Bacterial Contamination of Platelet Concentrates: Role of Biofilm Formation and Manufacturing Process

Mariam Taha

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Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

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ABSTRACT

Bacterial contamination of platelet concentrates (PCs) poses the highest transfusion-associated infectious risk with skin flora, such as *Staphylococcus epidermidis* and *Staphylococcus capitis,* being the predominant contaminants. These bacteria are able to form surface-attached aggregates or biofilms, which are present in the skin of healthy blood donors and can subsequently be isolated from contaminated PCs.

Disinfection of the venipuncture area before donation with a combination of 2% chlorhexidine-gluconate and 70% isopropanol is used at Canadian Blood Services. However, not all bacteria are eliminated during skin disinfection since contaminated PCs are still captured during routine PC screening. In this thesis, the ability of biofilm-forming *S. epidermidis* and *S. capitis* to resist the currently used disinfectants was explored. It was demonstrated that although a combination of chlorhexidine and isopropanol has a bactericidal effect, it is unable to completely eradicate skin flora biofilms.

Several countries have implemented Pathogen Inactivation Technologies (PITs) as a measure to help control transfusing bacterially-contaminated PCs by exposing PC units to ultra violet light. However, no investigations have been done to evaluate the ability of PITs against bacterial biofilms, which was one of the objectives of this thesis. Data revealed that the efficacy of a currently used PIT, the Mirasol® system, is similar for *S. epidermidis* present in PCs produced from whole blood inoculated with biofilm or non-biofilm cells. However, treatment effectiveness was strain dependent. In conclusion, further investigation to improve donor skin disinfection and PITs should be considered.

Surveillance at Canadian Blood Services shows that contamination rates in single-donor apheresis PCs (Aph-PCs) is generally higher than in four-donor buffy coat platelet pools (BC-PCs). This study investigated whether the BC-PC production method contributes to this observation as BC-PCs are derived from WB that is left to rest overnight while Aph-PCs are collected directly from the donor. Data showed that WB hold during BC-PC production does not have a broad-spectrum bactericidal effect and therefore other factors contribute to low rates of contamination in BC-PCs. The work presented in this thesis provides an insight to bacterial residence and persistence during blood product manufacturing and makes suggestions for PC safety improvements.

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“The best person is the one who benefit all human beings”-Prophet Mohamad.

“I would rather have it said, ‘He lived useful’ than ‘He died rich’”-Benjamin Franklin.

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LIST OF ABBREVIATIONS

Aae: Accumulation/adhesin protein
Aap: Accumulation-associated protein
ACD-A: adenine, citrate dextrose-formula A
ADP: Adenosine diphosphate
Aph: Apheresis
Aph-PC: Apheresis PCs
AtlE: autolysin E
ATRs: Adverse transfusion reactions
Bap: Biofilm-associated protein
BC: Buffy-coat
BC-PCs: Buffy coat platelet concentrates
BHI: Brain-heart infusion
BPA: Blood products aerobic
BPN: Blood products anaerobic
CD62P: P-selectin
CFU: Colony forming unit
CHG: Chlorhexidine-gluconate
CoNS: Coagulase negative staphylococcus
CPD: Citric acid, sodium citrate, monobasic sodium phosphate and dextrose
eDNA: extracellular DNA
ELISA: Enzyme-linked immunosorbent assay
Embp: Extracellular matrix-binding protein
IPA: Isopropyl alcohol
LPS: Lipopolysaccharide
MBEC: Minimal biofilm eradication concentration
MH: Müeller Hinton
MH-CA: Müeller Hinton cation adjusted
MIC: Minimal inhibitory concentration
MSCRAMM: Microbial surface components recognize adhesion matrix molecules
netCAD: Network Centre for Applied Development
OD: Optical density
P&G: Physiology & Genetics
PBS: Phosphate buffered saline
PCs: Platelet concentrates
PI: Pathogen inactivation
PIA: Polysaccharides intercellular adhesion
PIT: Pathogen inactivation technology
PLT: Platelets
PMNs: Polymorphonuclear leukocytes
PRP: Platelet-rich plasma
RBCs: Red blood cells

SAGM: Saline-adenine-glucose-mannitol
SEM: Scanning electron microscopy
TSB: Tryptic Soy Agar Broth
TSBg: Tryptic Soy Agar Broth supplemented with 0.5% dextrose
US: United States
UV: Ultra violet
WB: Whole blood
WB- biofilm: Whole blood units spiked with biofilm cells
WB- planktonic: Whole blood units spiked with planktonic cells

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CHAPTER 1.

General Introduction

1.1 HUMAN BLOOD COMPONENTS MANUFACTURED AT CANADIAN BLOOD SERVICES

Canadian Blood Services manufactures different blood products namely, red blood cell (RBC) concentrates (RBCs), platelet concentrates (PCs), and plasma components for therapeutic purposes (CBS, 2013). RBC units are leukocyte-, plasma- and platelet-reduced. This blood product is used for the treatment of anemic patients. RBCs are suspended in the additive solution saline-adenine-glucose-mannitol (SAGM) and are stored at 1–6°C for a maximum of 42 days (CBS, 2013). Plasma is used to treat individuals with deficient coagulation factors or plasma proteins. Plasma components produced by Canadian Blood Services include frozen plasma (frozen within 24 hours of donation), fresh frozen plasma (frozen within 8 hours of donation), cryosupernatant plasma and cryoprecipitate (CBS, 2013). They differ in their content of certain coagulation factors and plasma proteins. For example, fresh plasma contains an average of 0.91 IU of the coagulation factor VIII per mL whereas the factor VIII content in fresh frozen plasma has an average of 1.15 IU per mL (CBS, 2013). Plasma components are stored at -18˚C or lower. PCs manufactured at Canadian Blood Services are suspended in plasma and are RBC- and leukocyte-reduced. This blood product is transfused to treat platelet dysfunctionality or deficiencies in patients suffering from thrombocytopenia, significant hemorrhage, or undergoing chemotherapy (Perrotta et al., 2013). At Canadian Blood Services, PCs are stored at 20-24°C with a shelf life of 5 days (CBS, 2013).

1.2 PLATELET CONCENTRATES

1.2.1 Platelets

Platelets are 2-5 um discoid-shaped, granulated, anucleated cells primarily responsible for hemostasis (White, 2013). The normal platelet count in human whole blood (WB) ranges from 150×10^9/ L to 400×10^9/ L with a lifespan of 7- 10 days (Harrison and Briggs, 2013; White, 2013). Platelet activation and stable plug formation occurs in three stages: initiation, extension and stabilization. Vascular injury precedes the initiation step, where circulating platelets are exposed to collagen and collagen-von Willebrand factor (vWF), resulting in the entrapment and activation of platelets. This results in plug formation which consists of a monolayer of adhered platelet cells. Activated platelets recruit and activate more platelets by releasing agonists like adenosine diphosphate (ADP) and thrombin, thereby enlarging the plug. Intracellular signaling results in the activation of glycoprotein (GP) IIb-IIIa on the platelet surface which results in the structured aggregation of platelets. Additionally, CD62P (P-selectin), a cell adhesion molecule expressed on the surface of activated platelets, is thought to aid in platelet-fibrin and platelet-platelet binding and aggregation (Brass et al., 2013).

1.2.1.1 Platelets and the immune system

Platelets play a role in host immunity and clearance of infection. They contain intracellular granules that possess antimicrobial activities which come into effect upon degranulation either by releasing the granular content into the host circulation or by expressing the granular content on the platelet membrane. Some of these granules contain inflammatory chemokines or they express adhesive proteins (e.g., CD40L) that enhance neutrophil, monocyte and dendritic cell activation. Platelets also exert their antimicrobial activity by capturing bacteria upon activation

which are then eliminated by either the production of antimicrobial peptides or via phagocytosis (Yeaman, 2014; Semple et al., 2011).

Platelet-bacteria interaction results in platelet activation and degranulation and occurs in one of three ways: direct, indirect, or through secreted products. In direct interactions, bacteria bind to platelet glycoprotein receptors. This type of interaction is demonstrated by *Staphylococcus epidermidis* and *Staphylococcus aureus* which directly bind to the platelet receptor GP IIb-IIIa via the bacterial surface proteins SdrG (serine-aspartate repeat G) and IsdB (iron-regulated surface determinant), respectively (Brennan et al., 2009; Miajlovic et al., 2010). Indirect contact occurs when proteins (usually plasma proteins such as fibrinogen and fibronectin) bridge the interaction between bacteria and platelets (Hamzeh-Cognasse et al., 2015). Bacteria are capable of interacting with platelets via secreted products like bacterial toxins and shed lipopolysaccharide (LPS) (Clark et al., 2007). Examples of note are the interactions observed between the toxins produced by *S. aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* and the lipid bilayer of platelets which results in platelet activation and aggregation (Hamzeh-Cognasse et al., 2015). It is difficult to determine the precise mechanism of bacteria-platelet interactions as it varies between species and even within isolates of the same species (Cox et al., 2011; Kerrigan and Cox, 2010).

1.2.2 PC production processes

PCs are produced using three methods, namely: apheresis (Aph), buffy-coat (BC), and platelet-rich plasma (PRP) (Fig. 1.1). During apheresis PC (Aph-PC) production, blood is collected from a single donor in a closed sterile system and then blood is processed through an apheresis

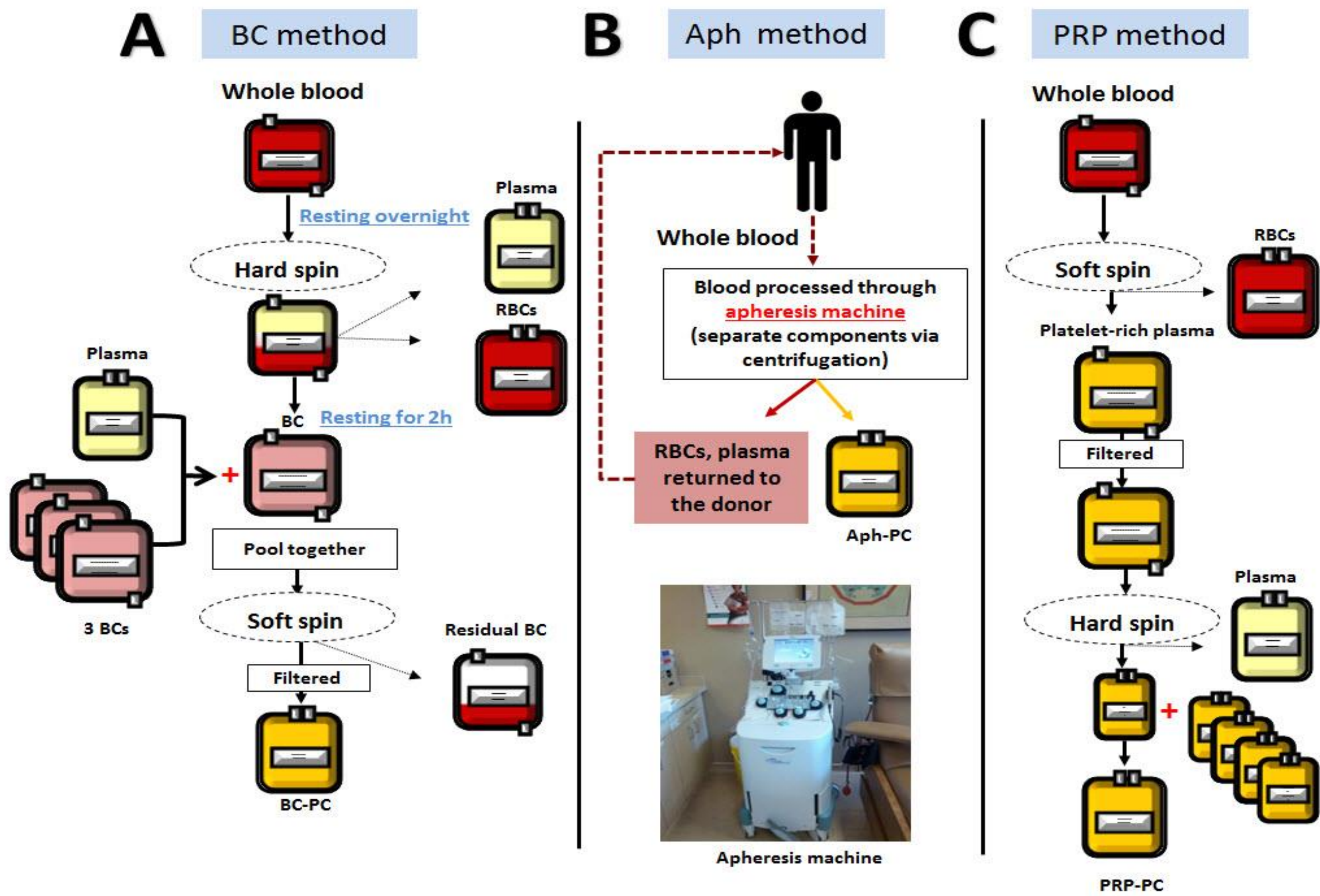

Figure 1.1. PC manufacturing methods. A) buffy-coat method (BC), B) apheresis method (Aph), and C) platelet-rich plasma method (PRP).

(centrifugation) machine separating the platelets from the rest of the blood and returning RBCs and most of the plasma fractions to the donor. Aph-PCs are stored in the ACD-A anticoagulant (adenine, citrate dextrose-formula A). The whole donation/production process takes up to approximately 1-2 hours. Aph-PCs that are matched-HLA (human leukocyte antigen) are preferentially used to treat patients who develop alloantibodies as a result of recurrent PC transfusions (Vamvakas, 2009).

Platelet-rich plasma PCs (PRP-PCs) are prepared from one single WB unit which is immediately processed into RBCs and PRP after a soft spin (Fig. 1.1). This is then followed by filtration of the PRP to remove leukocytes. Then, the PRP goes through a hard spin to separate platelet-poor plasma (PPP) from PRP. From four to six PRPs are then pooled together to produce the final PRP-PC product.

PCs prepared by the buffy coat method (BC-PCs) come from WB donations from four individuals collected in the presence of CPD anticoagulant (citrate, phosphate, dextrose) which allows maintaining the quality of RBCs manufactured from the WB donation. The whole BC-PC production process lasts 24-30 hours post blood donation. Briefly, after the blood donation, WB is allowed to rest overnight from eight to 24 hours on a cooling tray at 22± 2 ^{o}C, to decrease platelet aggregation and activation (Fig. 1.1) (Perrotta et al., 2013). WB then goes through a hard spin (3496 g) to separate RBCs and platelet poor plasma (PPP) from the buffy coat (BC) which mainly contains white blood cells (WBCs) and platelets (Levin et al., 2008). The BC is allowed to rest for a minimum of two hours at 22± 2 ^{o}C, after which four separate buffy coats (ABO-matched) and a plasma unit from one of the four donors are pooled. The pooled BCs then go through a soft spin (1258 g for 5 min) and a BC-PC pool is extracted through a leukoreduction filter, which eliminates most of the WBCs. Implementation of the BC-PC production method

started in 2005 and was completed in 2008 replacing PRP-PC production, which was terminated in 2010 The BC production method produces PCs of higher quality and is logistically more convenient and cost-effective (Jenkins et al., 2011, Levin et al., 2008). However, other countries like the United States (US) rely mainly on PRP and Aph PCs (Schrezenmeier and Seifried, 2010). Currently at Canadian Blood Services, PCs are produced either by Aph (30%) or the BC method (70%) and they have to meet standard quality criteria, which are described in Table 1.1.

1.2.3 PC storage

PCs are stored at 22± 2 ^{o}C with agitation for up to five or seven days depending on the blood component manufacturer. It has been shown that platelet functionality and viability are adversely affected when PCs are stored at 4 ^{o}C, and are rapidly removed from the recipient's circulation post-transfusion (Murphy and Gardner, 1969; Hoffmeister et al., 2003). Incubating PCs at the normal physiological temperature (37 ^{o}C) results in less viable platelet cells and a higher metabolic rate compared to PCs stored at 22± 2 ^{o}C (Badlou et al., 2005; Holme and Heaton, 1995). Interestingly, a recent study showed that storing Aph-PCs at 4°C in and 65% additive solution (Intersol® solution: sodium-citrate-acetate chloride solutions and phosphates for buffering) better maintains platelet viability and functionality compared to PCs stored at 22± 2 ^{o}C (Fenwal, 2014; Getz et al., 2016).

1.2.4 Platelet storage lesion

Collection, production and storage of PCs can induce platelet injury, which could potentially result in less than ideal post-transfusion outcomes. The *in vitro* quality of PCs and the damage

caused by storage can be monitored by examining platelet structure (such as platelet size, morphology and count), evaluating platelet function (such as hypotonic shock response and extent of shape change), assessing metabolic status (such as supernatant pH, pO_2, pCO_2, HCO_3, glucose consumption and lactate production), and measuring platelet activation (CD62P surface expression, platelet microparticle formation) (Perrotta et al., 2013). CD62P protein expression is measured by flow cytometry as an indicator of platelet activation during PC storage (Perrotta et al., 2013; Levin et al., 2012).

ThromboLUX® (LightIntegra Technology) is a commercialized device that rapidly (20 minutes) provides an indication of the *in vitro* quality of PCs using dynamic light scattering which determines the size and distribution of particles in PCs based on changes in the detected signals. In particular, it detects microparticles, and discoid and activated platelets by measuring their relative size change as a response to thermal stress (exposure to body temperature of 37 ^{o}C, then 20 ^{o}C and again to 37 ^{o}C) (Maurer-Spurej et al., 2006). Thermal stress activates functional platelets and leads to an increase in their size as a result of changes in their shape (pseudopods formation) and/or formation of microaggregates. The device also measures microparticle content which is shed from platelets upon activation due to exposure to agonists, stress, or as a result of aging (Italiano et al., 2010). ThromboLUX determines PC quality via a score that ranges from 0-40 (bad quality-good quality) with scoring $\geq$10 indicating acceptable PC quality (LightIntegra, 2015). The final represented score of a given PC sample takes into consideration platelet counts, response to thermal stress, and microparticle content (Labrie et al., 2013). Clinical studies have shown that ThromobLUX scores correlate with the clinical outcomes of transfusion (Maurer-Spurej et al., 2009).

Table 1.1. Quality control criteria of BC-PCs and Aph-PCs manufactured at Canadian Blood Services (CBS, 2015)

Criteria	BC-PCs	Aph-PCs
Platelet yield	≥240 $x10^9$ /unit*	≥300 $x10^9$ /unit*
Residual WBCs	< 5 $x10^6$/unit	< 55 $x10^6$/unit
pH	6.4-7.8	6.4-7.8
Other	• Donation frequency: every 56 days • About 2 times less expensive than Aph-PCs	• Donation frequency: every 14 days • Suitable to treat alloimmunized patients

* in ≥75% of tested units
Canadian Blood Services monitor 1% (or at least 10 units) of their monthly production of PCs from each production site after expiry (day 6).

1.3 BACTERIAL CONTAMINATION OF PCs

One of the major problems in transfusion medicine is bacterial contamination of PCs which is the predominant cause of infectious transfusion reactions in developed countries that can sometimes lead to fatal outcomes (Ramirez-Arcos and Goldman, 2012; FDA, 2015; Benjamin et al., 2014; Kou et al., 2015). Data from 21 blood centers around the world showed contamination rates in PCs ranging from 0.012% - 0.104% of donated units (Benjamin and McDonald, 2014). Interestingly, there is evidence that suggests that bacterial adverse transfusion reactions (ATRs) are underestimated, specifically in cases with mild symptoms (Walther-Wenke et al., 2010). Severe clinical reactions have been reported to occur at bacterial loads $\geq 10^5$ CFU/mL in PCs (Jacobs et al., 2008). PCs have the highest bacterial contamination rates when compared to other blood products like RBCs and plasma (Ramirez-Arcos and Goldman, 2012). Storage of PCs suspended in an additive solution that confers a neutral pH and a dextrose content of 500 mg/dL, in gas-permeable plastic containers, at $22 \pm 2\ ^{o}C$ under constant agitation offers an ideal environment for bacterial growth. (Ramirez-Arcos and Goldman, 2012; CBS, 2013).

1.3.1 Safety measures

Blood centers follow a number of standardized strategies to control bacterial contamination of blood products. It starts by an evaluation of the donor's health by measuring body temperature and asking the donor to fill in a mandatory questionnaire that is designed to exclude donors with possible bacteremia at the time of donation. Another important step is the disinfection of the phlebotomy site. Additionally, during blood donation, the first 20-40 mL of the donated blood is diverted as it has the highest probability of containing skin contaminant bacteria (Jenkins et al., 2011). These steps have led to a significant decrease in bacterial contamination of PCs

(McDonald, 2011; Benjamin et al., 2011; Wagner et al., 2000; Benjamin and McDonald, 2014). Prior to transfusion, PCs are screened for bacterial contamination and are visually inspected for the presence of clots and fibrin strands or gray discoloration, which can be indicative of bacterial contamination (CBS, 2009). Some blood centers also perform testing at the bed-site before transfusion (Pietersz et al., 2014). Despite the implementation of these mitigation strategies and screening tests, contaminated PC units are still detected and in some cases escape the screening process and are transfused causing ATRs and fatalities (Pietersz et al., 2014; Ramirez-Arcos and Goldman, 2012; FDA 2015; Kou et al., 2015).

1.3.1.1 Donor arm skin disinfection

Disinfecting the antecubital area before venipuncture is an important step to significantly reduce the contamination of blood products by skin bacteria (Ramirez-Arcos and Goldman, 2012). The type of skin disinfectant used, the concentration, volume, mode of application (e.g., scrub, swab or ampoule), and time of contact affects their efficiency (McDonald et al, 2001; McDonald et al, 2010). Therefore, before implementing any new skin disinfectant regiment, blood centers perform studies to evaluate the effectiveness of the candidate antiseptic. Moreover, the chosen skin disinfection method should be easy to use by the phlebotomists and suitable for donors in terms of waiting time and skin irritation and abrasiveness (Ramirez-Arcos and Goldman, 2010; McDonald, 2011). At Canadian Blood Services and other blood centers, the primary skin disinfectant is a one-step kit containing 70% isopropyl with 2% chlorhexidine (Jenkins et al., 2011; McDonald et al, 2010; Benjamin et al., 2011). For donors who react adversely to chlorhexidine, iodine-based disinfectants are used (Jenkins et al., 2011). Studies have shown that equal or better efficiency can be achieved with the application of a one-step 70% isopropyl with

2% chlorhexidine disinfectant versus iodine-based antiseptics in terms of bacterial reduction and contamination rates in blood products (Jenkins et al., 2011; McDonald et al, 2010; Benjamin et al., 2011).

1.3.1.2 Bacterial detection systems in PCs

Different bacterial detection systems used worldwide are described in Table 1.2. The BacT/ALERT® 3D culture system (bioMérieux, Marcy l'Etoile, France) is the most widely used screening system for bacterial contamination in blood banks (McDonald, 2013). At Canadian Blood Services, this system has been used to test PC products since 2004 (Jenkins et al., 2011). Screening is performed by inoculating 8-10 mL of the PC sample into aerobic (BPA) culture bottles containing 40 mL of media that supports growth of common bacterial contaminants. The inoculated bottle is then placed into the BacT/ALERT machine and incubated at 36°C for 6 days or until they turn positive. Once bacteria start proliferating and producing CO_2 in the bottles, a colorimetric sensor at the bottom of the bottle changes color from greenish to yellow as a result of a decrease in pH (Thorpe et al., 1990). This change in color is detected by the machine and the bottle is flagged as positive for the presence of bacteria. At Canadian Blood Services, PC units are placed in inventory after the BacT/ALERT sample is taken and results for other infectious diseases have been completed. At this time, PCs are labelled as "negative to date" for bacterial screening and can be released for transfusion.

The BacT/ALERT® system has a sensitivity of 1-10 CFU/mL of bacteria in PCs (Brecher, 2002; Brecher and Hay, 2004). Despite this high sensitivity, contaminated units are missed in some cases causing ATRs and fatalities (Pietersz et al., 2014, FDA 2012; Kou et al., 2015). Canadian Blood Services follows a defined protocol for identification of positive bacterial contamination of PCs (Fig. 1.2). Briefly, once a BPA bottle turns positive (initial positive), it is

sent to a microbiology laboratory for culturing and for bacterial identification. If the bottle reveals no bacterial growth then it is categorized as a *false positive due to machine failure*. In the case of bacterial growth, the corresponding PC unit is checked for availability and if it is not available, the initial result is considered *indeterminate (possible positive*). But if the unit is still accessible, it is re-sampled. If both bacteria identified from the first and second culture bottles are the same, the result is considered to be a *true positive*. However, if the second bottle of the PC unit yields negative growth, the result is considered *a false positive due to contamination*. If the initial culture yields a negative result but the PC unit is contaminated and causes a transfusion reaction, or results in positive growth when sampled later during storage, the initial result is considered a *false negative*. In the case of an ATR, the microorganism identified from the implicated PC unit and the patient should be the same.

Table 1.2. Bacterial detection systems in PCs

Method	Commercial assay	Principle	Properties	Reference
Culture methods	BacT/ALERT® 3D (Biomerieux)	Detects bacterial proliferation: PCs added to a special culture bottle and the drop in pH due to CO_2 production of growing bacteria changes the color of the indicator at the bottom of the bottle.	• Sensitivity: 1-10 CFU/mL	Benjamin, and McDonald 2014
	BACTEC 9240 (Decton Dickinson)		• Sensitivity: 1-10 CFU/mL • False-positive rate: 0.1%	Dunne et al., 2005
	VersaTREK® (TREK Diagnostics)	Detects bacterial proliferation: senses changes in the atmospheric pressure in the head space bottle as a result of gas consumption or production	• Sensitivity: 10-20 CFU/ml.	Nanua et al., 2009
Rapid methods	Pan Genera Technology (Verax Biomedical)	Lateral flow test that detects bacterial cell wall antigens: lipoteichoic acid in the cell wall of gram-positive bacteria and LPS of gram-negative cell wall get detected by double sandwich immune assay	• Rapid (20-30 min) • Sensitivity: 10^4-10^6 CFU/mL • False-positive rate: 0.51%	Jacobs et al., 2011
	BacTx™ (Immunetics)	Detect peptidoglycan of bacterial cell wall: series of enzymatic reactions are initiated by peptidoglycan binding protein resulting in colorimetric change	• Rapid (30 min + 15 min for sample preparation) • Sensitivity: 10^3-10^6 CFU/mL • No reported false-positives	Ramirez-Arcos et al., 2014
Flow cytometry	BactiFlow® (BioMerieux)	Detect Fluorescent labeled viable cells by flow cytometry: non-fluorescent fluorochrome get cleaved by the intracellular esterases of bacterial viable cells	• Rapid (0.75-1 hr for 12 samples) • Sensitivity 150 CFU/mL • False-positive rate 1.7%	Vollmer et al., 2011
Molecular techniques (Real-time PCR)	Not available, only *in-house*	Primers and probes specific for Staphylococcus genus 16S rRNA, *E. coli* 16S/23S rRNA, *S. aureus*, *S. epidermidis*, *Enterobacter aerogenes*, *Serratia marcescens* 23S rRNA	• <50 CFU/mL	Rood et al., 2011b; Zhang et al., 2015; Vollmer et al, 2012

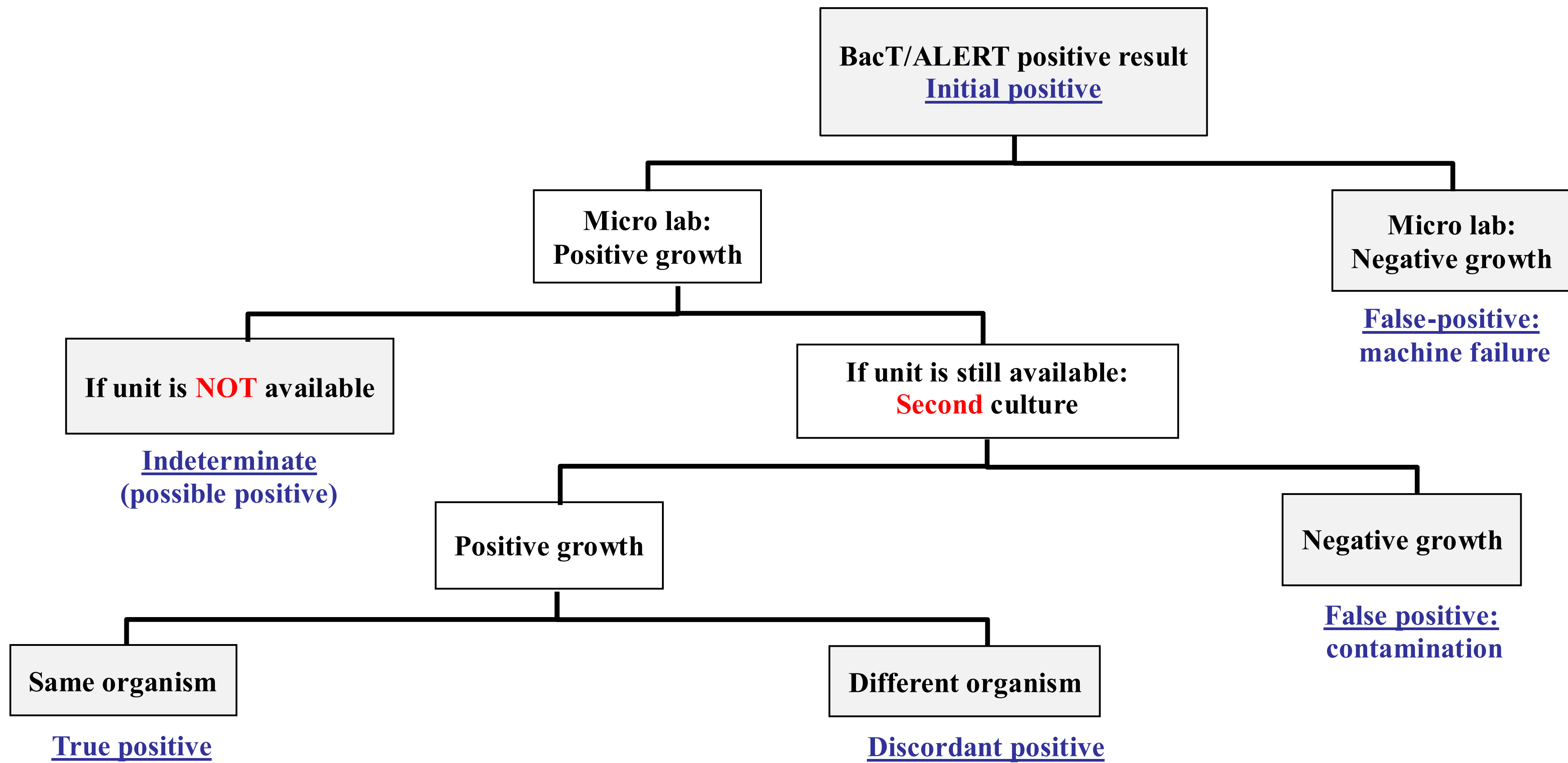

Figure 1.2. Flow chart of the identification of positive bacterial contamination of PCs at Canadian Blood Services (courtesy Dr. Ramirez-Arcos)

Table 1.3 summarizes bacterial contamination rates of the routinely screened PC units from January 2010 to December 2015 at Canadian Blood Services. Similar true positive rates were obtained for Aph-PC (~0.9 per 10,000) and BC-PCs (~0.8 per 10,000) screened during this period. However, a contamination rate approximately 1.7-fold higher in Aph-PCs than BC-PCs was observed when both confirmed and possible positive (indeterminate) results were included. As BC-PCs are prepared from four donors, it was expected that bacterial contamination rates would be higher in these products than in Aph-PCs, which are obtained from a single donor. Many factors may contribute to the removal of bacteria during the BC-PC production process such as the centrifugation steps used to separate the different blood fractions, killing by plasma proteins, phagocytosis during the resting periods, and bacterial adhesion to white blood cells (WBCs) or to activated platelets (Högman et al., 1991; Buchholz et al., 1994; Dzik, 1995; Mohr et al., 2006).

Table 1.3. Bacterial contamination rates of routinely screened PC units manufactured at Canadian Blood Services (Jan 2010-Dec 2015, courtesy Dr. Ramirez-Arcos).

Number	BC-PCs N = 520,815	Aph-PCs N = 167,724
Initial positives rate/10,000	324 6.22	247 14.73
True positives rate/10,000 (% initial positives)	42 0.81 (13.0)	15 0.89 (6.1)
Indeterminate (possible positive) results rate/10,000 (% initial positives)	25 0.48 (7.7)	25 1.49 (10.1)
False positives:		
• machine error rate/10,000 (% initial positives)	188 3.61 (58.0)	191 11.39 (77.3)
• contamination while sampling rate/10,000 (% initial positives)	69 1.32 (21.3)	16 0.95 (6.5)

Mathematical modeling has shown that the estimated initial concentration of bacteria in PC units that could lead to a false-negative BPA bottle is estimated to be approximately 1-60 CFU/product (~ 0.003-0.2 CFU/mL) with a 50% chance of detecting bacteria at a concentration of 0.09 CFU/mL in a PC unit with 8 mL inoculum (Benjamin and Wagner, 2007; Murphy et al., 2008). Missed detections could be caused as a result of sampling error due to very low initial bacterial concentrations in the PC unit at the time of sampling and due to the fact that only about 3-5% of the unit total volume is sampled (Benjamin and Wagner, 2007; McDonald, 2013). It had been shown that the detection rate of bacterially contaminated units is only 40% when screened on the same day of production (Pearce et al., 2011). Even if the sampling step gets delayed to allow for bacterial proliferation, slow growing bacteria can be missed during PC screening. For example, coagulase-negative staphylococci (CoNS) like *S. epidermidis* has a lag phase of up to three days in the PC environment and obligate anaerobic bacteria such as *Propionibacterium acnes* do not multiply in PCs and remain at low concentrations through PC storage (Ali et al., 2014; Störmer et al., 2008). Bacterial attachment to the inner surface of the bags (could take only 20 min) and biofilm-formation abilities also play a role in making bacterial cells less available for sampling (Greco-Stewart et al., 2012; Shanks et al., 2007). Table 1.4 lists bacteria that have been missed during PC screening at Canadian Blood Services and resulting in ATRs.

Table 1.4. Adverse transfusion reactions due to missed detection of bacterially-contaminated PC units at Canadian Blood Services (2005- 2016, courtesy Dr. Ramirez-Arcos).

Date	PC	Age (days)*	Organism	Comments
2005-06	Aph	5	*Serratia marcescens*	• Fatal reaction • False negative
2006-08	PRP	3	Group A *Streptococcus*	False negative
2010-03	BC	5	Coagulase negative *Staphylococcus*	False negative
2010-07	Aph	5	Coagulase negative Staphylococcus	False negative
2011-11	BC	5	*Staphylococcus aureus*	False negative
2012-01	Aph	3	*Staphylococcus aureus*	False negative
2014-09	BC	5	*Staphylococcus epidermidis*	• Fatal reaction • False negative
2016-05	BC	4	*Staphylococcus aureus*	False negative

*age when the PC product was transfused

1.3.1.3 Pathogen Inactivation Technologies

Pathogen inactivation technologies (PITs) have been implemented in several countries around the world to control the transfusion of contaminated PCs and improve blood safety. PIT systems use ultraviolet light (UV) in the presence or absence of a photosensitizer to treat PCs. This process induces irreversible damage to nucleic acids and prevents pathogens from proliferating. Three PITs are available in the market, Mirasol® (Terumo BCT, Lakewood, Colorado), Intercept™ (Cerus, Concord, California), and Theraflex (Macropharma, Tourcoing, France). Table 1.5 describes the UV light wavelengths, the photosensitizers used, and the mode of action for the three PITs. High pathogen inactivation abilities have been reported against parasites, viruses and bacteria (Devine and Schubert, 2016). However, these systems show reduced effectiveness against bacterial spores and some non-enveloped viruses (hepatitis A, hepatitis E, parvovirus B19) (Devine and Schubert, 2016). PITs are also capable of inactivating WBCs aiding to reduce the risk of graft-versus-host disease. Studies have indicated that treatment with PITs adversely affect PC quality *in vitro*. The illumination process has been shown to induce platelet activation, increase glucose metabolism, and affect platelet aggregation abilities (Picker et al., 2004; Reikvam et al., 2010; Devine and Schubert, 2016). However, studies examining clinical outcomes of patients treated with PI-inactivated PC units have not shown evidence of increased bleeding post transfusion (Lozano et al., 2011; Kaiser-Guignard et al., 2014; Devine and Schubert 2016).

Implementation of PITs in Canada is under consideration. The anticipated benefits of improving the safety of blood products need to be considered in the context of potential harm to PC quality and high cost of the technologies.

Table 1.5. Pathogen Inactivation Technologies

PIT system	UV light (nm)	Photosensitizer	Mode of action
Mirasol	280-360 (UV-A, B)	Riboflavin (vitamin B2)	Photoactivated riboflavin interacts with the nuclic acid and oxidase guanine causing irreversible damage
Intercept	320-400 (UV-A)	Psoralens amotosalen (remaining amotosalen and residual photoproduct are removed after UV treatment)	Crosslink pyrimidic base residues of the nucleic acids irreversibly
Theraflex	254 (UV-C)	None	Prevent nucleic acid replication by inducing pyrimidine dimers formation

This table is adopted from Devine and Schubert 2016 (Devine and Schubert, 2016) with modifications.

1.3.2 Bacterial PC contaminants

The major source of bacterial contamination in blood products is normal human skin flora which is introduced during venipuncture. Coagulase-negative staphylococci (CoNS), mainly *S. epidermidis,* and the anaerobic bacterium *P. acnes* are the most commonly isolated bacteria from contaminated PCs (Benjamin and McDonald, 2014; Ramirez-Arcos and Goldman, 2012). Blood products could also be contaminated due to transient bacteria present in the donor's skin (of enteric, nasopharyngeal or environmental origin) or from asymptomatic bacteremia. For example *S. aureus*, *Streptococcus* spp., *Bacillus* spp and *Corynebacterium* spp, just to name a few, have been isolated from contaminated PCs (Benjamin and McDonald, 2014; Pietersz et al., 2014).

1.3.2.1 Staphylococcus epidermidis

Staphylococcus spp. are among the most common commensal human skin flora residing in the different layers of the skin (Otto, 2010; Grice and Segre, 2011; Grice et al., 2009; Findley et al., 2013), with *S. epidermidis* being the most frequently isolated species (Kloos and Musselwhite, 1975). A number of factors contribute to the success of *S. epidermidis* to overcome the harsh environment of the human skin. For example, *S. epidermidis* is able to survive in the acidic and salty milieu of the skin and to withstand changing osmolarity (Gill et al., 2005). *S. epidermidis* possesses the capacity to resist cationic antimicrobial peptides (AMPs) (such as human-defensin 3 produced by skin keratinocytes) by decreasing the anionic nature of the bacterial surface or by expulsing or degrading the AMPs with proteases (Li et al., 2007; Otto 2009). Also, *S. epidermidis* can resist anionic AMPs (such as dermicidin, which is secreted by eccrine sweat glands) by increasing the activities of extracellular proteases (Lai et al., 2007). The secreted extracellular polymer poly-γ-DL-glutamic acid (PGA) had been reported to protect *S.*

epidermidis from phagocytosis and elimination by cationic AMPs (Kocianova et al., 2005). Also, PGA protects *S. epidermidis* from high concentrations of NaCl (Kocianova et al., 2005). *S. epidermidis* is capable as well of producing low levels of the toxin PSMδ (phenol-soluble modulin) that contributes to neutrophil destruction (Cheung et al., 2010).

1.3.2.1.1 Biofilm formation by S. epidermidis

S. epidermidis is well known for its role in nosocomial infections and bacteremia as a result of colonizing indwelling medical devices (Uckay et al., 2009). This organism is one of the major causes of endocarditis and it has been reported to resist multiple antibiotics including methicillin (Raad et al., 1998; Rupp, 2014; Uckay et al., 2009). *S. epidermidis* pathogenesis is manly linked to its ability to grow in multicellular surface- attached communities of cells, known as biofilms (Otto, 2009). Transmission electron microscopy images have shown that *S. epidermidis* can reside as a biofilm between the squamous cells of normal human skin (Costerton, 2007). *S. epidermidis* biofilms displayed increased resistance to antibiotics. The biofilm matrix acts as physical barrier preventing the antibiotics from reaching the cells. Additionally, cells in the center of a biofilm have decreased growth rate and metabolism and therefore antibiotics that target actively growing cells, such as penicillins and quinolones, have limited activity (Otto, 2009; Khardori et al., 1995; Hoiby et al., 2010). Biofilm formation by *S. epidermidis* is also responsible for evasion of the immune system with reports demonstrating a drop in the complement system clearance efficiency and decreased WBC phagocytosis and killing abilities. Biofilms prevent complement proteins and antibodies from deposition on the bacterial surface affecting opsonization (Vuong et al., 2004b; Kristian et al., 2008; Rohde et al., 2007).

There are four major stages in the *S. epidermidis* biofilm formation process including: attachment, accumulation, maturation and dispersal (Otto, 2009). *S. epidermidis* cells adherence

to abiotic surfaces is facilitated by the surface-associated proteins AtlE (autolysin E), the accumulation/adhesin protein Aae and teichoic acids (Heilmann et al., 1997; Heilmann et al., 2003; Otto 2009). When surfaces are coated with host proteins such as fibrinogen and collagen, the initial attachment process is mediated by the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) such as SdrG, SdrF (Otto, 2009). Bacterial cells then start accumulating by adhering to each other and multiplying through the synthesis of an adherence matrix, which could be made of polysaccharide, such as the polysaccharide intercellular adhesin (PIA) composed of poly-N-actylglucosamine, proteins (such as the accumulation-associated protein-Aap and the biofilm-associated protein-Bap), extracellular DNA (eDNA) and/or teichoic acids (Rohde et al., 2005; Qin et al., 2007a,b; Sadovskaya et al., 2005; Tormo et al., 2005; Christner et al., 2010). PIA is synthesized by enzymes encoded by the *icaADBC* operon which is regulated by the upstream *icaR* gene (Heilmann et al., 1996). As the biofilm matures, free-floating (planktonic) cells start to detach due to a mechanical effect or as a result of signaling of the *agr* quorum sensing system (Otto, 2009; Vuong et al., 2004a).

1.3.2.1.2 S. epidermidis biofilm formation in PCs

In the Ramirez' laboratory, PIA-biofilm forming isolates of *S. epidermidis* have been isolated from contaminated PC units (Greco et al., 2007; Greco et al., 2008; Ali et al., 2014). The effect of the PC storage conditions on *S. epidermidis* biofilm formation have been investigated as well. Interestingly, these studies have demonstrated that all tested *S. epidermidis* isolates recovered from contaminated PCs, including those categorized as non-biofilm formers, were capable of forming biofilms in PCs (Greco, 2011; Ali et al., 2014). The exact factors and genes that are involved in the process of turning non-biofilm formers to phenotypically biofilm-formers under the PC storage environment are not clear and are currently being investigated. Furthermore, the

Ramirez' group has shown that *S. epidermidis* grown in PCs displayed increased pathogenicity in a nematode killing assay (Hodgson et al., 2014).

1.3.2.2 PC contamination by Gram negative bacteria

Gram negative bacteria such as *Serratia marcescens, Escherichia coli, Klebsiella pneumoniae*, *Enterobacter cloacae* and *Salmonella* spp. have been isolated from contaminated PCs (FDA, 2015; Pietersz et al., 2014; Greco-Stewart et al., 2012). These Gram negative organisms could be of environmental or enteric origin (Jafari et al., 2002; Ramirez-Arcos et al., 2015). Some of these bacteria have been involved in fatal cases following the transfusion of contaminated PC units (Ramirez-Arcos et al., 2006; Niu et al., 2006; Jacob et al., 2008; FDA, 2015).The pathogenicity of Gram negative bacterial contaminants is increased due to endotoxin production by these organisms. Endotoxins are referred to the LPS complex present in the outer membrane of Gram-negative bacteria (Gorbet and Sefton, 2005). These heat-stable toxins are released during bacterial proliferation and bacterial death. LPS is made up of lipid A (responsible for much of the toxicity), core polysaccharides, and the O-antigen side chain (Gorbet and Sefton, 2005). Endotoxins stimulate strong immune response and inflammation. Clinically significant endotoxin levels in PCs were determined to be between 11,373 and 173,130 endotoxin units (associated with bacterial loads > 10^6 CFU/ml (Jacobs et al., 2008). Limulus amebocyte lysate (LAL) is the standard assay to detect endotoxin levels at very low concentrations. The assay uses the blood of horseshoe crabs which coagulates upon exposure to bacterial endotoxins as a result of stimulating the coagulation cascade (Iwanaga, 2007).

1.4 RELEVANCE TO TRANSFUSION MEDICINE

The Canadian Blood Services mission is to "*Operate Canada's blood supply in a manner that gains the trust, commitment and confidence of all Canadians by providing a safe, secure, cost-*

effective, affordable and accessible supply of quality blood, blood products and their alternatives". Unfortunately, bacterial contamination of PCs constitutes the greatest post-transfusion infectious risk in developed countries. Since safety and quality of blood products are paramount to the Canadian Blood Services mission, this study explored the efficiency of the currently used donor skin disinfectant, the role played by the BC-PC manufacturing process in PC bacterial contamination, and the potential use of the PIT Mirasol to inactivate bacterial biofilms. Understanding the mechanisms of bacterial survival during skin disinfection, BC-PC production and pathogen inactivation will aid in identifying steps in the blood donation and manufacturing processes that can be further optimized to minimize the risk of PC contamination, thereby increasing the safety of the platelet supply for Canadians (Fig. 1.3).

1.5 THESIS HYPOTHESES

It is hypothesized that biofilm-forming skin flora are resistant to the action of currently used donor skin disinfectants and the action of pathogen inactivation technologies. Furthermore, bacterial contamination of PCs is significantly reduced during the buffy coat production method due to the bactericidal action of immune factors present in whole blood.

1.6 OBJECTIVES OF THE THESIS

Objective 1. To evaluate the bactericidal efficacy of the arm disinfectants currently used at Canadian Blood Services against biofilms. Skin flora can exist in the skin as biofilms (Costerton, 2007). Interestingly, biofilm-forming bacteria of the human skin flora have been isolated from contaminated PC units (Greco et al., 2007; Greco et al., 2008; Ali et al., 2014). Therefore, this study was undertaken to investigate the efficacy of the arm disinfectants chlorhexidine and isopropanol against biofilm and planktonic (non-biofilm) bacterial cells of the skin flora species *S. epidermidis* and *Staphylococcus capitis*. The ability of PCs to support proliferation of disinfectant-treated biofilm cells of *S. epidermidis* and *S. capitis* was also assessed.

Objective 2. To study the prevalence of biofilm-forming *S. epidermidis* in human skin compared to PCs. Studies have demonstrated that the presence of the *ica* operon and biofilm-formation phenotype could serve as markers to differentiate between *S. epidermidis* isolated from normal human skin and clinical samples such as those isolated from joint and catheter related infections and blood cultures (Galdbart et al., 2000; Ziebuhr et al., 1997). In this work, the possibility of selecting for biofilm-positive *S. epidermidis* during the processes of blood

donation and blood product manufacturing was investigated. In particular, the prevalence of biofilm-forming *S. epidermidis* isolated from human skin was compared to the prevalence of biofilm-forming *S. epidermidis* isolated from contaminated PCs. In addition, the effect of the strain genetic background and isolation source on the ability to form biofilms in the PC milieu was explored.

Objective 3. To determine the efficiency of the pathogen inactivation technology - Mirasol® against *S. epidermidis* biofilms. The existence of bacterial cells in a biofilm increases their resistance to chemical and physical clearance. *S. epidermidis*, one of the predominant PC contaminants, can grow in the human skin forming biofilms, which are thought to be introduced into the blood collection bag during venipuncture (Rogers et al., 2008; Galdbart et al., 2000). The inactivation effectiveness of the PIT Mirasol system against *S. epidermidis* biofilms in PCs was evaluated in this thesis.

Objective 4. To investigate the bactericidal effect of the BC-PC production method. At Canadian Blood Services, BC-PCs show a generally lower bacterial contamination rate than apheresis PCs. This study investigated whether the PC production method contributes to this observation by examining the possible contribution of the different BC-PC manufacturing steps to bacterial reduction.

Objective 5. To examine the antibacterial activity of WB during the overnight hold time prior to BC-PC production. During the BC-PC production process, donated WB unit is allowed to rest at room temperature before it is processed to produce PCs. This hold time can

vary between 8 to 24 hours depending on the blood center (van der Meer and de Korte., 2015). Several reports discussed the potential benefits of this step on the safety of WB-driven products (such as BC-PCs) due to the presence of different immunological components such as WBCs (Sanz et al., 1997; van der Meer and de Korte., 2015). The aim of this study was to investigate bacterial survival in WB -outside the human body- of more than 20 bacteria at different time points during the overnight hold period and to explore the role of the different components of WB, such as plasma and neutrophils, in the elimination of selected bacteria.

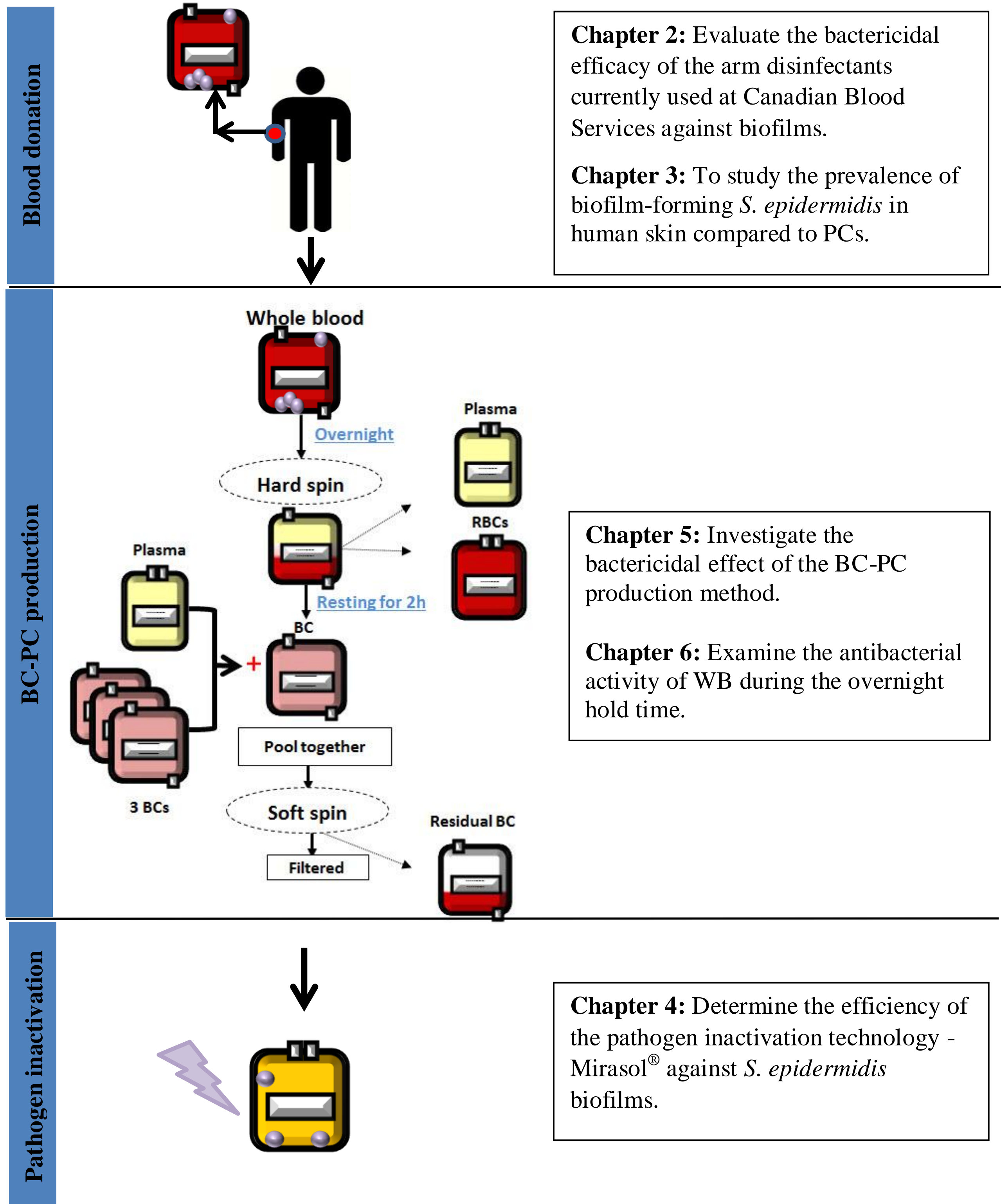

Figure 1.3. Flow chart summarizing the objectives in this thesis and how they relate to transfusion medicine.

CHAPTER 2.

Biofilm-Forming Skin Microflora Bacteria Are Resistant To The Bactericidal Action Of Disinfectants Used During Blood Donation

Mariam Taha[1], Miloslav Kalab[2], Qi-Long Yi[1], Carey Landry[1], Valerie Greco-Stewart[1],Ann Karen Brassinga[3], Costi D. Sifri[4], and Sandra Ramirez-Arcos[1]

[1]Canadian Blood Services,

[2]Agriculture and Agri-Food Canada,

[3]University of Manitoba,

[4]University of Virginia Health System

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2.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

The manuscript “Biofilm-forming skin microflora bacteria are resistant to the bactericidal action of disinfectants used during blood donation” has been published in *Transfusion* 2014; 54(11): 2974-2982.

M. Taha conducted all the experiments and prepared samples for scan electron microscopy examination. Dr. S. Ramirez-Arcos was the thesis’ supervisor providing guidance on the experimental design, troubleshooting and data interpretation. M. Taha and Dr. S. Ramirez-Arcos wrote the manuscript. Dr. M. Kalab performed scan electron microscopy examination. Dr. QL. Yi performed statistical analyses. Dr. C. Greco was an intellectual author; as a legacy of her PhD thesis at the Ramirez’ laboratory, she postulated the idea of biofilm resistance to donor skin disinfectants. Dr. V. Greco-Stewart, established the collaboration with Dr. K. Brassinga to obtain and characterize the *S. epidermidis* 9142 and *S. epidermidis* 9142 Δ*icaA* strains. Dr. C. Sifri is the owner of *S. epidermidis* 9142 and part of the research agreement with Drs. K. Brassinga and C. Sifri was that they were co-authors of this publication. Both of them read and provided comments on the manuscript.

2.2 ABSTRACT

A one-step skin disinfection method containing 2% chlorhexidine-gluconate (CHG) and 70% isopropyl alcohol (IPA) is currently used by blood suppliers worldwide. Reports of bacterially contaminated platelet concentrates (PCs) indicate that skin disinfection is not fully effective. Approximately 20% of skin microflora exist as surface-attached aggregates (biofilms), known for displaying increased resistance to disinfectants. This study was aimed at determining whether skin microflora biofilm-positive *Staphylococcus epidermidis* and *Staphylococcus capitis* are resistant to CHG and/or IPA. Free-floating cells and mono or dual (1:1 ratio) biofilms of *S. epidermidis* and *S. capitis* were exposed to CHG, IPA, or CHG/IPA for 30 seconds, simulating skin disinfection practices. Residual viable cells were quantified by colony counting. Morphology of disinfectant-treated *S. epidermidis* biofilms was examined by scanning electron microscopy. Treated *S. epidermidis* and *S. capitis* biofilms were inoculated into PCs and bacterial concentrations were determined on Days 0 and 5 of storage. Treatment of staphylococcal biofilm cells with all disinfectants caused cell damage and significant reduction in viability, with CHG/IPA being the most effective. However, biofilms were significantly more resistant to treatment than free-floating cells. Disinfectant-treated *S. epidermidis* proliferated better in PCs than *S. capitis,* especially when grown as monospecies biofilms. Although CHG/IPA is effective in reducing the viability of *S. epidermidis* and *S. capitis* biofilms, these organisms are not completely eliminated. Furthermore, disinfectant-treated staphylococcal biofilms multiply well in PCs. These results demonstrate that the biofilm-forming capability of the skin microflora reduces the bactericidal efficiency of blood donor skin disinfectants.

2.3 INTRODUCTION

Platelet concentrates (PCs) contaminated with bacteria are the major source of septic transfusion reactions in developed countries. The contamination rate in PC units ranges from approximately one in 100 to one in 7500 according to surveillance studies from 10 blood centers around the world (Ramirez-Arcos and Goldman, 2012). It has even been suggested that the risk of transfusing contaminated PC units can be as high as 1 in 150 for hematology-oncology patients receiving multiple PC units (Corash, 2011). In addition to causing severe morbidity in platelet (PLT) recipients, the transfusion of contaminated units also represent a financial burden to the health system. Angus and colleagues (Angus et al., 2001) have reported that the average cost of treating sepsis in the United States is $22,000 per patient. Skin microflora are the predominant bacteria isolated from PCs with *Staphylococcus epidermidis* and *Staphylococcus capitis* among the predominant organisms (Ramirez-Arcos and Goldman, 2012; Corash, 2011; Palavecino et al., 2010; Jacobs et al., 2008; Rood et al., 2011a). Both species have been reported to escape detection during routine PLT screening and *S. epidermidis* has been involved in severe transfusion reactions, in some cases resulting in fatalities (Murphy et al., 2008; Robillard et al., 2011; Goldman et al., 2001).

Strategies to prevent contamination of blood products include donor screening, proper disinfection of the venipuncture area before donation, diversion of the first 20 to 40 mL of the donated blood, and screening of PCs for bacterial contamination. A significant reduction in PLT contamination rates after the implementation of these strategies has been reported (Ramirez-Arcos and Goldman, 2012; Corash, 2011; Palavecino et al., 2010). However, bacterially contaminated PCs are still detected showing that in some cases they escape the screening process, which may lead to morbidity and mortality.

In 2009, Canadian Blood Services implemented a one-step 2% chlorhexidine-gluconate (CHG) and 70% isopropyl alcohol (IPA) kit for donor skin disinfection (Ramirez-Arcos and Goldman, 2010). Surveillance data showed that PLT contamination rates did not significantly change after 2 years of implementation of the new method compared to the previous two-step disinfectant method (IPA and iodine tincture solution) (Jenkins et al., 2011). Similar results were found at the English National Blood Services, where a combination of CHG/IPA was equally efficient as using IPA and iodine tincture for donor skin disinfection (McDonald, 2011). On the other hand, data from the American Red Cross showed that, compared to two-step povidone-iodine, a reduction in contamination rates in PCs after the usage of CHG/IPA was detected (Benjamin et al., 2011).

It has been shown that up to 20% of the bacteria present on the skin exist as surface-attached communities of cells known as biofilms (Ryder, 2005). We have recently shown that *S. epidermidis* and *S. capitis* are able to form biofilms during PLT storage (Greco et al., 2008; Greco-Stewart et al., 2013) which could make bacterial cells less available for sampling and likely contribute to missed detection during routine PLT screening. We have also demonstrated that PCs and their storage environment stimulate biofilm development of both biofilm and non–biofilm-forming bacteria (Greco-Stewart et al., 2013; Greco et al., 2007) resulting in increased pathogenicity (Hodgson et al., 2014). Since biofilms are highly resistant to antibiotic and disinfectant treatments and clearance by the immune system, biofilm formation is one of the major bacterial virulence factors associated with chronic infections (Valle et al., 20103).

The goal of this study was to evaluate the efficiency of CHG and IPA, each alone or in combination (CHG/IPA), against free-floating (planktonic) and biofilm cells

of *S. epidermidis* and *S. capitis*. Mono- and mixed (dual)-species biofilms were used in this study as a model of the skin microflora which is composed by a community of multiple (no single) interacting organisms. The ability of PCs to support proliferation of disinfectant-treated biofilm cells of *S. epidermidis* and *S. capitis* was also assessed herein.

2.4 MATERIALS AND METHODS

I. Bacterial strains and culture conditions

Two biofilm-positive strains were used: *S. epidermidis* 9142, isolated from a blood culture (Mack et al., 1992), and *S. capitis* CBS517, isolated from a contaminated PLT unit (Greco-Stewart et al., 2013). The strains were preserved in brain heart infusion broth (BD Biosciences, Bedford, MD) supplemented with 15% glycerol at −80°C. Bacteria were grown on trypticase soy agar (TSA) at 37°C for 24 hours, unless stated otherwise. Incubations in PCs were carried out at standard PLT storage conditions (22 ± 2°C with agitation). Sterile saline solution (0.9% wt/vol) was used for performing serial dilutions.

II. PLT units

Buffy coat outdated (8- to 12-day-old) PC units, provided by Canadian Blood Services sites, were used in this study. Each PC unit was tested for sterility upon arrival by inoculation of 8 to 10 mL into each of a blood products aerobic and a blood products anaerobic culture bottle (BacT/ALERT, bioMérieux, Marcy l'Etoile, France) followed by incubation in an automated microbial detection system (BacT/ALERT 3D system, bioMérieux) for a maximum of 6 days as per Canadian Blood Services standard procedures (Jenkins et al., 2011). Ethical approval for this study was granted by the Canadian Blood Services Research Ethical Board.

III. Disinfectants and neutralizer

Formulations of 2% (wt/vol) CHG, 70% (vol/vol) IPA, and 2% (wt/vol) CHG in 70% (vol/vol) IPA were prepared from 20% (wt/vol) CHG (Sigma-Aldrich, Oakville, Ontario, Canada) and 100% (vol/vol) IPA (Fisher Scientific, Whitby, Ontario, Canada) in sterile distilled water. The activities of the disinfectants were neutralized by the addition of 0.5% lecithin (Fisher Scientific) and 4% Tween 20 (Fisher Scientific) in distilled water according to established procedures (Kampf et al., 2005; Sutton, 1996). The neutralizer toxicity and efficiency were validated following the "standard test methods for evaluation of inactivators of antimicrobial agents" (ASTM, 2008).

IV. Minimal inhibitory concentration and minimal biofilm eradication concentration for CHG

Minimal biofilm eradication concentration (MBEC) Physiology & Genetics (P&G) assay (Innovotech, Inc., Edmonton, Alberta, Canada) was used to obtain the minimal inhibitory concentration (MIC) and MBEC for CHG of planktonic and biofilm cells, respectively, of *S. epidermidis* and *S. capitis*. MBEC P&G consists of 96 polystyrene pegs, which fit in a 96-well MBEC microtiter plate. The assay was performed according to the manufacturer's protocol (Innovotech, 2013). Briefly, suspensions of *S. epidermidis* or *S. capitis* containing approximately 10^7 colony-forming units (CFUs)/mL were prepared in Müeller Hinton cation-adjusted (MH-CA) broth (Fisher Scientific) and inoculated into the 96-well microtiter plate followed by incubation at 37°C with agitation at 130 rpm. After 24 hours, the lids with pegs carrying the biofilms were exposed to a gradient of CHG (0.25-128 μg/mL) for 24 hours. Initial CHG concentrations were prepared in sterile distilled water and brought to their final concentrations in MH-CA. CHG concentrations higher than 128 μg/mL were not prepared due to precipitation of this disinfectant

in MH-CA. MIC results were documented (lowest concentrations with no visible growth) from the exposure plate, and after that, the pegs were rinsed in saline solution (0.9% wt/vol) and added to a 96-well recovery plate containing the neutralizer (0.5% lecithin and 4% Tween 20) prepared in MH-CA. Biofilms were dislodged by sonicating the pegs in a water bath for 30 minutes. The recovery plate was incubated at 37°C for 72 hours. The assay was repeated at least three times independently with eight replicates (different rows of the MBEC plate) for each concentration in each repetition. Because of the volatile nature of alcohols, MIC and MBEC assays for IPA or the CHG/IPA combination were not performed.

V. Eradication abilities of CHG, IPA, and CHG/IPA

Suspensions corresponding to 1.0 McFarland (approx. 3×10^8 CFUs/mL) in Müeller Hinton (MH) broth (Fisher Scientific) were prepared for *S. epidermidis* and *S. capitis* from colonies grown overnight on TSA. The suspensions were diluted in MH (1/30) to a final concentration of approximately 10^7 CFUs/mL for each of the two species separately or in combination (1 : 1 ratio) for inoculation of six tissue culture–treated polystyrene plates (Fisher Scientific). After an overnight incubation period, planktonic cells were harvested from the supernatant and biofilm cells were left attached to the bottom of the wells.

Eradication of planktonic cells. To test the action of the disinfectants against planktonic cells, an eradication assay was performed according to Adams and coworkers (Adams et al., 2005) with some modifications. Collected planktonic cells were washed three times with saline and centrifuged for 5 minutes at $3270 \times g$. The suspensions were serially diluted and plated to confirm initial concentration (before exposure) of approximately 10^8 CFUs/mL. Approximately 10^6 CFUs were then mixed with CHG, IPA, or CHG/IPA for 30 seconds (simulating donor skin disinfection practices followed by Canadian Blood Services). The activity of the disinfectants

was neutralized by addition of 9.9 mL of neutralizer (0.5% lecithin, 4% Tween 20) to 100 uL of the bacteria-disinfectant mix. After 5 minutes of neutralization, the solution was centrifuged (3270 × *g* for 10 min). The pellet, suspended in 1 mL of the supernatant, was plated to determine viable cell counts (post exposure values). Each disinfectant assay for each bacterial species was repeated in duplicate three independent times.

Eradication of biofilm cells. Mono- or dual-biofilms, attached to the bottom of the wells of the six-well plates, were washed three times with saline solution to remove residual planktonic cells. Washed biofilms were exposed to either sterile dH_2O (negative control), CHG, IPA, or combined CHG/IPA for 30 seconds. The disinfectants were neutralized for 5 minutes. After neutralization, biofilm cells were scraped, collected, and vigorously mixed in saline solution followed by plating for colony counts determination. The two strains used in this study were distinguishable by colony color, *S. capitis* colonies are yellow whereas *S. epidermidis* colonies are white. Each assay was repeated at least four independent times.

VI. Growth of disinfectant-treated biofilms in PCs

Scraped biofilms obtained after treatment with all CHG, IPA, and CHG/IPA were inoculated into PC units. After 20 minutes of PLT agitation, 8 to 10 mL of PCs was inoculated into BacT/ALERT blood products aerobic bottles followed by incubation in the BacT/ALERT 3D system to assess survival to the disinfectant treatment. Spiked PC units were also sampled at Days 0 and 5 for bacterial quantification by serial dilution and colony counting on TSA. *S. epidermidis* and *S. capitis* survival from the single-specie biofilms was tested using three PC units for each organism per treatment. Dual-species biofilms were assayed in at least five PC units for each treatment.

VII. Scanning electron microscopy

S. epidermidis and *S. capitis* biofilm cells were examined before and after exposure to disinfectants. Biofilms were grown on six-well polystyrene plates and 1-cm^2 coupons were cut from the bottom of the wells. The coupons were then fixed in 0.1 mol/L cacodylate ($Na(CH3)_2AsO_2 \cdot 3H_2O$ (Sigma-Aldrich, Inc., St Louis, MO) containing 2.5% glutaraldehyde (J.B. EM Services, Inc., Dorval, Quebec, Canada) and stored at 4°C overnight. The coupons were then dehydrated in an ethanol gradient (20, 50, 70, 95, or 100%) followed by critical-point drying (Biodynamic Research Corp., San Antonio, TX). The dried coupons were coated with a 20 nm gold layer with a sputter coater and photographed using a microscope (XL30 ESEM, Philips, Eindhoven, the Netherlands) operated at an accelerating voltage of 7.5 kV and spot size 2.

VIII. Statistical analysis

Log transformation (base 10) was performed for the number of CFUs to obtain a better property for the statistical comparison. The efficiency of disinfectants was expressed as relative reduction (%), which was calculated by subtracting the log of viable cells in treated cultures from the log of viable cells in the control (untreated) cultures. Mixed-model analysis was performed to test differences in efficacy between disinfectants, between species, and between biofilm and planktonic cells. For the adjustment of multiple comparisons, Scheffe's method was applied. The t test (paired) was used to compare bacterial proliferation rates between Day 0 and Day 5 in PCs. A p value of not more than 0.05 was considered to be significant and all the analyses were performed with computer software (Statistical Analysis System, SAS, 2000-2004, SAS Institute, Inc., Cary, NC).

2.5 RESULTS

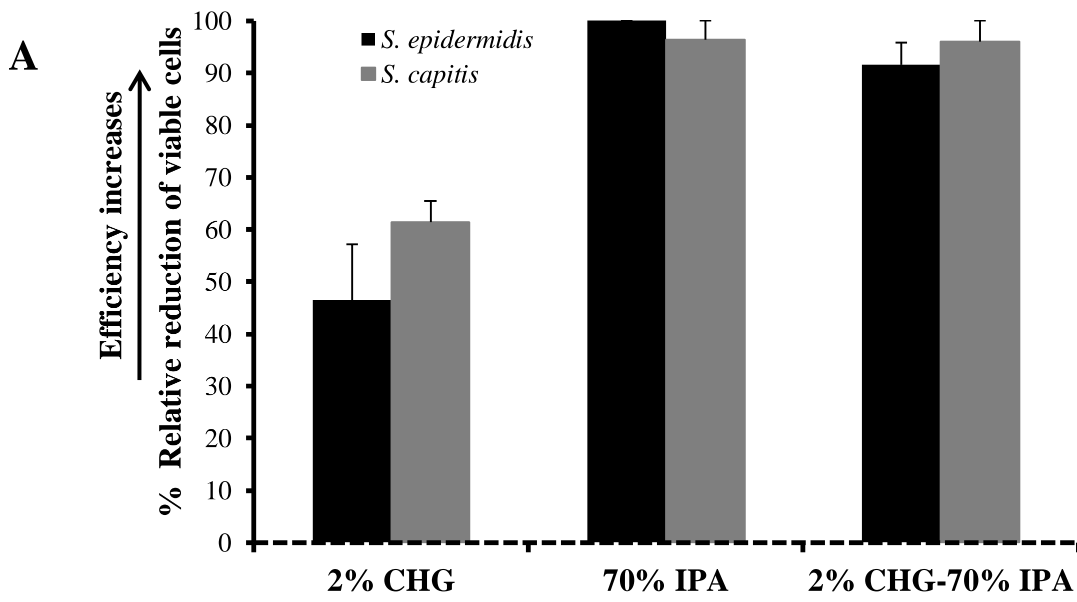
I. Staphylococcal biofilms are more resistant than their planktonic counterparts to the bactericidal action of blood donor skin disinfectants

Biofilms of both organisms, *S. epidermidis* and *S. capitis*, were at least 64-fold more resistant to CHG than their planktonic counterparts with MIC and MBEC values of 2 and more than 128 ug/mL, respectively. These data were confirmed using eradication assays. The viability of planktonic *S. epidermidis* and *S. capitis* after treatment (after exposure) with CHG, IPA, and the CHG/IPA combination was significantly reduced ($p < 0.01$) in comparison to their initial counts (before exposure). Figure 2.1A shows that IPA alone and IPA combined with CHG reduced the viability of planktonic *S. epidermidis* and *S. capitis* very efficiently (>90%) while the treatment with CHG alone eradicated 50% to 60% of bacteria. Viability of single-species biofilms of *S. epidermidis* and *S. capitis* treated with all disinfectants was also significantly reduced in comparison to the negative control ($p = 0.000$). However, biofilm cells were more resistant to disinfectant treatment than their planktonic counterparts resulting in lower reduction rates of viable cells: 30% to 40% with CHG, 40% to 50% with IPA, and 60% to 80% with CHG/IPA (Fig. 2.1 B).

II. IPA is more effective against *S. epidermidis* biofilms than *S. capitis* biofilms

Interestingly, IPA alone or in combination with CHG was highly effective in reducing the viability of planktonic cells of both *S. epidermidis* and *S. capitis* (Fig 2.1 A). However, the effect of IPA on the viability of staphylococcal biofilms varied with the species and presence of CHG. While IPA and CHG had a comparable bactericidal effect against *S. capitis* biofilms (30%-40% reduction, respectively; Fig 2.1 B), IPA was significantly more effective in

A



B

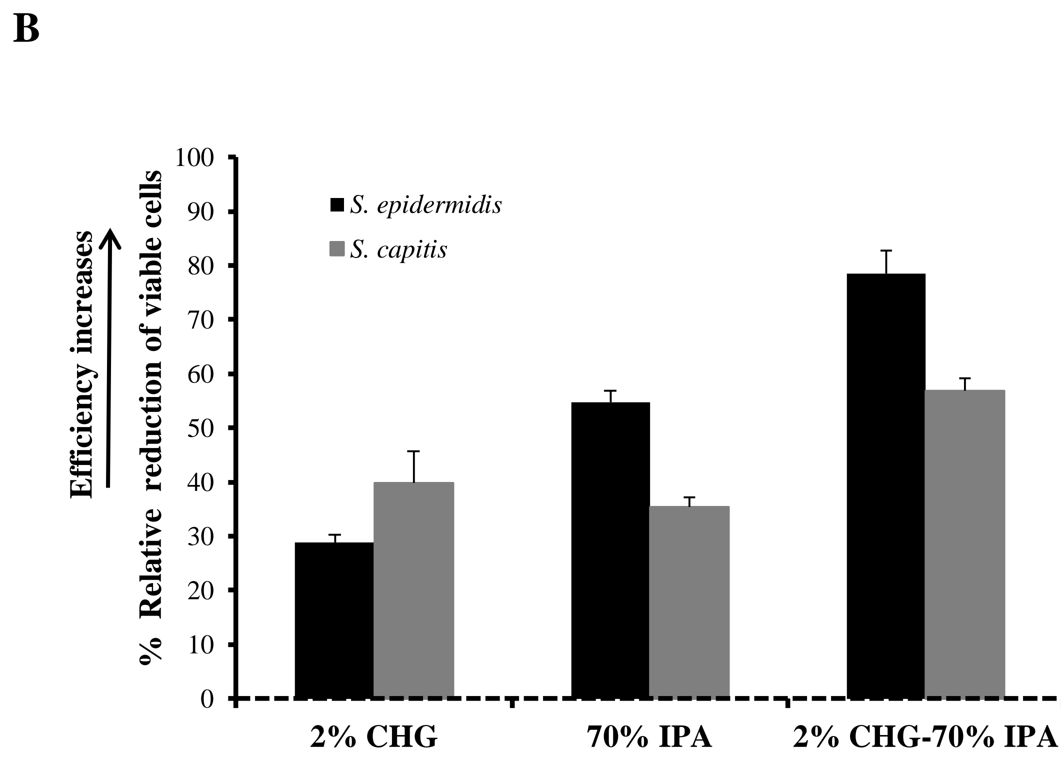


Figure 2.1. Efficacy CHG, IPA, and CHG/IPA against planktonic (A) and biofilm (B) cells of *S. epidermidis* 9142 (■) and *S. capitis* CBS517 (■) after exposure for 30 seconds (relative to the pre-exposure reduction value of 0%, dotted line). N = 3, ±SE.

eliminating *S. epidermidis* biofilms than CHG ($p = 0.000$, Fig. 2.1 B). The deleterious effect of IPA on *S. epidermidis* biofilms was also observed when in combination with CHG. *S. capitis* biofilms were significantly less sensitive to the action of CHG/IPA than *S. epidermidis* biofilms ($p = 0.012$, Fig 2.1 B). This could be related to an apparent greater cell damage due to the action of IPA on *S. epidermidis* biofilm cells (Fig. 2.2 B) than in *S. capitis* biofilms (Fig. 2.2 D). While control biofilms (treated with water) showed a smooth cell surface (Figs 2.2 A and 2.2 C), samples treated with IPA displayed lysed and collapsed cells (Figs 2.2 B and 2.2 D). Notably, *S. epidermidis* biofilms were denser and therefore estimation of cell damage by just scanning electron microscopy (SEM) could be misleading due to different biofilm architecture of the two bacteria. The SEM results should be analyzed in junction with viable cells counts (Fig 2.1 B).

III. Disinfectant efficacy changes are observed in mono- versus dual-species biofilms

The skin microbiota is a community of cohabiting organisms, and it is therefore expected that microbial interactions affect their responses to the external stress posed by skin disinfection. To evaluate this concept, dual- versus monospecies biofilms were used as model growth systems. Results of these experiments showed that the sensitivity of *S. epidermidis* to CHG was slightly increased when grown in dual-species biofilms versus monospecies biofilms, but its sensitivity to IPA or CHG/IPA was not affected. In contrast, when present in dual-species biofilms, *S. capitis* became more sensitive to the action of all disinfectants than when grown in monospecies biofilms (Fig 2.3).

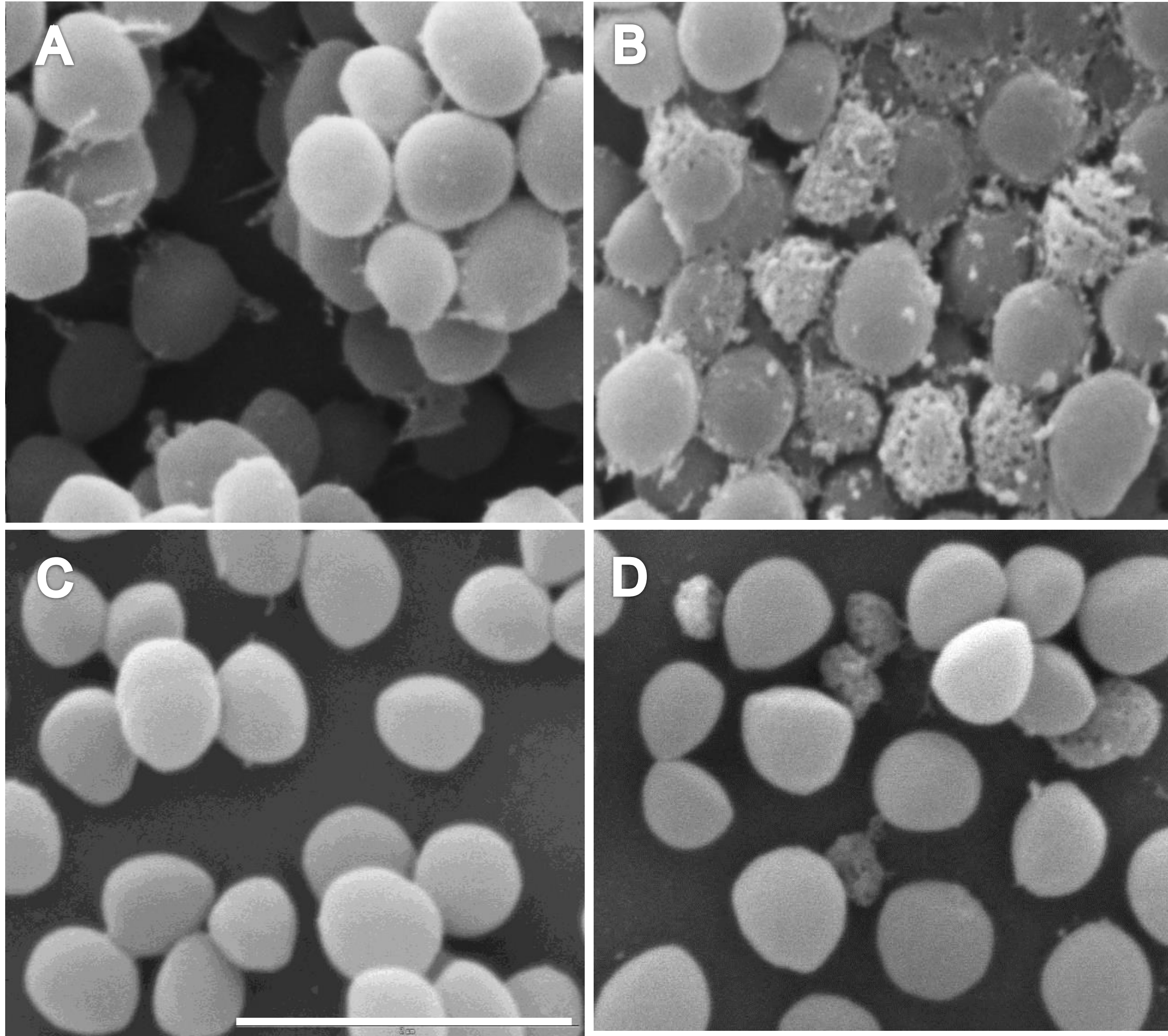


Figure 2.2. SEM images of *S. epidermidis* 9142 and *S. capitis* CBS517 control biofilm cells. (A and C, respectively) and cells treated with IPA (B and D, correspondingly) at a magnification of 14,000×. Scale bar = 2 um. These images are representatives of several microscopic fields. Control biofilms (treated with water) showed normal structure and smooth cell surface (A and C) whereas bacteria treated with IPA displayed bulged, lysed, and collapsed cells (B and D).

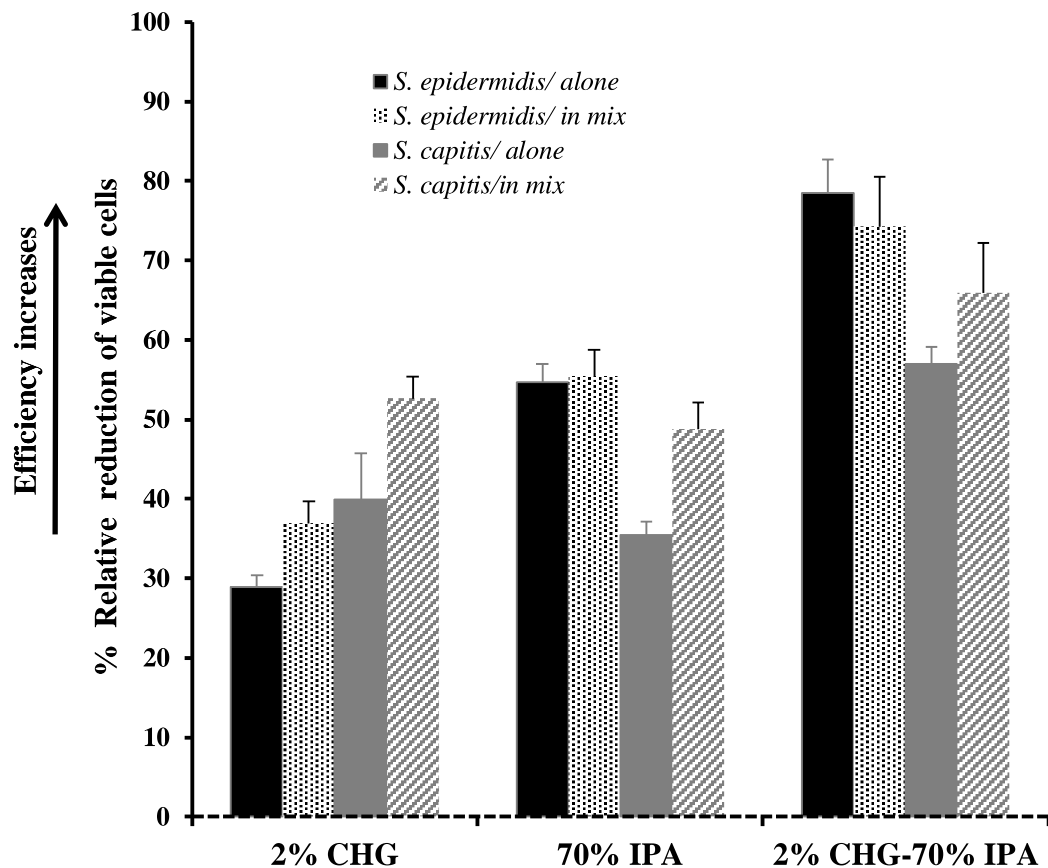


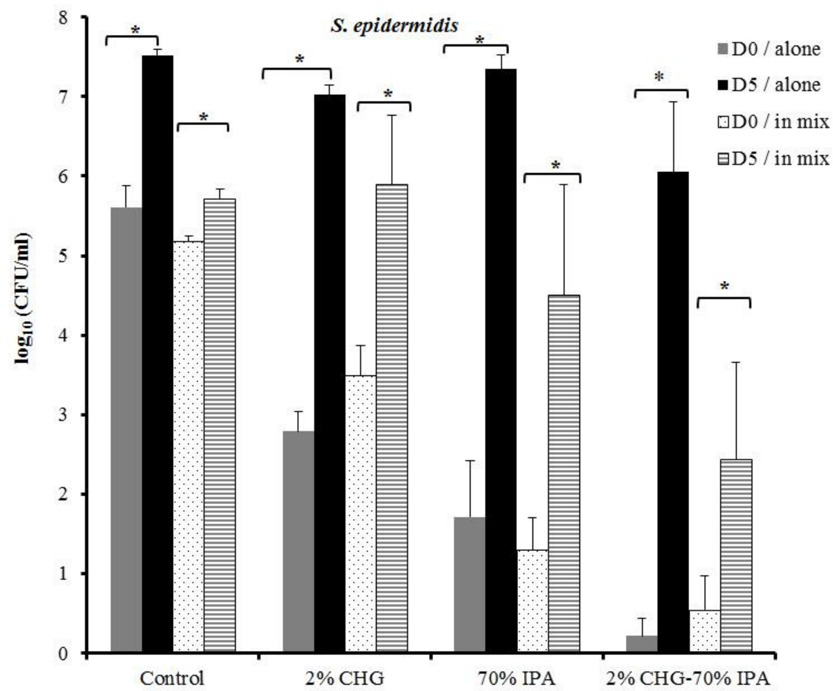
Figure 2.3. Efficacy of CHG, IPA, and CHG/IPA against biofilm cells of *S. epidermidis* 9142 and *S. capitis* CBS517 on monospecies and dual-species biofilms after exposure for 30 seconds (relative to the control value of 0%, dotted line). N ≥ 4, ±SE.

IV. Disinfectant-treated staphylococcal biofilms recover and proliferate when grown in PCs

The ability of biofilm cells that escaped eradication by CHG, IPA, and CHG/IPA to survive in PCs was also evaluated. Figure 2.4 A shows that *S. epidermidis* cells, which had initially been treated with the disinfectants, recovered rapidly when allowed to grow in PCs and reached concentrations of up to 1×10^6 CFUs/mL after 5 days of incubation. Although *S. capitis* cells that survived disinfectant treatment were also able to grow in PCs, their recovery was not as efficient as that demonstrated by *S. epidermidis,* since treated biofilm cells of this organism only reached concentrations of 10^3 to 10^5 CFUs/mL at the end of storage (Fig. 2.4 B). Notably, IPA-treated *S. capitis* biofilms were less efficient in recovering in PCs than cells treated with the other disinfectants, CHG and IPA/CHG (Fig. 2.4 B).

The growth dynamics of disinfectant-treated *S. epidermidis* and *S. capitis* changed when grown in dual-species biofilms. While monospecies biofilms, treated with all disinfectants, were efficiently able to proliferate in PCs, this capacity was decreased when grown in dual-species biofilms (Fig 2.4). As noted with monospecies biofilms, *S. capitis* was less efficient than *S. epidermidis* in recovering and growing in PCs when present in mixed biofilms. Interestingly, *S. capitis* treated with CHG remained viable but did not proliferate while biofilms treated with IPA and CHG/IPA did show a significant increase in concentration by Day 5 of storage (Fig. 2.4 B).

A.



B.

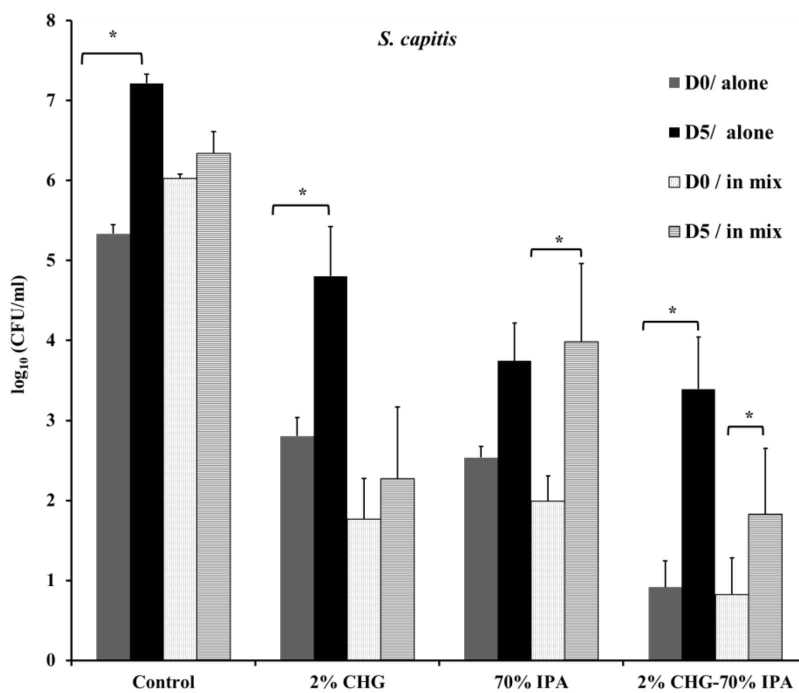


Figure 2.4. Viability of mono- and dual-species biofilms of *S. epidermidis* 9142 (A) and *S. capitis* CBS517 (B) in PCs after treatment with CHG, IPA, and CHG/IPA. Day 0 (D0) = counts on the day of spiking; Day 5 (D5), counts after 5 days of incubation under PLT storage conditions. *Significant difference between D0 and D5 ($p < 0.05$). $N \geq 5$, ±SE.

2.6 DISCUSSION

In this study we have demonstrated that biofilm-forming skin microflora bacteria, represented by *S. epidermidis* and *S. capitis*, have increased resistance to the skin disinfectants CHG and IPA used during blood donations worldwide. The results obtained herein show that the most effective disinfectant against biofilms of both organisms was the combination CHG/IPA. Both CHG and IPA have a broad spectrum of action and act mainly by disrupting cell membrane and precipitating proteins (McDonnell and Russell, 1999).While CHG remains on the skin for up to 48 hours, its activity is pH dependent and is reduced in the presence of organic matter. In contrast, IPA evaporates quickly once in contact with the skin. It is therefore known that a CHG/IPA solution is more effective than each disinfectant alone due to a longer duration and action in contact with skin, blood, and body fluids (CDC, 2008).

Although CHG/IPA effectively decreased the viability of staphylococcal biofilms and disrupted their cellular morphology, it did not eliminate the microorganisms completely. It is known that biofilms present up to 1000-fold increased antimicrobial resistance than their planktonic counterparts (Mah and O'Toole, 2001). The increased resistance can be linked to the biofilm matrix composed of extracellular materials that cover mature biofilms, which prevents antibiotics and disinfectants penetration by forming physical or chemical diffusion barriers (McDonnell and Russell, 1999; Mah and O'Toole, 2001). Genotypic variation within the biofilm and/or production of neutralizing enzymes or physiological changes, including pH gradient, have been documented to protect against the action of antimicrobial agents (McDonnell and Russell, 1999). Although increased resistance of *S. epidermidis* biofilms to CHG/IPA has been reported by Adams and his group, (Adams et al., 2005) to our knowledge, no similar data has been reported for *S. capitis*.

Biofilm cells of *S. epidermidis* and *S. capitis* were found to be sensitive to the three disinfectants tested. There were differences, however, between the sensitivities of the two species to the various disinfectants. *S. epidermidis* biofilms were more sensitive to the actions of IPA and CHG/IPA than *S. capitis*, while the latter showed increased sensitivity of CHG alone. This might be due to the differences in the biofilm architectures and/or composition of the biofilm matrices in the two organisms. We have recently demonstrated that while the *S. epidermidis* matrix is mainly composed of polysaccharides, proteins are a major component of *S. capitis* biofilms (Greco-Stewart et al., 2013). Other factors such as hydrophobic properties and/or the presence of DNA in the biofilm matrix could also affect the penetration properties of each of the disinfectants in a different manner for each organism. Since it has been shown that microflora bacteria exist as biofilms in the deeper layers of the skin, the action of the disinfectants could be improved if the penetration of the antimicrobials is enhanced. It has been shown that eucalyptus oil increases CHG skin penetration (Karpanen et al., 2010). Further research is needed to test natural oils or other components that increase CHG penetration into the skin, which would result in reduced number of bacteria being introduced during venipuncture.

We studied dual-species biofilms because most microorganisms cohabit with multiple species in natural niches. Studying mixed staphylococcal biofilms is not easy due to their similarities in growth and morphology; however, we had the advantage of using a strain of *S. capitis* that grows as yellow colonies allowing for differentiation from the white colonies of *S. epidermidis*. Data obtained in this study show that *S. capitis* has reduced capacity to recover and proliferate in the PLT storage environment compared to *S. epidermidis* (Fig. 2.4). These results correlate well with surveillance data obtained during PLT screening for bacterial contamination, which shows *S. epidermidis* as the predominant PLT contaminant (Palavecino et

al., 2010; Jacobs et al., 2008; Greco et al., 2008). The advantage that *S. epidermidis* has over other staphylococci has also been observed in natural environments. Skin microbiome research has recently demonstrated that within the staphylococci group, *S. epidermidis* is the predominant skin organism; other *Staphylococcus* species including *S. capitis, S. hominis*, and *S. sapprohyticus* are also present although in lower numbers (Scharschmidt and Fischbach, 2013). Predominance of *S. epidermidis* might be due to different factors including halotolerance and the production of skin adhesins and proteases (Scharschmidt and Fischbach, 2013). Interestingly, it has been demonstrated that *S. epidermidis* modulates the host skin immune response to its advantage. This bacterium produces phenol-soluble modulins to inhibit skin pathogens, triggers the expression of antimicrobial peptides for which it is resistant, and inhibits skin inflammation (Grice and Segre, 2011).

Within the staphylococci present on human skin, *S. epidermidis* is the most frequently isolated species from contaminated PCs followed by others including *S. capitis*, *S. warneri, S. saccharolyticus, S. hominis, S. pasteuri, S. saprohyticus*, and *S. lugdunensis (*Rood et al., 2011). At present, it is unknown which factor(s) favor *S. epidermidis* growth in PCs over other bacteria, which could be the subject of future investigation.

Microbial interaction in mixed-species biofilms could also affect their sensitivity to antimicrobials. Data presented here showed that *S. capitis* became more sensitive to the action of IPA and CHG/IPA when grown as dual-species biofilms with *S. epidermidis* (Fig. 2.3). This might be due to the antagonist effect that *S. epidermidis* has over other species as described above. This organism may produce a specific compound that makes *S. capitis* weaker and less resistant to disinfectant treatment. Giaouris and colleagues (Giaouris et al., 2013) showed that when *Listeria monocytogenes* was grown in a dual-biofilm with *Pseudomonas putida*, the

resistance of the latter organism to benzalkonium chloride was strongly increased probably due to the production of biosurfactants, which inhibit biofilm formation and promote dispersal of established biofilms. To our knowledge, ours is the first report addressing sensitivity to disinfectants in mixed-species staphylococcal biofilms.

In conclusion, disinfecting the skin of blood donors is a crucial step to decrease blood contamination by resident and transient skin microflora. Special attention should be paid to biofilm forming organisms as it is known that 20% of the skin microflora exist as biofilms (Ryder, 2005). Furthermore, we and others have demonstrated that biofilm-forming *S. epidermidis* are prevalent on the skin of healthy individuals who represent blood donor communities (Greco et al., 2008; Farran et al., 2013). Skin disinfectants composed of the CHG/IPA combination are currently broadly used by blood suppliers for donor skin disinfection during venipuncture. Results from this study showed that the combination of these two antimicrobials is more effective in destroying biofilm cells of *S. epidermidis* and *S. capitis* than each disinfectant alone. Nevertheless, biofilm cells cannot be completely removed meriting investigation to increase the efficacy of these disinfectants or finding antiseptics with better activity. Approaches to improve skin disinfection efficacy should include studying organisms in the biofilm default growth mode and using polymicrobial populations, which represent the true nature of the microbial skin residents. Moreover, enhancing disinfectant penetration into the lower skin layers should be considered to further reduce the levels of skin microflora bacterial contamination of blood products.

2.7 ACKNOWLEDGMENTS

The authors thank staff at Canadian Blood Services sites for PLT collection and shipping. We also acknowledge Ms. Emily Mastronardi (Honours student, Canadian Blood Services) for contributing in obtaining primary data for MBEC experiments. Ms. Heather Perkins and Mr. Yuntong Kou (senior research assistants, Canadian Blood Services) critically revised the manuscript.

CHAPTER 3.

Comparative Characterization Of *S. epidermidis* Isolates Obtained From Human Skin And Contaminated PCs

3.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

The manuscript "Comparative characterization of *S. epidermidis* isolates obtained from human skin and contaminated platelet concentrates" will be submitted for peer-review.

M. Taha conducted all the experiments and data analysis. Dr. S. Ramirez-Arcos was the thesis' supervisor providing guidance on the experimental design, troubleshooting and data interpretation. The Honours students Ms. S. Mallya and Ms. C. Kohnen (University of Ottawa) assisted with crystal violet assays, *icaA* and *icaD* PCR detection and Congo red agar assays for PC and skin isolates, respectively. The research assistants Mr. Y. Kou, Ms. A. Zapata and Ms. H. Perkins (Canadian Blood Services) aied in the isolation and characterization of the skin isolates. Dr. QL. Yi, (Canadian Blood Services) helped with statistical analyses. The *divIVA, icaA* and *icaD* primers were designed by previous members of Dr. Ramirez' team.

3.2 ABSTRACT

The skin flora *S. epidermidis* possess the ability to form biofilms which are present in the skin of healthy blood donors and can subsequently be isolated from contaminated PCs. This study was aimed at exploring the abundance of biofilm-formers in contaminated PCs compared to skin isolates. Furthermore, the potential positive selection of *S. epidermidis* biofilm-formers during donor skin disinfection, blood processing and PCs storage resulting in a higher prevalence of this organism in contaminated PCs was explored. Twenty-four *S. epidermidis* isolates obtained from contaminated PCs at Canadian Blood Services and 48 *S. epidermidis* isolates obtained from the venipuncture area of human skin were compared for their biofilm-formation abilities in laboratory media and the PC milieu. To assess biofilm formation by *S. epidermidis,* a semi-quantitative crystal violet assay, the presence of the biofilm-associated *icaA* and *icaD* genes and slime production on Congo red agar plates were used. Results revealed that skin-derived *S. epidermidis* isolates had a higher abundance (43.7 %) of the biofilm-forming phenotype compared to PC-derived isolates (25%). The prevalence of *ica*-positive biofilm-former isolates was similar in PC and skin isolates (16.6% and 18.7%, respectively), however, the abundance of *ica*-negative biofilm-formers was lower in isolates obtained from contaminated PCs in comparison to skin isolates (8% versus 25%). All the tested skin and PC isolates formed biofilms in the PC storage environment. In this study, it had been shown that biofilm-formers are not being favoured during the skin disinfection, blood processing or PCs storage. However, *ica*-negative biofilm-formers are negatively affected by these processes resulting in a lower abundance of this phenotype among PC contaminants compared to skin isolates. Furthermore, *S. epidermidis* can adopt a biofilm-forming phenotype in PCs regardless of their genetic background or source of origin.

3.3 INTRODUCTION

Staphylococcus spp., which includes *S. epidermidis,* are the most ubiquitous bacteria in moist areas of the human skin including the antecubital fossa (Grice et al., 2009; Grice and, Segre, 2011). *S. epidermidis* is also a major contaminant of PCs and is thought to be introduced into blood collection bags through venipuncture during the donation process (Hong et al., 2016; Walther-Wenke et al., 2010; Corash, 2011). *S. epidermidis* is considered an opportunistic pathogen and plays a major role in nosocomial infections (Otto, 2009; Uckay et al., 2009) and its ability to form biofilms is known to contribute to its virulence profile (Fey and Olson, 2010). Biofilms confer elevated resistance to antibiotics and immune clearance (Vuong et al., 2004c; Kristian et al., 2008; Khardori et al., 1995). A major component of *S. epidermidis* biofilms is the extracellular polysaccharide matrix called polysaccharide intercellular adhesin (PIA). PIA is comprised of poly-beta-1,6-N-acetyl-D-glucosamine and is actively involved in the biofilm aggregation step during biofilm formation. The *icaADBC* operon contains genes that encode for the IcaA and IcaD transferase membrane proteins responsible for the biosynthesis of PIA which is then exported by IcaC and altered by the IcaB de-acetylase protein (Heilmann et al., 1996; Mack et al., 1996). Apart from its contribution to the structural integrity of biofilms, PIA has been reported to play a role in *S. epidermidis* resistance to clearance by polymorphonuclear leucocytes (PMNs), antimicrobial peptides (AMPs) and deposition of C3b of the complement system (Vuong et al., 2004b; Kristian et al., 2008). Though *S. epidermidis ica*-positive isolates are usually associated with biofilm formation, *ica*-negative biofilm-formers have been reported (Rohde et al., 2005; Rohde et al., 2007). Multiple studies have demonstrated that some *ica*-negative biofilm formers have proteinaceous matrices (Hussain et al., 1997; Tormo et al., 2005;

Los et al., 2010; Christner et al., 2010), and that proteins such as the accumulation-associated protein (Aap), the biofilm-associated protein (Bap), and the extracellular matrix-binding protein (Embp) have been identified as playing a pivotal role in the aggregation step during biofilm formation. Furthermore, teichoic acids and extracellular DNA (eDNA) have also been reported to be involved in the accumulation process during biofilm formation of some *S. epidermidis* strains (Otto, 2009; Qin et al., 2007a,b; Sadovskaya et al., 2005; Tormo et al., 2005; Christner et al., 2010). *S. epidermidis* resides in human skin forming biofilms (Costerton, 2007; Rogers et al., 2008; Galdbart et al., 2000). The Ramirez' laboratory has previously reported that *S. epidermidis* recovered from PCs demonstrate biofilm forming capabilities in classic laboratory media (Greco et al., 2007; Greco et al., 2008; Ali et al., 2014). Interestingly, *S. epidermidis* recovered from PCs were capable of forming biofilms under PC storage conditions, despite the fact that some isolates lacked the *ica* genes and were incapable of forming biofilms in classic laboratory medium (Greco, 2011; Ali et al., 2014). The exact mechanism by which this occurs is yet to be elucidated.

Studies have demonstrated that there are phenotypic and genotypic differences between commensal isolates of *S. epidermidis* and isolates obtained from sites of infection (Galdbart et al., 2000; Ziebuhr et al., 1997). In particular, it was demonstrated that the presence of a biofilm-formation phenotype and the *ica* operon could serve as markers to differentiate between *S. epidermidis* isolated from normal human skin and *S. epidermidis* isolated from clinical samples. This suggests that an infectious environment may be exerting selection pressure to favor biofilm-former *S. epidermidis* isolates (Galdbart et al., 2000; Ziebuhr et al., 1997; Frebourg et al., 2000).

The goal of this chapter is to compare the prevalence of biofilm-formers between 24 *S. epidermidis* isolates obtained from contaminated PCs at Canadian Blood Services and 48 *S.*

epidermidis isolates acquired from the antecubital area of human skin (strains that have not been in contact with blood products). This study also explored the possibility that the source of isolates and their genetic background could affect the ability of the strains to form biofilms in the PC milieu. To that end, the objectives defined in this chapter are i) to obtain *S. epidermidis* isolates directly from human skin and ii) to compare the biofilm genotypic and phenotypic characteristics (in classic laboratory media and in PCs) of *S. epidermidis* isolated from human skin and contaminated PCs.

3.4 MATERIALS AND METHODS

I. Platelet concentrates (PCs)

Aph-PCs or BC-PCs were used for biofilm formation assays. PCs were collected and processed at the Canadian Blood Services Network Centre for Applied Development (netCAD; Vancouver, BC) according to Canadian Blood Services' procedures (Levin et al., 2008). All PCs were obtained from consenting healthy volunteers. The research design was approved by the Canadian Blood Services Research Ethics Board. Sterility testing was performed using BacT/ALERT BPA and BPN culture bottles as described before (Mastronardi et al., 2012).

II. Bacterial strains

PC contaminant isolates. Twenty-four *S. epidermidis* isolates obtained from either contaminated BC-PCs or Aph-PCs at Canadian Blood Services over a 5-year period (2008-2013) and detected by the BacT/ALERT system (Appendix A.1) were used in this chapter.

Human skin isolates. Forty-eight isolates of *S. epidermidis* were obtained from the antecubital fossa area of 48 healthy volunteers. Briefly, over 130 volunteers were screened for *S. epidermidis* isolation where 65 mm diameter contact plates (Oxoid Inc., Nepean, ON, Canada or

BBL D/E Neutralizing Agar; BD, Sparks, MD, USA) were applied to the antecubital fossa for 30 seconds and then the plates were left to incubate at 37°C overnight. White smooth and elevated colonies were selected to be subcultured on mannitol salt agar (MSA) plates along with the control *S. epidermidis* ATCC 12228 to isolate non-mannitol fermenters. MSA selectively supports the isolation of *Staphylococcus* and *Micrococcus*. Any white, red or pink colonies (non-mannitol fermenters) were further subcultured on TSA to perform Gram staining and oxidase test (positive and negative controls were *Pseudomonas* aeruginosa ATCC 27853 and *E. coli* ATCC 25922, respectively). Frozen stocks were prepared for isolates that were non-mannitol fermenters, oxidase negative and Gram positive cocci in clusters. Oxidase test helped to exclude *Micrococcus* spp which are oxidase positive. Pure colonies were added to BHI (Brain-heart infusion) with 15% glycerol and stored at -83°C.

S. epidermidis isolates were identified by PCR amplification of the cell division *divIVA* gene, which allows the differentiation of this species from other CoNS, an assay previously optimized in the Ramirez' laboratory (Mastronardi and Ramirez-Arcos, 2007). Each PCR reaction (50 uL) consisted of 5 uL of bacterial cell suspension, 31.75 uL of sterile ddH_2O, 10 uL 5X PCR buffer containing 15 mM $MgCl_2$ (OneTaq® Buffer,New England BioLabs Inc., Ipswich, MA), 1 uL dNTP mix (10 mM of each dNTP; New England Biolabs, Ipswich, MA), 1 uL of each primer (0.2 ug/mL) (Appendix 3.3), and 0.25 uL of One Taq® DNA polymerase (5,000 U/mL; New England BioLabs, Ipswich, MA). The bacterial suspensions were prepared by inoculating ddH_2O with bacterial colonies at about 10^7 CFU/mL (0.5 McFarland turbidity standard; Hardy Diagnostics, Santa Maria, CA). *S. epidermidis* ATCC 12228 was used as a positive control for *divIVA* screening while *S. aureus* ATCC 27664 served as negative control. The Analytical Profile Index test (API Staph, bioMérieux, Marcy l'Etoile, France) was performed on the *divIVA*

positive isolates to confirm their identity as *S. epidermidis* with a cut-off value of > 85% identification accuracy (Renneberg et al., 1995).

III. Presence of the *icaA* and *icaD* genes

The *ica* primers are listed in Appendix A.2 and were designed using the genome sequence information of *S. epidermidis* strain ATCC 35984 (GeneBank Accession No. NC 002976) and synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA). A reaction to a final volume of 22.5 uL was prepared for each of the *icaA* and *icaD* genes: 16.4 uL of sterile ddH_2O, 5 µL OneTaq® Buffer (5X) (New England BioLabs Inc., Ipswich, MA), 0.5 uL dNTP mix (10 mM of each dNTP; New England Biolabs Inc., Ipswich, MA), 0.25 uL of forward primer, 0.25 uL reverse primer, 0.125 uL OneTaq® DNA polymerase (5,000 U/mL; New England BioLabs, Ipswich, MA). Bacterial suspension of 2.5 uL was added to each 22.5 uL individual aliquot reaction. Bacterial suspensions were prepared as mentioned above. For *icaA, S. epidermidis* Hamburg 9142 served as the positive control strain, while *S. epidermidis* Hamburg 9142 Δ*icaA* served as the negative control strain. As for *icaD*, the strains 9142 and 9142 Δ*icaA* served as the positive controls and ATCC 12228 was the negative control. The PCR products were run on a 1% agaraose gel in 1X tris-acetate-EDTA buffer with drops of 0.625 mg/mL EtBr at 100 mV. Along with all the tested samples and the controls, a molecular weight marker (1kb Plus DNA ladder, New England BioLabs Inc., Ipswich, MA) was included. MultiImage™ Light Cabinet and AlphaImager™ 2200 software (Fisher Scientific, St-Laurent, Quebec) was used to visualize and capture resulting images.

IV. Slime (PIA) formation

Congo red agar plates were prepared with sterile BHI agar and then a mixture of filter-sterilized (0.2 nm pore filter) Congo red stain (Sigma-Aldrich Canada Ltd., Oakville, Canada) and

saccharose (Sigma-Aldrich Canada Ltd., Oakville, Canada) in ddH_2O was added to final concentrations of 0.8 g/L and 36 g/L, respectively. To test for slime production (Freeman et al., 1989), *S. epidermidis* isolates were streaked on Congo red agar plates and left to incubate for approximately 40 hours at 37 ^oC. Each strain was streaked at least two independent times. Slime-producing isolates appear as black, dry, crusty colonies as a result of hyperpigmentation due to the interaction of Congo red dye with the extracellular polysaccharide matrix, while slime-negative isolates result on pink, creamy colonies. *S. epidermidis* Hamburg 9142 and Hamburg 9142 Δ*icaA* served as the positive and negative controls, respectively.

V. Biofilm formation

In media. One colony of *S. epidermidis* was added to 3 mL of TSB and incubated for 17-18 hours at 37 ^oC, the liquid culture was then adjusted to OD_{600} = 0.1 in TSB supplemented with 0.5% glucose (TSBg), which approximately corresponds to 1.0 x10^7 CFU/mL. The wells of polysterene 6-well or 12-well TC plates (Falcon, Corning Inc., Durham, NC) were inoculated with 3 mL of the bacterial suspension, and 2 wells per plate were filled with sterile TSBg to serve as background. The plates were incubated statically at 37 ^oC for 24 hours to allow for biofilm formation. The supernatant was then removed and the wells were washed 3 times with 3 mL of 1X PBS (pH 7.4) to remove any free-floating cells. Three mL /well of Gram crystal violet solution (BD Biosciences, MD, USA) was then added to all the wells and incubated at room temperature for 30 minutes. The crystal violet dye was removed and the wells were rinsed with 1X PBS. The biofilms were destained using 3 mL/well of 80% ethanol: 20% acetone (vol:vol) and incubated at room temperature for 15 minutes on the orbital shaker at ~120 rpm. Two-hundred uL of the eluted crystal violet solution from each well was added to 6 wells of a 96-well plate (Falcon, Corning Inc., Durham, NC) and the intensity of the purple color was measured by

quantifying the absorbance at a wavelength of 492 nm using a plate reader (Expert Plus microplate reader, Biotech, Montreal, Canada). The blank wells (TSBg only) were processed identically to the test wells. For each strain, the crystal violet assay was repeated at least two independent times in duplicate. The biofilm former strain of *S. epidermidis* Hamburg 9142 was used as the positive control and its isogenic non-biofilm former strain Hamburg 9142 Δ*icaA* was used as the negative control (Marck et al., 1992).

In PCs. Biofilm formation assays in polystyrene plates were performed as described above with the exception that PCs were utilized as the bacterial culture media instead of TSBg. The plates were incubated for 5 days under PCs storage conditions at 22± 2°C with agitation for 120 hours. Although all 48 skin isolates were tested in PCs, only nine out of the 24 PC isolates were tested for biofilm formation in PCs due to limited PC availability at the time of the study development. Six of these nine isolates were biofilm negative in TSBg. All assays were performed at least three independent times (different PC units) in duplicate. *S. epidermidis* Hamburg 9142 was used as the positive control. Since all the tested *S. epidermidis* (including Hamburg 9142 Δ*icaA*) adopted a biofilm-positive phenotype in PCs, results of biofilm formation in PCs were compared to those of the negative control (*S. epidermidis* Hamburg 9142 Δ*icaA*) in TSBg.

VI. Statistical analyses

The final absorbance reading for each test well was corrected by subtracting the average absorbance value of the blank wells (only TSBg or PCs) from the absorbance value of each test well. To assess the biofilm formation abilities of the *S. epidermidis* skin-derived and PC-derived isolates, the average corrected absorbance at 492 nm of each isolate in TSBg or PCs was compared to three standard deviations above the average corrected absorbance of the negative control (*S. epidermidis* Hamburg 9142 Δ*icaA*) in TSBg (OD-neg). Any isolate with an average

corrected absorbance value greater than the cut-off OD-neg value was considered biofilm-positive (Stepanović et al., 2007; Fox et al., 2005; Mulder et al., 1998). Chi-square test was used to compare biofilm formation prevalence between skin-derived and PC-derived isolates. To compare the abundance of *ica*-positive and *ica*-negative biofilm-former isolates from the same environment (skin or PCs), binomial test was used and comparisons between different environments (skin versus PCs) were performed using a Fisher's exact test. A p-value of <0.05 was considered statistically significant. Analyses were performed with the Statistical Analysis System (SAS, 2000-2004, SAS Institute, Inc., Cary, NC).

3.5 RESULTS

I. Forty eight *S. epidermidis* isolates were obtained from the antecubital area of the skin of healthy volunteers

Figure 3.1 represent a gel electrophoresis of the PCR amplification of *S. epidermidis divIVA* from a subset of skin isolates. A total of 48 skin-derived isolates possessed the *divIVA* gene and their identity was confirmed by biochemical profiling using the API Staph kit.

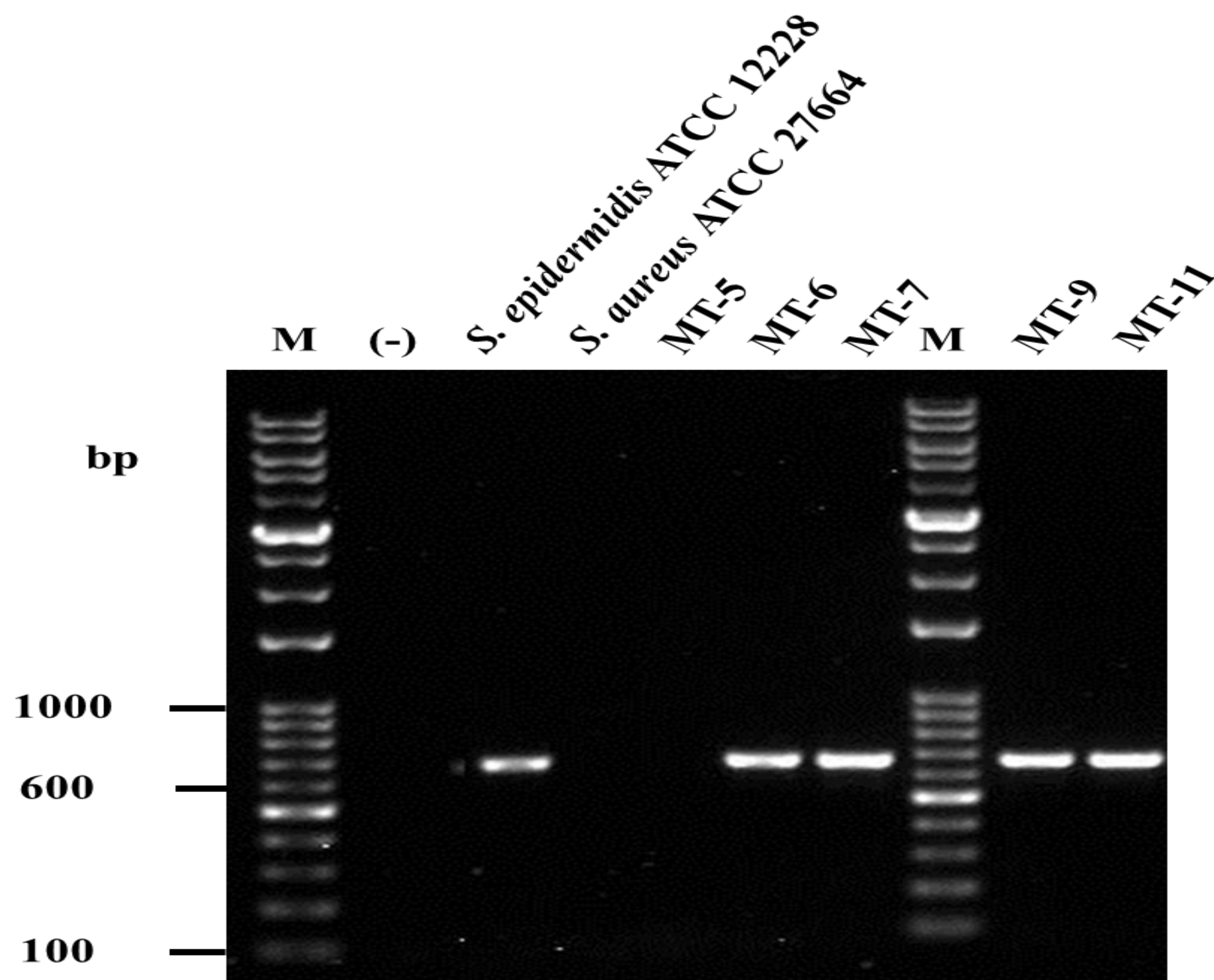


Figure 3.1. Gel electrophoresis of PCR to detect *S. epidermidis divIVA* in skin isolates (MT-5, MT-6, MT-7, MT-9, MT-11). M, molecular weight marker; (-) negative control (no DNA template), *S. epidermidis* ATCC 12228 is the positive-*divIVA* control and *S. aureus* ATCC 27664 is the *divIVA* negative control. The expected size of the *S. epidermidis divIVA* fragment is 656 base pairs (bp). The represented isolates are a subset of the 48 skin-derived *S. epidermidis* isolates where all the isolates possessed *divIV* gene.

II. *ica*-positive and *ica*-negative biofilm-forming *S. epidermidis* were isolated from human skin

Biofilm formation abilities of 48 skin isolates of *S. epidermidis* in TSBg and PCs were assessed using crystal violet assay. Also, the presence of the *icaA* and *icaD* genes was studied using PCR. Results indicate that 21 out of the 48 screened isolates (43.7%) were able to form biofilms in TSBg (Fig. 3.2). Out of the 48 isolates, 12 (25%) were biofilm-positive but *ica*-negative while nine (18.7%) were biofilm formers and *ica*-positive ($p > 0.05$) (Table 3.1, Fig. 3.3). When only the 21 biofilm formers were considered, the ratio of *ica*-negative to *ica*-positive was approximately 1.3:1.

III. PC storage triggers the phenotypic conversion of biofilm-negative to biofilm-positive in *S. epidermidis* skin isolates

All *S. epidermidis* skin isolates of (48/48, 100%) formed biofilms in PCs (Fig 3.2) regardless of their slime production properties and the presence of the *ica* genes and all biofilm-forming isolates in TSBg maintained their phenotype when assayed in PCs. As every biological replicate in the biofilm formation assays in PCs was performed in a different PC unit, the results obtained herein show large standard deviation error bars owing to donor variation. A large variation in the properties of PCs collected from healthy individuals has been reported (Kunicki and Nugent, 2010).

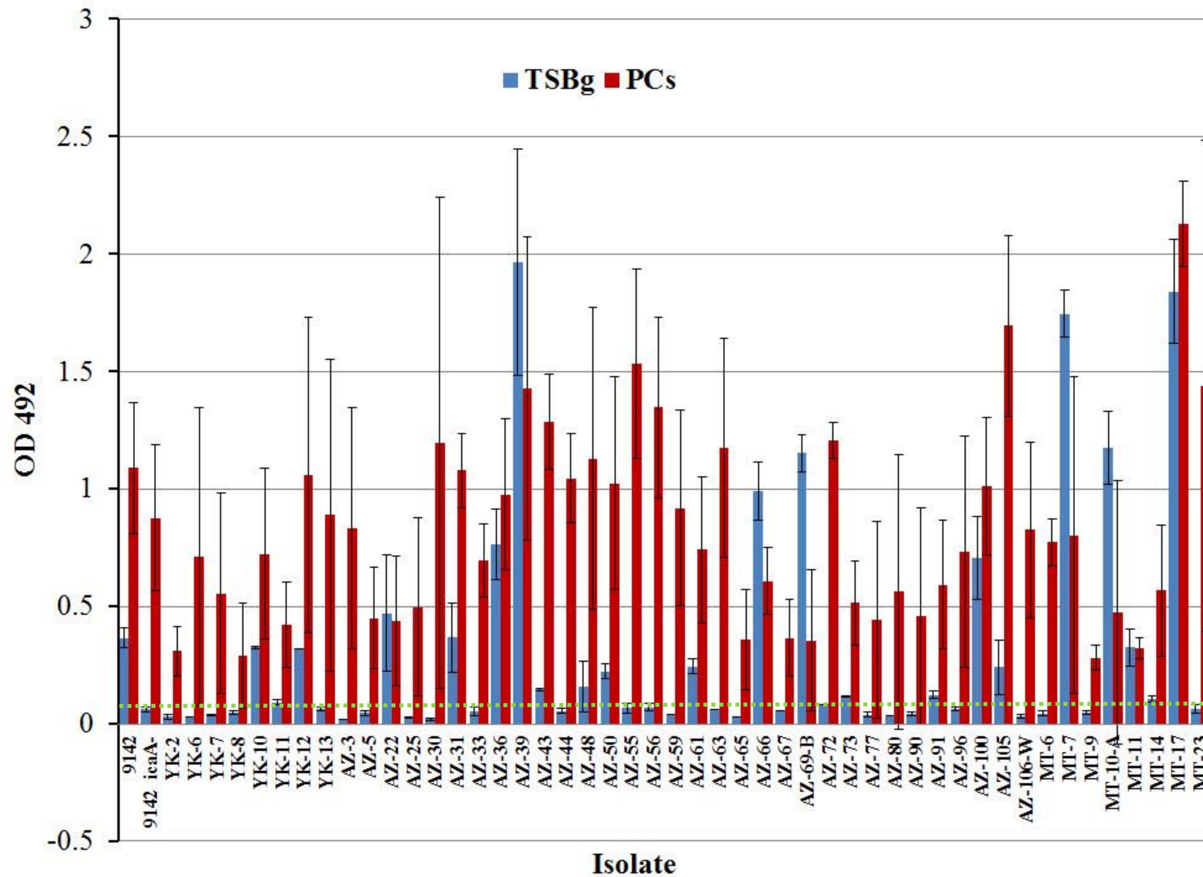


Figure 3.2. Biofilm formation abilities in TSBg and PCs of 48 skin-derived *S. epidermidis* isolates evaluated using a semi-quantitative crystal violet assay. Mean absorbance at wavelength 492 nm ±SD. *S. epidermidis* Hamburg 9142 is the positive control and *S. epidermidis* Hamburg 9142 *icaA*- (Δ *icaA*) is the negative control. Biofilms were allowed to grow statically at 37 °C for 24 hours for the TSBg experiments (two or more independent experiments and each was performed in duplicate), and at 22± 2 °C for 5 days with agitation for the PC experiments (three or more independent experiments and each was performed in duplicate). The dashed green line represents the cut-off value of the negative control *S. epidermidis* Hamburg 9142 *icaA*- (Δ *icaA*) in TSBg.

Table 3.1. Phenotypic and genotypic biofilm-formation abilities of 48 *S. epidermidis* isolates obtained from the antecubital fossa area of human skin.

Number of skin isolates	**Presence of *ica* genes:**		**Slime***	**Biofilm formation**	
	icaA	***icaD***		**TSBg**	**PCs**
27	No	No	No	No	**Yes**
12	No	No	No	**Yes**	**Yes**
9	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Controls					
Hamburg 9142	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Hamburg 9142 Δ *icaA*	No	No	No	No	**Yes**

*Slime production was assessed by Congo red agar assay.

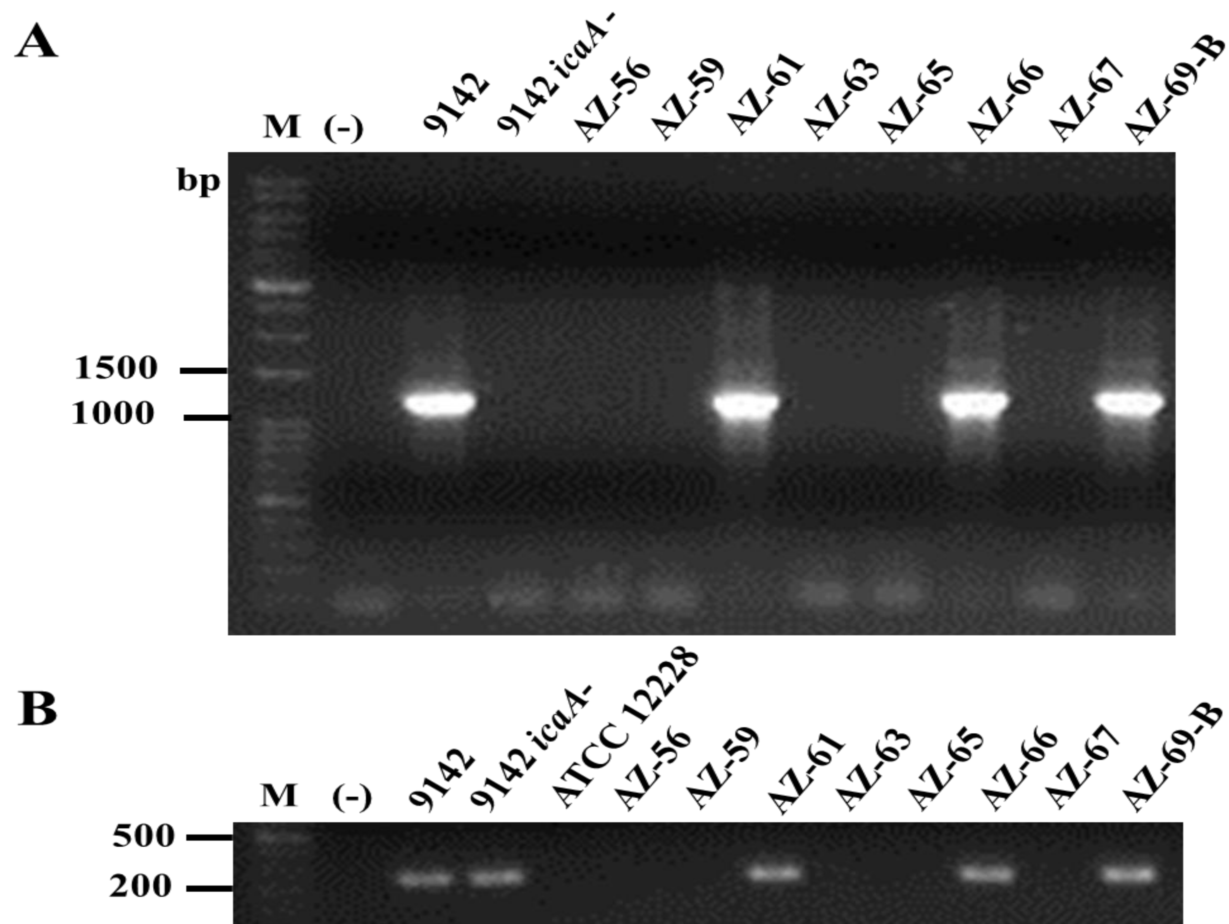


Figure 3.3. Gel electrophoresis of PCR to detect *icaA* (A) and *icaD* (B) in *S. epidermidis* (AZ-56,AZ-59,AZ-61,AZ-65,AZ-66,AZ-67,AZ-69-B). M, molecular weight marker; (-) negative control (no DNA template); (A) *S. epidermidis* 9142 and 9142 *icaA*- are positive and negative controls, respectively, for *icaA* (B) ATCC 12228 is the negative control for *icaD* and *epidermidis* 9142 and 9142 *icaA*- are positive controls. The expected sizes of the *icaA* and *icaD* fragments are 1189 and 239 bp, respectively. The represented isolates in this figure are a subset of 72 screened *S. epidermidis* isolates.

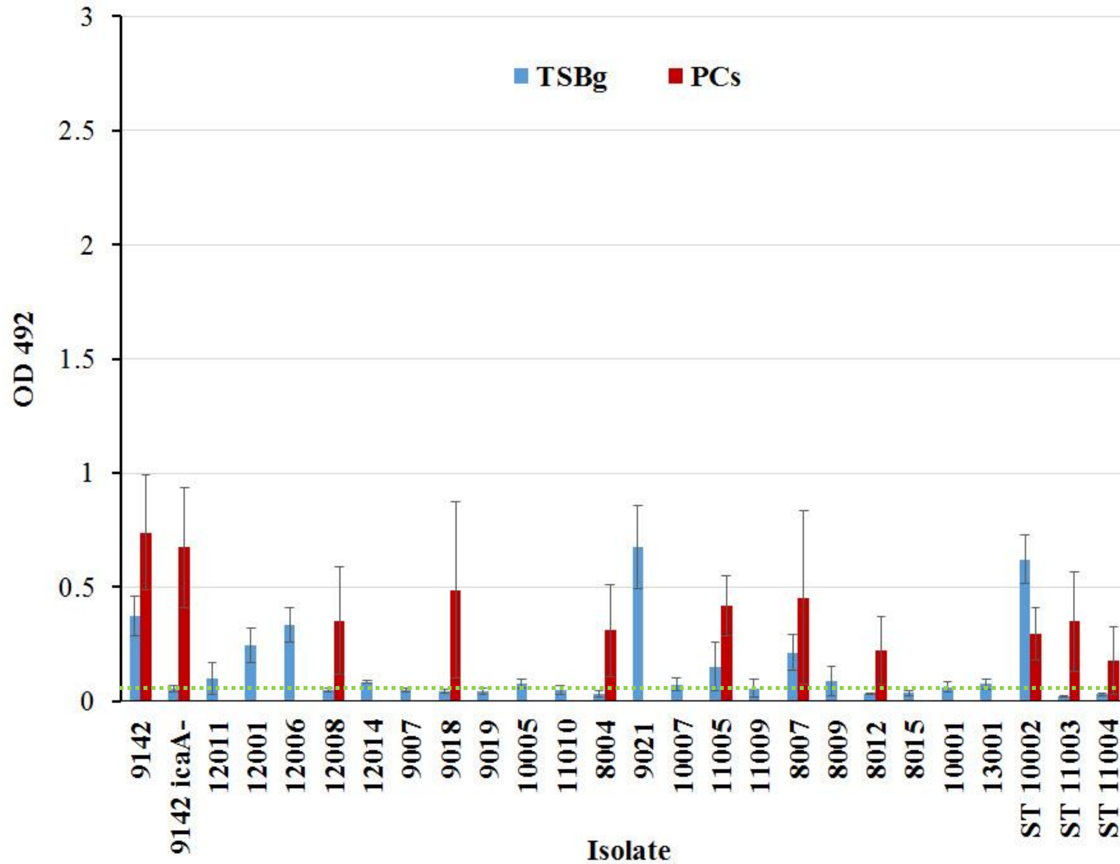


Figure 3.4. Biofilm formation abilities in TSBg and PCs of *S. epidermidis* isolates recovered from contaminated PCs evaluated using a semi-quantitative crystal violet assay. Mean absorbance of crystal violet dye at wavelength 492 nm, ±SD. *S. epidermidis* Hamburg 9142 is the positive control and *S. epidermidis* Hamburg 9142 icaA- (Δ*icaA)* is the negative control. Biofilms were allowed to grow statically at 37 ^{o}C for 24 hours for the TSBg experiments (three or more independent experiments, each performed in duplicates), and at 22± 2 ^{o}C for 5 days with agitation for the PC experiments (nine strains were screened, five independent experiments, each performed in duplicates). The dashed green line represents the cut-off value of the negative control.

IV. *ica*-positive and *ica*-negative biofilm-forming *S. epidermidis* were isolated from PCs with higher prevalence of *ica*-positive

Data showed that six out of the 24 *S. epidermidis* isolates obtained from PCs (25%) were able to form biofilms in TSBg. Only two of the 24 (8%) isolates showed *ica*-negative biofilm-formation abilities, while the remaining four (16.6%) were *ica*-positive biofilm-formers ($p > 0.05$) (Fig. 3.4, Table 3.2). When only the six biofilm formers were considered, the ratio of *ica*-negative to *ica*-positive was 1:2.

V. PC storage triggers the phenotypic conversion of biofilm-negative to biofilm-positive in *S. epidermidis* PC isolates

Six of the chosen nine isolates that did not form biofilms in TSBg, displayed a biofilm positive phenotype in the PCs environment (Fig 3.4). All the biofilm-forming isolates in TSBg maintained their phenotype when assayed in PCs. For the majority of the data presented herein, there is a link between the presence of *icaA* and *icaD* genes, slime production (PIA) on Congo red agar plates (Appendix A.3) and biofilm formation by crystal violet assay in laboratory media (Table 3.2). An exception is the isolate CBPA-BT 10001, which is a slime producer but lacks the *ica*A and *ica*D genes and is not able to form biofilms in media (Table 3.2).

Table 3.2. Phenotypic and genotypic biofilm-formation abilities of 24 *S. epidermidis* isolates recovered from contaminated PCs.

Number of PC contaminant isolates	**Presence of *ica* genes:**		**Slime***	**Biofilm formation**	
	icaA	***icaD***		**TSBg**	**PCs**
6	No	No	No	No	**Yes**
11	No	No	No	No	NT
2	No	No	No	**Yes**	**Yes**
1	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
3	**Yes**	**Yes**	**Yes**	**Yes**	NT
1†	No	No	**Yes**	No	NT
Controls					
Hamburg 9142	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Hamburg 9142 Δ*icaA*	No	No	No	No	**Yes**

NT; not tested

*Slime production was assessed by the Congo red agar assay.

† Isolate CBPA-BT 10001

3.6 DISCUSSION

This chapter highlights the genotypic and phenotypic characteristics associated with the biofilm-forming abilities of *S. epidermidis* in commensal isolates obtained from human skin in comparison to *S. epidermidis* isolates recovered from contaminated PCs. It also investigates if their site of isolation (antecubital fossa skin versus contaminated PCs) and genetic background affect their ability to adopt a biofilm-forming phenotype under the PC storage environment. *S. epidermidis* biofilm-formers were detected in both groups (i.e., skin isolates and contaminated PCs) where both, *ica*-positive and *ica*-negative strains, were obtained. This is consistent with previously published studies where both types of biofilm-formers have been isolated from clinical samples and healthy human skin (Ziebuhr et al., 1997; Galdbart et al., 2000; Qin et al., 2007b; Ninin et al., 2006; Vandecasteel et al., 2003; Los et al., 2010; Rohde et al., 2007).

Overall, skin derived *S. epidermidis* had a higher prevalence of biofilm-forming isolates compared to PC contaminants. No difference in the percentage of *ica*-positive biofilm formers among skin and contaminated PCs isolates was observed in this study. On the contrary previous studies have demonstrated that the biofilm-forming phenotype and/or the *ica* operon were more prevalent in *S. epidermidis* isolates obtained from clinical samples such as infected joint and catheters and patient blood cultures than from normal human mucosa and skin (Galdbart et al., 2000; Frebourg et al., 2000; Ziebuhr et al., 1997; Arciola et al., 2001). Ziebuhr and co-workers showed a significant higher prevalence of biofilm formation (86.5 %) and presence of the *ica* operon (84.6%) in sepsis-related *S. epidermidis* isolates compared to skin flora (Ziebuhr et al., 1997). In this study, it was hypothesized that the PC collection, production and storage processes might exert a positive selection pressure for biofilm-positive *S. epidermidis* isolates.

However, the data obtained in this study demonstrate the opposite results and therefore such preferential selection for a biofilm-forming phenotype does not exist.

Interestingly, a three-time higher percentage of *ica*-negative biofilm-forming isolates was found within the skin isolates compared to PC-derived *S. epidermidis.* These *ica*-negative biofilm producing isolates likely have a matrix composed of proteins, teichoic acids and/or eDNA (Qin et al., 2007b; Sadovskaya et al., 2005; Tormo et al., 2005; Christner et al., 2010). Since the disinfectants chlorohexidine and isopropanol alcohol act by causing cell membrane disruption, protein and nucleic acid precipitation and denaturation (McDonnell and Russell, 1999), it is likely that *ica*-negative biofilm forming isolates are negatively selected during skin disinfection due to their matrix composition. Qin and colleagues reported a higher sensitivity to the antibiotics vancomycin in *ica*-negative, protein/eDNA-based biofilms compared to *ica*-positive PIA-based biofilms of *S. epidermidis* (Qin et al., 2007b). The exact mechanism behind these patterns has not been defined; however, these differential sensitivities indicate that the type of biofilm matrix plays a role in the organism's susceptibility to elimination by chemical agents.

Data presented in this chapter showed a correspondence between the presence of *icaA* and *icaD* genes, slime production on Congo red agar and biofilm formation by crystal violet assay in laboratory media. However, the isolate CBPA-BT 10001 was a slime producer but it lacked the *icaA* and *icaD* genes and was not able to form biofilms in TSBg. This could be due to the interaction between the Congo red dye and other polysaccharides, such as the 20-kDa polysaccharide also produced by certain *S. epidermidis* isolates (Teather et al., 1982; Spiliopoulou et al., 2012).

Our laboratory had documented the ability of the PC milieu to support a conversion of *S. epidermidis* isolates from a biofilm-negative to a biofilm-positive phenotype (Greco, 2011; Ali

et al., 2014). In this study, those previous studies were expanded and a large collection of *S. epidermidis* isolated from PCs but also from human skin, which had not been tested before, were included. Excitingly, all 72 isolates tested herein, regardless of their source of origin, genetic background or ability to form biofilms in laboratory media, were able to produce a biofilm-forming phenotype in the PC storage environment. Although the mechanism involved in the stimulation of the biofilm-forming phenotype of *S. epidermidis* during PC storage is not fully understood, the Ramirez' group has demonstrated that biofilm forming abilities of this bacterium are significantly reduced when PCs are prepared with 70% of an additive solution (SSP+, MacoPharma) and 30% plasma or when platelet-poor-plasma is used (Greco et al., 2011; Greco et al., 2007). This indicates that both, plasma factors and platelets, are needed by *S. epidermidis* to adopt a biofilm-forming phenotype in PCs regardless of the organism's ability to form biofilms in laboratory media. Some plasma proteins such as albumin, fibrinogen, fibronectin, and vitronectin have been shown to rapidly adsorb and condition abiotic surfaces enhancing *S. epidermidis* adhesion to these surfaces by bacteria-protein interactions (Xu and Siedlecki, 2012; Paulsson et al., 1993; Mohammad et al., 1988). As a matter of fact, *S. epidermidis* possess surface proteins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that allow it to interact with host proteins (Patti and Höök, 1994; Nilsson et al., 1998). The *S. epidermidis* fibrinogen-binding protein SdrG (serine-aspartate repeat G) interacts with the platelet's receptor glycoprotein (GP) IIb-IIIa using fibrinogen as a bridging molecule (Brennan et al., 2009; Cox et al., 2011). SdrG also acts by cross-linking the GP IIb-IIIa and FCγRIIa platelet receptors or by interacting indirectly with GP IIb-IIIa. This bacterium-platelet interaction would lead to platelet activation and aggregation. Cox et al. explained that it is difficult to specify the exact way of interaction between bacteria and platelets due to the presence

of different adhesion mechanisms and due to inter and intra species variations (Cox et al., 2011). Moreover, it has been shown that the contact between a biomedical polymer surface with activated platelets promotes *S. epidermidis* adhesion to the surface (Wang et al., 1993a; Wang et al., 1993b). Taken together, a strong case can be made suggesting that the presence of plasma proteins and platelets trigger the biofilm formation phenotype of *S. epidermidis* observed in the PC milieu. However, the exact mechanism of biofilm formation and maturation is unknown.

This work has highlighted that the prevalence of the biofilm-forming *S. epidermidis* phenotype is not higher among isolates recovered from contaminated PCs compared to isolates obtained from healthy skin. Interestingly, the abundance of *ica*-negative biofilm-former isolates was lower in isolates obtained from contaminated PCs, suggesting that the donation process negatively affects the survival of *ica*-negative biofilm-formers. Furthermore, this work has confirmed previous observations that *S. epidermidis* can adopt a biofilm-forming phenotype in PCs regardless of their genetic background or source of origin.

Currently at Dr. Ramirez' laboratory, *S. epidermidis* isolates studied in this thesis are being used for the characterization of the biofilm matrix and cell wall structure of *ica*-negative biofilm formers. Moreover, transcriptome work is being performed to unravel the mechanisms involved in the enhancement of biofilm formation by *S. epidermidis* in PCs. Our laboratory has previously shown that *S. epidermidis* biofilms in PCs increase the virulence of the organism when tested in a nematode killing assay (Hodgson et al., 2014). A similar approach would allow for the comparison of the virulence profiles of isolates displaying different biofilm matrix compositions. Understanding the nature of the biofilm matrix and the mechanisms associated

with biofilm formation could help to target appropriate pathways and/or their components to control biofilm formation in PCs thereby improving the safety of this blood product.

As it has been shown that the abundance of *ica*-negative biofilm-former isolates was lower in PC than skin *S. epidermidis* isolates, it would be recommended to assess the efficiency of currently used skin disinfectants against *S. epidermidis* isolates displaying different biofilm matrices. This would provide insights into the ability of the skin disinfectants to eradicate *S. epidermidis* biofilms carrying structurally different matrices present in the donor skin and eventually would provide information to increase the efficiency of the current donor skin disinfection process.

3.7 ACKNOWLEDGMENTS

Many thanks for the volunteer blood donors and netCAD staff for blood collection and PC manufacturing.

CHAPTER 4.

Efficiency Of Riboflavin And Ultraviolet Light Treatment To Inactivate *S. epidermidis* Biofilms In Buffy Coat Platelet Concentrates

4.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

The manuscript "Efficiency of riboflavin and ultraviolet light treatment to inactivate *S. epidermidis* biofilms in buffy coat platelet concentrates" will be submitted for peer-review.

M. Taha conducted all the experiments, analysed data and prepared samples for scan electron microscopy examination. Dr. S. Ramirez-Arcos was the thesis' supervisor providing guidance on the experimental design, troubleshooting and data interpretation. Flow cytometery (CD62P) experiments (day 1 of storage) and PC units splitting and illumination were performed with the support of Ms. B. Culibrk and Dr. P. Schubert (Canadian Blood Services). Statistical analyses were performed by Dr. QL.Yi (Canadian Blood Services). Scanning electron microscopy was performed by Dr. M. Kalab (Agriculture and Agri-Food Canada). Dr. D. Mack kindly provided the anti-PIA antibodies.

4.2 ABSTRACT

Pathogen inactivation technologies (PITs) are effective against clinically-relevant bacteria in PCs. *S. epidermidis*, the predominant PC contaminant, forms biofilms in PCs, which have been linked to missed detection during routine PC screening. This study was aimed at testing the efficacy of riboflavin-UV treatment (the Mirasol® PIT system) to inactivate *S. epidermidis* biofilms in buffy coat PCs (BC-PCs). Two biofilm-positive *S. epidermidis* strains (ST1-0002 and AZ-66) were used in this study. Biofilm and planktonic (non-biofilm) cells were collected from both strains and used to individually inoculate whole blood (WB) units at a concentration of ~10^6 CFU/mL (N≥4). Each spiked WB unit was processed through the BC production method to obtain a PC pool. Riboflavin was then added to the BC-PC and split into two bags: UV-treated and untreated (control). Bacterial concentration was measured before and after treatment. On days 1 and 5 of PC storage, samples were taken for *in vitro* quality analyses monitoring platelet activation by flow cytometry (CD62P expression) and changes in dynamic light scattering using the ThromboLUX system. Results showed that bacterial concentration was reduced during BC-PC production from ~10^6 CFU/mL in WB to 10^3-10^4 CFU/mL in PCs ($p<0.0001$). Treatment of these PCs with Mirasol did not completely inactivate bacteria. There was a ≥3.5 log reduction of *S. epidermidis* AZ-66 (≥96%), which was higher ($p<0.0001$) than the 2.6-2.8 log reduction obtained for strain ST-10002 (70-80%). No differences in *S. epidermidis* inactivation were observed in PCs produced from WB inoculated with biofilm or planktonic cells ($p>0.05$) for both strains. At the end of the 5-day PC storage, platelet activation measured by flow cytometry was increased due to Mirasol treatment ($p<0.05$) whereas ThromboLUX scores did not indicate changes in *in vitro* PC quality. Platelet activation was enhanced in PCs produced with WB inoculated with biofilm cells compared to planktonic cells ($p<0.05$), which should be further

investigated. In conclusion, the efficacy of the Mirasol treatment was similar for *S. epidermidis* present in PCs produced from WB inoculated with biofilm or planktonic cells. Interestingly, treatment effectiveness was strain dependent with *S. epidermidis* ST-10002 showing higher resistance to inactivation than *S. epidermidis* AZ-66.

4.3 INTRODUCTION

The safety of PCs is one of the major concerns in transfusion medicine since bacterially-contaminated PC units have been reported periodically by different blood centers (Ramirez-Arcos and Goldman, 2012; Pietersz et al., 2014; Hong et al., 2016). A number of practices have been implemented to improve the safety of the PC supply and to minimize the risk of transfusing bacterially–contaminated blood products. Canadian Blood Services screens 100% of its PC products for bacterial contamination using the BacT/ALERT® 3D automated blood culture system. However, bacterially-contaminated PC units are still missed and in some cases they are transfused leading to fatalities (FDA, 2015; Pietersz et al., 2014; Kou et al., 2015). Several European, Latin American and Middle-Eastern countries have implemented Pathogen Inactivation (PI) Technologies (PITs) where PCs are exposed to ultraviolet (UV) light preventing the replication of microorganisms thereby controlling the transfusion of bacterially-contaminated PCs (Prowse, 2013). The Mirasol®, Intercept™ and Theraflex PI systems are described in Table 1.5. Mirasol uses riboflavin as a photosensitizer which modifies nucleic acids and causes irreversible changes upon exposure to UV light (A and B, 280-360 nm) resulting in inactivation of microorganisms (Marschner and Goodrich, 2011). Mirasol has been evaluated for the inactivation of a broad spectrum of bacteria including Gram positive and Gram negative organisms. In published studies, it has been shown that the Mirasol PI system was able to

inactivate 22 clinically-relevant bacterial strains that belong to 13 species with 66-100% effectiveness when present at an initial concentration ≤100 CFU/ PC product (Goodrich et al., 2009; Marschner and Goodrich, 2011).

S. epidermidis, one of the predominant PC contaminants, can grow in the human skin forming biofilms and is thought to be introduced to the blood collection bag during venipuncture (Rogers et al., 2008; Galdbart et al., 2000). This was demonstrated by the isolation of biofilm forming *S. epidermidis* strains from contaminated PCs (Greco et al., 2008; Ali et al., 2014; chapter 3 of this thesis). The existence of bacterial cells in a biofilm increases their resistance to chemical and physical clearance. It has been shown that the *P. aeruginosa* biofilm matrix physically shields cells within it against elimination by UV light compared to their planktonic counterparts. It has been reported however, that the addition of a photosensitizer increases the sensitivity of biofilm associated cells to UV-A light treatment (Elasri and Miller, 1999). To our knowledge, no investigations have been performed to evaluate the inactivation of bacterial biofilms by PITs used in transfusion medicine.

The main objective of this chapter was to evaluate the inactivation abilities of the Mirasol PI system against *S. epidermidis* biofilms present in PCs. To simulate a realistic contamination event, whole blood (WB) was spiked with *S. epidermidis* cells that were either biofilm-associated or planktonic in nature. The spiked WB was subjected to the BC- PC production process. The resulting BC-PC pools were then treated with Mirasol and bacterial concentrations were determined before and after treatment and during 5 days of PC incubation. Additionally, the quality of the PCs that had undertaken Mirasol treatment was monitored.

4.4 MATERIALS AND METHODS

I. Bacterial strains

Two biofilm-positive *S. epidermidis* strains were used in this study, *S. epidermidis* ST-10002 (PC isolate) and AZ-66 (skin isolate). One colony of *S. epidermidis* was added to 3 mL of TSB and incubated for 17-18 hours at 37°C, the liquid culture was then adjusted to OD_{600} = 0.1, which approximately corresponds to 1.0 x10^{7} CFU/mL in TSBg. The wells of polysterene 6-well tissue culture plates (Falcon, Corning Inc., Durham, NC) were inoculated with 3 mL of the bacterial suspensions. The plates were incubated statically at 37°C for 24 hours to allow for biofilm formation. The 3 mL supernatant that contained planktonic cells was removed and cells were washed 3x with a 0.9% NaCl solution and were then suspended in 3 mL 0.9% NaCl + 15% glycerol. The bacterial concentration was determined by plating on TSA and the remaining planktonic cells were added to a 10 mL syringe which was kept frozen at -83°C until it was needed. The wells that contained the attached biofilms were washed with 3 mL of 0.9% NaCl to remove any free floating cells. Three mL of 0.9% NaCl + 15% glycerol was added to each well, the biofilms were scrapped off and collected in a 10 mL syringe which was frozen at -83°C until it was needed. Two of the 6 wells served as controls to check for bacterial concentration in the biofilms. Theses biofilm (aggregated) and planktonic cells were collected from both strains and used to individually inoculate WB units.

II. WB Spiking and BC-PC treatment with Mirasol®

The research design was approved by the Canadian Blood Services Research Ethics Board. WB was collected from healthy consenting volunteers and processed to manufacture BC-PCs at the Network Centre for Applied Development, Canadian Blood Services (netCAD; Vancouver, BC, Canada) in accordance with Canadian Blood Services' procedures (Levin et al., 2008).

Immediately after WB collection in a Macopharma 'top-and-bottom' collection set, WB unit was sampled and tested for sterility using the BacT/ALERT system as described previously (Mastronardi et al., 2012). The WB unit was then spiked with biofilm (WB-biofilm) or planktonic (WB-planktonic) cells to a target concentration of ~10^6 CFU/mL. Each bacterial strain (ST-10002 or AZ-66) for each type of cells (biofilm or planktonic) was tested in at least four separated WB units. The initial bacterial concentration in the spiked WB unit was verified by plating serial dilutions onto blood agar (BA) plates, which were incubated overnight at 37°C. High spiking concentrations were chosen to monitor bacterial distribution and/or elimination during BC- PC production. After spiking, the WB unit was held at 22 ± 2°C on a cooling tray (CompoCool, Fresenius-Kabi, Bothell, WA) for 16 – 23 hours (overnight hold). After spinning the inoculated WB unit, the BC fraction was pooled with 3 non-spiked ABO matched BCs and suspended in an autologous plasma unit to produce the final BC-PC product. Riboflavin was then added (500 uM, 35±5 mL) to the PC pool and split into two Mirasol illumination bags (TerumoBCT, Lakewood, CO), one of which served as the UV-treated unit, while the other served as an internal control and was left untreated (Fig. 4.1). Bacterial concentrations were measured on the day of production (day 1) in control-untreated units, before and after Mirasol treatment for the test units, and throughout PC storage (days 1-5) for both treated and untreated pools. BPA bottles of the BacT/ALERT system were inoculated with Mirasol- treated and

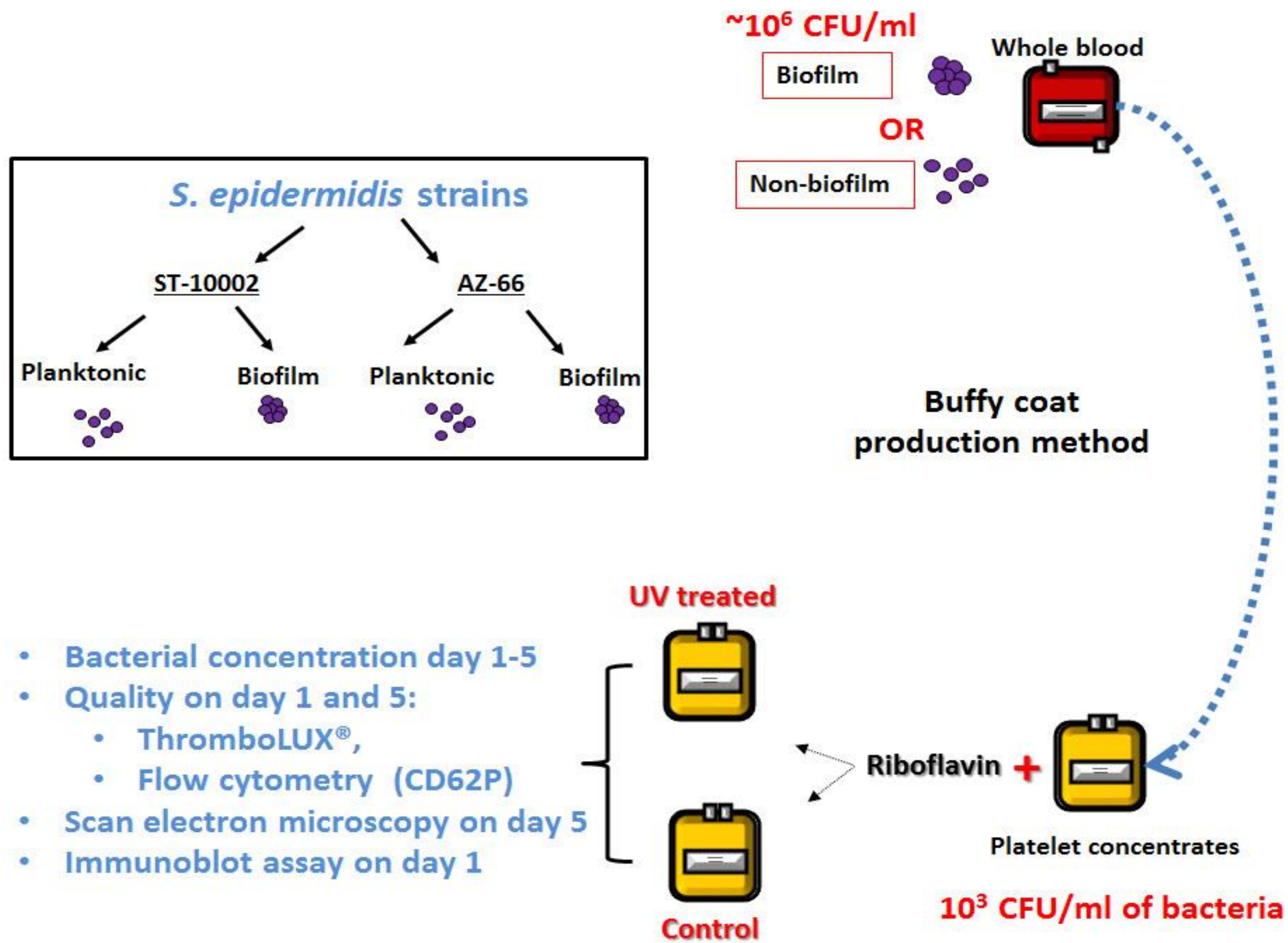


Figure 4.1. **Flow chart showing the protocol used for the Mirasol® experiments.** WB was spiked with ~10^6CFU/mL of planktonic or biofilm cells of *S. epidermidis* (ST-10002, AZ-66). WB was subjected to BC-PC production.

untreated PCs immediately after the Mirasol treatment and on day 5 of storage. Positive bottles were plated on BA plates to confirm bacterial growth and colony morphology. BC-PC samples were taken from untreated and treated pools on day 1 (production day) and day 5 for *in vitro* quality analyses.

III. *In vitro* PC quality

Flow cytometry. Platelet degranulation capacity as a response to 10 uM of the agonist ADP was determined by CD62P (platelet activation marker) as follows: diluted PCs (200-300 X 10^9 platelets/L) with or without ADP were incubated for 15 minutes followed by addition of anti-CD42 (platelet marker) and anti-CD62P antibodies (Beckman-Coulter, Mississauga, Ontario, Canada). After 15 minutes of incubation, the samples were analysed by flow cytometry (FACS Canto II, BD Biosciences, Mississauga, ON, Canada). Response to ADP was determined as the difference between the percentages of CD62P expression with and without ADP. Due to the nature of the study design, it was not possible to obtain an unspiked control, therefore historical control data collected at Canadian Blood Services were obtained for unspiked PCs.

Dynamic light scattering. ThromboLUX® (LightIntegra Technology, Vancouver, BC, Canada) is a device that assesses PC quality by measuring platelet count, size, response to thermal stress, and presence of microparticles. PC quality is expressed as a score ranging from 0 to 40 with a scoring ≥10 indicating good quality (Lightintegra, 2015). Samples of 100 uL of PCs were collected on days 1 and 5 of storage in microcapillaries and tested using the ThromboLUX device. Data were analysed using the ThromboSight v3 software. Due to the nature of the study design, it was not possible to obtain unspiked controls, therefore historical control data were obtained of unspiked PCs.

IV. Scanning electron microscopy

At the end of the PC shelf life, PC containers were emptied and 1 cm^2 coupons were cut out and washed in 1x PBS , fixed in 2% formaldehyde (Canemco and Marivac Inc, Quebec, Canada) and 2.5% glutaraldehyde (Polysciences, Inc., Warrington, PA) for 2 hours, and dehydrated in an ethanol gradient followed by critical-point drying (Biodynamic Research Corp., San Antonio, TX). The dried coupons were coated with a 20 nm gold layer with a sputter coater and photographed using a microscope (XL30 ESEM, Philips, Eindhoven,The Netherlands) operated at an accelerating voltage of 7.5 kV and spot size 2.

V. Immunoblot assay

An immunoblot assay was performed to detect the *S. epidermidis* biofilm matrix PIA in samples obtained from PC pools according to procedures described before (Sadykov et al., 2008; Vuong et al., 2003) with modifications. Briefly, 1 mL samples were collected from PC pools directly after production and frozen at -83°C until they were tested. To lyse cells and release any cell wall-bound PIA into the supernatant, the samples were thawed and centrifuged at 5000 g for 10 minutes, and the pellet was resuspend in 0.5 M EDTA (pH 8.0). After that, the cells were boiled at 100 °C for 5 minutes, and centrifuged at 5000 g for 10 minutes. Ten microliters of 10 mg/mL proteinase K (Sigma-Aldrich, Oakville, Canada) was added to 40 uL of the supernatant and incubated at 37 °C for 1 hour. The proteinase K was then inactivated at 95 °C for 5 minutes. Samples were spotted (2 uL/spot) onto a nitrocellulose membrane (NC) and allowed to dry. The membrane was blocked at room temperature for 1 hour on a rotator with 0.5% (w/v) bovine serum albumin (BSA, Bio-Rad, Saint-Laurent, Canada) prepared in TBS-T (Tris-buffered saline, 0.05% tween 20) buffer. A 1:5000 dilution of anti- *S. epidermidis* PIA antibody (Mack et al., 2001) in 0.5% BSA/TBS-T was then added to the membrane and incubated for 1 hour at room

temperature. The membrane was washed 3x with TBS-T, and a 1:5000 dilution of alkaline-phosphatase conjugated anti-rabbit IgG (Sigma-Aldrich, Oakville, Canada) in 0.5% BSA/TBS-T was added to the membrane and incubated for 1 hour. The membrane was again washed 3x in TSB-T and a solution of BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, Sigma-Aldrich, Oakville, Canada) was added and the membrane was agitated for 20 minutes. Color development was stopped by the incubation of the membrane in ddH_2O for 10 minutes. Images of the membrane were taken using MultiImage™ Light Cabinet and AlphaImager™ 2200 software (Fisher Scientific, St-Laurent, Quebec). Controls of PIA production levels were prepared by growing *S. epidermidis* AZ-66, ST-10002, Hamburg 9142 (PIA-positive) and Hamburg 9142 Δ*icaA* (PIA-negative) under standard conditions in TSBg at 37 ^{o}C for 24 hours.

VI. Statistical analyses

The numbers of CFU were log transformed (base 10) for statistical comparisons. No bacterial growth was represented as 0 CFU/ml (0 log 10). A p-value of <0.05 was considered statistically significant. Analyses were performed with the Statistical Analysis System (SAS, 2000-2004, SAS Institute, Inc., Cary, NC). Paired t-test was used to assess bacterial reduction during BC-PC production and bacterial inactivation in PCs derived from WB-biofilm or from WB-planktonic. Also, it was used to compare the response to ADP of Mirasol treated PCs to unspiked untreated units (historical data) on days 1 and 5. Moreover, paired t-test was used to compare ThromboLUX scores of Mirasol treated PCs to control unspiked untreated units (historical data) on days 1 and 5. Untreated control PCs derived from WB-biofilm were compared to WB-planktonic in terms of their response to ADP on day 5 of storage using paired t-test. Linear mixed model regression analysis was used to compare the efficiency of the treatment between

the two strains. ANOVA was used to compare bacterial distribution and concentration in the different fractions of the BC-PC production process.

4.5 RESULTS

I. *S. epidermidis* concentrations are reduced during BC-PC production and the microorganism segregates towards cellular fractions

Viable bacterial counts were tracked in the different fractions of the BC-PC production process. The initial bacterial concentrations in WB in all the repetitions for both planktonic and biofilm cells were comparable (approximately 6 log CFU/mL in WB) (Table 4.1). Bacterial concentrations were reduced 2-3 logs CFU/mL during BC-PC production from ~ 6 log CFU/mL in WB to 3-4 log CFU/mL in PCs ($p<0.0001$) for both *S. epidermidis* strains (Table 4.1). Moreover, the data indicate that *S. epidermidis* segregates toward the cellular fractions (i.e., BC and RBCs) rather than the plasma fraction ($p<0.01$) during the BC-PC process independently of the nature of the bacterial inoculum in WB (planktonic or biofilm cells) (Table 4.1).

II. Mirasol reduces but does not eliminate *S. epidermidis*

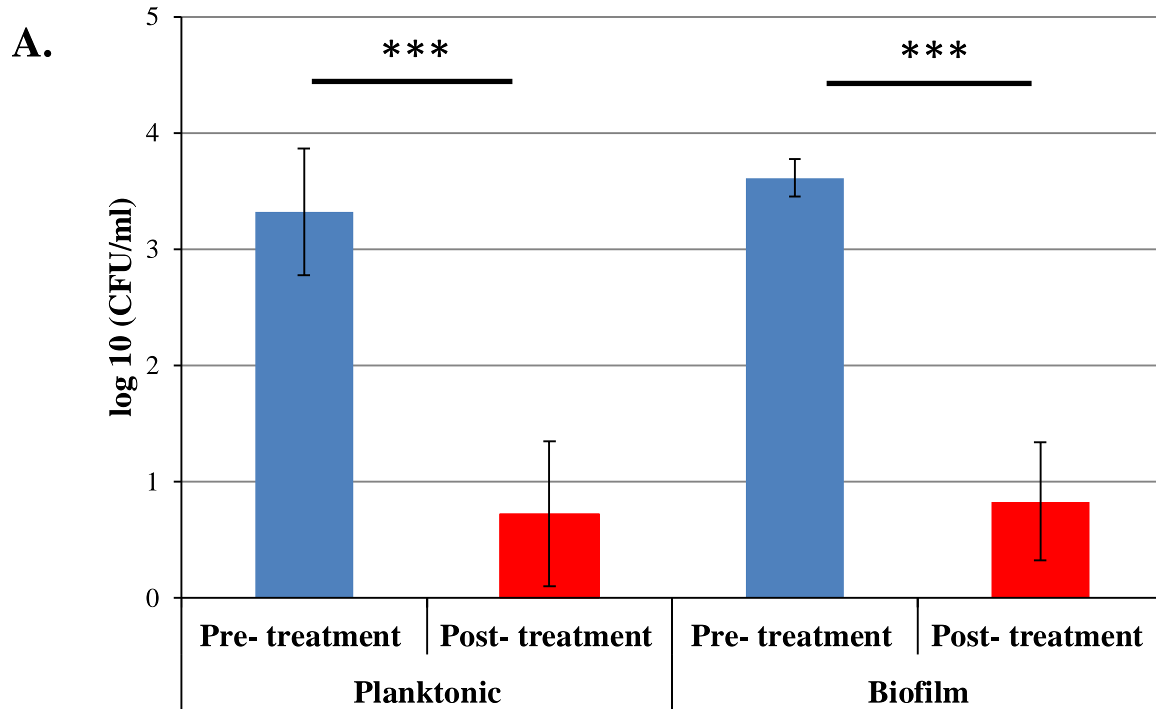
Viable bacterial counts were compared before and after illumination to check for treatment efficiency. Mirasol treatment of PC units, obtained from WB-biofilm or WB-planktonic cells, did not completely inactivate bacteria (Fig. 4.2). Even though, *S. epidermidis* AZ-66 was not detected after treatment by plating (500 uL/plate), all the BacT/ALERT culture bottles inoculated immediately after treatment and at the end of PC storage turned positive indicating the presence of viable bacteria (Appendix B.1).. The identity of the organism recovered in the culture bottles was confirmed by plating on BA plaelets

Table 4.1. *S. epidermidis* distribution in the different fraction during the BC-PC production method after spiking WB with either planktonic or biofilm cells of the strains ST-10002 (clinical isolate) or AZ-66 (skin isolate).

Strain	**ST10002**		**AZ-66**	
	Planktonic	**Biofilm**	**Planktonic**	**Biofilm**
	Mean (±SD) Log 10 (CFU/mL)			
N	**4**	**5**	**4**	**4**
WB t0	5.93 (0.16)	5.98 (0.35)	5.78 (0.41)	6.40 (0.48)
WB overnight	5.60 (0.09)	5.82 (0.17)	5.18 (0.41)	5.69 (0.17)
RBC	5.28 (0.28)	5.75 (0.43)	5 (0.5)	5.67 (0.22)
Plasma t0	3.74 (0.42)	4.51 (0.32)	3.55 (0.45)	3.97 (0.39)
Plasma t2	3.78 (0.42)	4.50 (0.34)	3.52 (0.42)	4 (0.39)
BC t0	5.46 (0.21)	5.92 (0.24)	5.53 (0.44)	6.46 (0.65)
BC t2	5.34 (0.13)	5.70 (0.31)	5.34 (0.26)	5.95 (0.34)
Pool	4.30 (0.29)	4.75 (0.51)	4.26 (0.33)	4.52 (0.32)
Residual	4.42 (0.09)	4.85 (0.43)	4.80 (0.53)	5.03 (0.34)
PC	3.37 (0.54)	3.68 (0.09)	3.70 (0.5)	3.61 (0.11)

N; sample size
t0, time zero, prior to incubation at room temperature
t2, after 2 hours of incubation at room temperature

A.



B.

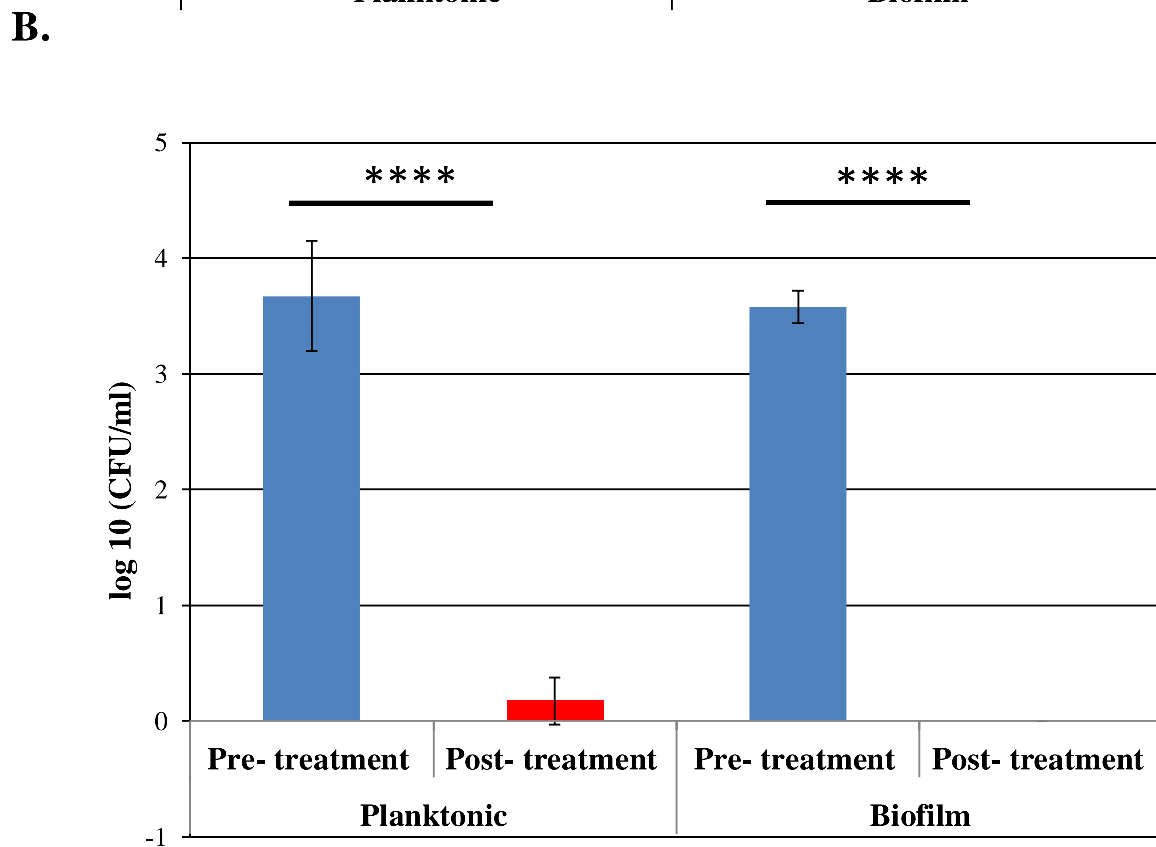


Figure 4.2. Viable cell counts in PCs pre- and post- Mirasol® treatment. A) Viable counts of *S. epidermidis* ST-10002 in PCs derived from WB spiked with planktonic or biofilm cells. B) Viable counts of *S. epidermidis* AZ-66 in PCs derived from WB spiked with planktonic or biofilm cells. N ≥4, ±SD, ***P<0.001,****P<0.0001.

III. Similar Mirasol efficacy was observed in PCs derived from WB-biofilm or WB-planktonic

No differences in *S. epidermidis* inactivation were observed in PCs produced from WB-biofilm or WB-planktonic ($p=0.467$, $p= 0.67$, respectively) for both strains. There was a ≥3.5 log reduction of *S. epidermidis* AZ-66 (≥96%), which was higher ($p<0.0001$) than the 2.6-2.8 log reduction of strain ST10002 (70-80%) (Fig. 4.2, Appendix B.1).

IV. Mirasol-treated *S. epidermidis* proliferated during PC storage and attached to the inner surface of the PC containers

Bacterial survival and proliferation of Mirasol-treated *S. epidermidis* during PC storage was monitored. Data show that both strains ST-10002 and AZ-66 remained viable and proliferated in the control and treated PC pools during PC incubation (Appendix B.2). SEM images display bacterial attachment to the inner surface of the bags from Mirasol treated units of both strains (Fig. 4.3).

V. Mirasol treatment affected *in vitro* PC quality

To check for deleterious effects of the Mirasol treatment, PC quality was monitored by testing the ability of the platelets to respond to ADP and express CD62P and using the ThromboLUX device. No differences were observed between any of the treated groups on day 1 of PC storage compared to the unspiked historical controls ($p>0.05$). Interestingly, on day 5, platelet response to ADP was lower in untreated PCs derived from WB- biofilm compared to untreated PCs produced with WB- planktonic attaining significance difference for strain ST-10002 (Fig. 4.4). Similarly, untreated PCs derived from WB-biofilm showed a lower response to ADP than the

unspiked historical data ($p<0.001$), while no differences were observed between untreated PCs WB-planktonic compared to the unspiked historical controls ($p>0.05$) (Fig. 4.3). These results indicate that the presence of biofilms in WB induced platelet activation in the final BC-PC product. Mirasol-induced platelet damage was observed on day 5 as significantly lower ($p<0.0001$) platelet response to ADP was obtained for Mirasol-treated PCs, derived from both WB-biofilm and WB-planktonic, compared to the unspiked historical controls (Fig. 4.4). This observation is supported by the fact that Mirasol treatment induced a decrease in quality in PCs produced from WB-planktonic compared to untreated WB-planktonic ($p<0.01$) (Fig. 4.4). A similar effect was not observed in PCs derived from WB-biofilm likely due to the biofilm-induced platelet damage described above. ThromboLUX scores were above the cut-off point of ≥10 indicating unchanged PC quality of all tested products and historical controls (Fig. 4.5).

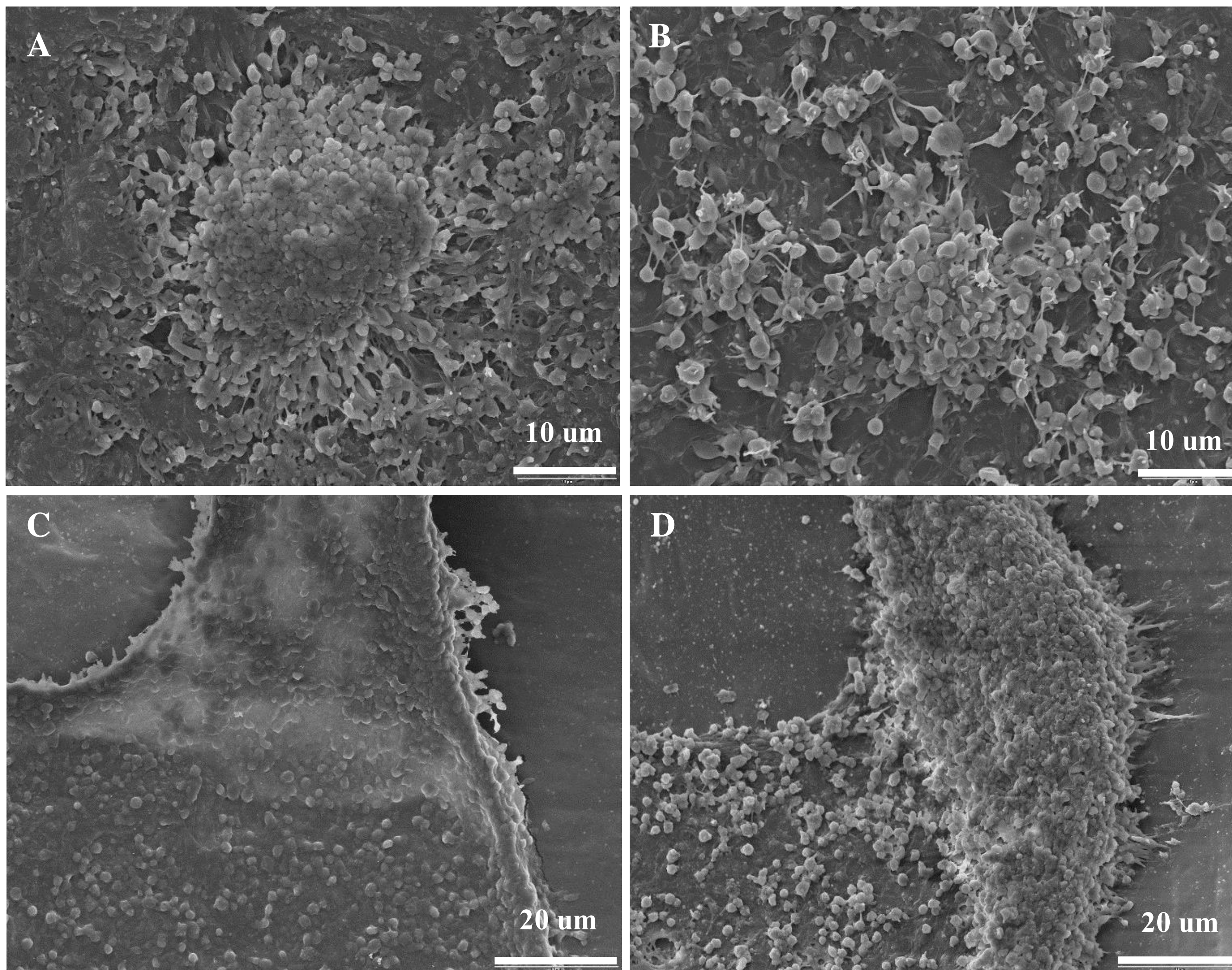
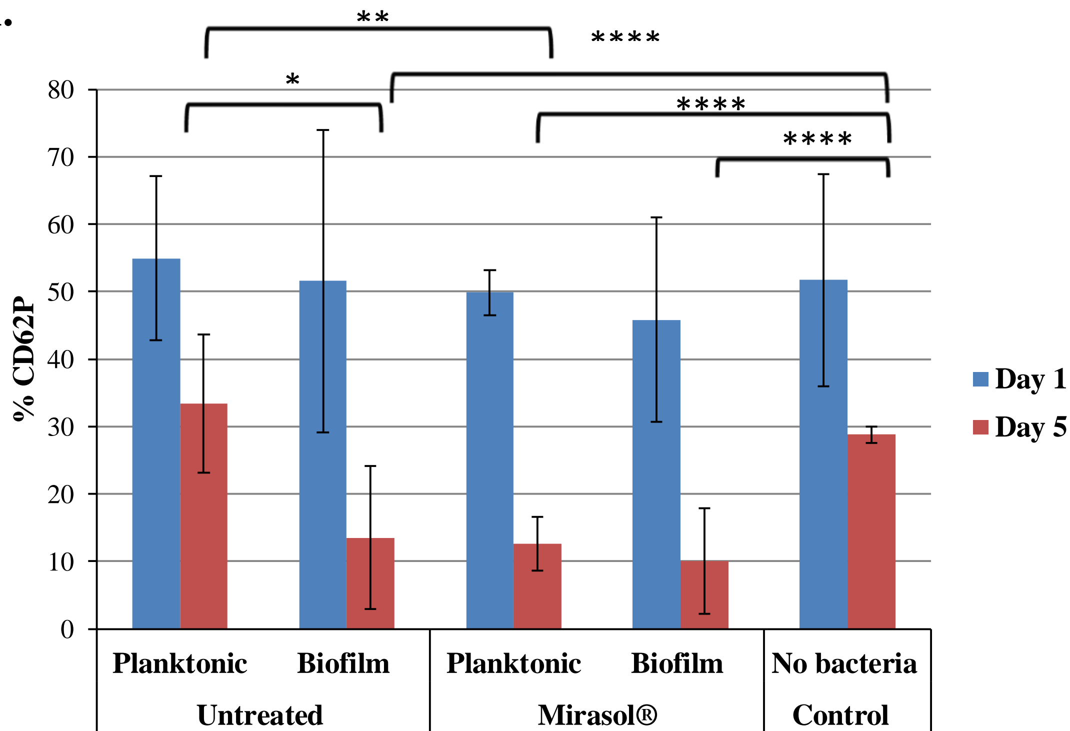


Figure 4.3. Scanning electron micrographs of 1 x 1cm^2 coupons of the inner surface of Mirasol-treated PC containers: A, B) *S. epidermidis* ST-10002 derived from WB spiked with biofilm and planktonic cells, respectively, at a magnification of 2000x. C, D) *S. epidermidis* AZ-66 derived from WB spiked with biofilm and planktonic cells, respectively, at a magnification of 1250X.



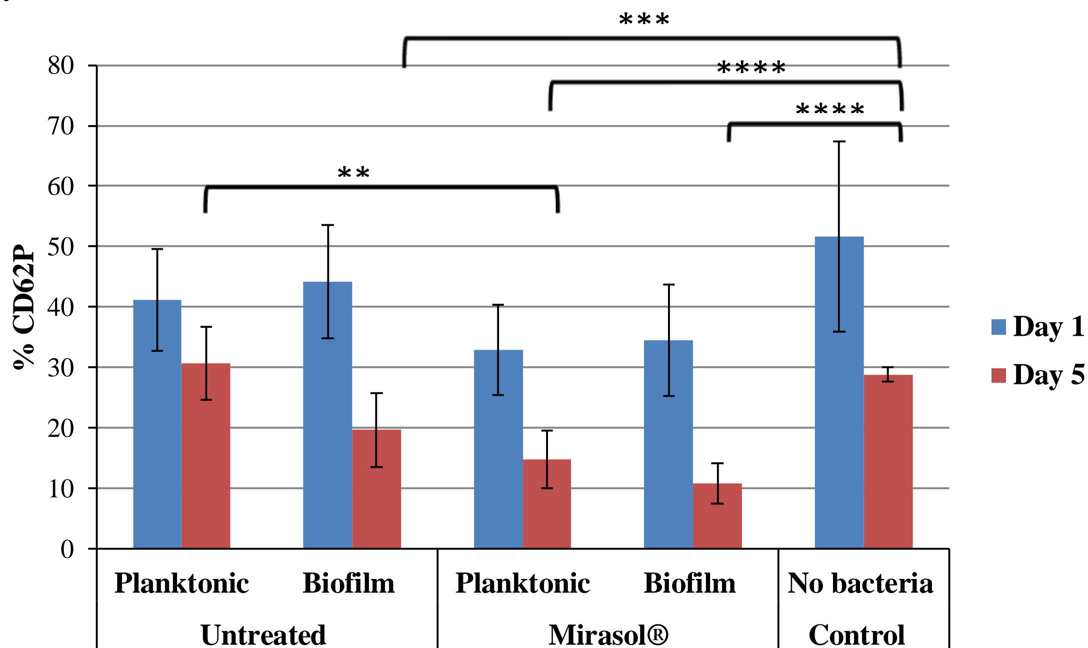
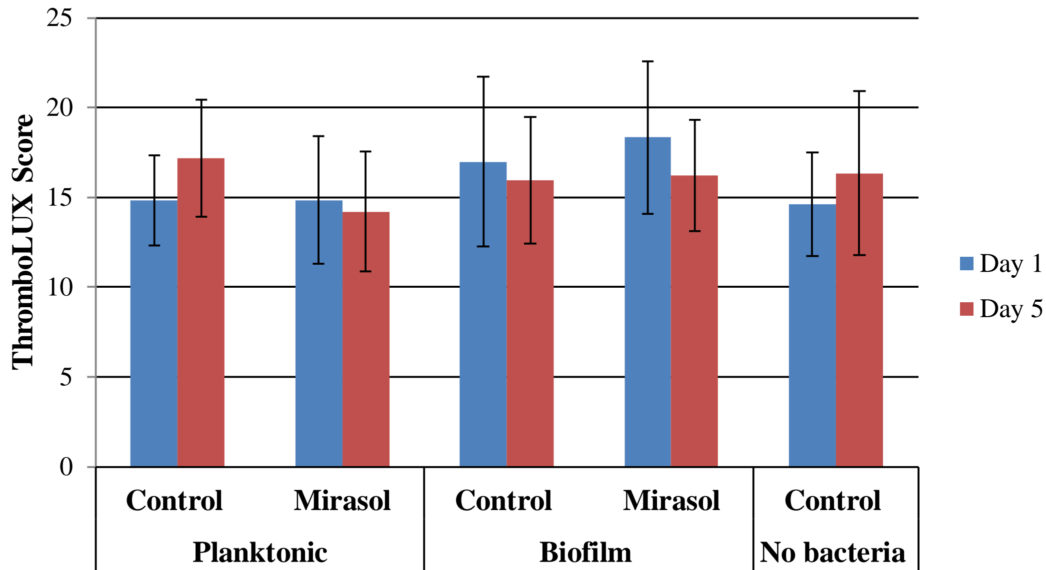


Figure 4.4. ***In vitro*** **quality of PCs using flow cytometry (%CD62P).** CD62P expression percentage as a response ADP for PCs derived from WB spiked with planktonic or biofilm cells: A) *S. epidermidis* ST-10002. B) *S. epidermidis* AZ-66.N≥4, ±SD. Controls: not treated unspiked PCs, N=12, historical data (*P<0.05,***P≤0.001, ****P≤0.0001).

A.



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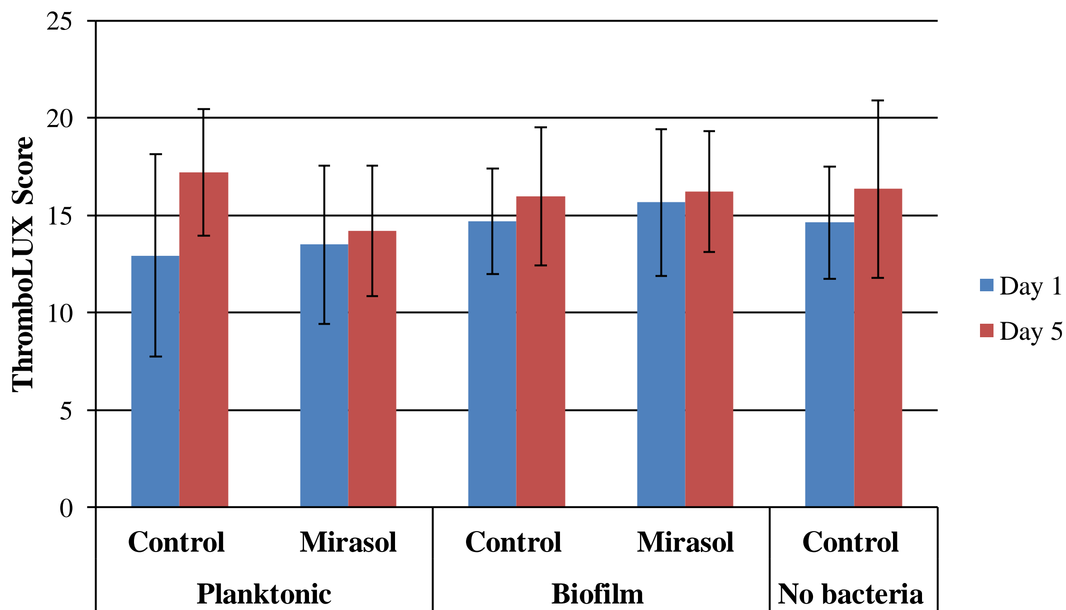

Figure 4.5. ***In vitro*** **quality of PCs using ThromboLUX®.** A) *S. epidermidis* ST-10002. B) *S. epidermidis* AZ-66.N≥4, ±SD. Controls: not treated unspiked PCs, N=10, historical data.

VI. The effect of the BC production on biofilm integrity in the final PC product is unknown

Biofilm cells might have been dispersed due to mechanical stress during BC-PC production from WB-biofilm. Therefore, the presence of bacteria in biofilms in PCs was investigated. Immunoblot assays were performed to detect levels of the biofilm matrix PIA in PCs on the production day assuming that biofilm cells would have higher levels of PIA than planktonic cells. Unfortunately, the assay was not sensitive enough to detect PIA at concentration of ~ 10^3 CFU/mL in PC samples on day 1 of storage (Appendix B.3). The detection limit of the assay for PIA levels in planktonic cells in TSBg was determined to be ~10^6 CFU/mL for control samples AZ-66, ST-10002 and Hamburg 9142 (Appendix B.3).

4.6 DISCUSSION

In this chapter, the efficiency of the Mirasol PI system against *S. epidermidis* biofilms was investigated. Mirasol treatment was capable of significantly inactivating both strains of *S. epidermidis*, one isolated from human skin and the other from contaminated PCs; however, the treatment could not completely eliminate the bacteria present at concentrations $\geq 10^3$ CFU/mL. The surviving viable cells in treated PCs were able to proliferate under PC storage conditions. Kwon and colleagues reported a similar pattern of incomplete inactivation of *S. aureus* and *E. coli* by Mirasol when PC units were spiked with 10^7-10^8 CFU/mL (Kwon et al., 2014). They reported 4-5 logs reduction which was in line with previous reports of incomplete inactivation for a variety of Gram positive and Gram negative bacteria (*S. epidermidis*, *S. aureus*, *S. marcescens*, *E. coli, K. pneumoniae* and *P. aeruginosa*) when present at high concentrations (Goodrich et al., 2006; Keil et al., 2015).

It is acknowledged that the high levels of *S. epidermidis* in PCs in this work (≥ 10^3 CFU/mL) and in other studies are not clinically relevant since it had been estimated that initial bacterial contamination levels in PC units range from 1 to 60 CFU/ PC unit (approximately 0.003–0.2 CFU/mL) (Pearce et al., 2001; Murphy et al., 2008; Benjamin and Wagner, 2007). However, contamination with fast growing bacteria could be a problem with PITs. A delay in the illumination step while using the PI technology Intercept has resulted incomplete elimination of *K. pneumoniae* when present at concentrations of ~ 10^8 CFU/mL (Schmidt et al., 2015; Wagner et al., 2016). Additionally, as shown in chapter 5, when plasma-resistant Gram negative bacteria are present at low initial concentrations in collected WB, they can multiply reaching high concentrations during the overnight hold of WB during BC-PC production (chapter 5 of this thesis; Taha et al., 2016). It is expected that bacteria present at high concentrations would be captured during PC screening with culture methods; however, it would pose a challenge for PITs. Taken together, these results indicate that it is crucial to minimize the time between donation and illumination during PI to avoid bacterial multiplication. More work should be undertaken to investigate the efficiency with which PI systems inactivate fast growing bacteria.

The Mirasol system was equally efficient against *S. epidermidis* derived from WB-biofilm or WB-planktonic. Unfortunately, it was not possible to confirm the presence of biofilms in the final BC-PC product. Interestingly, a difference in the efficiency of the system was observed between strains of *S. epidermidis* for both WB-biofilm and WB-planktonic. The skin isolate AZ-66 showed a higher sensitivity to inactivation than the clinical isolate ST-10002. Other studies have reported different PI efficiencies against different strains of *S. aureus* and *S. epidermidis*. Kwon et al. and Goodrich et al. showed incomplete inactivation with Mirasol of the strain *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 700578 in PCs; however, the other

tested ATCC strains of the same species were completely inactivated at comparable concentrations (Kwon at al., 2014; Goodrich et al., 2009). Goodrich and co-workers postulated that because some bacterial strains can interact and adhere to platelets, this interaction could serve as a shield that protects bacteria from UV light and photosensitizer uptake (Goodrich et al., 2009). They presented experimental data demonstrating complete inactivation of these strains in plasma when platelets were removed. This could be one reason behind the different efficacy in PI observed for the two *S. epidermidis* strains used in this study. Therefore, it is possible that the clinical isolate ST-10002 had a higher interaction with platelets during the production process that provided better protection against the riboflavin-UV treatment which merits further investigation.

The quality of the PC units was also monitored in this study, and as shown in other studies, the riboflavin-UV light treatment resulted in a deleterious effect on platelet quality as indicated by flow cytometry results. PITs work by damaging nucleic acids which inactivate pathogens and other cellular components such as platelets. Therefore, it is not surprising to detect platelet activation after Mirasol treatment. Similar patterns of drop in PC quality (including PC degranulation as response to ADP) after Mirasol treatment have been well documented (Ruane et al., 2004; Li et al., 2004; Castrillo et al., 2013; Schubert et al., 2015). Interestingly, untreated PC units derived from WB- biofilm, showed a significant drop in quality (lower response to ADP measured by flow cytometry) compared to untreated pools derived from WB-planktonic after 5 days of storage. It is hypothesized that increased platelet – bacteria interactions due to the presence of biofilm aggregates in these PCs led to higher platelet activation.

The efficacy of riboflavin-UV PI treatment is similar for *S. epidermidis* present in PCs produced from WB inoculated with biofilm or planktonic cells. The system did not completely inactivate *S. epidermidis* at high concentrations and, interestingly, the treatment effectiveness was strain dependent.

The Mirasol® vendor recommends that the units be treated within 8 hours of production. Based on the findings presented in this chapter, chapter 5 (Taha et al., 2016), and chapter 6 , as well as the outcomes of other studies (Know et al., 2014; Schmidt et al., 2015) demonstration of incomplete inactivation at high bacterial concentrations is known to occur.It would be of a great value to investigate the ability of the Mirasol system to inactivate several fast growing organisms when PCs are spiked at clinical-relevant concentrations and there is a delay in the illumination step. Moreover, since PI systems do not inactivate endotoxins, it will be crucial to determine whether clinically-significant endotoxin levels are present in PCs contaminated with fast growing Gram negative organisms pre-PI treatment.

In this study, the efficiency of the Mirasol system against *S. epidermidis* in PCs was investigated, however, it was not possible to determine whether biofilms used to spike WB units remained as biofilm aggregates following the production process and were present in the final PC product. Therefore, to determine whether biofilm integrity gets affected during the BC-PC production process, samples of fresh PC derived from WB spiked with biofilm cells samples should be obtained for scanning electron microscopy examination. Another approach to test the efficiency of PITs against biofilms is to directly spike PC with biofilm aggregates and then subject them to PI treatment.

In this study, a deleterious effect on the quality of PCs was observed when biofilm cells existed in WB. This observation needs to be further studied and confirmed. A possible approach is to directly spike PC units with planktonic or biofilm cells and monitor bacteria-platelet interactions and activation using CD62P expression during PC storage.

4.7 ACKNOWLEDGMENTS

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CHAPTER 5.

Bacterial Survival And Distribution During Buffy Coat Platelet Production

Mariam Taha[1], Miloslav Kalab[2], Qi-Long Yi[1], Elisabeth Maurer[3], Craig Jenkins[1], Peter Schubert[1] and Sandra Ramirez-Arcos[1]

[1]Canadian Blood Services

[2]Agriculture and Agri-Food Canada

[3]LightIntegra

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5.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

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M. Taha conducted all the experiments and prepared samples for scan electron microscopy examination. Dr. S. Ramirez-Arcos was the thesis' supervisor providing guidance on the experimental design, troubleshooting and data interpretation. M. Taha and Dr. S. Ramirez-Arcos wrote the manuscript. Dr. M. Kalab performed scan electron microscopy examination. Dr. QL. Yi performed statistical analyses. Dr. E. Maurer and Dr. P. Schubert contributed during the study design and data interpretation for the PC quality experiments. Mr. C. Jenkins provided guidance for the BC-PC production study design.

5.2 ABSTRACT

At Canadian Blood Services, buffy coat (BC) platelet concentrates (BC-PCs) show a generally lower bacterial contamination rate than apheresis PCs. This study investigated whether the PC production method contributes to this observation. Whole blood (WB) inoculated with eight bacterial strains was processed using the BC method. Bacteria were enumerated throughout BC-PC production and subsequent PC storage. Endotoxin production and bacterial adhesion to PC bags were evaluated during PC storage. PC quality was monitored by CD62P expression (flow cytometry) and changes in dynamic light scattering (ThromboLUX®). During overnight WB hold, *Staphylococcus epidermidis* titres remained unchanged, commercial *Escherichia coli* and *Klebsiella pneumoniae* were eliminated and the remaining organisms proliferated to high concentrations. Through BC-PC production, bacteria segregated preferentially towards the cellular fractions compared to plasma ($P < 0{\cdot}05$). During PC storage, most bacteria adhered to the PC bags and Gram negatives produced clinically significant endotoxin levels. Changes in CD62P expression or ThromboLUX scoring did not consistently reflect bacterial contamination in BC-PCs. WB hold during BC-PC production does not have a broad-spectrum bactericidal effect, and therefore, other factors contribute to low rates of contamination in BC-PCs.

5.3 INTRODUCTION

Septic transfusion events continue to pose the major infectious risk for transfusion patients in developed countries as demonstrated by recent active surveillance data (Hong et al, 2016). Despite all the mitigation strategies implemented to reduce this risk, reports of fatal transfusion reactions involving bacterially contaminated PCs continue to be reported (Kou et al., 2015; FDA, 2015). Prior to venipuncture, donors are screened by a questionnaire and undergo a

skin disinfection process. Postdonation, the first 20–40 ml of donated blood is diverted and produced PCs are screened for bacterial contamination (Benjamin and McDonald, 2014).

At Canadian Blood Services, PCs are produced by single-donor apheresis (Aph-PCs) and by the buffy coat production method (BC-PCs). BC-PCs are manufactured by pooling four buffy coats and suspending the pool in an autologous plasma unit. Both Aph-PCs and BC-PC pools are screened for bacterial contamination after 24–28 hours of the stop bleed time using the BacT/ALERT 3D system as described by Jenkins et al. (Jenkins et al., 2011). From January 2010 to March 2016, 173 223 Aph-PCs and 543 615 BC-PCs were screened for bacterial contamination at Canadian Blood Services. Data collected during that period show that the rate of confirmed positive cultures is similar for both PC types: one in 11 548 for Aph-PCs and one in 11 817 for BC-PCs. In some cases, initial positive results cannot be confirmed and these indeterminate results are classified as 'suspected positives'. Screening results show a rate of bacterial contamination approximately 1·7-fold higher in Aph-PCs than BC-PCs when both confirmed and suspected positive cultures are included. As the majority of published reports document higher contamination rates in BC-PCs compared to Aph-PCs (Macauley et al., 2003; Larsen et al., 2005; Pearce et al., 2011) , we were intrigued by our PC testing results and have conducted the study reported herein.

PC testing for bacterial contamination has been implemented in several developed countries mostly using culture-based methods, which are known to detect only 40–50% of contaminated PCs (Benjamin and Wagner, 2007). Units with undetectable levels of contamination can be transfused, and even if they do not cause a transfusion septic event, the impact on PC quality and function is unknown.

The majority of studies addressing bacterial contamination in PCs are performed by spiking the PCs with bacteria. As reported here, we have taken a real-life mimicking approach and inoculated WB with bacteria, and subsequently subjected the spiked WB to the BC-PC manufacturing process. Bacteria were tracked from beginning to end of PC production and during PC storage. Additionally, changes in PC quality markers were evaluated as potential indicators of bacterial contamination.

5.4 MATERIAL AND METHODS

I. Bacterial strains

Bacterial strains used in this study included *E. coli* ATCC 10798 and *K. pneumoniae* ATCC 10031, purchased from the American Type Culture Collection (ATCC); *S. marcescens* CBS12/2010 and *E. coli* CBS11001, isolated during routine PC screening at Canadian Blood Services; *S. epidermidis* ST-11003 and *S. epidermidis* ST-10002, missed during routine PC screening and isolated during quality control testing of expired products; and *S. marcescens* CBS07/2005 and *K. pneumoniae* PEI-B-P-08-1, both implicated in fatal transfusion events (Greco-Stewart et al., 2012; Graul et al., 2003). Gram-positive and Gram-negative bacteria were grown in Tryptic Soy Broth and Luria Bertani, respectively, and harvested at OD_{600} = 0·05 (*S. marcescens* and *K. pneumoniae)* or 0·1 (*S. epidermidis* and *E. coli*) corresponding to approximately 10^8 colony-forming units (CFU)/ml. The bacterial suspensions were stored at −85°C after the addition of glycerol to a final concentration of 15%.

II. Whole blood spiking and bacteria tracking during BC-PC production

Whole blood was collected and processed to manufacture BC-PCs at the Network Centre for Applied Development, Canadian Blood Services (netCAD; Vancouver, BC, Canada) in

accordance with Canadian Blood Services' procedures (Levin et al., 2008). The donors were healthy volunteers and signed consent before blood collection. Research design was approved by the Canadian Blood Services Research Ethics Board. Immediately after WB collection using a MacoPharma 'top-and-bottom' collection set, samples were taken for sterility testing as described previously (Mastronardi et al., 2012). WB units were inoculated to a target concentration of 10^2 CFU/mL and held at 22 ± 2 °C on a cooling tray (CompoCool, Fresenius-Kabi, Bothell, WA, USA) for 16 – 23 hours (overnight hold). This concentration was chosen to allow bacterial tracking during buffy coat PC production. Throughout BC-PC production, samples of about 1 ml were collected from all the fractions. These samples were plated onto blood agar (BA) plates followed by colony counting after overnight incubation at 30 °C (*S. marcescens*) or 37 °C (other species). Bacterial proliferation was monitored for 5 days of PC storage by daily sampling, and then, serial dilutions, plating and incubation were performed as described above. BC-PC production day was designated as day 1 of storage, and therefore, end of PC shelf life was designated as day 6 of storage. PCs were stored for an extra day (day 7 of storage) for electron microscopy samples. All assays were repeated at least twice. For PC pools containing Gram-negative bacteria, 1 mL samples were collected in endotoxin-free tubes on days 1 and 5 of storage. As a negative control, a sample was obtained from a sterile BC-PC. All samples were stored at −85 °C until shipped to the Charles River Laboratories (Wilmington, Massachusetts, US) for determination of endotoxin levels (measured as endotoxin units-EU).

III. Scanning electron microscopy

On day 7 of storage, PC containers were emptied and 1 × 1 cm^2 bag sections (coupons) were cut and washed in sterile phosphate-buffered saline, fixed in 2% formaldehyde (Canemco and Marivac Inc, Quebec, Canada) and 2·5% glutaraldehyde (Polysciences, Inc., Warrington, PA,

USA) for 2 hours and dehydrated in an ethanol gradient followed by critical-point drying (Biodynamic Research Corp., San Antonio, TX, USA). The dried coupons were coated with a 20 nm gold layer with a sputter coater and photographed using a microscope (XL30 ESEM, Philips, Eindhoven, The Netherlands) operated at an accelerating voltage of 7·5 kV and spot size 2.

IV. *In vitro* PC quality

Flow cytometry. On days 1 and 5 of storage, platelet activation and platelet response to 10 µm ADP was determined by P-selectin expression (CD62P) as follows: diluted PCs (200–300 × 10^9 platelets/l) with or without ADP were incubated for 15 minutes followed by addition of anti-CD42 and anti-CD62P antibodies (Beckman-Coulter, Mississauga, Ontario, Canada). After a 15-min incubation, the samples were analysed by flow cytometry (FACS Canto II, BD Biosciences, Mississauga, ON, Canada). Response to ADP was determined as the difference between the percentages of CD62P expression with and without ADP. Historical control data were obtained for non-spiked PCs.

Dynamic light scattering. ThromboLUX® (LightIntegra Technology, Vancouver, BC, Canada) is a device that assesses PC quality by measuring platelet count, size, response to thermal stress and presence of microparticles. The quality is expressed as a score ranging from 0 to 40 with a scoring ≥10 indicating good PC quality (Labrie et al., 2013). Samples of 100 ul of PCs were collected on days 1 and 5 of storage in microcapillaries and tested using the ThromboLUX® device. Data were analysed using the ThromboSight v3 software. Historical control data were obtained for non-spiked PCs.

V. Statistical analyses

The numbers of CFU were log-transformed (base 10) for statistical comparisons. No bacterial growth was represented as 0 CFU/mL(0 log 10). A group t-test was used to compare the

ThrombloLUX® score and CD62P values of contaminated PC units with historical controls on days 1 and 5 of storage. A paired t-test was used to analyse changes in bacterial concentration after the overnight hold of WB and after the 2 hours BC and plasma rest periods. Analysis of variance (anova) was used to compare bacterial segregation into the RBC, plasma and BC fractions. A two-sided *P* value of $<0{\cdot}05$ was considered statistically significant. Analyses were performed with the Statistical Analysis System (sas, 2000–2004, SAS Institute, Inc., Cary, NC, USA).

5.5 RESULTS

I. The effect of an overnight hold of WB differs depending on the bacterial strain

The initial bacterial concentration introduced into WB units was between 10^2 and 10^3 CFU/ml (WB t0, Table 5.1). After the overnight hold of WB at 22 ± 2°C, three different bacterial survival scenarios were observed (Fig. 5.1): (1) The concentration of both *S. epidermidis* strains ST-10002 and ST-11003 remained unchanged; (2) the two commercial bacteria *E. coli* ATCC 10798 and *K. pneumoniae* ATCC 10031 vanished almost immediately after spiking, and the clinical Gram-negative strains *E. coli* CBS11001, *S. marcescens* CBS12/2010, *S. marcescens* CBS07/2005 and *K. pneumoniae* PEI-B-P-08-1 proliferated overnight reaching concentrations ranging from 10^3 to 10^7 CFU/mL.

Table 5.1. Bacterial distribution during BC production

	Bacterial concentration - Log $_{10}$ CFU/mL (SD)					
	S. epidermidis		*E. coli*	*S. marcescens*		*K. pneumoniae*
	ST-10002	ST-11003	CBS 11001	CBS12/2010	CBS07/2005	PEI-B-P-08-1
N	4	3	2	3	4	2
WB t0	2.32 (0.56)	2.89 (0.55)	2.38 (0.16)	2.49 (0.13)	2.59 (0.26)	3.30 (0.23)
WB overnight	2.22 (0.48)	2.90 (0.49)	3.22 (0.10)	5.76 (0.47)	5.72 (1.45)	7.27 (0.44)
RBC	1.68 (0.51)	2.19 (0.75)	2.62 (0.14)	5.35 (0.12)	5.18 (1.62)	5.62 (0.27)
Plasma t0	0.93 (0.62)[a,b]	1.03 (1.27)[a]	0.00 (0.00)[a,b]	2.79 (0.34) [a,b]	3.69 (1.25)	4.02 (1.13)
Plasma t2	0.92 (0.62)	0.96 (1.13)	0.66 (0.93)	2.97 (0.42)	4.40 (1.42)	4.36 (1.06)
BC t0	2.21 (0.56)	3.13 (0.34)	3.04 (0.58)	5.86 (0.45)	5.17 (1.88)	7.49 (0.43)
BC t2	2.18 (0.71)	2.83 (0.11)	2.82 (0.47)	5.86 (0.39)	5.23 (1.99)	8.20 (0.68)
Residual BC	1.62 (0.68)	2.43 (0.57)	2.83 (0.59)	5.17 (0.24)	5.09 (1.78)	6.74 (0.65)
Pool	1.34 (0.99)	1.73 (0.38)	1.74 (0.80)	4.25 (0.05)	4.48 (1.62)	7.25 (0.64)
PCs day 1	0.91 (0.67)	1.09 (0.30)	0.54 (0.34)	3.77 (1.15)	4.38 (1.79)	7.53 (0.46)
Total titer reduction[c]	1.31 (0.42) p=0.0081	1.82 (0.79) p=0.0577	2.68 (0.43) p=0.0724	1.99 (0.94) p=0.0669	1.35 (1.12) p=0.0958	-0.25 (0.02)[d] p=0.042

N, sample size
t0, time zero, prior to incubation at room temperature
t2, after 2 hours of incubation at room temperature
[a]Significantly lower bacterial concentration compared to BC fraction (p<0.05)
[b]Significantly lower bacterial concentration compared to RBC fraction (p<0.05)
[c]Difference in bacterial concentration between “WB overnight” and “PCs day 1”
[d]Significant increase in bacterial concentration during PC production

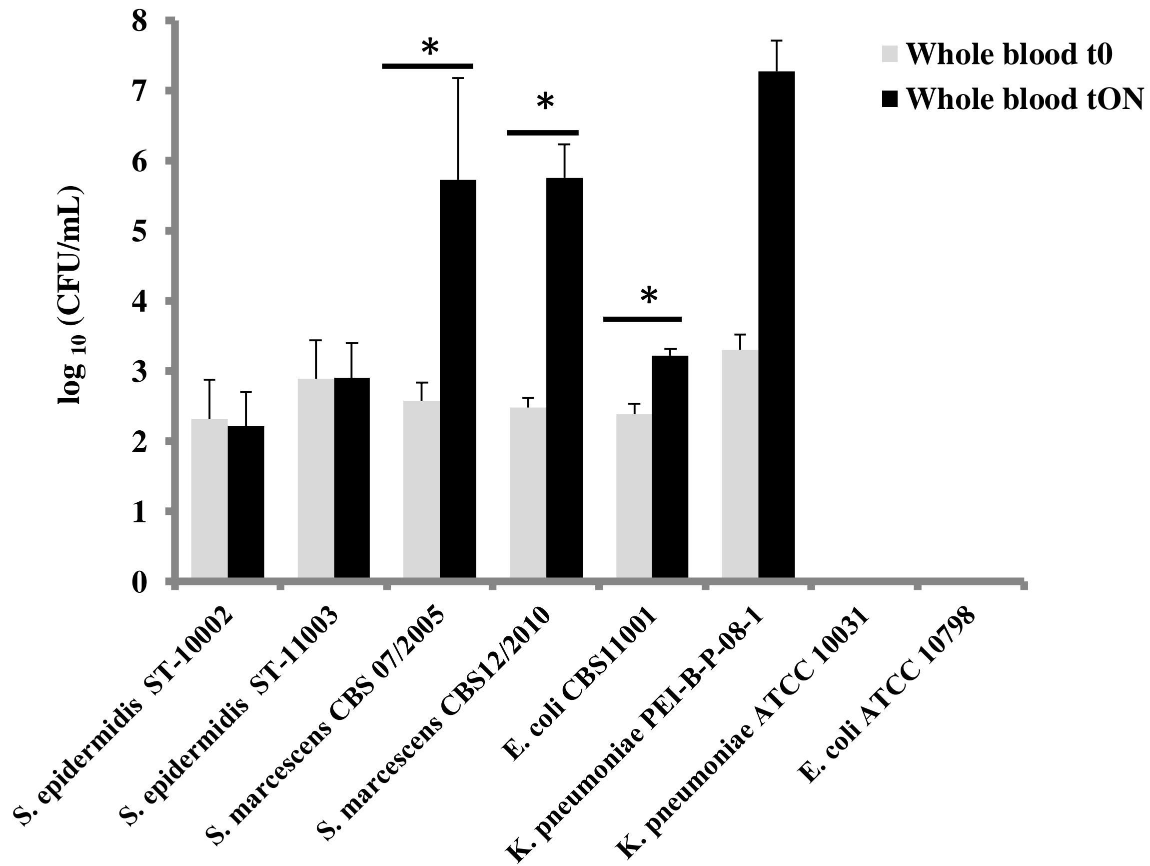


Figure 5.1. Bacterial concentration in WB units after spiking (time zero, t0, grey bars) and after an overnight hold (time overnight, tON, black bars) of WB at 22±2 °C. *$p < 0.05$, significant difference. N≥2 ± SD.

II. Bacteria segregate preferentially towards the cellular fractions during BC-PC production

Bacteria segregated preferentially into the cellular fractions during BC-PC production showing higher concentrations in the BC and RBC fractions compared to plasma, with most bacteria segregating into the BC fraction ($P < 0{\cdot}05$ for both *S. epidermidis* strains, *E. coli* CBS11001 and *S. marcescens* CBS12/2010, Table 5.1). No bactericidal effect was observed in BC or plasma as bacterial concentrations were comparable after a 2 hours resting of these fractions (plasma t0 vs. t2 and BC t0 vs. t2, Table 5.1). With the exception of *K. pneumoniae* PEI-B-P-08-1, all bacteria showed a significant reduction during the BC-PCs production process, which was most pronounced after pooling the four BC fractions with one plasma unit and during leucocyte reduction (BC t2 compared to pool and residual BC compared to PCs day 1, respectively, Table 5.1).

III. Bacteria proliferated during PC storage and attached to the inner surface of the PC containers

Bacteria which survived the BC-PC production process, regardless of their concentration in PCs on day 1, were able to proliferate after 5 days of incubation under standard PC storage conditions (Fig 5.2). This growth was associated with visible clumping in the supernatant of the BC-PCs containing *E. coli* CBS11001, *S. marcescens* CBS12/2010 and CBS07/2005 (data not shown). Bacterial adhesion to the inner surface of platelet containers was evaluated on day 7 of storage. Scanning electron microscopy showed that *S. epidermidis, S. marcescens* and *K. pneumoniae* formed surface-attached aggregates or biofilms on the PC container surface (Fig 5.3 A-C). While the images showed interaction between bacteria and platelets in the biofilms of *S. epidermidis* and *S. marcescens* (Fig 5.3 A and B), *K. pneumoniae* biofilms were mainly formed of bacterial

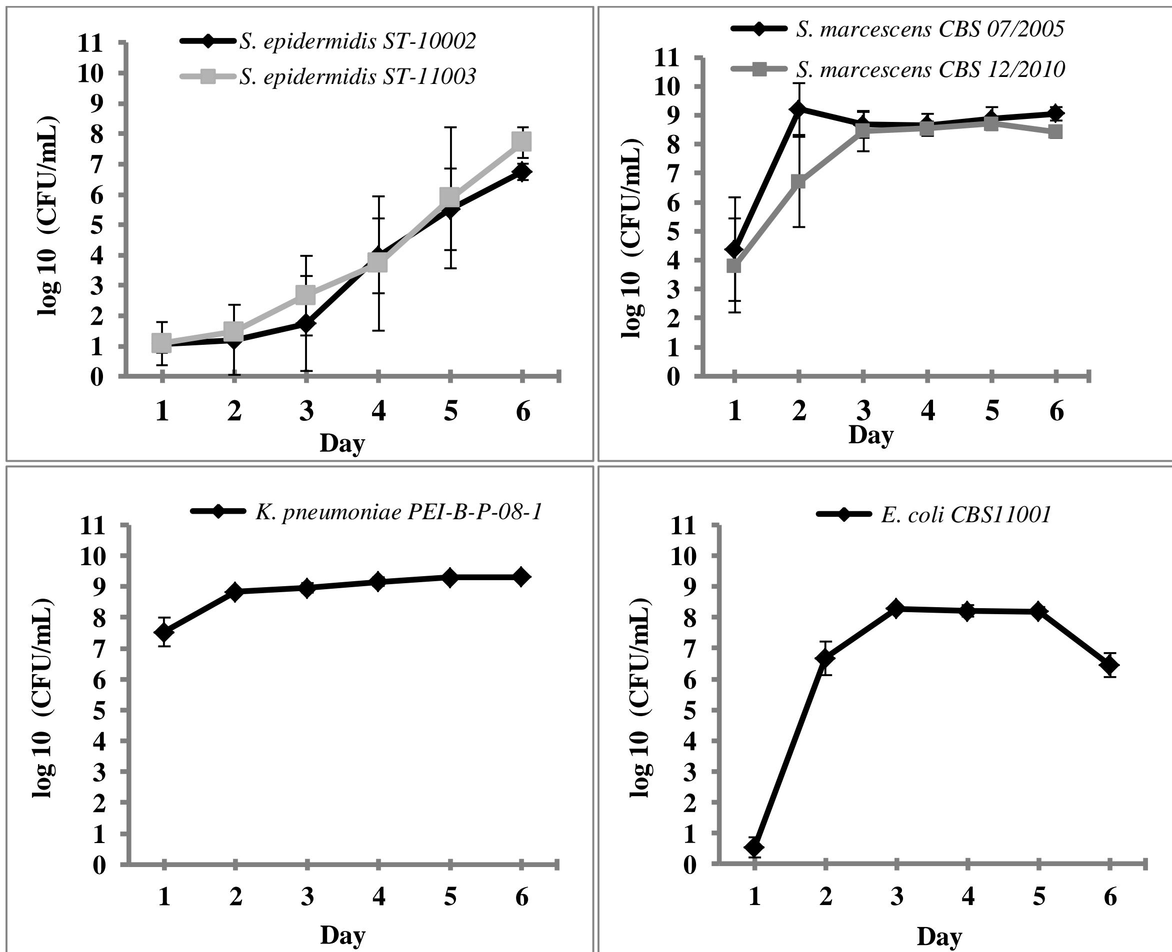


Figure 5.2. Bacterial growth in PCs under standard storage conditions over 5 days. N ≥ 2 ± SD.

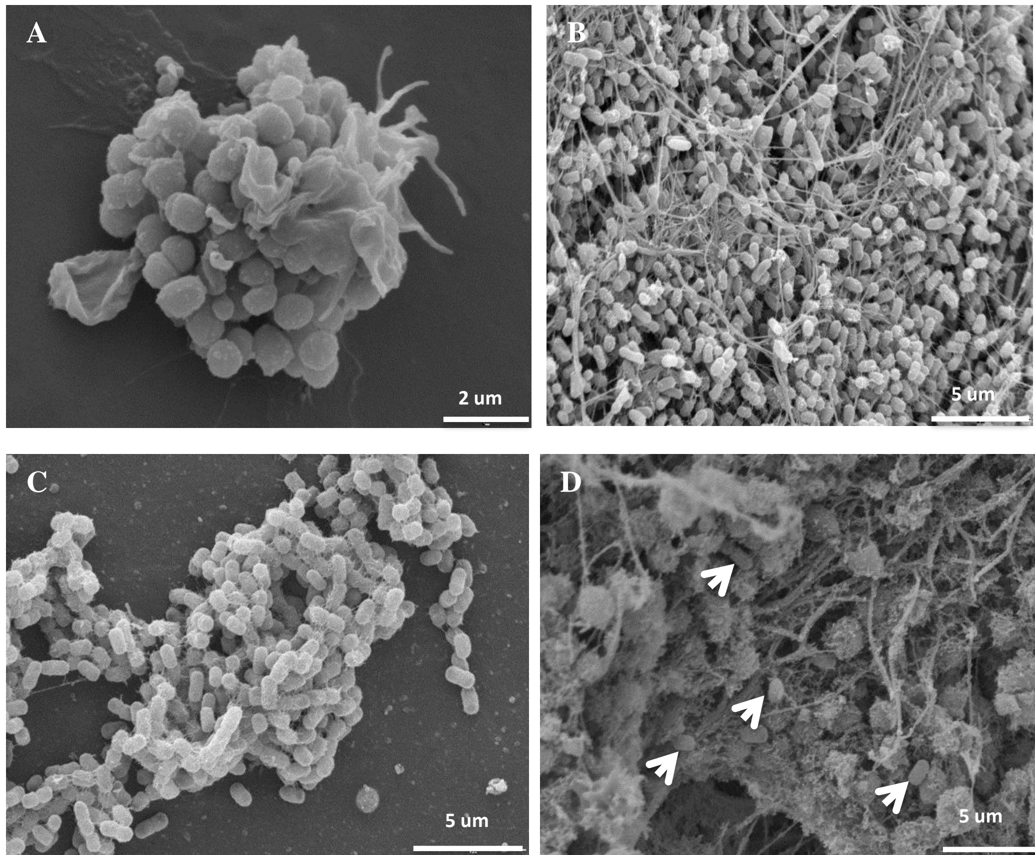


Figure 5.3. Scanning electron micrographs of $1 \times 1\ cm^2$ coupons of the inner surface of PC containers contaminated with: (a) *S. epidermidis* ST-10002, (b) *S. marcescens* CBS12/2010, (c) *K. pneumoniae* PEI-B-P-08-1 and (d) *E. coli* CBS11001; the arrows indicate scarce bacterial cells.

cells (Fig 5.3 C). In contrast, *E. coli* did not show attachment to the PC containers although scarce bacterial cells were observed trapped in a mesh of what seems to be platelet debris (Fig. 5.3 D).

IV. Clinically significant levels of endotoxins are associated with high titers of Gram-negative bacteria in BC-PCs

Endotoxin levels were determined on day 1 and day 5 of PC storage for PC units containing Gram-negative bacteria. Clinically significant endotoxin levels (>11 000 EU) (Jacobs et al., 2008) were detected in BC-PCs carrying *K. pneumoniae* PEI-B-P-08-1 (23 577 200 EU) and *S. marcescens* CBS12/2010 (12 580 EU) on day 1 of PC storage demonstrating endotoxin production during BC-PC manufacturing, likely during the overnight hold of WB. On day 5 of PC storage, clinically significant endotoxin levels were detected for all strains ranging from 4 988 976 EU for *S. marcescens* CBS 07/2005 to 74 384 474 EU for *S. marcescens* CBS12/2010. No detectable endotoxin levels were found in the BC-PC units prepared with WB units spiked with *E. coli* ATCC 10798 and *K. pneumoniae* ATCC 10031, which did not survive the overnight hold of WB.

V. *In vitro* PC quality testing does not consistently reflect bacterial contamination in BC-PCs

Comparison results of CD62P expression in response to ADP and ThromboLUX scoring are shown in Table 5.2. On day 1 of storage, BC-PCs containing *S. marcescens* CBS12/2010 and *S. marcescens* CBS07/2005 showed significant differences in CD62P expression and ThromboLUX scoring, respectively. On day 5 of storage, all BC-PCs spiked with Gram-negative

bacteria that survived overnight WB incubation showed significant changes in CD62P expression. ThromboLUX scoring also decreased significantly for these strains on day 5 of storage with the exception of pools containing *K. pneumoniae* PEI-B-P-08-1. None of the two *S. epidermidis* strains induced a significant change in quality measured by either CD62P expression or ThromboLUX scoring. Similarly, the presence and elimination of *E. coli* ATCC 10798 and *K. pneumoniae* ATCC 10031 during the overnight hold of WB did not induce any quality changes in the BC-PCs produced with these WB units.

Table 5.2. Changes in *in vitro* PC quality throughout PC storage

Bacteria	Day 1 of storage				Day 5 of storage			
	Mean % CD62P[a] (SD)	**Difference (p value)[b]**	**Mean TLX scoring (SD)**	**Difference (p value)[b]**	**Mean % CD62P[a] (SD)**	**Difference (p value)[b]**	**Mean TLX scoring (SD)**	**Difference (p value)[b]**
S. marcescens CBS07/2005	43.84 (17.02)	0.4089	18.75 (2.69)	0.0296	-0.72 (3.48)	0.0026	3.84 (3.57)	0.0003
S. marcescens CBS12/2010	22.23 (13.53)	0.0109	17.67 (3.53)	0.1808	2.67 (2.41)	0.0151	6.55 (5.45)	0.0095
E. coli ATCC 10798	73.08 (0.47)	0.0872	17.75 (0.77)	0.1581	34.62 (4.31)	0.6226	19.83 (1.33)	0.3405
E. coli CBS11001	57.32 (3.65)	0.6340	15.20 (0.96)	0.7910	0.45 (0.34)	0.0295	0.96 (1.28)	0.0393
K. pneumoniae ATCC 10031	56.96 (6.13)	0.5873	16.12 (12.02)	0.7118	41.10 (2.08)	0.2105	16.33 (10.26)	0.9877
K. pneumoniae PEI-B-P-08-1	37.25 (3.40)	0.2329	21.63 (0.26)	0.0599	0.31 (0.28)	0.0289	18.61 (1.48)	0.4919
S. epidermidis ST-10002	56.33 (10.30)	0.5944	15.07 (5.70)	0.8381	38.42 (9.29)	0.2719	18.09 (5.87)	0.5000
S. epidermidis ST-11003	58.58 (12.23)	0.4963	14.48 (2.24)	0.9488	35.09 (9.16)	0.5985	17.72 (3.85)	0.6193
Historical controls	51.7 (15.7)	NA	14.6 (2.88)	NA	28.8 (1.2)	NA	16.35 (4.56)	NA

NA, Not applicable. TLX, ThromboLUX®

[a] Percentage of CD62P expression in response to ADP treatment (difference of CD62P expression with ADP and without ADP)

[b] $p < 0.05$ shows significant difference between spiked BC-PCs and historical control data. N≥2 for spiked BC-PCs; N= 12 for BC-PC controls for %CD62P; and N = 10 and 38 for days 1 and 5 of storage, respectively, for TLX BC-PC controls.

5.6 DISCUSSION

In this study, we have taken a real-life mimicking approach producing BC-PCs from WB spiked with transfusion-relevant bacteria. Our results demonstrated that the BC process does not have a widespread bactericidal effect. Additionally, we have provided insights on the bacterial distribution during BC-PC production with preferential segregation towards cellular fractions. The two commercial *E. coli* and *K. pneumoniae* strains used in our study were eliminated in the first few minutes of exposure to WB. Mohr et al. conducted a study also imitating real-life conditions with BC-PC production using bacterially inoculated WB units (Mohr et al., 2006). However, in their study, WB was spiked 8 hours postblood donation and not immediately as done in our study. In agreement to our results, *E. coli* and *Pseudomonas aeruginosa* were also eliminated immediately after WB spiking. One of the major bactericidal immune components in WB is neutrophils. Although neutrophils have been considered short-lived cells with a half-life circulation time of 6–8 hours (Summers et al., 2010). Bashir et al. reported >90% viability of neutrophils from pooled buffy coats which had been stored for up 18 h at 22 ± 2°C without agitation (Bashir et al., 2008*)*. Immediate killing of bacteria when in contact with WB may therefore be due to the action of neutrophils in addition to other systems such as the complement membrane attack complex (Joiner et al., 1984; Taylor, 1983). Survival of the bactericidal activity of WB by Gram-negative bacteria is mainly linked to serum resistance provided by the OmpA protein and changes in the O antigen of the cell wall or capsular polysaccharide (Weiser and Gotschlich, 1991; Cortés et al., 2002). *S. epidermidis* resistance to WB immune factors such as antimicrobial peptides has been reported (Cheung et al., 2010). Additionally, biofilm-positive *S. epidermidis* produces a polysaccharide matrix, which also confers resistance to immune clearance (Kristian et al., 2008).

In the aforementioned study by Mohr, spiked WB was stored for 8 hours at 22 ± 2°C and not up to 23 hours as in our study and still, similar bacterial survival scenarios were observed. In addition to the bacterial killing of two bacteria, other species showed significant titre reductions, one remained at a constant concentration and another multiplied significantly during the 8 h hold of WB. de Korte et al. (De Korte et al., 2001) reported that an overnight hold of WB does not have a significant effect on bacterial contamination levels. These studies support our major conclusion that lower bacterial concentrations in WB-derived PCs, in comparison to Aph-PCs, are not necessarily due to their prolonged contact with WB immune factors. Bactericidal effects of the BC-PC manufacturing process cannot be predicted and depends on the intrinsic characteristics of the bacterial isolates.

Other factors that may contribute to the difference in bacterial levels of contamination between Aph-PCs and BC-PCs include the PC containers, anticoagulants, additive solutions and the manufacturing processes. In our study, we detected bacterial 'dilution' during pooling of the contaminated BC fraction with three other uncontaminated BC fractions suspended with one plasma unit. Furthermore, a decrease in bacterial levels was observed during leucocyte reduction of the BC pool to produce the final PCs. Bacteria could have been removed during the filtration process either by being trapped in the filter or adhered to the removed white blood cells (Dzik, 1995; Buchholz et al., 1994; Holden et al., 2000). PC manufacturing technology affecting rates of bacterial contamination has been recently reported for Aph-PCs (Bravo et al., 2015). Interestingly, we observed preferential bacterial segregation towards the cellular fractions (RBC and BC) in comparison to the plasma unit through BC-PC production. During the centrifugation steps, bacteria can be separated depending on their density, size and sedimentation rates along

with blood cells. Bacteria segregation towards RBC can also be mediated by immune adherence (Damgaard et al., 2015).

In our study, bacteria that survived the BC-PC production process proliferated to high concentration levels, displaying increased virulence by biofilm formation and/or endotoxin production. Despite these safety risk factors, only the Gram-negative organisms decreased *in vitro* PC quality when present at high concentrations. These results support our previous findings that *in vitro* quality markers are not reliable indicators of bacterial contamination in PCs (Greco et al., 2010).

It is expected that contaminated BC-PCs derived from WB containing bacteria that proliferate overnight in WB are captured during PC testing with culture methods. However, these organisms pose a challenge for pathogen inactivation technologies. Keil *et al*. have reported that the Mirasol system (Terumo Inc.) was unable to completely inactivate high titres of bacteria (Keil et al., 2015). Similarly, Schmidt et al. demonstrated that the Intercept Blood System (Cerus Inc.) was unable to inactivate high concentrations of *K. pneumoniae* (Schmidt et al., 2015). A study comparing both pathogen inactivation systems has shown that intercept is more effective for inactivation of bacteria when present at high concentrations (Kwon et al., 2014). In addition, pathogen inactivation technologies are ineffective against endotoxins produced during BC-PC manufacturing. Therefore, the conflicting evidence regarding the effectiveness of pathogen inactivation against high titres of bacteria should be carefully investigated in view of our findings and those of Mohr et al. of bacterial proliferation during WB hold at the beginning of BC-PC production (Mohr et al., 2006). It is important to carefully select bacterial strains for the type of experiments described herein as we demonstrated that commercial strains are not always capable of growing in blood components.

Overall, we have shown that the BC-PC production process does not have a broad bactericidal effect and that bacterial sensitivity to WB immune factors is strain dependent. The fact that pathogenic bacteria can proliferate in WB is an important aspect when considering the implementation of pathogen inactivation technologies. As we and others have shown that bacteria segregate preferentially towards cellular fractions, consideration should be given to screen or pathogen inactivate WB before overnight hold during the buffy coat PC manufacturing process to minimize or eliminate contamination of the final PC product.

5.7 ACKNOWLEDGMENTS

Ms. M. Taha performed all the experiments described in this study. Dr. M. Kalab performed electron microscopy while Dr. QL Yi conducted statistical analyses. Drs. P. Schubert and E. Maurer assisted with the study design and data interpretation for the flow cytometry and ThromboLUX experiments, respectively. M. C. Jenkins helped with the BC-PC production study design. Dr. S. Ramirez-Arcos and Ms. M. Taha designed the study, analysed the data and wrote the manuscript. The authors thank the volunteer blood donors and netCAD staff for blood collection and PC manufacturing. We are also thankful to Ms. J. Leung (LightIntegra, Vancouver, Canada) and staff of Drs. M. Scott and D. Devine (UBC Centre for Blood Research, Vancouver, Canada) for their invaluable support and technical assistance. We extend our gratitude to Dr. G. Walsh (Scientific Writer, Canadian Blood Services) for critical review of this manuscript. Ms. M. Taha holds a Graduate Student Fellowship awarded by Canadian Blood Services and Health Canada. Funding for this study was provided by Canadian Blood Services and Health Canada. The views expressed herein do not necessarily represent the view of the federal government.

CHAPTER 6.

Bacterial Survival And Elimination During Overnight Hold Of Whole Blood

6.1 STATEMENT OF MANUSCRIPT STATUS AND CONTRIBUTIONS

The manuscript "Bacterial survival and elimination during overnight hold of whole blood" will be submitted for peer-review.

M. Taha conducted all the experiments and data analysis. Dr. S. Ramirez-Arcos was the thesis' supervisor providing guidance on the experimental design, troubleshooting and data interpretation. Dr. D. Kyluik-Price (University of British Columbia, Vancouver) assisted with the bacterial growth assays in WB. Ms. D. Kumaran (Canadian Blood Services, Ottawa) provided technical assistance during the plasma, CPD and neutrophil assays. Ms. R. Pasha (Canadian Blood Services, Ottawa) facilitated training for the flow cytometer assays used during the neutrophil killing studies.

6.2 ABSTRACT

Collected whole blood (WB) for BC-PC production is left to rest overnight at room temperature. WB is known for its antimicrobial activities, therefore, this study was aimed at investigating the contribution of the WB resting period on bacterial elimination. A panel of 21 Gram positive (*S. epidermidis, S. aureus, S. capitis* and *S. agalactiae*) and Gram negative (*Serratia liquefaciens, S. marcescens, K. pneumoniae E. coli and Yersinia enterocolitica*) bacteria were individually inoculated in WB at a target concentration of 500 CFU/mL. Spiked WB was held overnight at room temperature and bacterial viability and growth were monitored at 3, 8 and 24 hours during this period. Furthermore, the role of plasma and neutrophils in the elimination process of selected bacteria was explored by performing a plasma-sensitivity assay and a neutrophil killing assay, respectively. There were three different scenarios for bacterial behavior in WB during the overnight hold: some bacteria grew overnight, others remained viable but did not proliferate, and a third group had a drastic decreased in viability with some of them being completely eliminated. Different patterns of sensitivity for different strains that belongs to the same species were observed. It was also found that the antibacterial activity of the overnight hold period of WB is strain dependent with some bacteria being able to grow to high levels towards the end of the overnight incubation. Consideration should be given to screening WB for bacterial contamination after the overnight hold in order to minimize or eliminate bacterial contamination of the final PC product.

6.3 INTRODUCTION

Following the blood donation procedure, the WB unit is allowed to rest at room temperature before it is processed into RBC, plasma, and PC components via the BC production

method, and this hold time can vary between 8 to 24 hours depending on the blood center (van der Meer and de Korte, 2015). In addition to logistical and financial benefits, it has been reported that this hold period allows for some blood components to rest, resulting in better quality products (van der Meer and de Korte, 2015). For example, platelet counts in PCs were reported to be higher following a 24 hours hold period at room temperature when compared to counts obtained following an 8 hours hold period (Lu et al., 2011; Dijkstra-Tiekstra et al., 2008). Overnight WB resting periods results in the production of PCs with lower platelet activation and apoptosis (Lu et al., 2011; Dijkstra-Tiekstra et al., 2008). Moreover, increased safety of BC-PCs due to the bactericidal action of immunological components such as WBCs during the overnight hold of WB has been reported (Sanz et al., 1997; van der Meer and de Korte, 2015). In chapter 5 of this thesis (Taha et al., 2016) the antibacterial activity of WB during the overnight hold was examined against eight bacteria, and it was established that WB storage contributes to bacterial elimination of some bacteria. However, other bacteria were able to proliferate during the overnight storage period at room temperature even in the presence of WBCs. Similar outcomes have been documented by Mohr et al following 8 hours of WB storage at room temperature (Mohr et al., 2008). Therefore, it was of interest to screen more bacterial species and strains and monitor their growth at different time points of the overnight incubation period to strengthen the observations described in chapter 5 of this thesis.

In this chapter, the objective was to test 21 bacteria for thier survival in WB -outside the human body- during an overnight hold period at room temperature. Furthermore, the role of plasma and neutrophils in the elimination of selected bacteria was explored.

6.4 MATERIALS AND METHODS

I. Bacterial strains

Table 6.1 lists the bacteria that were used in this study. Bacteria were streaked from frozen stocks on BA plates and were incubated at 30 ^{o}C (*S. marcescens* and *S. liquefaciens*) or 37°C (other species). After overnight incubation, colonies were used to prepare suspensions adjusted to Densimat 0.5 (approximately 10^8 CFU/mL) in BHI+ 15% glycerol. Adjusted stocks were kept frozen at -83 ^{o}C until further use.

II. WB and plasma collection

WB donors were healthy volunteers who signed consent before blood collection. Research design was approved by the Canadian Blood Services Research Ethics Board. For the plasma experiments described below, WB was collected and processed to manufacture plasma through the BC-PC method at netCAD in accordance with procedures established at Canadian Blood Services (Levin et al., 2008).

III. Bacterial spiking of WB

WB was collected in a 'top-and-bottom' MacoPharma (Mouvaux, France) collection set with the anticoagulant CPD (citrate, phosphate, dextrose) which contains 26.3 mg/mL sodium citrate, 3.27 mg/mL citric acid, 25.5 mg/mL dextrose. Samples were immediately taken for sterility testing using BacT/ALERT BPA and BPN culture bottles as described previously (Mastronardi et al., 2012). As logistically it was not possible to spike whole WB bags with each of the 21 bacteria with three replicates for each experiment, WB aliquots were used for this study. Seven mL of WB or TSB (control medium) were added to 15 mL polystyrene tubes (Falcon, Corning Inc., Durham, NC). Bacterial frozen stocks were thawed and serially-diluted in sterile 0.9% saline and appropriate dilutions were used to inoculate 7 mL of WB and TSB at a target

concentration of 500 CFU/mL. The tubes containing WB inoculated with bacteria were tilted and incubated at room temperature (22± 2 ^{o}C) overnight (for 24 hours). At Canadian Blood Services, the standard operation procedure prescribes an 8- 24 hours hold period for WB, therefore bacterial viability was monitored at time points 0, 3, 8 and 24 hours by plating 100 uL of each WB sample on BA plates after gently inverting the tubes 10 times. Plates were incubated at the appropriate temperature for 24 hours followed by colony counting. When bacteria were not detected by plating on BA plates, the spiked WB (~ 7 mL) was inoculated into a BPA bottle and incubated for 6 days using the BacT/ALERT system. Samples from positive culture bottles were taken and plated on BA plates and Gram staining was performed to confirm the colony morphology of the inoculated organism. Each experiment was performed using three different WB units.

IV. Plasma-sensitivity assay

Bacteria that showed elimination or drop in counts during the 24 hours hold of WB were tested for sensitivity to plasma. Fresh plasma units were tested for sterility using the BacT/ALERT system (Mastronardi et al., 2012). A fraction of the plasma unit was heat-inactivated for 1 hour at 56 ^{o}C and the rest was left untreated. Bacterial frozen stocks of seven bacteria from Table 6.1 were serially-diluted in 0.9% sterile saline and appropriate dilutions were used to inoculate 7 mL of plasma, 7 mL of heat-inactivated plasma and 7 mL of TSB at a target concentration of ~500

Table 6.1. Bacterial strains used in this study

Species	Strain	Origin
S. epidermidis	CBS 2014-09[a]	PC contaminants
	CBS 6038	
	AZ-25	Skin isolates
	AZ-69	
	AZ-66	
	AZ-80	
	AZ-90	
	AZ-106-w[b]	
S. aureus	CBS 12003	PC contaminant
	ATCC 27217	ATCC commercial (nasal origin)
S. agalactiae	CBS 12005	PC contaminant
Y. enterocolitica	ATCC 49397[b]	ATCC commercial
S. liquefaciens	CBS 0602	PC contaminant
	ATCC 27592	ATCC commercial (milk isolate)
S. marcescens	ATCC 13880[b]	ATCC commercial (pond water)
	ATCC 43862	ATCC commercial
	CBS 07/2005[a]	PC contaminant
S. capitis	517	PC contaminants
	512[b]	
E. coli	ATCC 25922	ATCC commercial (clinical isolate)
	ATCC 10798 [b,c]	ATCC commercial (human feces)
K. pneumoniae	ATCC 13883[b]	ATCC commercial
	ATCC 10031[b, c]	

[a] Involved in fatal transfusion reactions
[b] Tested for plasma sensitivity
[c] Tested in WB in chapter 5

CFU/mL. The tubes were tilted and incubated at room temperature (22±2 ^{o}C) for 24 hours. Bacterial viability was monitored at time points 0, 3, 8 and 24 hours by plating 100 uL of the sample on BA plates after gently inverting the tubes 10 times. Bacterial colonies were counted after 24 hours of incubation at the appropriate temperature. When bacteria were not detected by plating on BA plates, the spiked plasma samples (~ 7 mL) were added to BPA bottles and incubated for 6 days in the BacT/ALERT system at 36 ^{o}C. Samples from positive culture bottles were withdrawn and plated on BA plates and Gram staining was performed to confirm colony morphology of the inoculated organism. Each experiment was performed using at least three different plasma units.

V. Bacterial survival in CPD

The antibacterial activity of the anticoagulant CPD, which is used for WB collection, was tested against bacteria that showed elimination or a drop in viability in WB. Sterile CPD was obtained from unused WB collection bags (MacoPharma, Mouvaux, France). Table 6.2 describes the media used in these assays. Media were aliquoted into 4 mL in 14 mL polystyrene tubes. Pre-adjusted bacterial frozen stocks were serially-diluted and the appropriate dilution was used to inoculate 4 mL of media at a final concentration of ~ 500 CFU/ml. The tubes were tilted and incubated at room temperature (22±2 ^{o}C) for 24 hours. Bacterial viability was monitored at time points 0, 3, 8 and 24 hours by plating 100 uL of the samples on TSA plates. Bacterial colonies were counted following 24 hours of incubation at the appropriate temperature. This experiment was conducted three independent times.

Table 6.2. Media used to test for CPD antibacterial activity.

	Media					
	TSB	**CPD + 0.9% saline**	**0.9 % Saline + dextrose**	**[b]CPD+TSB**	**[b]0.9 % Saline+ dextrose+TSB**	**[b,c]CPD+TSB pH adjusted**
[a]Final CPD (%)	N/A	14.4	N/A	14.4	N/A	14.4
Dextrose concentration (mg/mL)	2.5	3.68	3.68	6.18	6.18	6.18
Purpose	**Control:** -optimal media for bacteria	**Test** CPD bactericidal effect	**Control** for CPD/saline: - contains the same dextrose concentration as CPD/saline - uses saline instead of CPD	**Test** CPD bactericidal effect while accounting for possible effect of lack of nutrient on bacterial survival	**Control** for CPD/TSB: - contains the same dextrose concentration as CPD/TSB - uses saline instead of CPD	**Test** CPD bactericidal effect while accounting for possible effect of media pH on bacterial survival

[a] Approximately the same CPD% in blood collection bag after WB donation.
[b,c] To assay *S. epidermidis* AZ-106-w and *S. capitis* 512
[c] pH was adjusted from 6.57 to 7.1.
N/A; not applicable

VI. Neutrophil killing assay

Bacterial elimination by neutrophils, which are the most abundant type of WBCs in human blood and play a major role in bacterial eradication (Nauseef, 2007), was assessed for *S. epidermidis* AZ-106-w and *S. capitis* 512.

Neutrophil isolation. WB donors were healthy volunteers and signed consent before blood collection. Research design was approved by The Ottawa Hospital Research Institute (OHRI) Research Ethics Board. WB was collected in 10 mL ethylenediaminetetraacetic acid (EDTA)-coated tubes (60 mL in total) and processed within 2 hours of donation. Neutrophils were isolated using Lympholyte®-poly (density separation, Cedarlane, ON, Canada) according to the vendor's instructions and according to the protocol by Oh et al., 2008 with some modifications. The WB was layered slowly over Lympholyte®-poly in 50 mL tubes with a 1:1 ratio and centrifuged for 35 min at 500 g at room temperature (18-22 ^{o}C). Six distinct layers were obtained and the neutrophil-rich layer was removed and placed in another tube where it was washed with 1x Hanks' Balanced Salt solution (HBSS, GIBCO, Burlington, Canada) without $Ca^{2}+/Mg^{2}+$ and then centrifuged at 350 g for 10 minutes. RBCs contaminating the pellet were lysed using Red Cell Lysis Buffer (Roche, Germany) and removed by centrifugation at 250 g for 5 minutes. The pellet was then washed again with HBSS (without $Ca^{2}+/Mg^{2}+$) and centrifuging at 250 g for 5 minutes and resuspended in 1 mL of plasma.

Neutrophil enumeration, viability and purity. Cells were diluted (1:50) in HBSS and 10 uL was added to 10 uL of 0.4% trypan blue solution. Ten microliters of this suspension was added to a heamocytometer and viable cells (colorless, trypan blue exclusion) were counted using an inverted microscope at 10 X magnification (Olympus CKX41). Based on the cell count obtained on the heamocytometer, cells were diluted in 2% fetal bovine serum/Dulbecco's PBS

(Invitrogen, Burlington, Canada) to prepare a cell suspension of ~200,000 cells /100 uL. One-hundred uL of the diluted cells was added to an eppendorf tube containing anti-human WBCs antibodies: 2.5 uL mouse anti-human CD45- allophycocyanine (APC) and 20 uL mouse anti-human CD16b- phycoerythrin (PE) antibodies, respectively, were added. This mix was incubated for 20 minutes in the dark at room temperature followed by a spin at 300 g for 6 minutes. The pellet was then resuspended in 100 uL 1X Annexin binding buffer followed by addition of 5 uL Annexin V Alexa Fluor 488 conjugate and 3 uL Sytox AADvanced™ dead cell stain, incubation for 15 minutes at room temperature and finally addition of 400 uL Annexin buffer. Annexin V and Sytox were added to measure cell apoptosis and death, respectively. Samples were analyzed using Attune® Acoustic Focusing Cytometer (AB applied biosystems, Life Technology). Apoptotic and dead cell events were excluded from analysis by gating out Annexin V and Sytox positive cells. The antibodies used in this experiment were purchased from Becton Dickinson Biosciences. Annexin binding buffer, Annexin V and Sytox were obtained from Life Technologies. This separation process yielded a cell suspension of $\geq$ 85% purity for neutrophils with $\geq$ 89% viability.

***Neutrophil killing assay*.** Isolated neutrophils were diluted in plasma to a final concentration of 5-6 X 10^6 cells/mL, which corresponds to the neutrophil concentration found in WB. These dilutions were prepared by taking the percentage of viable neutrophils obtained from flow data into account. Then, 80 uL of neutrophils/plasma, TSB, plasma or heat-activated plasma were added to a 96-well plate in triplicate (2 separate 96-well plates for time points 3 and 24 hours were prepared) plus 20 uL of the bacterial suspension for a final concentration of 10^3 CFU/mL (~1 CFU per 6000 neutrophils). The 96-well plates were incubated at room temperature at 22± 2 ^{o}C (stimulating the WB experiment incubation settings) on a shaker at 60 rpm. At time points 3

and 24 hours, plates were put on ice to stop neutrophil killing activities. Suspensions of neutrophils + bacteria were removed and added to eppendorf tubes along with 200 uL of ice-cold 0.9% saline. The mix was centrifuged for 5 minutes at 250 g at 4 ^{o}C and the supernatant was collected in a separate tube. The pellet was resuspended in ice-cold saline (300 uL) and centrifuged for 5 minutes at 250 g at 4 ^{o}C and the supernatant was collected. This washing step was performed three times to recover extracellular bacteria. The collected supernatant (900 uL) of extracellular bacteria was plated on large TSA plates (150 X 15 mm). The pellet that contained the neutrophils was incubated at room temperature for 10 minutes with a neutrophil lysing solution: 400 uL of 4% Tween 20 followed by adding 600 uL of ddH_2O and plating the 1 mL sample on TSA to recover intracellular bacteria. Preliminary control experiments indicated that there was no deleterious effect of 4% Tween 20 on the viability of *S. epidermidis* AZ-106-w and *S.capitis* 512 (Appendix C.1).

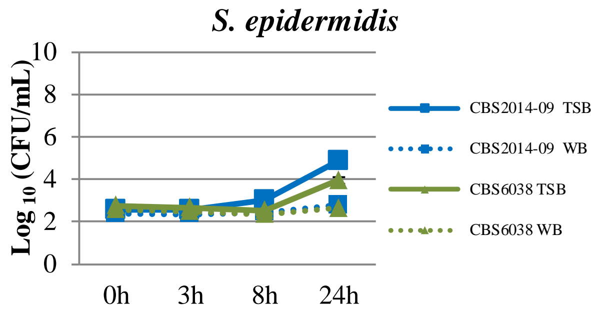
VII. Statistical analyses

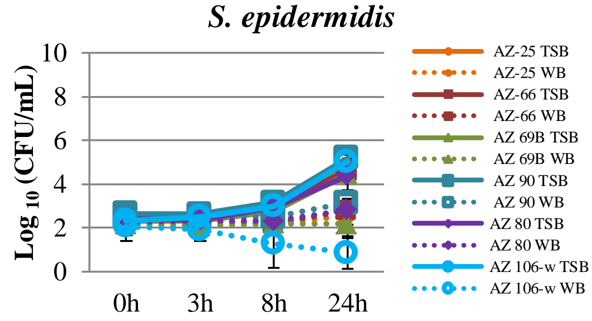
The numbers of CFU were log transformed (base 10) for statistical comparisons. Effect of 4% Tween 20 on bacterial viability was evaluated by comparing viable bacterial counts with Tween to the 0.9% saline control after incubation using t-test (paired). The effect of CPD on bacterial viability was checked by comparing counts at 0 to 24 hours (paired t-test). The killing percentage of *S. capitis* 512 and *S. epidermidis* AZ-106-w by neutrophils was calculated according to the following formula: $[1\text{-}(CFU_{\text{intracellular+ extracellular}}/ CFU_{\text{plasma without neutrophils}}]$ X 100 (Vuong et al., 2004c). The significance of the neutrophils' killing ability was calculated by comparing killing % to 0% (when no neutrophils were added to plasma) using t-test. A p-value of <0.05 was considered statistically significant. Analyses were performed with the Statistical Analysis System (SAS, 2000-2004, SAS Institute, Inc., Cary, NC).

6.5 RESULTS

I. No universal bactericidal effect of WB was observed during overnight hold at room temperature

The bactericidal effect of WB during the overnight hold time was tested with 21 bacteria (including Gram positive and Gram negative organisms) (Fig. 6.1). Bacteria displayed different degrees of sensitivity to WB and were categorized in three groups. The first group of bacteria grew overnight, other bacteria remained viable but did not proliferate, and bacteria in the third group had a drastic decreased in viability with some of them being completely eliminated. In the first group, *E. coli* ATCC 25922, *S. liquefaciens* CBS 0602 and ATCC 27592, *S. marcescens* ATCC 43862 and CBS 07/2005 grew to very high levels (up to 5 logs CFU/mL increase)) with concentrations starting to increase after 8 hours of WB storage at room temperature (Fig. 6.1). In contrast, the second group of bacteria including *S. capitis* 517, seven out of the eight *S. epidermidis* strains, the two *S. aureus* strains and *S. agalactiae,* remained viable with no or very low increase in concentration . In the last group, *S. capitis* 512 and *S. epidermidis* AZ-106-w remained viable but showed a decrease in concentration (Fig. 6.1). These two bacteria were not detected by plating but grew in the BPA bottles from the 3 different tested WB units (Appendix C.2). The strains *S. marcescens* ATCC 13880 (in two out of three repetitions), *Y. enterocolitica* ATCC 49397 and *K. pneumoniae* ATCC13883 were completely eliminated at 24 hours of incubation (Fig 6.1, Appendix C.2)


S. epidermidis
Log 10 (CFU/mL)
0h
3h
8h
24h
CBS2014-09 TSB
CBS2014-09 WB
CBS6038 TSB
CBS6038 WB


S. epidermidis
Log 10 (CFU/mL)
0h
3h
8h
24h
AZ-25 TSB
AZ-25 WB
AZ-66 TSB
AZ-66 WB
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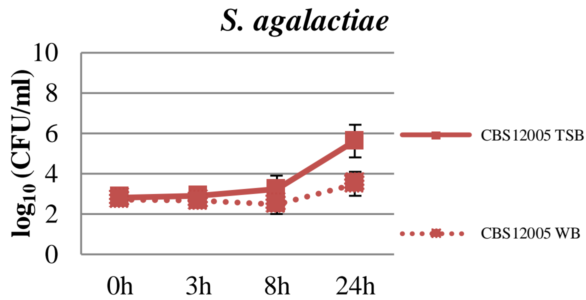
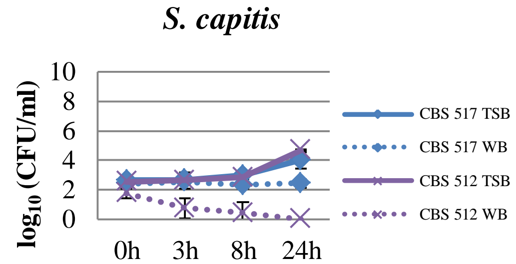
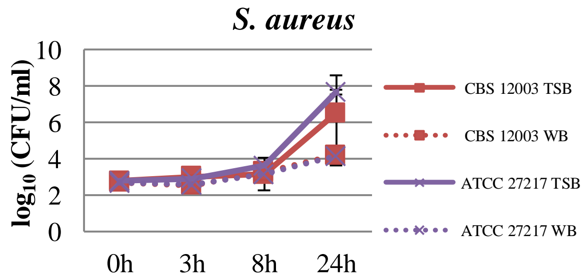
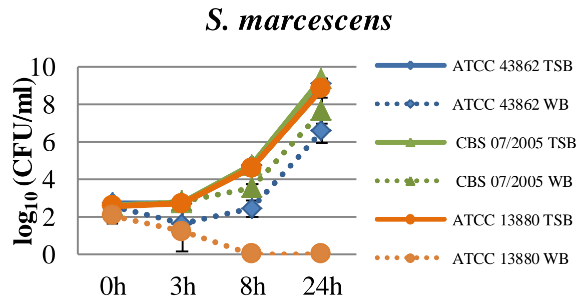
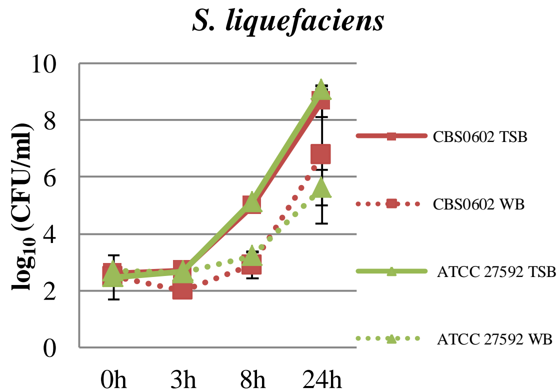
AZ 90 WB
AZ 80 TSB
AZ 80 WB
AZ 106-w TSB
AZ 106-w WB


S. agalactiae
log10 (CFU/ml)
0h
3h
8h
24h
CBS12005 TSB
CBS12005 WB


S. capitis
log10 (CFU/ml)
0h
3h
8h
24h
CBS 517 TSB
CBS 517 WB
CBS 512 TSB
CBS 512 WB


S. aureus
log10 (CFU/ml)
0h
3h
8h
24h
CBS 12003 TSB
CBS 12003 WB
ATCC 27217 TSB
ATCC 27217 WB


S. marcescens
log10 (CFU/ml)
0h
3h
8h
24h
ATCC 43862 TSB
ATCC 43862 WB
CBS 07/2005 TSB
CBS 07/2005 WB
ATCC 13880 TSB
ATCC 13880 WB


S. liquefaciens
log10 (CFU/ml)
0h
3h
8h
24h
CBS0602 TSB
CBS0602 WB
ATCC 27592 TSB
ATCC 27592 WB

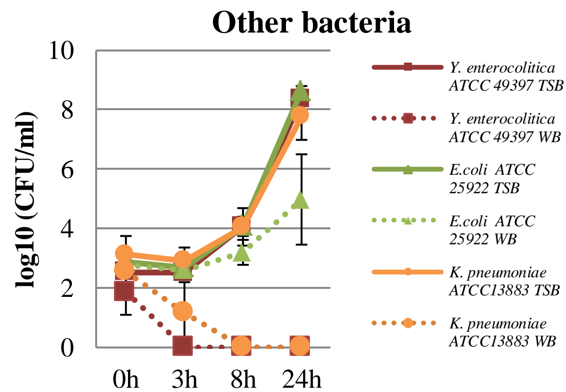

Other bacteria
log10 (CFU/ml)
0h
3h
8h
24h
Y. enterocolitica ATCC 49397 TSB
Y. enterocolitica ATCC 49397 WB
E.coli ATCC 25922 TSB
E.coli ATCC 25922 WB
K. pneumoniae ATCC13883 TSB
K. pneumoniae ATCC13883 WB

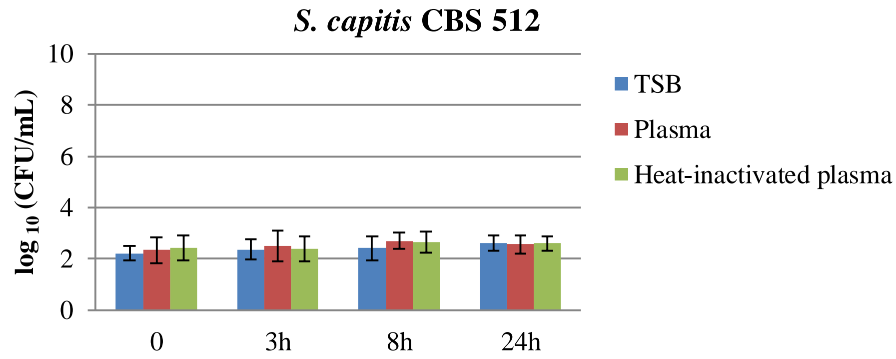
Figure 6.1. Growth dynamics of 21 bacteria in WB (dashed line) and TSB (solid line) at 22± 2°C for 24 hours. N=3, ±SD.

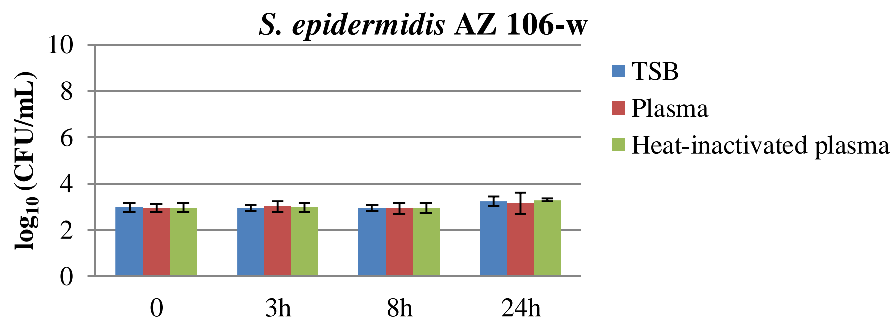
II. Detection of plasma-sensitive bacteria

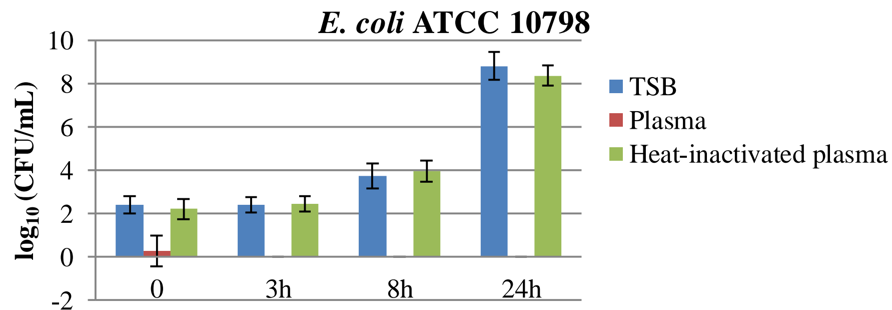
Five bacteria (*K. pneumoniae* ATCC 13883, *S. capitis* 512, *S. epidermidis* AZ-106-w, *S. marcescens* ATCC 13880 and *Y. enterocolitica* ATCC 49397) were further tested for their plasma sensitivity due to their drastic decreased in viability or elimination during the overnight hold of WB. *E. coli* ATCC 10798 and *K. pneumoniae* ATCC 10031 (eliminated in WB in chapter 5 experiment Fig. 5.1 , Taha et al., 2016) were also tested for their plasma-sensitivity. Results showed that five of the seven examined bacteria were plasma-sensitive and were eliminated almost immediately after incubation in untreated plasma while they survived and even multiplied in heat-inactivated plasma and TSB (Fig. 6.2). Both of the Gram positive bacteria *S. capitis* 512 and *S. epidermidis* AZ-106-w, were plasma-resistant and remained at the same concentration in the three tested media (plasma, heat-inactivated plasma and TSB).

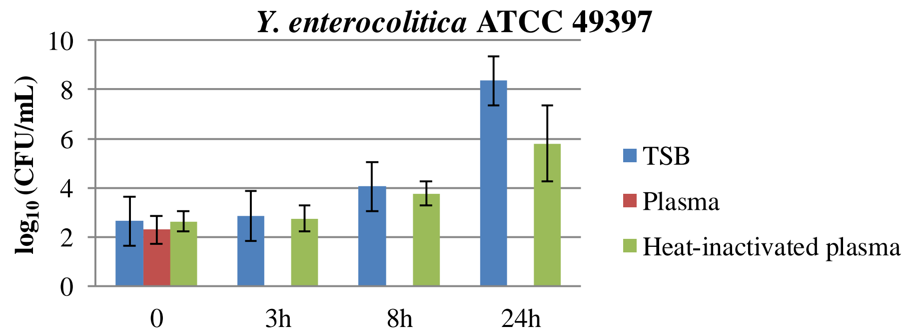
III. CPD has a bactericidal effect on selected strains

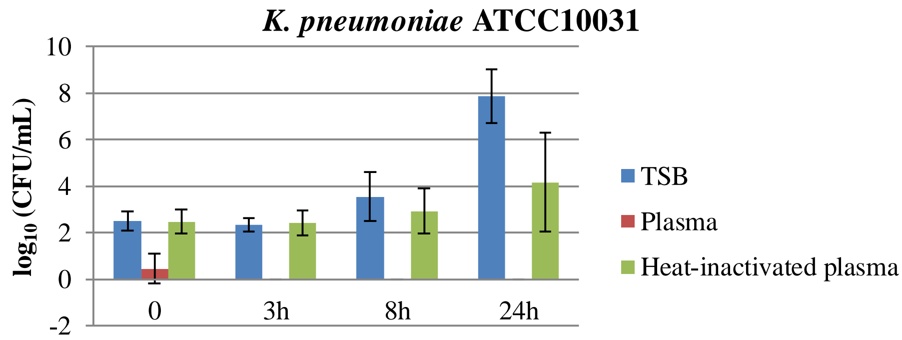
Seven bacteria (*K. pneumoniae* ATCC 13883, *S. capitis* 512, *S. epidermidis* AZ-106-w, *S. marcescens* ATCC 13880, *Y. enterocolitica* ATCC 49397, *E. coli* ATCC 10798 and *K. pneumoniae* ATCC 10031) were tested for their growth characteristics in CPD since they were either eliminated or showed a significant decrease in concentration during the overnight hold of WB. CPD (CDP+ saline) had no bactericidal effect on all the tested five Gram negative bacteria (Fig. 6.3) with viable counts remaining the same ($p>0.05$) after 24 hours of incubation at room temperature. On the other hand, Gram positive *S. capitis* 512 and *S. epidermidis* AZ-106-w showed a significant drop in viable cell counts at 24 hours ($\geq$ 1.5 log CFU/mL, $p<0.05$) in both CPD+ saline and control (saline+ dextrose) (Fig. C.3). Therefore, they were tested in CPD+TSB to rule out a lack of nutrients effect. Interestingly, *S. epidermidis* AZ-106-w was not eliminated

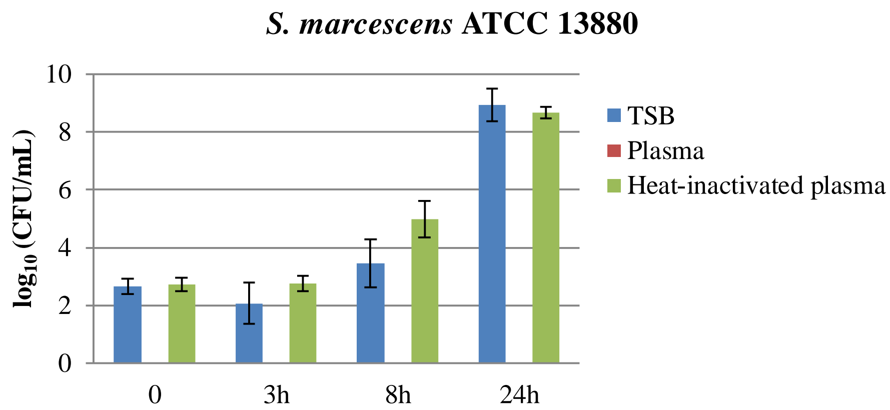

S. capitis CBS 512
log 10 (CFU/mL)
10
8
6
4
2
0
0
3h
8h
24h
TSB
Plasma
Heat-inactivated plasma


S. epidermidis AZ 106-w
log10 (CFU/mL)
10
8
6
4
2
0
0
3h
8h
24h
TSB
Plasma
Heat-inactivated plasma


E. coli ATCC 10798
log10 (CFU/mL)
10
8
6
4
2
0
-2
0
3h
8h
24h
TSB
Plasma
Heat-inactivated plasma


Y. enterocolitica ATCC 49397
log10 (CFU/mL)
10
8
6
4
2
0
0
3h
8h
24h
TSB
Plasma
Heat-inactivated plasma





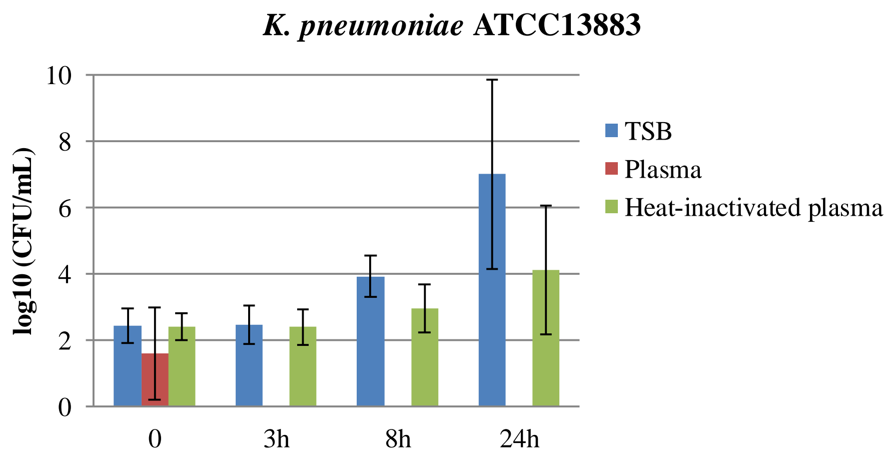
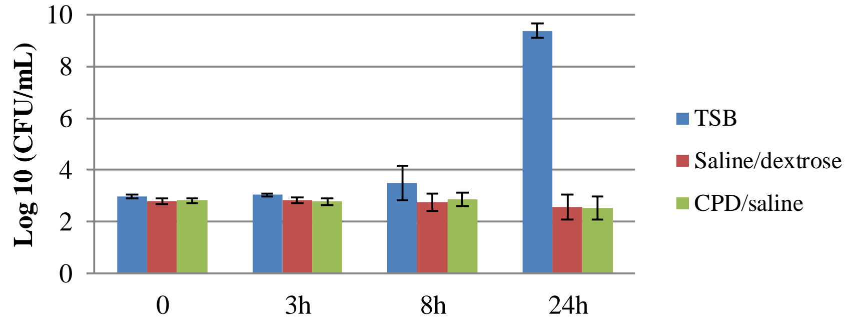
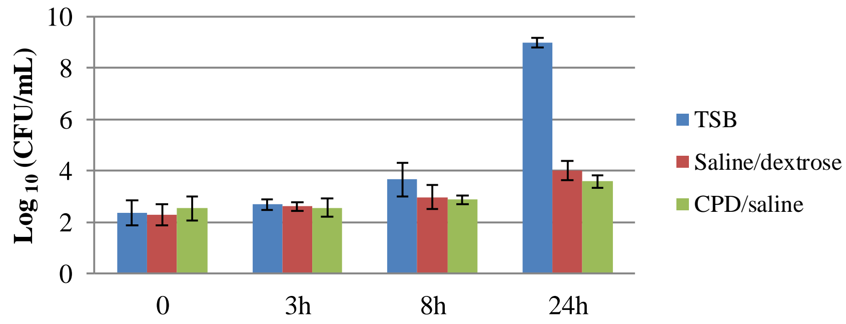
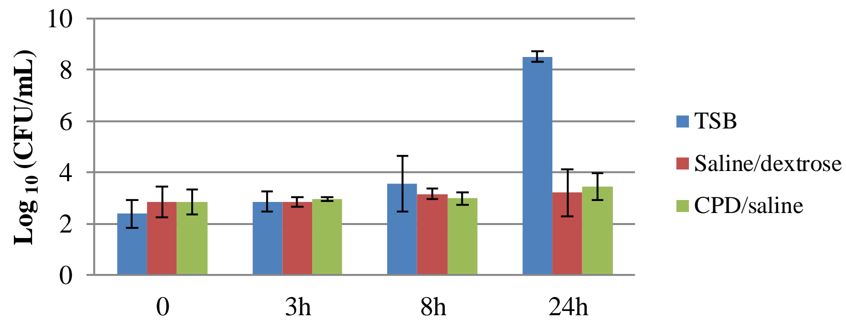
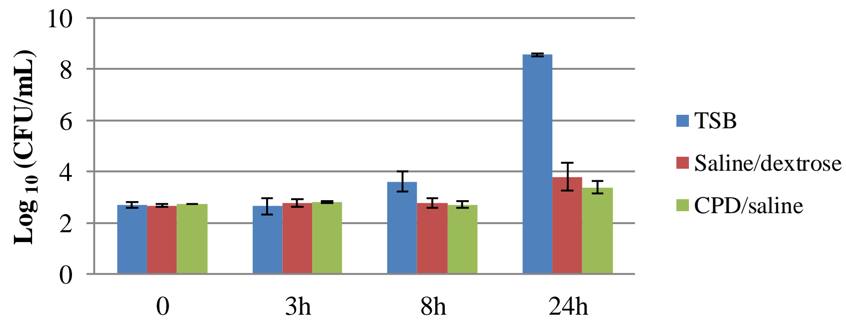


Figure 6.2. Bacterial growth in plasma, heat-inactivated plasma and TSB at 22± 2°C for 24 hours. N≥3, ±SD.


S. marcescens ATCC 13880
Log 10 (CFU/mL)
10
8
6
4
2
0
0
3h
8h
24h
TSB
Saline/dextrose
CPD/saline


E. coli ATCC 10798
Log 10 (CFU/mL)
10
8
6
4
2
0
0
3h
8h
24h
TSB
Saline/dextrose
CPD/saline


Y. enterocolitica ATCC 49397
Log 10 (CFU/mL)
10
8
6
4
2
0
0
3h
8h
24h
TSB
Saline/dextrose
CPD/saline


K. pneumoniae ATCC13883
Log 10 (CFU/mL)
10
8
6
4
2
0
0
3h
8h
24h
TSB
Saline/dextrose
CPD/saline

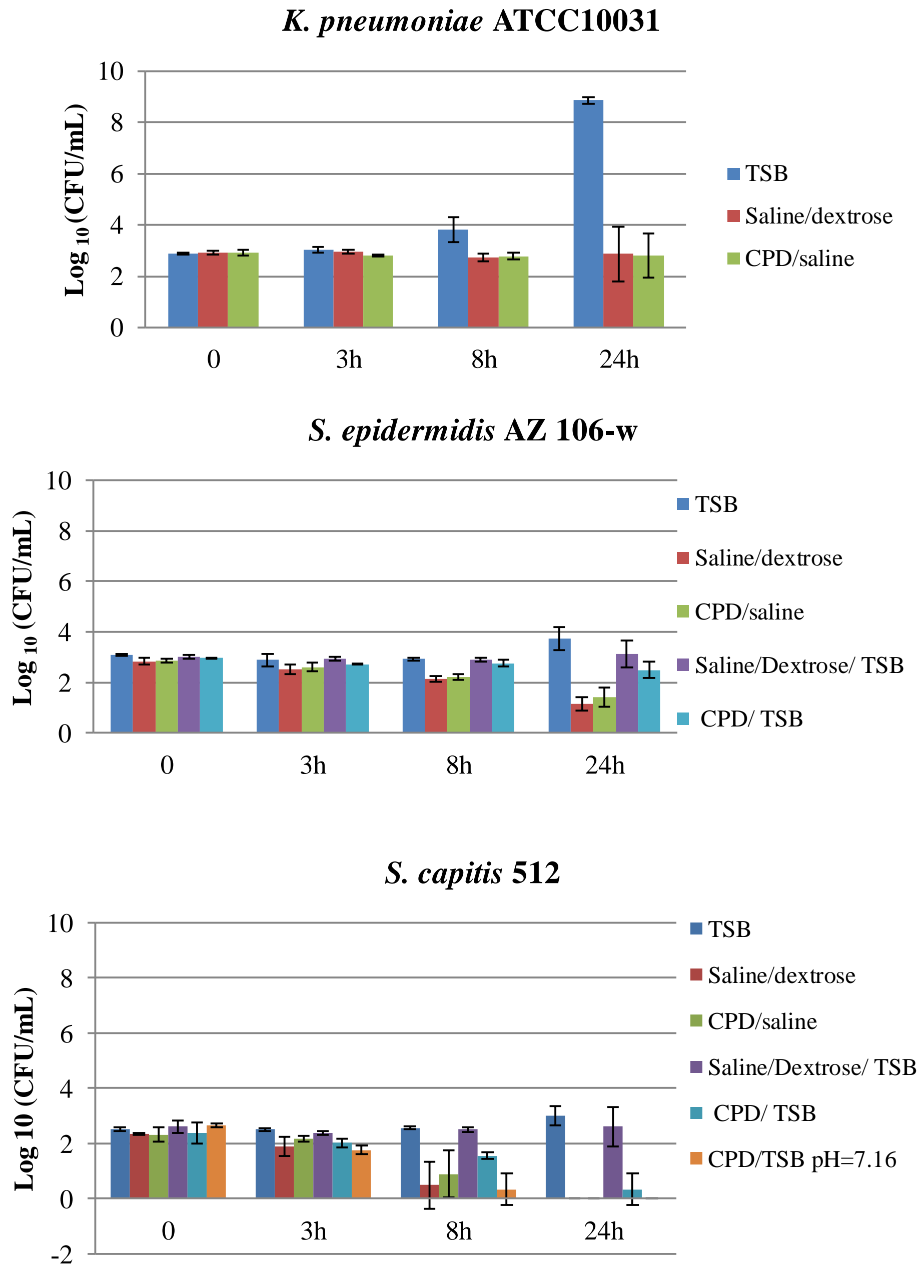


Figure 6.3. Antibacterial activities of CPD. Seven bacteria were tested in CPD and other media (described in Table 6.2) at 22± 2 ^{o}C and for 24 hours. N=3, ±SD.

by the end of the incubation period in CPD/TSB ($p>0.05$), however, *S. capitis* 512 was still susceptible to CPD (≥ 2 log CFU/mL reduction, $p=0.0646$) at 24 hours. *S. capitis* 512 was also susceptible to CPD even after the pH of CPD/TSB was adjusted from pH=6.57 to pH=7.1($p=0.003$). The above results confirmed the deleterious effect of CPD for *S. capitis* 512.

IV. Susceptibility to killing by neutrophils

Neutrophil killing assay was performed to determine the susceptibility of *S. capitis* 512 and *S. epidermidis* AZ-106-w to elimination by neutrophils. Both bacteria eliminated by neutrophils overtime (time points 3 and 24 hours) and were significantly ($p<0.05$) with a decrease in viable counts of 57.7% and 60.2%, respectively towards the end of the storage period at 24 hours (Fig. 6.4, Appendix C.3).

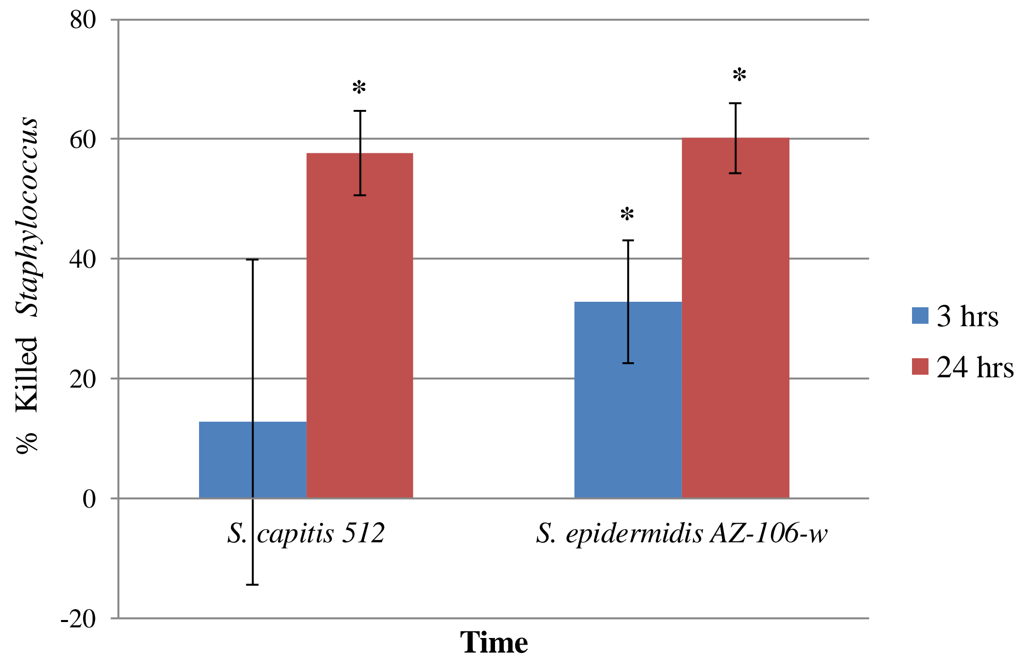


Figure 6.4. Neutrophil killing % of *S. capitis* 512 and *S. epidermidis* AZ-106-w at 3 and 24 hours of incubation at 22±2°C. Killing % calculated according to the following formula: [1-(CFU $_{intracellular+ extracellular}$/ CFU $_{plasma\ without\ neutrophils}$] X 100. N=3, ±SD. (*$p<0.05$).

6.6 DISCUSSION

Blood is known for its antimicrobial activity owing to the fact that it contains different immunological components such as WBCs, platelets, complement system, and antimicrobial peptides (Nauseef, 2007; Yeaman, 2014; Walport et al., 2011). In this study, the antibacterial activities of WB, used for blood component production, were investigated over time during an overnight hold period at room temperature. Results of the study revealed three different scenarios for bacterial survival/growth in WB during overnight hold: bacteria that grew overnight, others that remained viable but did not proliferate, and bacteria that displayed a drastic decreased in viability with some of them being completely eliminated. Different patterns of sensitivity for different strains that belong to the same species were observed.

Among the 21 screened bacteria, five Gram negative organisms, *S. marcescens* ATCC 13880, *Y. enterocolitica* ATCC 49397, *K. pneumoniae* ATCC 13883, *K. pneumoniae* ATCC 10031 and *E. coli* ATCC 10798 (the last two bacteria had been previously tested in WB in chapter 5, Taha et al., 2016) were eliminated following exposure to WB and were plasma-sensitive. The Complement Membrane Attack Complex (MAC) was likely responsible for the fast elimination of these organisms. The MAC targets the bacterial cell membrane forming pores that lead to bacterial lysis and death (Walport et al., 2001, Joiner et al., 1984; Taylor ,1983). Recent work published by Berends and colleagues showed that MAC-independent killing mechanisms can also contribute to the rapid killing of *E. coli* and *K. pneumoniae* (Berends et al., 2015). The complement system is proposed herein to be the main immunological component responsible for the fast elimination of Gram negative bacteria, but it is important to note that WB is collected in containers containing the anticoagulant CPD, which has chelating properties. Since the full activation of the complement system is dependent on the presence of Ca^2+ and

Mg^2+, and CPD chelates these ions, it is expected that collected WB has reduced complement activities. However, there are reports showing that complement activities are not completely lost but are reduced up to 60% in citrate plasma compared to serum (Seelen et al., 2005; Huang et al., 2014). Therefore, it seems that the remaining complement system activity in WB collected with CPD was enough to inactivate plasma-sensitive bacteria that were tested in this study.

It has been reported that CPD has antibacterial activities due to its chelation properties, which affect bacterial growth, survival and cell wall integrity (Lee et al., 2001; Ash et al., 2000; Helander et al., 2000). However, none of the plasma-sensitive bacteria were affected by CPD which rules out the contribution of CPD to the bacterial elimination in WB and plasma.

The other five tested Gram negative bacteria in this study showed proliferation during overnight hold of WB, specifically, after 8 hours of storage bacterial growth for all of them (*E. coli* ATCC 25922, *S. liquefaciens* CBS 0602 and ATCC 27592, and *S. marcescens* ATCC 43862 and CBS 07/2005) was observed. Buchholz and colleagues showed a similar pattern of multiplication of Gram negative bacteria in WB. In their experiments, *E. coli*, *E. cloacae* and *S. marcescens,* inoculated in WB at an initial concentration of 1 CFU/mL, reached high concentrations after 7 hours of incubation at room temperature (Buchholz et al., 1994).

The above described data showed that bacterial sensitivity to WB and plasma varied within strains of the same species. Previous reports have also documented strain dependent sensitivity to serum elimination (Benge, 1988; Jessop and Lambert, 1986). For example, Benge reported different degrees of sensitivity to serum after screening 120 strains of *K. pneumoniae* (Benge,1988). Immune system evasion and complement-resistance of Gram negative bacteria is linked to certain bacterial properties. For example, the presence of the outer membrane protein A acts by stopping the complement system through binding to C4bp, one of the complement

regulatory proteins, resulting in preventing the MAC formation as well as weakening opsonization for phagocytosis (Prasadarao et al., 2002; Taylor, 1974; Weiser and Gotschlich, 1991). Also, the capsular polysaccharide of certain bacteria acts as a physical barrier against phagocytosis by PMNs and prevents complement deposition (Benge, 1988; Cortés et al., 2002; El Fertas-Aissani et al., 2013). Moreover, if bacteria are replicating at a rate that it is higher than the ingestion capacity of neutrophils, it becomes harder for them to catch up (Leijh et al., 1979).

Different patterns of sensitivity for *Staphylococcus* strains were observed in this work. *S. capitis* 517 and seven out of the eight tested *S. epidermidis* strains were able to survive the overnight hold time of WB, which is consistent with previous findings that show that *S. epidermidis* is able to resist immune evasion. *S. epidermidis* produces low levels of the toxin PSMδ which lyses neutrophils. This bacterium also produces the exopolymer PGA which confers protection against the bactericidal action of antimicrobial peptides (AMPs) and phagocytosis (Kocianova et al., 2005; Li et al., 2007; Otto, 2009). Moreover, the production of PIA (polysaccharide-based biofilm matrix) by some *S. epidermidis* has been reported to provide a better resistance against AMPs, phagocytosis and killing by PMNs as well as to influence complement activity by preventing complement proteins and antibodies from the deposition on the bacterial surface inhibiting opsonization (Vuong et al., 2004c; Kristian et al., 2008; Otto, 2010). Also, PIA has a positive net charge which is thought to contribute to the repulsion of cationic AMPs from the bacterial biofilm surface (Vuong et al., 2004b).

In contrast to the other staphylococcal strains described above, *S. capitis* 512 and *S. epidermidis* AZ-106-w showed a >1.2 log CFU/mL reduction by the end of the storage period in WB. Neutrophil killing assays revealed significant elimination of *S. capitis* 512 and *S. epidermidis* AZ-106-w during the overnight incubation period, whereas, in plasma, these bacteria

remained viable. Although *S. epidermidis* AZ-106-w was not affected by CPD, the anticoagulant showed a bactericidal effect against *S. capitis* 512. It is believed that the anticoagulant was not responsible for the decrease in viability of the latter bacterium in WB since its viability was not affected in CPD-containing plasma. Therefore, it is concluded that the mechanism responsible for elimination of *S. capitis* 512 and *S. epidermidis* AZ-106-w in WB is neutrophil-mediated killing. It is still unknown if other cellular components, such as monocytes, could have also played a role in the elimination process of the two bacteria (Strunk et al., 2012).

The antibacterial activity of the overnight hold period of WB does not have broad bactericidal properties and is strain dependent. Towards the end of the overnight incubation time in WB, some bacteria were able to grow to high titers, others did not proliferation but remained viable while other bacteria drastically decreased in viability or got completely eliminated. Consideration should be given to screen WB during BC-PC production for bacterial contamination after the overnight hold in order to minimize bacterial contamination of the final PC product.

Studies can be performed to have a deeper understanding of what components were responsible for the elimination of certain bacterial strains used in this work. The direct role of the complement system can be assessed by detecting and measuring complement activation using ELISA kits (Huang et al., 2014) or by performing a killing assay in serum or in WB treated with the anticoagulant lepirudin (inhibit thrombin) that does not chelate $Ca^{2}+$ /$Mg^{2}+$ in order to obtain full complement system activities. Also, MAC activity could be inhibited by blocking

either C3b or mannose binding lectin to investigate whether it has role in bacterial elimination or whether MAC-independent killing mechanisms are involved.

S. capitis sensitivity to the anticoagulant CPD was demonstrated in this work, however, it does not seem to play a major role in the elimination of this strain in WB. For a better understanding of the bactericidal CPD effect in WB, assays with WB prepared with different anticoagulants, which do not chelate $Ca^{2}+$ or $Mg^{2}+$ such as lepirudin, could be performed.

6.7 ACKNOWLEDGMENTS

Many thanks go to volunteer blood donors. Also, Dr. Subash Sad, Dr. Angela Crawley (University of Ottawa), Dr. William Cameron and the nurses Danielle Tardiff and Isabelle Seguin (Ottawa Hospital Research Institute) are acknowledged for facilitating and helping with the procurement of blood for this research.

CHAPTER 7.

Conclusions and Future Directions

Bacterial contamination of PCs is still one of the outstanding challenges in the field of transfusion medicine (McDonald, 2013; Hong et al., 2016). The work described in this thesis aimed to address this problem by looking at bacterial survival, resistance, and elimination during the different stages of blood processing that culminates in a PC product (Fig. 1.3). In particular, this thesis explored bacterial resistance to skin disinfectants during blood donation, bacterial elimination and survival during the BC-PC production process, and the efficiency of the pathogen inactivation technology Mirasol against skin bacteria. The major findings of this thesis have been listed in Table 7.1.

Disinfecting the skin prior to venipuncture is a crucial step that contributes to lowering contamination rates. Nonetheless, contaminated PC units are still captured where skin flora are the major contaminants, and of these contaminants, some have been shown to be biofilm-formers (Greco et al., 2008; Ali et al., 2014). The work described herein demonstrates that the skin disinfectants (CHG/IPA) currently used during venipuncture at Canadian Blood Services are incapable of completely eliminating bacteria when they exist in a biofilm. The work provided evidence that the biofilm-forming capability of the skin microflora reduces the bactericidal efficiency of blood donor skin disinfectants. It was also shown that disinfectant-treated biofilm cells are capable of proliferating in PCs during storage. Approaches to improve the current skin disinfection method could include increasing the efficacy of the disinfectants and enhancing their ability to penetrate into the lower skin layers thereby further reducing the likelihood of contaminating blood products. It is also possible to combine disinfectants with natural oils to

increase skin penetration or to investigate the efficacy of other available antiseptics with enhanced antibacterial activity.

Studies have demonstrated that the biofilm-formation phenotype and/or the presence of the *ica* operon could serve as markers to differentiate between *S. epidermidis* isolated from normal human skin and clinical samples such as those isolated from blood cultures (Galdbart et al., 2000; Ziebuhr et al., 1997). In this work, it has been shown that the prevalence of the biofilm-forming *S. epidermidis* phenotype is similar for isolates obtained from healthy skin and those recovered from contaminated PCs. Although the sample size was small and the data would be improved by increasing the number of isolates, these finding suggests that there is no selective pressure towards biofilm-formers during the donation and PC production processes. Both *ica*-negative and *ica*-positive biofilm formers have been isolated from human skin as well as contaminated PCs. Current initiatives at Dr. Ramirez' laboratory are aimed at characterizing the biofilm matrix of *ica*-negative *S. epidermidis* biofilm formers. The work could help shed some light on the major components of the matrix and allow for the development of skin disinfectants that target specific components of the biofilm-matrices present on donor skin, thereby increasing their efficacy. Furthermore, the work in this thesis verified previous observations that *S. epidermidis* adopts a biofilm-forming phenotype in PCs regardless of their genetic background or source of origin. Work is being performed at Dr. Ramirez' laboratory to unravel the mechanisms involved in the enhancement of biofilm formation of *S. epidermidis* in PCs.

Biofilm-forming bacteria have been isolated from human skin (Costerton, 2007), and from contaminated PCs (Greco et al., 2007). Biofilms offer a distinct advantage of increased resistance to chemical and physical clearance (Otto, 2009). In this study, the efficacy of riboflavin-UV PI treatment against *S. epidermidis* biofilm cells in PCs was investigated. When

PCs produced from WB inoculated with *S. epidermidis* biofilm or planktonic cells were inactivated using the PIT-Mirasol, similar efficiencies of bacterial elimination were observed. Future work should look at the efficacy of the technology against biofilms spiked directly into PC units to confirm this observation and to mimic the production settings for Aph-PCs. Interestingly, the technology is not 100% efficient to eliminate *S. epidermidis* at concentrations $\geq 10^3$ CFU/mL in PCs. It is acknowledged that *S. epidermidis* will not reach these concentrations during BC-PC production, however, contamination with fast growing bacteria would be a problem if there is a delay in the PI illumination step (Schmidt et al., 2015).

At Canadian Blood Services, BC-PCs show a generally lower bacterial contamination rate than Aph- PCs. This work investigated whether the BC-PC production method contributes to this observation by tracking bacteria through the different BC-PC manufacturing steps with a special focus on the initial overnight incubation time of WB. The findings of this study indicate that the BC-PC process can contribute to bacterial removal ranging from 1-3 logs (CFU/mL) depending on the strain; however, it does not exhibit a broad-spectrum bactericidal effect. It was also shown that the overnight hold of WB allows for the rapid proliferation of certain bacteria. Therefore, the screening of WB units following the overnight hold, and the implementation of PIT soon after the donation process should be considered to minimize or eliminate bacterial contamination of the PC final product.

Table 7.1. Major findings of this thesis and their relevance to transfusion medicine field

<table>
<tr><td colspan="4">Hypotheses. It is hypothesized that biofilm-forming skin flora are more resistant to the action of currently used donor skin disinfectants and the action of pathogen inactivation technologies. Furthermore, bacterial contamination of PCs is significantly reduced during the BC production method due to the bactericidal action of immune factors present in WB.</td></tr>
<tr><th>Objective</th><th>Conclusion</th><th>Suggestions to transfusion medicine practices on the light of these findings</th><th>Chapter</th></tr>
<tr><td>The arm disinfectants (CHG/IPA) currently used at Canadian Blood Services are not completely efficient against:
• S. epidermidis biofilms
• S. capitis biofilms
• Dual biofilms of S. capitis and S. epidermidis

Disinfectant-treated biofilm cells are able to grow in PCs:
• S. epidermidis biofilms
• S. capitis biofilms
• Dual biofilms of S. capitis and S. epidermidis</td><td>Yes
Yes
Yes

Yes
Yes
Yes</td><td>Investing in finding more efficient disinfectants or improving the quality of the currently used ones</td><td>2</td></tr>
<tr><td>There is more prevalence of biofilm-forming S. epidermidis in PCs isolates compared to human skin isolates:
• Biofilm-forming phenotype

Both ica-positive and ica-negative biofilm-forming S. epidermidis were isolated from:
• Skin isolates
• PC contaminants

• The isolation source (PCs vs skin) of S. epidermidis, genetic (ica-positive vs ica-negative) and phenotypic (biofilm-former vs non-biofilm former) background do not affect biofilm-formation phenotype in the PC milieu</td><td>No

Yes
Yes[a]

Yes[b]</td><td>Evaluating the composition of biofilm matrices of S. epidermidis ica-negative and ica -positive in skin isolates. This would allow for the development/ improvement of skin disinfectants that target specific biofilm- matrices components on the skin</td><td>3</td></tr>
<tr><td>PCs produced from WB containing biofilm cells of S. epidermidis are</td><td>Yes[c]</td><td>PI should be performed as</td><td>4</td></tr>
</table>

capable of resisting PI treatment in PCs		early as possible after PCs production	
Bactericidal effect of the BC-PCs production method: • Overall effect of the BC method • Overnight hold of WB • 2 hours hold of BC • 2 hours hold of plasma **Bacteria fractionate towards cellular fractions** **Platelets activation (CD62P expression, ThromboLUX scoring) can be used as indicator for bacterial contamination of PCs**	Yes[d] Yes[e] No No Yes No	The overnight hold of WB provides some bacteria with the opportunity to proliferate, therefore, consideration should be given to screening WB after overnight hold or to implement PI, as early as possible after donation, in order to minimize or eliminate bacterial contamination of the final product of PCs	5
Antibacterial activity of WB overtime during the overnight hold time: • WB • Plasma • Neutrophils	Yes[e] Yes[e] Yes[f]		6

[a] Lower rates of *ica*-negative compared to the skin isolates
[b] All tested isolates formed biofilm in PCs
[c] At concentrations $\geq$ 3 logs (CFU/mL) in PCs. Similar efficiency against *S. epidermidis* present in PCs produced from WB inoculated with biofilm or planktonic cells
[d] 1-3 logs (CFU/mL)
[e] Species and strain dependent
[f] Only 2 bacteria were tested

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Appendix A.1. *S. epidermidis* isolates recovered from contaminated PCs.

Origin	Type of PC unit	Strain ID
Routine PC screening	Buffy coat	CBP-A-08004
		CBPA-08007
		CBPA-08009
		CBPA-08015
		CBPA-09007
		CBPA-09019
		CBPA-09021
		CBPA-BT-10001
		CBPA-BT-10005
		CBPA-BT-10007
		CBPA-BT-11005
		CBPA-BT-11009
		CBPA-BT-11010
		CBPA-BT-12001
		CBPA-BT-12006
		CBPA-BT-12011
		CBPA-BT-12014
		CBPA-BT-13001
	Apheresis	CBPA-08012
		CBPA-09018
		CBPA-BT-12008
QC Sterility Testing*	Buffy coat	CBPA-ST 11004
	Apheresis	CBPA-ST 10002
		CBPA-ST 11003

* PC units screened at expiry on day 6

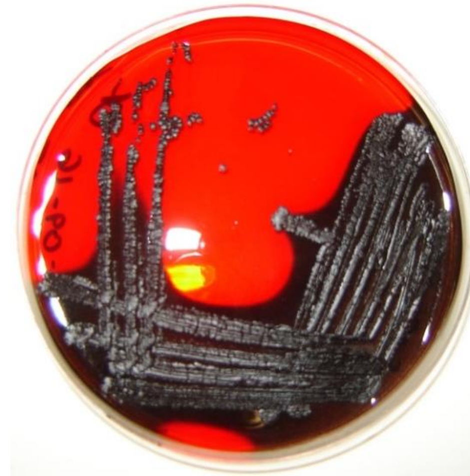
Appendix A.2. Primers and PCR conditions used to amplify *S. epidermidis divIVA, icaA* and *icaD* genes

***S. epidermidis* gene**	**Primer Pair**	**Primers sequence**	**Amplification settings** [b]
divIVA	Staphdiv-Fw	5'-gcgcgtcgac[a]- ATG CCT TTT ACA CCA AGT G-3'	15 min at 95°C; 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 46°C, and extension for 1 min at 72°C; 10 min at 72°C;and hold at 4°C
	Sepdiv-Rev	5'- gcgcggatcc[a]- TTA ATT ATTT GAT GTT GAT TG-3'	
icaA	Sepi_*icaA*-FW	5'-GCG CCT GGA TAG TAG GAT CGA TTT AC -3'	15 min at 95°C; 50 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 58°C, and extension for 30s at 72°C; 5 min at 72°C; and hold at 4°C.
	Sepi_*icaA*-REV	5'- GCG CTT ACC GTT GGA TAT TGC CTC T - 3'	
icaD	Sep*icaD*-FW	5'-AAG CCC AGA CAG AGG CAA TAT CCA -3'	5 min at 95°C; 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 47°C, and extension for 30s at 72°C; 5 min at 72°C; and hold at 4°C
	Sep*icaD*-REV	5'-AGT ACA AAC AAA CTC ATC CAT CCG A-3'	

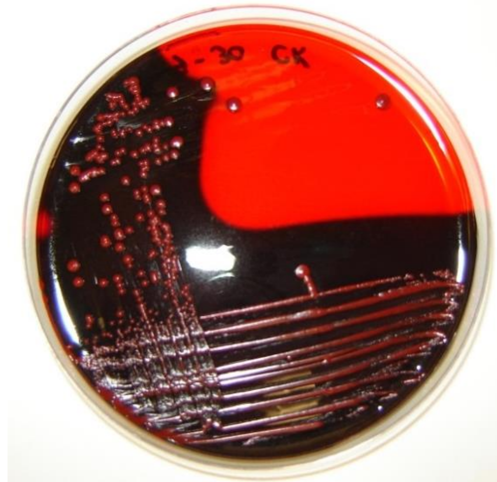
[a] The sequence in small case is an extension sequence that was added for cloning purposes and is not part of the *divIVA* gene.
[b] A Mastercycler ep Thermal Cycler PCR system (Eppendorf, Hamburg, Germany) was used.

A.

B.



C.



D.

Appendix A.3. Assessment of slime production ability of *S. epidermidis* by Congo red agar. A) negative control (non-slime producer) *S. eppidermidis* Hamburg 9142 Δ*icaA*, smooth red-to- pink colonies, B) positive control (slime producer) Hamburg 9142, rough, dull black colonies, c) skin isolate AZ-3, non-slime producer D) skin isolate YK-12, slime producer. C and D are a subset from the 48 *S. epidermidis* skin isolates studies in chapter 3 of this thesis.

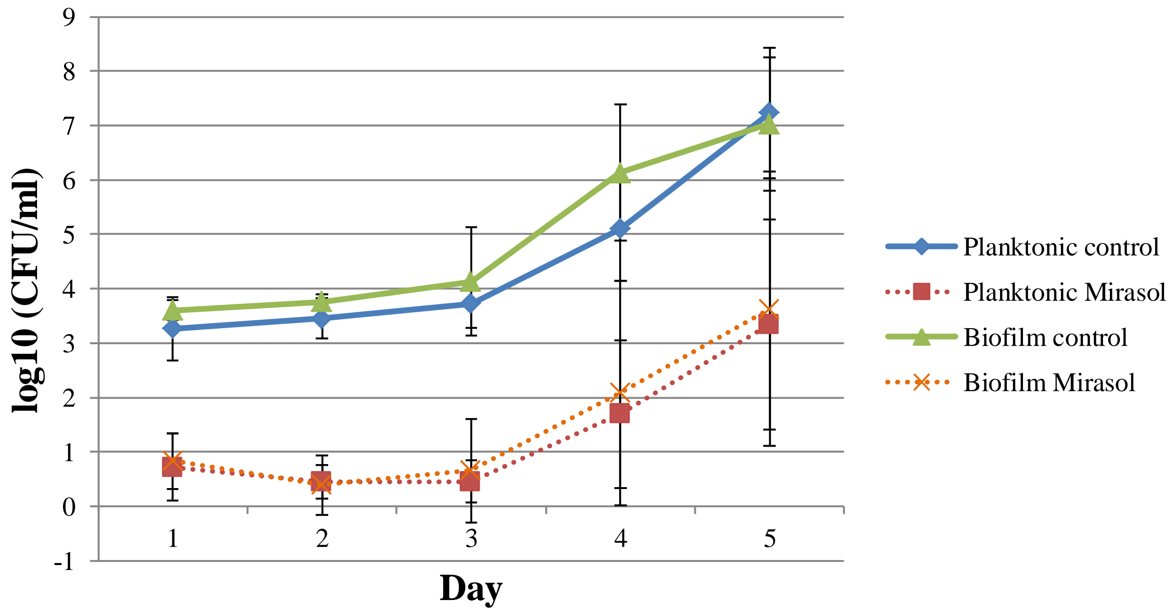
Appendix B.1. Inactivation efficacy of the Mirasol system against two strains of *S. epidermidis*, ST-10002 and AZ-66, in BC-PCs derived from WB spiked with planktonic or biofilm cells.

		Bacterial titer log $_{10}$(CFU/ml)				
ST-10002	**Replicate**	**Pre- Mirasol treatment**	**Post- Mirasol treatment**	**BPA bottle [a]**	**Log reduction factor [b]**	**Mean log reduction factor**
Planktonic	1	2.66	ND	Positive	≥2.66	**2.60**
	2	3.94	1.51	Positive	2.42	
	3	3.54	0.78	Positive	2.76	
	4	3.14	0.60	Positive	2.54	
Biofilm	1	3.85	1.48	Positive	2.37	**2.78**
	2	3.67	0.30	Positive	3.37	
	3	3.56	1.26	Positive	2.30	
	4	3.41	0.60	Positive	2.80	
	5	3.58	0.52	Positive	3.06	

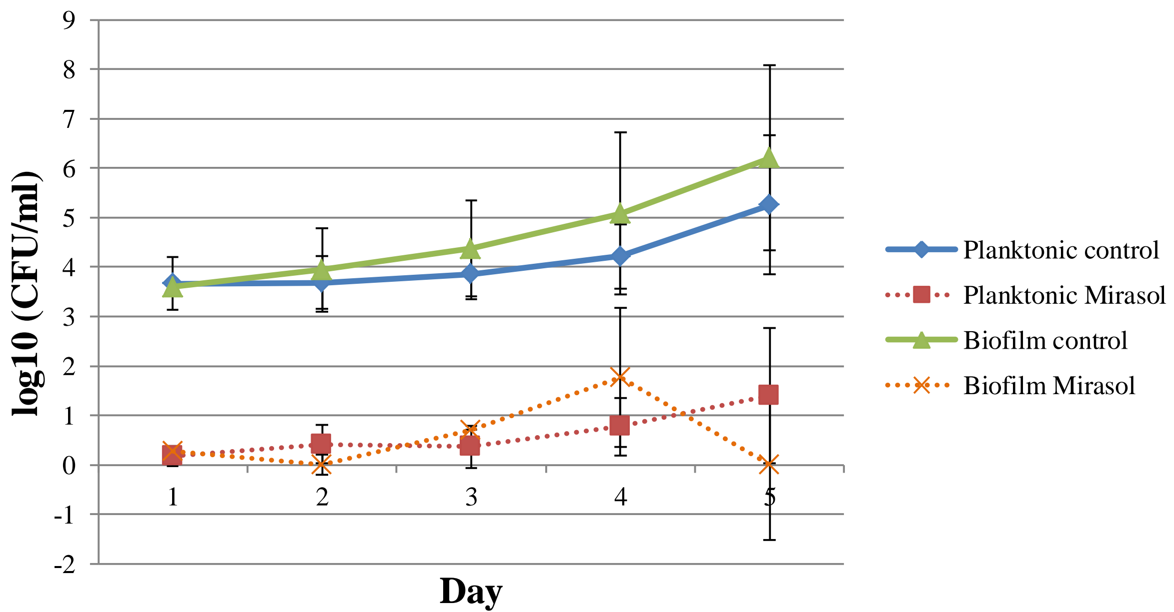
		Bacterial titer log $_{10}$(CFU/ml)				
AZ-66	**Replicate**	**Pre- Mirasol treatment**	**Post- Mirasol treatment**	**BPA bottle [a]**	**Log reduction factor [b]**	**Mean log reduction factor**
Planktonic	1	3.57	ND	Positive	≥3.57	**3.50**
	2	4.33	0.4	Positive	3.93	
	3	3.59	0.3	Positive	3.29	
	4	3.20	ND	Positive	≥3.20	
Biofilm	1	3.74	ND	Positive	≥3.74	**3.58**
	2	3.65	ND	Positive	≥3.65	
	3	3.50	ND	Positive	≥3.50	
	4	3.43	ND	Positive	≥3.43	

ND; bacteria no detected by plating on BA agar (below detection limit <1 CFU/mL). [a] Bottle of BacT/ALERT® 3D system, inoculated with 8 mL PCs directly after Mirasol treatment [b] ≥ bacterial titer before treatment (when bacteria are not detected by plating).

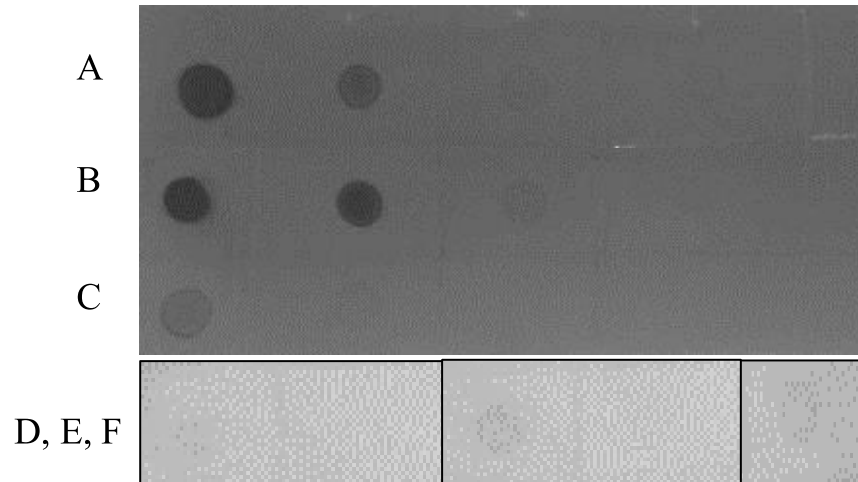
A.



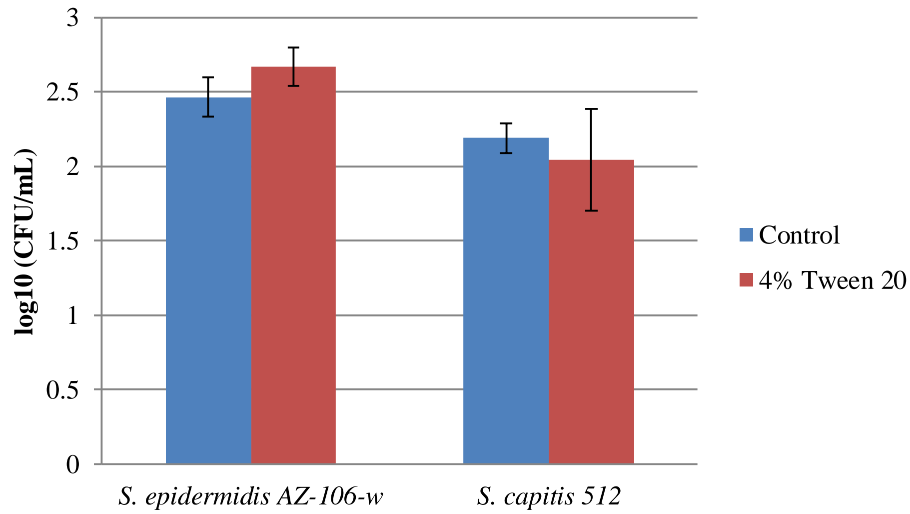
B.



Appendix B.2. *S. epidermidis* growth in Mirasol® treated and untreated BC-PC units over a period of 5 days at 22± 2°C with agitation. A) Strain ST-10002. B) Strain AZ-66. BC-PC units obtained from WB units spiked with planktonic or biofilm cells. N≥4, ±SD.



Appendix B.3. Immunoblot assay to detect PIA in *S. epidermidis*. A) Planktonic cells of 9142 grown in TSBg (10^8-10^4 CFU/mL). B) Planktonic cells of AZ66 grown in TSBg (10^8-10^4 CFU/mL). C) Planktonic cells of ST10002 grown in TSBg (10^8-10^4 CFU/mL). D) ST-10002 in PC units derived from WB-biofilm (10^4 CFU/mL) and WB-planktonic (10^4 CFU/mL). E) AZ-66 in PC units driven from derived from WB-biofilm (10^3 CFU/mL) and WB-planktonic (10^3CFU/mL) F) Negative control 9142 Δ*icaA*.

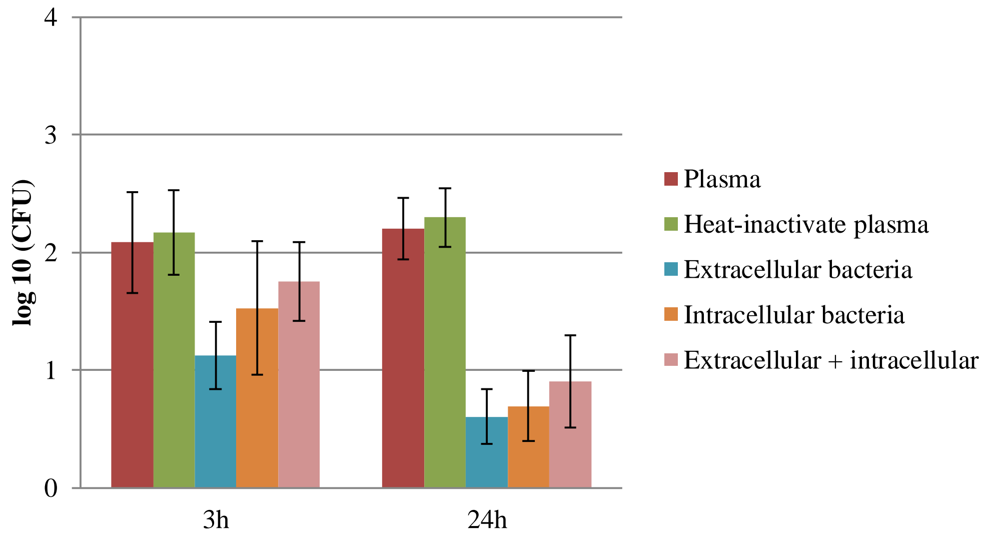


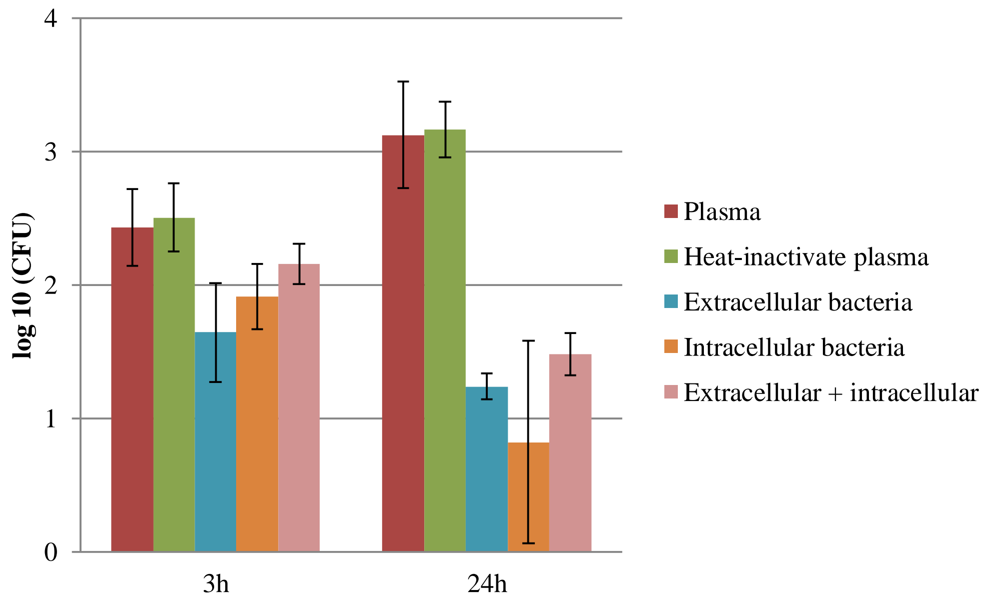
Appendix C.1. No effect of 4% Tween 20 on *S. epidermidis* AZ-106-w (p=0.1260) or *S. capitis* 512 (p=0.6064) viability during incubation for 10 minutes at room temperature compared to control (0.9% saline). N≥3, ±SD.

Appendix C.2. Bacterial survival in WB and plasma detected by BPA bottles of the BacT/ALERT.

Bacteria	Tested fraction	Negative BPA bottles/total tested*
S. capitis 512	WB	0/3
	Plasma	N/A
S. epidermidis AZ-106-w	WB	0/2
	Plasma	N/A
Y. enterocolitica ATCC 49397	WB	3/3
	Plasma	3/3
S. marcescens ATCC 13880	WB	2/3
	Plasma	4/4
E. coli ATCC 10798	WB	N/A
	Plasma	6/6
K. pneumoniae ATCC10031	WB	N/A
	Plasma	5/5
K. pneumoniae ATCC13883	WB	3/3
	Plasma	3/3

*Each bottle represents one independent repetition. Negative bottle represents no growth after 6 days of incubation in the BacT/ALERT system.
N/A; not applicable





Appendix C.3. Bacterial viability intracellular and extracellular at 3 and 24 hours of incubation with neutrophils at 22±2°C. N=3, ±SD.

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  Biofilm-Forming Skin Microflora Bacteria Are Resistant To The Bactericidal Action Of Disinfectants Used During Blood Donation

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Billing Address	Ottawa, ON k1v 8y5 Canada Attn: Mariam Taha
Total	0.00 USD
Terms and Conditions	

TERMS AND CONDITIONS