growth curves of several bacterial strains in TSB media. Overnight cultures were adjusted to optical density (OD_{600nm}) = 0.1 in TSB, and were incubated for 24 hours with a constant agitation at ~260 rpm at 37ºC. Results are presented as mean ±SD of three separate experiments.



Appendix D.9. Construction of a *S. epidermidis sepA* deletion mutant