Growth and biofilm formation by *Staphylococcus epidermidis* and other relevant contaminant bacteria during storage of platelet concentrates

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ABSTRACT

Coagulase negative staphylococci (CoNS) are the most prevalent bacterial contaminants of platelet concentrates (PCs), and have been implicated in severe and fatal adverse transfusion reactions. Of this group, *Staphylococcus epidermidis* is most frequently identified. Its ability to cause chronic nosocomial infections by forming surface-attached communities of organisms known as biofilms has imparted the status of major opportunistic pathogen to this otherwise innocuous, Gram-positive skin commensal. However, its virulence in the context of transfusable blood products has not been extensively studied. In particular, biofilm formation during platelet storage may be fundamental to missed bacterial detection by current screening methods. The preliminary objective of this thesis was to confirm that biofilm formation could be exhibited by *S. epidermidis* under platelet storage conditions. This was achieved using a modified crystal violet staining assay to detect plastic-adherent bacterial cells and examination of attachment processes by scanning electron microscopy. A collection of CoNS isolated from PCs obtained from reportedly healthy donors was then assessed for biofilm-forming potential. Confirmatory markers included (a) presence of the biofilm *icaD* gene (b) slime production on Congo red agar, (c) biofilm formation on polystyrene, and (d) polysaccharide intercellular adhesin detection via indirect immunoassay. Despite the presumable commensal origin of these isolates, a high proportion of *S. epidermidis* strains displayed a biofilm phenotype.

Validation of the threat of *S. epidermidis* biofilm formation during platelet storage signifies that any modifications to current platelet storage protocols should be rigorously evaluated with consideration of this risk. The advent of platelet additive solutions (PASs) as an alternative to plasma for PC storage provides a relevant example. Little is known about the effect of PAS on the growth dynamics of bacteria, and there have been no studies on the influence of bacteria on platelet quality when suspended in PAS. To this end, PCs suspended in either plasma or PAS were inoculated with *S. epidermidis* or the Gram-negative bacterium *Serratia liquefaciens*. Growth rates and biofilm formation were assayed for 5 days. Concurrently, platelet markers were measured by an assay panel and flow cytometry. Only *S. liquefaciens* exhibited a slower doubling time in plasma-PCs. Reduced biofilm formation was exhibited by both species during storage in PAS-PCs, and is advantageous since it increases bacteria availability for sampling detection. Although *S. liquefaciens* altered several platelet quality markers by day 3-4 post-inoculation in both PAS-PCs and plasma-PCs, *S. epidermidis* contamination did not produce measurable platelet changes. Ultimately, culture-based detection remains the earliest indicator of bacterial presence in PAS-PCs, while changes of platelet quality can herald *S. liquefaciens* contamination in excess of 10^8 CFU/mL.

The findings of this thesis research endorse novel proposals to minimize biofilm occurrence during platelet storage. Since formation of platelet-bacteria aggregates is largely based on receptor-ligand interactions, it was postulated that biofilm formation by contaminant bacteria could be abrogated by receptor shielding. As a final, proof-of-principle objective, methoxypoly(ethylene glycol) was applied to covalently modify the platelet surface using a process termed ‘PEGylation’. It is herein demonstrated that PEGylation of PCs inoculated with *S. epidermidis* results in significantly reduced bacterial binding and biofilm formation during platelet storage.

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α	alpha, anti
β	beta
γ	gamma
κ	kappa, coefficient of agreement
λ	lambda, wavelength
μ	mu, micro-
σ	sigma
°	degree

LIST OF ABBREVIATIONS

aa	Amino acid
AABB	American Association of Blood Banks
Aap	Accumulation-associated protein
Aae	Accumulation/adhesin protein
ADP	Adenosine diphosphate
AFM	Atomic force microscopy
Agr	Accessory gene regulator
AHL	Acylated homoserine lactone
AI-2	Autoinducer-2
AP	Alkaline phosphatase
Arg	Arginine
ATCC	American type culture collection
AtlE	Autolysin E
ATR	Adverse transfusion reaction
Amp	Ampicillin
A492nm	Absorbance values at a 492 nm wavelength
B_2	Riboflavin
BC	Buffy coat
BF	Biofilm formation
BHI	Brain heart infusion
Bhp	Bap-homologous protein
C	Cytosine
Ca^{2+}	Calcium

CAM	Cell adhesion molecule
CBS	Canadian Blood Services
CD9	Cluster of differentiation protein 9 (tetraspanin)
CD62P	Cell adhesion molecule P-selectin (Cluster of differentiation protein 62P)
CFU	Colony forming units
CO_2	Carbon dioxide
Co^{2+}	Cobalt
CoNS	Coagulase negative staphylococci
CSLM	Confocal scanning laser microscopy
CTD	C-terminal domain
d	Day
D	Aspartic acid/Aspartate
Da	Dalton
ddH_2O	Distilled, deionized water
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Embp	Extracellular matrix binding protein
Eph	Ephrin
FACS	Fluorescence activated cell sorter
FDA	Food and Drug Administration
Fg	Fibrinogen
FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate

FPLC	Fast protein liquid chromatography
g	Gravitational force
G	Guanine
Gly	Glycine
Gmk	Guanylate methyl kinase
GMP	Good manufacturing practice
GP	Glycoprotein
h	Hour
H^+	Hydrogen ion
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HLA	Human leukocyte antigen
Ica	Intercellular adhesin
IgG	Immunoglobulin-G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kbp	Kilo-base pair
kDa	Kilodalton
KLH	Keyhole limpet hemocyanin
K_2HPO_4	Potassium phosphate, dibasic
LB	Luria-Bertani
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
$MgCl_2$	Magnesium chloride
mM	Milimolar

M	Molar
min	Minute
mPEG	Methoxy-polyethylene glycol
MPV	Mean platelet volume
mRNA	Messenger RNA
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MWCO	Molecular weight cut-off
n	Sample size
N	No
NA	Not applicable
Neg	Negative
NaCl	Sodium chloride
ND	Not done, not determined
netCAD	Network centre for applied development
NIR	Near infrared
NTD	N-terminal domain
O_2	Oxygen
OD	Optical density
ONPG	*ortho*-nitrophenyl-β-galactoside
OsO_4	Osmium tetroxide
P	Probability
Pall eBDS	Pall enhanced bacterial detection system
PAS	Platelet additive solution
PC	Platelet concentrate

PCR	Polymerase chain reaction
pCO_2	Partial pressure of carbon dioxide
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEG	Polyethylene glycol
PIA	Polysaccharide intercellular adhesin
pO_2	partial pressure of oxygen
PPP	Platelet-poor plasma
Pro	Proline
PRP	Platelet-rich plasma
PRT	Pathogen reduction technology
PSL	Platelet storage lesion
PVC	Polyvinyl chloride
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
rDNA	Ribosomal DNA
Rg	Radius of gyration
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
s	Second
S	Serine
SC	Succinimidyl carbonate
SD	Standard deviation

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SdrG	Serine-aspartate repeat protein G
Se, *S. epi*	*Staphylococcus epidermidis*
SEM	Scanning electron microscopy
Sl, *S. liq*	*Serratia liquefaciens*
Spp	Species
TBS	Tris buffer saline
TBST	TBS+0.05% Tween-20
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSBg	Tryptic soy broth + 0.5% glucose
TxA_2	Thromboxane A2
U	Unit
UVA	Ultraviolet-A
vWF	von Willebrand Factor
vol	Volume
Y	Yes

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1. INTRODUCTION

1.1 PLATELETS

Platelets are anucleate cells approximately 2-5 microns in diameter which are derived from the megakaryocyte lineage of haematopoietic stem cells (White, 2007). The major bodily compartment for platelets is the bloodstream, although the spleen and liver house significant reserves of this cell type (Scheffel et al., 1982; Hill-Zobel et al., 1983). The normal platelet count for peripheral blood is 150-400 x 10^9 per litre (Briggs et al., 2007). Significantly, platelets have been detected at various developmental stages in the bone marrow, bloodstream and lungs; thus, platelet biogenesis is thought to be independent of a specific bodily compartment, with differentiation occurring either before or after megakaryocyte secretion from the bone marrow (Italiano and Hartwig, 2007). Once in circulation, platelets survive for approximately 5-9 days, after which point they are cleared by liver hepatocytes or macrophages (Grozovsky et al., 2010).

Platelets participate in normal haemostatic processes, preservation of vascular integrity, clot retraction, and wound healing. Although platelets are discoid at origin, they become rounded and exhibit spreading after substrate adherence in a process called activation. *In vivo*, activation is mainly induced by collagen or thrombin (Brass et al., 2007); however, leukocytes, activated complement components, cytokines, histamines, and other platelet-derived metabolites all contribute to platelet activation and microvesicle formation (Seghatchian and Krailadsiri, 1997). Additional factors which render platelets susceptible to activation include shear stress (Yin et al., 2011), cold temperature (Maurer-Spurej et al., 2001), and contact with negatively-charged surfaces (Sperling et al., 2009). Microscopically, extrusion of finger-like filopodia is often apparent, particularly during activation with a non-continuous surface, which is crucial for the formation of a haemostatic plug (White, 2007).

The mechanism of platelet-mediated control of vascular bleeding is shown in Figure 1. Damaged endothelial cells lining blood vessels present exposed collagen, which can activate platelets directly via platelet surface receptors α2β1 and glycoprotein (GP)V1 (Brass et al., 2007). Collagen complexed with circulating von Willebrand factor (vWF) provides additional potentiation of platelet activation upon direct contact via platelet receptors GPIIb-IIIa and GP1b/IX (Brass et al., 2007). This comprises the stage of platelet adhesion (Figure 1A). Once activated platelets have started to accumulate at this site of vascular injury, they release intracellular compartments containing adenosine diphosphate (ADP), thromboxane A2 (TxA_2), and calcium (Ca^{2+}) in a process termed degranulation, which is sufficient for activation of circulating platelets (Brass et al., 2007). Platelet aggregation that follows can be mediated by numerous factors, including integrins such as GPIIb-IIIa, cell adhesion molecules (CAMs) such as CD62P (P-selectin), and phosphorylation of exposed ephrin by ephrin kinase (Brass et al., 2007; Figure 1B). As well, alpha-granules containing large intracellular stores of Ca^{2+} which are released upon platelet activation are essential cofactors in GPIIb-IIIa bridging with vWF, fibrin or the matrix molecule fibrinogen, Fg (Brass et al., 2007; Figure 1A).

1.1.1 Therapeutic Use

Evaluation of platelet transfusion for treatment of acute leukemia-induced hemorrhage was met with success in the 1950s (Hersh et al., 1965), and the efficacy of platelet therapy in medical practice was greatly enhanced in subsequent years by innovations

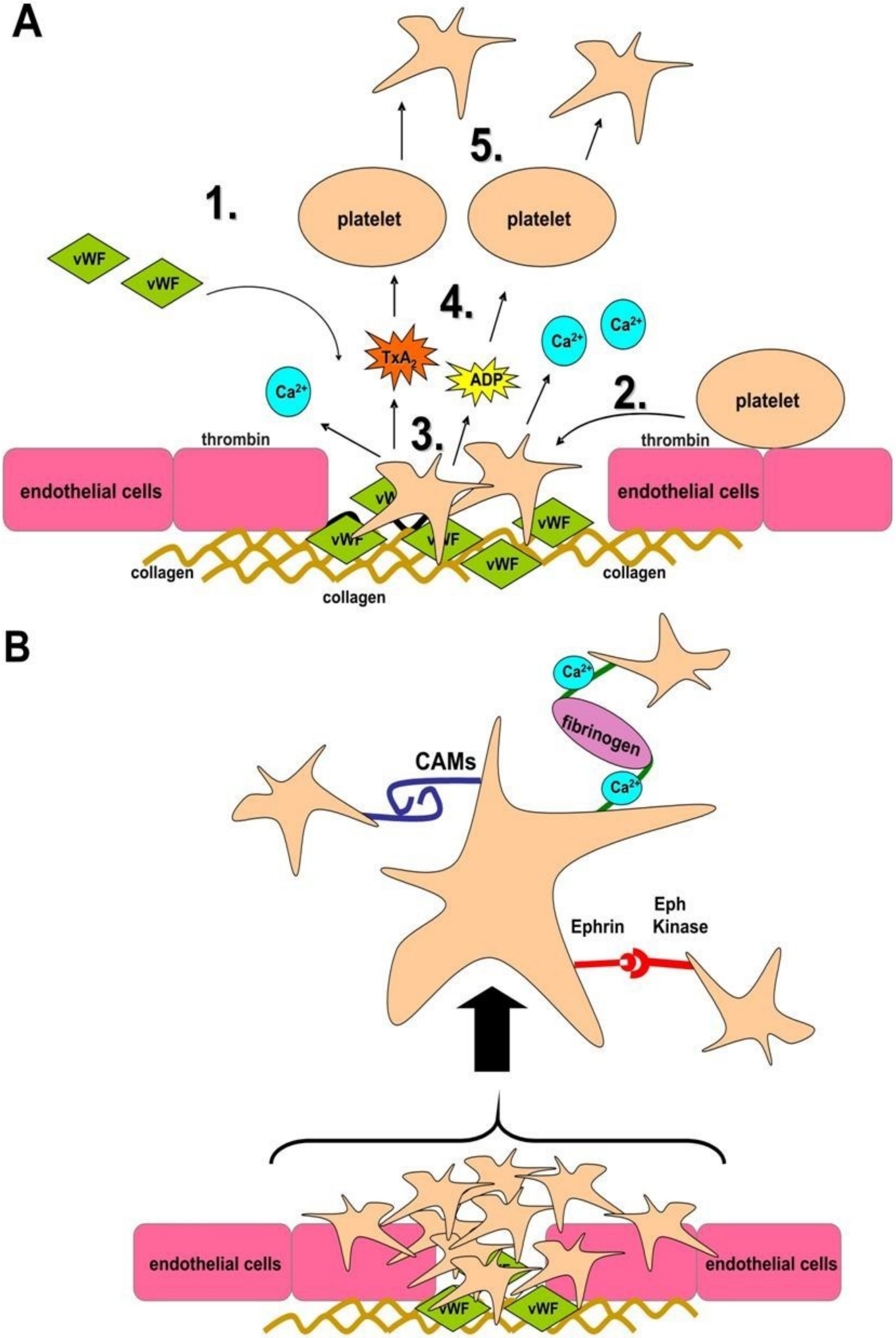
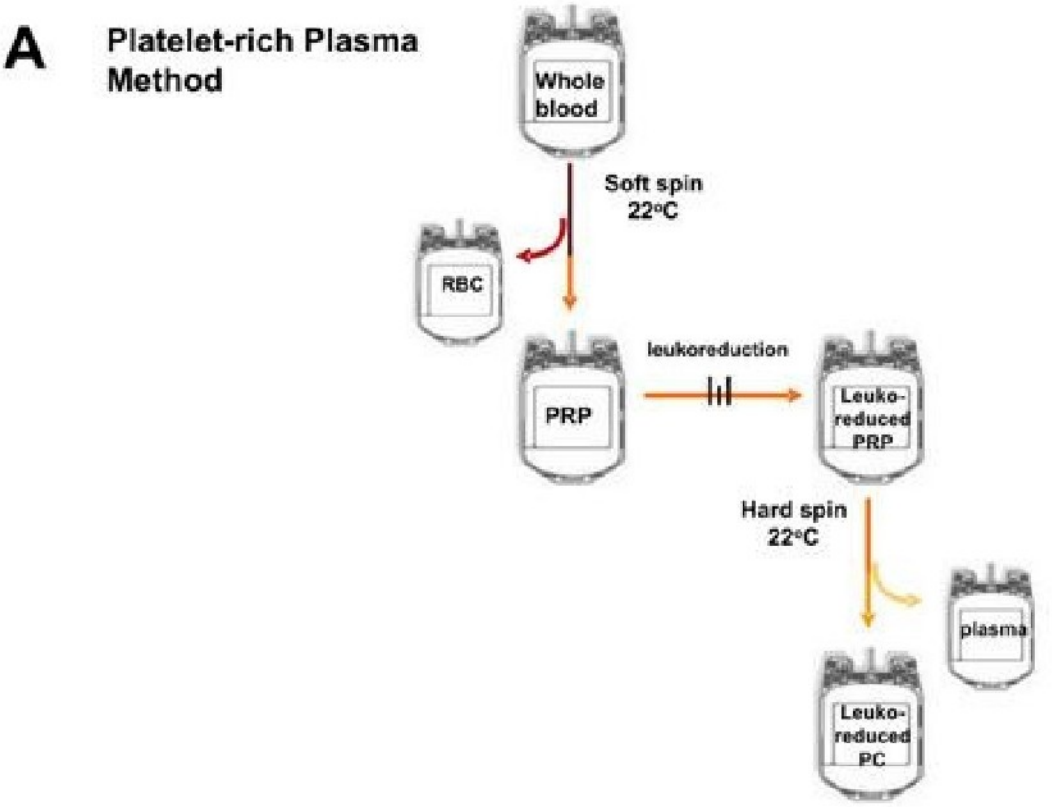
A
5.
1.
platelet
platelet
vWF
vWF
4.
TxA2
ADP
Ca2+
Ca2+
Ca2+
2.
platelet
3.
thrombin
thrombin
endothelial cells
endothelial cells
vWF
vWF
vWF
vWF
vWF
collagen
collagen
collagen
B
Ca2+
fibrinogen
Ca2+
CAMs
Eph
Kinase
Ephrin
endothelial cells
endothelial cells
vWF
vWF
collagen

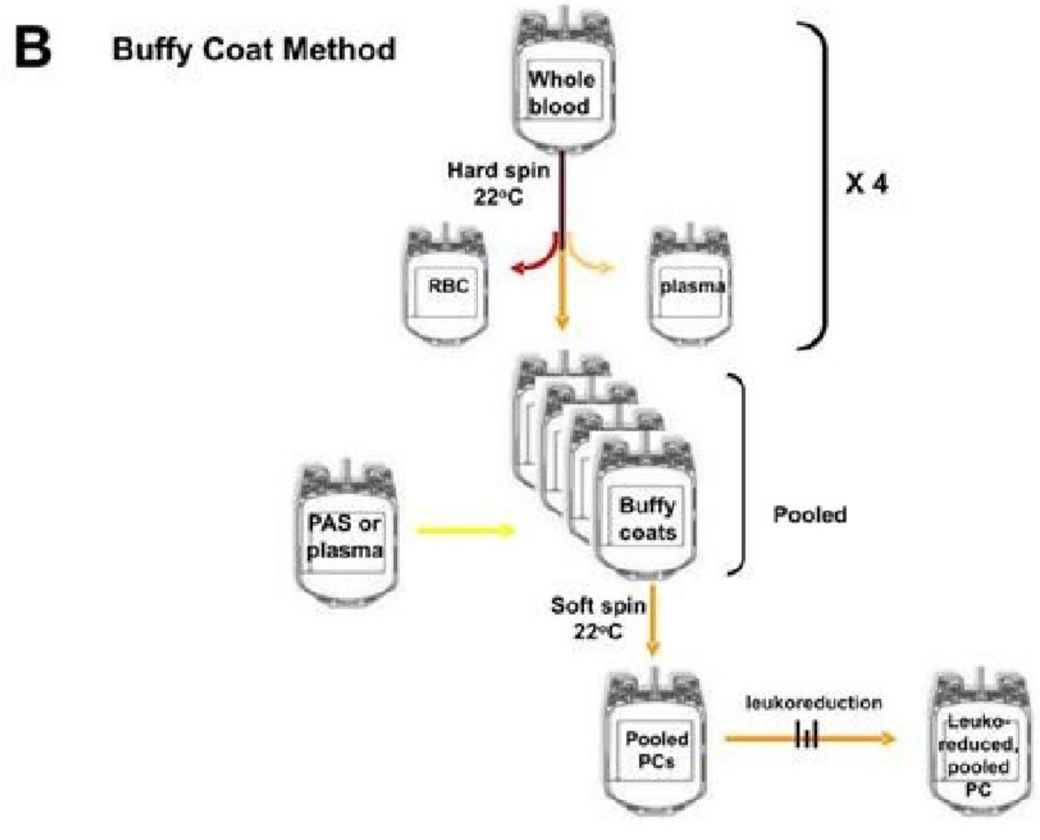
Figure 1. The process of platelet activation and clot formation. (A) Simplified mechanism of platelet activation pertaining to adhesion and degranulation. (1) At the site of vascular injury collagen becomes exposed and can become complexed with circulating von Willebrand factor (vWF). Simultaneously, prothrombin is converted to its active form, thrombin, by factor X at sites of vascular damage. Thrombin, in turn, converts fibrinogen into insoluble fibrin, and potentiates the coagulation cascade. (2) Platelets can become activated by direct contact with collagen, the collagen-vWF complex, and/or thrombin in a process mediated by various cell surface receptors. (3) Activated platelets secrete adenosine diphosphate (ADP), intracellular calcium stores (Ca^{2+}), and thromboxane A2 (TxA_2), which interact with circulating platelets (4), causing further platelet activation (5). (B) Platelet aggregation generates a hemostatic plug. Activated platelets which have bound to a site of vascular injury can further interact with neighbouring platelets by a variety of mechanisms including direct binding via cell adhesion molecules (CAMs) such as C D62P; Ca^{2+} dependent cell-cell bridging via integrins (green) complexed with fibrinogen, and phosphorylation of Ephrin (Eph) by exposed Eph kinase (Brass et al., 2007).

such as plastic platelet bag sets, blood cell separators for high yield platelet retention, and optimization of ideal storage conditions (Stroncek and Rebulla, 2007). Platelet concentrates (PCs) are transfused today to treat bleeding diatheses due to thrombocytopenia (low platelet counts) which may arise from inherited or acquired disease. Frequently, prophalactic platelet transfusions are performed to provide prothrombotic support after chemotherapeutic treatments (Slichter et al., 2010). Diseases for which platelet transfusion therapy is most frequently indicated include haematological malignancies (eg, acute leukemia), solid tumor chemotherapy, cardiopulmonary bypass surgery, and immune thrombocytopenic purpura (Slichter et al., 2010). A platelet level of < 10,000 platelets/μL is a positive indicator for platelet transfusion, and a formula of 50-100x10^9 platelets per 10 kg of adult bodyweight is generally accepted (Stroncek and Rebulla, 2007).

1.1.2 Production of Platelet Concentrates (PCs)

Platelets can be separated from whole blood and concentrated by various means. Most commonly used methods are outlined in Figure 2 and include the platelet-rich plasma (PRP) method (Figure 2A), the buffy coat (BC) method (Figure 2B) and plateletpheresis, or apheresis platelet derivation (Figure 2C; Levin et al., 2008). The first two methods typically require the pooling of four to five donations, and involve differential centrifugation steps to isolate the platelet component of the product. The PRP method is characterized by an intermediate stage of platelet suspension in plasma after the removal of red blood cells (RBCs). In contrast, BC platelets are prepared by an initial 'hard spin' which enables the separation of RBCs and plasma from the product at the same stage of


A
Platelet-rich Plasma Method
Whole blood
Soft spin 22°C
RBC
PRP
leukoreduction
Leuko-reduced PRP
Hard spin 22°C
plasma
Leuko-reduced PC


B
Buffy Coat Method
Whole blood
Hard spin 22°C
RBC
plasma
X 4
Buffy coats
Pooled
PAS or plasma
Soft spin 22°C
Pooled PCs
leukoreduction
Leuko-reduced, pooled PC

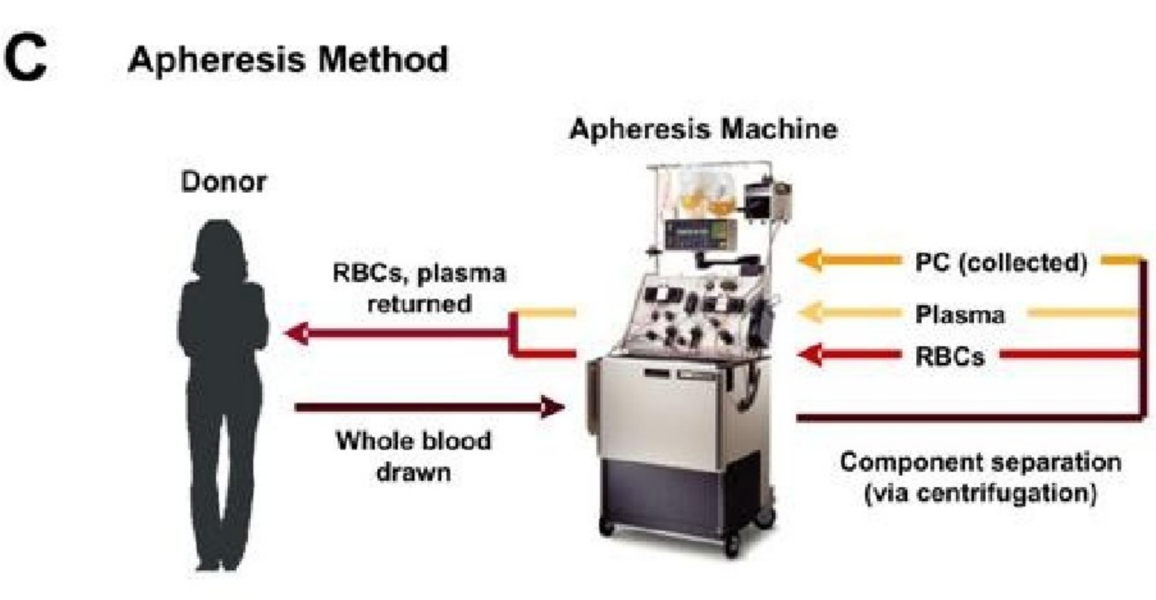

C
Apheresis Method
Apheresis Machine
Donor
RBCs, plasma returned
Whole blood drawn
PC (collected)
Plasma
RBCs
Component separation (via centrifugation)

Figure 2. Methods for preparing PCs. Diagrams depict (A) the platelet-rich plasma (PRP) method (B), the buffy coat method and (C) the apheresis method of component production. Note that during PRP production, the single-donor PC (shown in A) is subsequently pooled with four additional ABO-matched single-donor PCs.

processing, once a buffy coat layer has been established. Apheresis platelet derivation is a single-donor, closed, sterile system whereby RBCs and most of the plasma fraction of the donation are returned to the donor. Not only does this enable more frequent donations (every 14 days, compared to 56 days for whole blood donation), but this method is ideal for chronically transfused patients who have become alloimmunized and thus require human leukocyte antigen (HLA)-compatible units from matched donors to minimize immune rejection of the product (Vamvakas, 2009). In terms of composition, apheresis platelet bag kits are supplemented with acid-citrate-dextrose, while platelet units derived from whole blood collection contain citrate-phosphate-dextrose due to better compatibility with residual RBCs which are retained for alternative therapeutic uses.

1.1.3 Platelet Storage Lesion (PSL)

PCs are stored at room temperature (22 $\pm 2^{o}$C) with constant agitation in oxygen permeable polyvinyl chloride storage containers for up to 5 days prior to transfusion. The shelf-life of PCs is limited by the phenomenon called platelet storage lesion (PSL) which is defined as the alterations to platelet structure and function which accumulate between the time of donation and the time of transfusion to a recipient (Seghatchian and Krailadsiri, 1997; Devine and Serrano, 2010). This process can be mediated by several factors, ranging from normal platelet metabolism to bacterial contamination-induced platelet injury (Shrivastava, 2009). Several methods have been identified for detection of platelet storage lesion including (but not limited to) visible swirl, pH change, glucose consumption, lactate production, change of shape or mean platelet volume (MPV), O_2 consumption, CO_2 production, hypotonic shock response (Snyder, 1992), increased expression of platelet activation marker P-selectin (CD62P; Devine and Serrano, 2010), or exposure of phosphatidylserine detected via annexin V binding (Cookson et al., 2010). Specifically, phosphatidylserine has procoagulant properties due to the presence of factor V binding sites important to the prothrombinase clotting complex (Seghatchian and Krailadsiri, 1997).

PSL results from a combination of factors. Accumulation of metabolic end products (including proteolytic enzymes and clotting factors) within the platelet unit compromise platelet viability over time during storage, increasing the risk of an adverse transfusion reaction, and reducing post-transfusion, *in vivo* platelet recovery and hemostatic efficacy (Seghatchian and Krailadsiri, 1997). Oxidative stress, ATP depletion, platelet apoptosis, and activation by residual leukocytes are also associated with the evolution of PSL over time (Seghatchian and Krailadsiri, 1997).

1.1.4 Platelet-Bacteria Interactions

Mammalian platelets are not only important for hemostasis, but have demonstrated roles in innate and adaptive immune responses, ranging from storage and secretion of antimicrobial peptides to expression of immune mediating cytokines and chemokines in response to infection (Semple et al., 2011). It is believed that platelets evolved from invertebrate hemocytes, which is a cel l lineage characterized by phagocytic and hemostatic properties (Yeaman, 2010). Platelet are the quickest blood cells to accumulate at sites of vascular damage, and release of chemokines and cytokines stored in platelet alpha-granules is an effective means of enhancing recruitment of macrophages and neutrophils to sites of infection (Yeaman, 2010).

The ability of platelets to clump with bacteria was first described in a rabbit model of *Vibrio cholerae* infection in 1901, and the dependence of this aggregative effect was further characterized in 1971 to vary based on bacterial species, load, and direct cell-cell interaction (Clawson and White, 1971). It is now known that bacterial interaction with platelets can be direct, indirect, or mediated via secreted products (Cox, 2009).

Some examples of direct platelet-bacteria binding include streptococcal adherence to platelet GPIb via SrpA (Plummer et al., 2005), and Hsa (Kerrigan et al., 2007), and *Staphylococcus aureus* binding to GPIIb-IIIa via IsdB (Miajlovic et al., 2010).

A seemingly more common technique of bacteria-platelet binding involves an indirect, or bridged interaction, via plasma proteins. This is observed with *S. aureus* ClfA binding to GPIIb-IIIa via fibrinogen (Siboo et al., 2001). The same platelet-binding mechanism has been substantiated for *Staphylococcus epidermidis* with homologous protein SdrG (Brennan et al., 2009). Interestingly, it has been shown that secreted capsular polysaccharide from *S. aureus* blocks ClfA-mediated adhesion to platelets (Risley et al., 2007). GPIIb-IIIa additionally binds fibronectin, and *S. aureus* fibronectin binding protein Fnbp can facilitate platelet binding in this manner (Fitzgerald et al., 2006a). *S. aureus* and *Helicobacter pylori* also bind platelet GPIb via Protein A using the bridging molecule vWF (Cox, 2009). Also, bacteria can bind to antibody or complement, which is recognized by the platelet IgG Fc receptor FcγRIIa (Fitzgerald et al., 2006a).

Whether secreted or cell-wall associated, lipopolysaccharide (LPS) from Gram-negative bacteria, and lipoteichoic acid (LTA) from Gram-positive species bind to platelets (Kerrigan and Cox, 2010). LPS has been proven to directly interact with TLR4 present on the platelet surface, although does not necessarily produce a platelet aggregative response (Kerrigan and Cox, 2010). LTA is recognized by both the TLR2 receptor (Kerrington and Cox, 2010), and platelet activating factor receptor, PAF-R, also present on the surface of platelets (Zhang et al., 2005). The interaction of pathogen-associated molecular patterns with platelet interaction is well-reviewed by Kerrigan and Cox (2009), and a more comprehensive review of bacterial-platelet interactions is provided by Fitzgerald et al. (2006b).

1.2 BACTERIAL CONTAMINATION OF PCs

Of all blood products, PCs are the most susceptible to bacterial contamination (Brecher and Hay, 2005; Ramirez-Arcos et al., 2007a). As previously stated, PCs are stored with constant agitation at room temperature for up to five days in bags designed to be oxygen permeable for optimal aeration. As well, this blood product is characterized by an ideal physiological pH and high glucose content (~500mg/dL) provided in part by an anticoagulant solution containing dextrose. Consequently, bacterial contamination of PCs constitutes the highest post-transfusion infectious risk in current times (Hillyer et al. 2003; Blajchman et al., 2005; Walther-Wenke, 2008; Palavecino et al., 2010). Recent estimates suggest that as many as 0.1% of platelet units are contaminated with bacteria (Walther-Wenke, 2008; Pearce et al., 2011), and approximately 1/100 000 units cause septic transfusion reactions due to bacterial presence (Walther-Wenke, 2008; Eder et al., 2009; Ramirez-Arcos, 2010a).

Coagulase negative staphylococci (CoNS) are the predominant bacterial contaminants of PCs with *S. epidermidis* being the species that is most commonly isolated (Brecher and Hay, 2005; Palavecino et al., 2010; Rood et al., 2011). This can be attributed to the ubiquitous presence of CoNS on human skin, combined with their ability to survive and proliferate in PCs during storage. Other Gram-positive bacteria which are often identified include *Staphylococcus aureus*, *Bacillus* spp., and *Streptococcus* spp. (Ramirez-Arcos et al., 2007a; Jacobs et al., 2008; Eder et al., 2009; Pearce et al., 2011; Lafeuillade et al., 2010).

Gram-negative bacterial contaminants are typically derived from transient colonization of donor skin or silent donor bacteremia (Palavecino et al., 2010). Due to the potent septic shock reaction induced by Gram-negative LPS, these species are responsible for a greater proportion of life-threatening or fatal transfusion reactions in comparison to Gram-positive bacteria (Jacobs et al., 2008). The most frequently identified Gram-negative PC contaminants include *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Serratia* spp. (Ramirez-Arcos et al., 2007a; Jacobs et al., 2008; Eder et al., 2009; Pearce et al., 2011; Lafeuillade et al., 2010).

1.2.1 Coagulase Negative Staphylococci (CoNS)

Bacteria of the genus *Staphylococcus* belong to the phylum *Firmicutes*, and are distinguished by a G + C genome content of less than 50% (Schleifer and Bell, 2009). Clinical microbiology laboratories typically differentiate the Staphylococcaceae by their ability to coagulate plasma since these species, particularly *S. aureus*, are considered to have greater pathogenic potential (Mack et al., 2004). It is for this reason that CoNS are often not distinguished further. Rapid identification systems are commercially available which facilitate species distinction via miniaturized biochemical tests with considerable success. Examples include the API Staph and Vitek Gram-positive identification systems (Both by bioMérieux, Marcy l'Etoile, France). Apart from direct sequencing, methods such as pulsed-field gel electrophoresis (Goering, 2010) or mass spectrometry (Rood et al., 2011), albeit time-consuming, are accurate and beneficial for epidemiological purposes including source tracking and studies of CoNS strain relatedness.

1.2.1.1 *Staphylococcus epidermidis*

A part of the normal skin microbiota, coagulase negative *S. epidermidis* is an opportunistic nosocomial pathogen responsible for sepsis and chronic biomedical device-related infections (von Eiff et al., 2000). The high affinity that *S. epidermidis* displays for abiotic surfaces is demonstrated with persistent cases of device contamination including colonization of intraocular implants, prosthetic joints, and indwelling catheters (Vadyvaloo and Otto, 2005; Shanks et al., 2006).

1.2.1.1.1 Cell Division

Cell division processes have not been characterized in *S. epidermidis*; however, studies in other Gram-positive bacteria have been informative. The division cell wall (*dcw*) genomic cluster of Gram-positive cocci has a widespread connection to Gram-positive bacterial cell division, yet its different homologs manifest diverse interspecies functionality (Pucci et al., 1997).

The *dcw* cluster gene product FtsZ is well characterized for its role in bacterial cell division site selection and presents a high level of sequence conservation within the kingdom bacteria, with homology to eukaryotic tubulin (Corton et al., 1987; Pinho and Errington 2003; Weart and Levin, 2003; Rothfield et al., 2005). In terms of functionality, FtsZ is essential to formation of the cell division septum by forming a filamentous scaffold known as a Z-ring for the recruitment of other cell division proteins (Rothfield et al., 2005). Studies in *Bacillus subtilis* which examined the link between growth rate and

Z-ring formation revealed that cytosolic levels of FtsZ are independent of bacterial doubling time, and induction of constitutive FtsZ expression has no effect on the progress or morphology of cytokinesis (Weart and Levin, 2003). In *Staphylococcus aureus*, FtsZ depletion results in large, aberrant cells prone to lysis (Pinho and Errington, 2003).

Another *dcw*-attributed gene linked to cell division site selection is *divIVA*. Homologous to eukaryotic tropomyosin, it is essential to *Corynebacterium glutamicum* (Valbuena et al., 2006), *Streptomyces coelicolor* (Hempel et al., 2008), *Mycobacterium tuberculosis* (Wag31; Kang et al., 2008), and *Enterococcus faecalis* (Ramirez-Arcos et al., 2005). Although it is non-essential in *B. subtilis* (Perry and Edwards, 2004) and *Streptococcus pneumoniae* (Fadda et al., 2007), it is required for normal cell division in these organisms. Conversely, this gene is completely dispensable for viability and cell division in *S. aureus* (Pinho and Errington, 2004). *E. faecalis* DivIVA cannot be used to complement its counterparts in *B. subtilis* and *S. pneumoniae* despite significant, shared amino acid identity (Ramirez-Arcos et al., 2005). Likewise, DivIVA from *C. glutamicum* could not restore DivIVA function in *B. subtilis* or *S. pneumoniae*, although it could compensate for loss of the ortholog in heterologous hosts *S. coelicolor* and *Brevibacterium lactofermentum* (Letek et al., 2008). These findings present compelling evidence for DivIVA functional divergence despite high conservation of protein sequence and coiled-coil structural motifs amongst bacterial species (Rigden et al., 2008). In the process of establishing a basis for these distinctions, it has been noted that the only region of DivIVA which consistently displays interspecies variability is the C-terminal domain.

Of particular interest, our laboratory has discovered that the C-terminal domain of *S. aureus* DivIVA differs drastically from that of the orthologous protein in *S.*

epidermidis. This occurs despite an otherwise similar protein sequence and conserved *dcw* cell division repertoire within the respective genomes. Specifically, *S. epidermidis divIVA* encodes a protein with a highly polar, serine-rich C terminus which is absent from the *S. aureus* counterpart (Greco et al., 2005). Through construction of a knock-out mutant, our group has confirmed that like *S. aureus divIVA*, the *S. epidermidis* ortholog is non-essential for growth or cell division (unpublished results). However, the localization of this protein is markedly different in both hosts. *S. epidermidis* DivIVA was found to bind to specific cell membrane phospholipids phosphatidylserine and cardiolipin, with uniform distribution around the staphylococcal cell periphery (unpublished results). Contrarily, *S. aureus divIVA* is targeted to the cell septum (Pinho and Errington, 2005). Since *S. epidermidis* grows significantly slower as compared to *S. aureus* in complex environments such as stored PCs (Mohr et al., 2006), it is possible that different cell division regulatory mechanisms are in place in these two species of the same genus.

1.2.1.1.2 Biofilm Formation

The ability to establish multicellular, slime-encased bacterial communities is referred to as biofilm formation (Monds and O'Toole, 2009). The biofilm environment provides protection from antimicrobial compounds and reduces detection by the innate immune system which hampers eradication and often results in chronic host infections (Mah and O'Toole, 2001; Vuong et al., 2004a; Monds and O'Toole, 2009). Importantly, biofilms cause an estimated 65% of all human infections, and can exhibit 1000-fold greater resistance to antimicrobial therapies compared to planktonic, or free-floating cells (Mah and O'Toole, 2001). Thus, biofilm formation can be designated as a virulent phenotype.

Skin flora bacteria have been documented to form biofilms on the skin surface, stratum corneum, underlying epidermis, sebaceous glands and hair follicles (Ryder, 2005). Despite the frequency of mixed microbial biofilms in nature, *S. epidermidis* biofilm infections are typically singled-species (von Eiff et al., 2000). All *S. epidermidis* strains vary in their ability to form biofilms, and several genes and regulatory pathways have been shown to influence biofilm-forming ability (Mack et al., 2004; Otto, 2009). Biofilm formation can be distinguished by four phases: adsorption, attachment, accumulation, and dispersal (Figure 3), with discrete molecular and biochemical characteristics which may or may not be species-specific (Cramton and Götz, 2003).

The adsorption phase of biofilm formation is characterized by transient bacteria-surface interactions due to van der Waals and electrostatic forces, and is often referred to as a reversible attachment phase (Monds and O'Toole, 2009). In terms of irreversible attachment, *S. epidermidis* is adept at colonizing both biotic and abiotic surfaces. Complementation studies of transposon mutants defective in biofilm formation showed that autolysin E (AtlE) is essential for biofilm initiation (Heilmann et al., 1997). This enzyme breaks down cell wall peptidoglycan for cell division (Biswas et al., 2006), and also enables bacterial attachment to surfaces such as plastics via release of DNA which provides a scaffold for microcolony formation (Qin et al., 2007). Bap-homologous protein (Bhp) is also involved in *S. epidermidis* primary attachment (Tormo et al., 2005a). *S. epidermidis* adherence to biotic surfaces, including surfaces coated with biological molecules, is more complex. It is known that adsorption of plasma proteins to

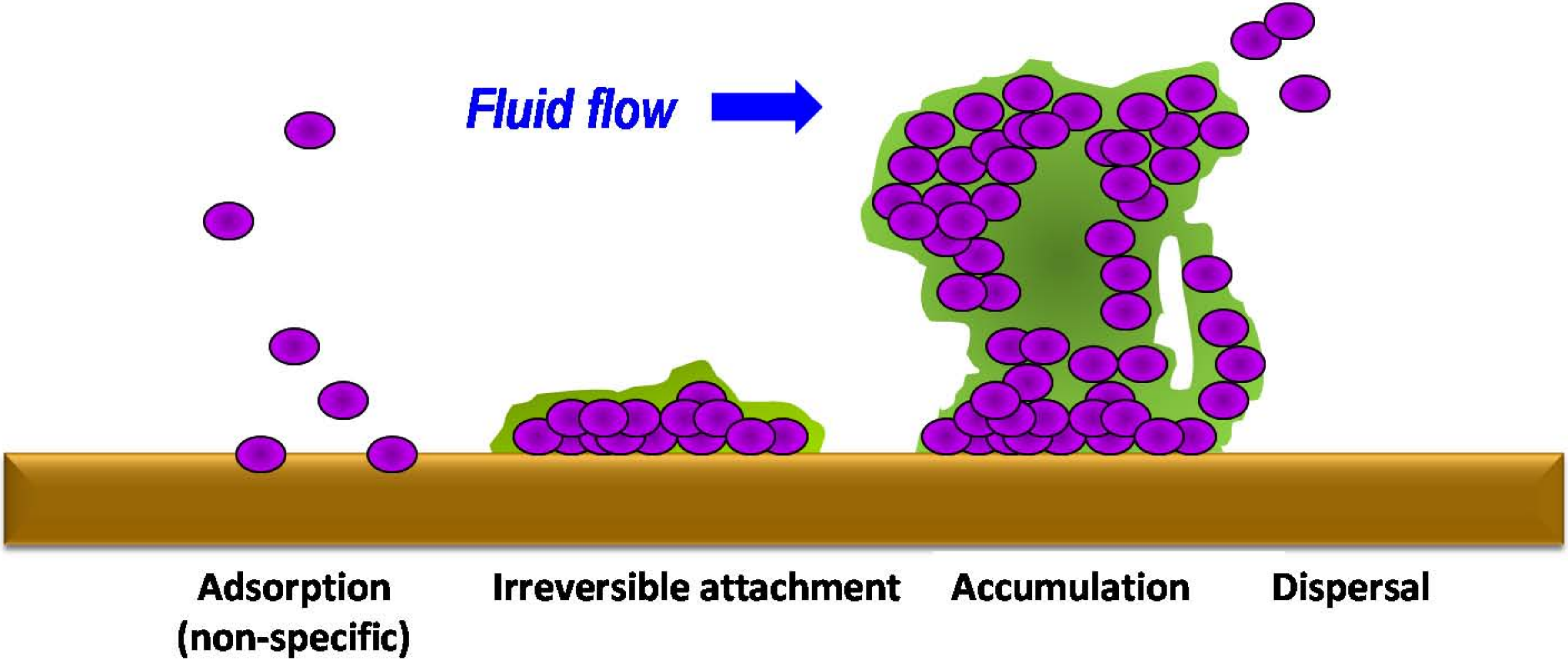

Fluid flow
Adsorption
(non-specific)
Irreversible attachment
Accumulation
Dispersal

Figure 3. Stages of bacterial biofilm formation. The four stages of biofilm formation shown are (1) adsorption of microbial cells, which is transient and reversible; (2) irreversible attachment, whereby adhesive factors mediate cell-to-surface binding; (3) accumulation of bulk biofilm, normally characterized by extensive extracellular matrix production. This phase is sometimes subdivided with biofilm maturation as an additional stage; and (4) biofilm cell dispersal, where single cells or cell aggregates become dislodged and can colonize a downstream location.

most hydrophobic plastic polymers, along with shear force, decreases initial adherence of this bacterium (Wang et al., 1995). Subsequent research demonstrated that this effect is transient, and that deposited serum proteins actually result in increased bulk biofilm formation on hydrophobic surfaces over time due to seemingly increased slime production resulting in improved bacterial aggregation (Patel et al., 2007). Likewise, initial attachment of *S. epidermidis* to charged surfaces (anionic and cationic) is increased in the presence of serum proteins (MacKintosh et al., 2006). *S. epidermidis* adhesion to hydrophobic plastic is also facilitated by pre-conditioning with platelets (Wang et al., 1993a and 1993b), and deposition of platelets on hydrophobic surfaces contributes to increased bacterial adherence (Sapatnekar et al., 1995). A more detailed review of *S. epidermidis*-platelet interactions is given in Section 1.2.1.1.3.

Biofilm accumulation by *S. epidermidis* has been linked to the presence of many factors, including accumulation-associated proteins Aap and Aae (Banner et al. 2007; Heilmann et al., 2003), cell wall techoic acids (Holland et al., 2011), and enzymes of the intercellular adhesion (*ica*) locus (Heilmann et al., 1996). In particular, strong biofilm producing strains most often harbour the *icaADBC* operon in their genomes (Heilmann et al., 1996; Zhang et al., 2003). This genetic locus encodes enzymes which facilitate formation of the extracellular matrix material called polysaccharide intercellular adhesin (PIA), important for biofilm accumulation by these strains (Heilmann et al., 1996; Mack et al., 1996). Enzymes IcaA and IcaD, when co-expressed, will exhibit optimal activity for the assembly of β(1-6)-linked glucosaminoglycans which comprise PIA (Gerke et al., 1998). Absence of *icaD* is associated with a striking decrease in PIA production (Gerke et al., 1998), and previous studies have indicated that all strains positive for *icaA* are similarly positive for *icaD* (Arciola et al., 2001; Arciola et al., 2003). IcaC likely functions in extrusion of PIA from the bacterial cell (Otto, 2009), while IcaB appears to function as a deacetylase which enables the so-called ‘activation’ of an otherwise neutral PIA molecule by conferring a cationic character necessary for retention at the negatively-charged bacterial cell surface (Vuong et al., 2004b). PIA was once suspected to facilitate initial attachment to plastics; however, this was disproved by Higashi et al. (1998).

Less is known about *S. epidermidis* mechanisms for biofilm dispersal; however, this biofilm stage was found to be *agr*-dependent (Vuong et al., 2003), and can be triggered by bacterial concentration-dependent communication using auto-inducing peptides in a process known as quorum sensing (Vuong et al., 2003; Xu et al., 2006). *In vitro* biofilm simulations have helped to uncover several mechanisms which regulate *S. epidermidis* biofilm development. Particularly, it has been shown that quorum sensing via the accessory gene regulator (Agr) and LuxS systems not only cause dispersal, but also repress *S. epidermidis* biofilm formation (Yao et al., 2006; Xu et al., 2006). Also, TcaR and IcaR act as negative regulators of PIA synthesis (Conlon et al., 2002; Chang et al., 2010). In contrast, the global gene regulators SarA and SarZ positively regulate biofilm development by this bacterium (Tormo et al., 2005b; Wang et al., 2008). Similarly, stress (pH, heat, osmolarity, ethanol) responsive elements σ-factor B and Ygs exert positive influence on biofilm formation (Pintens et al., 2008; Wang et al., 2011a). The *S. epidermidis* protease ClpP degrades negative biofilm regulator Spx, demonstrating a unique interplay affecting biofilm dynamics (Wang et al., 2010). Conditions of iron starvation exert influence on iron-associated proteins SirR and SitABC, leading to sessile *S. epidermidis* growth (Massonet et al., 2006). The mobile genetic element IS256 is linked to insertional inactivation of biofilm genes (Ziebuhr et al., 1999). As well, *S. epidermidis* produces peptides termed phenol soluble modulins, or delta toxins, which act as surfactants to facilitate biofilm dispersal (Wang et al., 2011b). Finally, environmental factors linked to increased *S. epidermidis* biofilm production include low temperature (Fitzpatrick et al., 2005), ethanol (Conlan et al., 2002), glucose (Jäger et al., 2005), NaCl (Knobloch et al., 2001), exposure to inotropic (muscle contracting) drugs like adrenaline, dobutamine, and dopamine (Lyte et al., 2003), and subinhibitory concentrations of antimicrobials (Rachid et al., 2000). The molecular genetics of *S. epidermidis* biofilms is reviewed extensively by Otto (2009).

1.2.1.1.3 Host Interaction

As previously stated, *S. epidermidis* biofilm formation can be facilitated by binding biotic surfaces. This includes substrates presenting deposits of matrix molecules present in serum and interstitial fluid including fibrinogen, fibronectin, vitronectin and collagen. *S. epidermidis* microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) comprise the best-described mechanism for host tissue binding. In particular, the Serine (S)-Aspartate (D) repeat protein SdrG, formerly known as FbeA, is a proven fibrinogen-binding receptor, employing the ‘dock-lock and latch’ model for ligand interaction (Bowden et al., 2008), and is sufficient for induction of *in vitro* platelet activation (Brennan et al., 2009). Additional *S. epidermidis* MSCRAMMs include the collagen adhesin GehD (Bowden et al., 2002), type I collagen receptor SdrF (Arrecubieta et al., 2007), and the extracellular matrix binding protein Embp (Christner et al., 2010). Mechanisms for bacterial anchoring to the platelet surface have been described in Section

1.1.4. It is likely that the ongoing process of decoding the *S. epidermidis* genome for putative virulence genes will uncover even more host binding factors.

1.2.1.1.4 Adverse Transfusion Reactions (ATRs)

S. epidermidis infections have been identified in severe and fatal transfusion reactions (Palavecino et al., 2010). In 2001, a case of fatal septic shock was reported in Canada after the transfusion of PCs contaminated with *S. epidermidis* (Goldman et al., 2001). The 74-year-old patient was undergoing chemotherapy for acute myelogenous leukemia. A hemovigillance study in Quebec reported four severe ATRs caused by *S. epidermidis* between 2000 and 2003 (Robillard and Nawej, 2004). Data compiled by the Food and Drugs Administration showed that 10% of transfusion-transmitted fatalities in the United States between 1976 and 1998 were caused by *S. epidermidis* (Goldman et al., 2003). Similarly, it has been reported that this microorganism was responsible for 26.7% of the fatalities involving neonatal blood transfusions over a three-year period in Brazil (Pessoa-Silva et al., 2001). A 15 year study of bacteria-induced ATRs at an Ohio tertiary care hospital revealed that 9 out of the 30 *S. epidermidis*-tainted platelet units that were transfused resulted in ATRs ranging from mild to life-threatening (Jacobs et al., 2008). Between 2004 and 2008 the American Red Cross reported 31 septic transfusion reactions, of which 21 were caused by 18 strains of CoNS (Eder et al., 2009). In France, the hemovigilance systematic review of ATRs which occurred between 2000 and 2007 identified 11 reactions due to CoNS (Lafeuillade et al., 2010). A Taiwanese study evaluating the risk of septic events associated with platelet transfusion confirmed at least one case of *S. epidermidis* sepsis out of 2,338 platelet units tested (Hsueh et al., 2009).

1.2.1.2 Other CoNS

Apart from *S. epidermidis*, some other common CoNS which are human skin colonizers include *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus hominis*, *Staphylococcus lugdenensis* and *Staphylococcus haemolyticus* (Schleifer and Bell, 2009), and clinical isolates of each species have demonstrated *in vitro* biofilm-forming ability (Hall et al., 1987; de Silva et al., 2002; Chaieb et al., 2006; Frank et al., 2007; Fredheim et al., 2009).

1.2.2 *Serratia* Species

Bacteria of the genus *Serratia* are Gram-negative bacilli of the Enterobacteriaceae family with an environmental distribution in water and soil. Like *S. epidermidis*, *Serratia* species have emerged as nosocomial pathogens despite their saprophytic associations in nature. Although *Serratia* infections are most frequently associated with an immuno-compromised state, pathogenesis in healthy individuals can result from colonization of the respiratory tract, bloodstream, urinary tract and wounds, and may cause life-threatening inflammatory processes such as endocarditis and meningitis (Hijazi and Falkiner, 1997). *Serratia* are known to produce a variety of extracellular proteins which are implicated in virulence (Hines et al., 1988). Of importance to the present study, severe and fatal reactions as a consequence of blood product contamination have led to the identification of *Serratia liquefaciens* (Jeppsson et al., 1984; Woodfield, 1991; Boulton et al., 1998; Roth et al., 2000; Brecher and Hay 2005; Ramirez-Arcos et al., 2007a) and *Serratia marcescens* (Ramirez-Arcos et al., 2006) as causative bacteria. Basic research has demonstrated that *Serratia* growth is enhanced by blood bag material and its constituents when cultured in deionized water (Szewzyk et al., 1993), and these bacteria also proliferate well in leukoreduced platelet-rich plasma (Gong et al., 1993).

S. liquefaciens is primarily distinguished from *S. marcescens* by its ability to ferment arabinose (Hijazi and Falkiner, 1997). Both species present similar etiology, although *S. marcescens* appears to be more prevalent in nature, and is a likely explanation for why disease caused by this species is more frequently reported (Traub, 2000; Stock et al., 2003). Biofilm formation in *Serratia* species is modestly described in the literature, but it is known that quorum sensing via acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2) mediates biofilm development, including substrate attachment and dispersal (Labbate et al., 2004; Rice et al., 2005; Labbate et al., 2007; Van Houdt et al., 2007). A major quorum-sensing independent mechanism enabling *Serratia* biofilm formation involves expression of type 1 fimbriae which anchor the bacteria to biotic substrates (Labbate et al., 2007; Kalivoda et al., 2008).

1.3 CONTROL OF PLATELET CONTAMINATION

Adverse transfusion reactions due to bacterial contamination of PCs can be reduced and/or eliminated at several stages during the process of collection, production, and finally transfusion. The problem of bacterial contamination is the major focus of this thesis, and so current and future methods employed to avoid, detect and potentially eliminate bacteria from PCs will be presented.

1.3.1 Avoidance

The first and most simple means to avoid a transfusion-associated bacteremic event is by preventing initial introduction of bacteria to the blood component. Risk of contamination is minimized by the use of sterile collection supplies such as those obtained from Good Manufacturing Practice (GMP)-certified companies. As well, needle packaging should be opened only immediately prior to use, and needles should never be reused. The phlebotomists tasked with collecting donor blood products should have completed standard, mandatory bloodborne pathogen training and sterile phlebotomy technique, and should wear personal protective equipment (for example, gloves, scrubs) at all times during the procedure. In developed countries, preliminary evaluation of donor health is performed using a mandatory questionnaire which includes questions of a general nature (eg, "Are you feeling well today") and questions aimed at identifying conditions associated with increased risk for sepsis (eg, "In the last 6 months have you had a tattoo, ear or skin piercing"). Donors whose responses reveal risk factors for transfusion-transmissible disease are deferred either temporarily or permanently, depending on the etiology of the (potential) disease. For instance, a person with a confirmed history of syphilitic disease will be permanently deferred from all donations, while dental surgery entails only a 72-hour deferral, provided the donor is asymptomatic for infection. Elevated body temperature (eg, $> 37.5^{o}C$) will also result in temporary deferral due to risk of active infection. Skin disinfection is an added safeguard which prevents bacteria present at the site of phlebotomy from entering the blood or platelet collection set (Ramirez-Arcos and Goldman, 2010). CBS recently implemented the use of a one-step swabstick with 2% chlorhexidine gluconate and 70% isopropyl alcohol as its sole method of skin disinfection (Ramirez-Arcos and Goldman, 2010), similar to the United Kingdom

McDonald et al., 2010). Other disinfection methods which are used throughout the world are described elsewhere (McDonald et al., 2006).

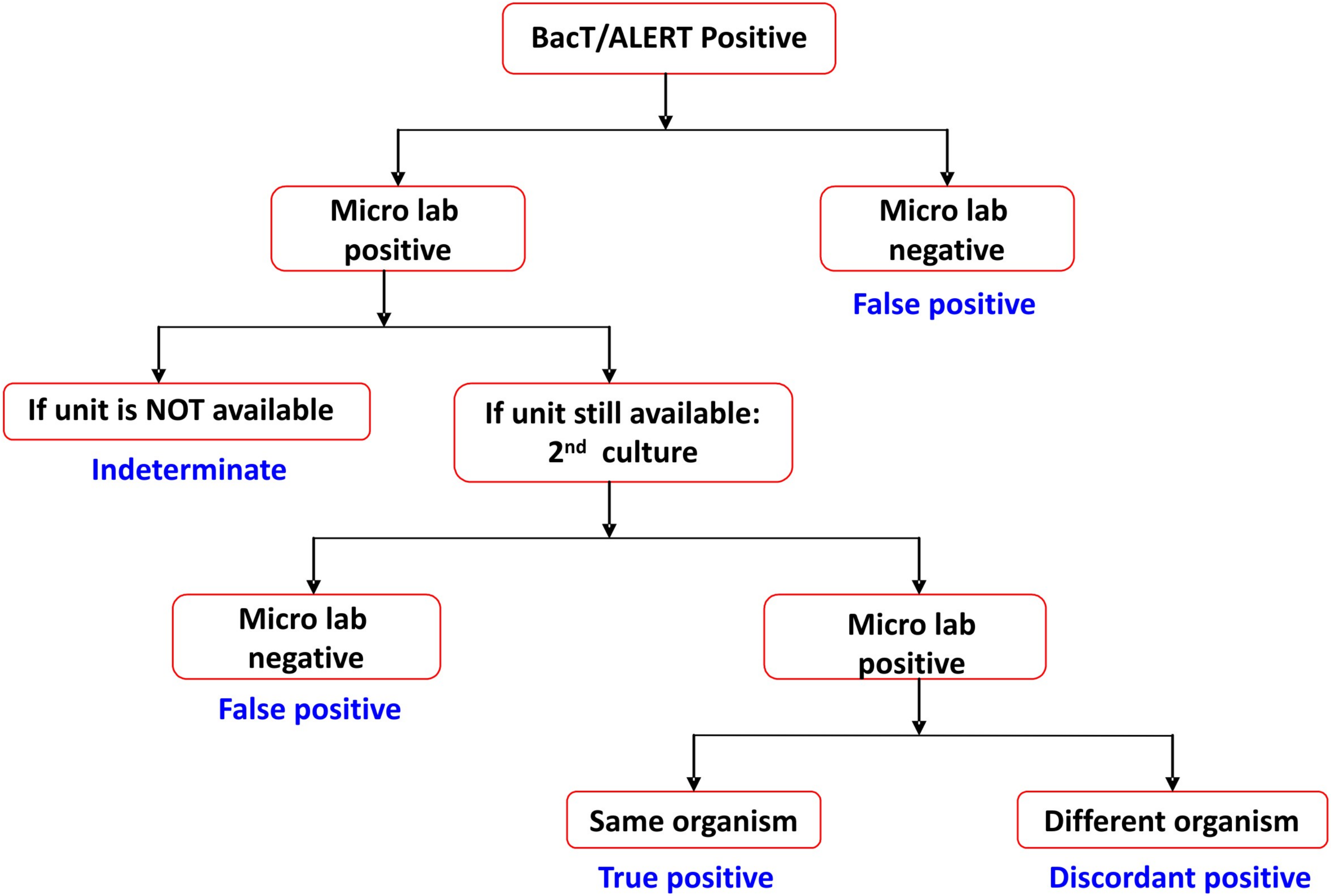
It is presumed that the first 10 to 20 mL of a blood donation has the highest probability of harbouring skin contaminant bacteria, and is also likely to contain the 'skin plug' produced upon needle insertion into through the donor's skin as an added source of bacteria. Accordingly, diversion of this first aliquot has been proven to reduce instances of bacterial transmission from transfusion, and has been implemented at many blood centres, including all in Canada, as a final safeguard for bacterial avoidance during blood collection (Eglin et al., 2004; McDonald, 2006; Ramirez-Arcos et al., 2007b).

1.3.2 Surveillance

Pre-transfusion bacterial detection was implemented in Europe in the late 1990s and in Canada and the US in 2004 in order to reduce instances of bacterial sepsis caused by transfusion of PCs (Blajchman et al., 2005). Loss of platelet swirling and marked decreases in glucose concentration and pH are good indicators of high levels of contamination (eg, $>10^6$ CFU/mL), but may result in excess product wastage due to high false-positive rates, as well as missed detection of lower inocula (Yomtovian et al., 2006). Direct microscopic examination of Gram or Acridine Orange stains may be useful for detecting moderate contamination (eg, $>10^4$ and $>10^3$ CFU/mL, respectively); however, these methods demand the time of skilled staff to stain and examine the samples (Yomtovian et al., 2006). High-throughput bacterial detection systems validated for PC screening are the preferred option for blood banking organizations due to their relatively high sensitivities and specificities. Available platforms which have been approved in North America for initial screening of PCs include the BacT/ALERT$^{®}$ 3D system (bioMérieux, Marcy l'Etoile, France) and the enhanced Bacterial Detection System (eBDS; Pall Corp, East Hills, NY). Other systems used for blood culture such as BacTec (BD, Sparks, MD) and VersaTREK (Trek Dignostics, Cleveland, OH) must be validated in-house prior to use for platelet testing (Palavecino et al., 2010). Of these platforms, BacT/ALERT$^{®}$ has been implemented at CBS and by the majority of US blood centres (Mastronardi et al., 2007). BacT/ALERT$^{®}$ is an automated culture-based incubation system that continuously scans culture bottles which are housed at 35-37^{o}C from 24 h post-collection until discard. Specifically, colour change of a patented substrate contained at the base of an inoculated bottle reflects decreased pH due to production of CO_2, and thus is suggestive of aerobic bacterial growth (Thorpe et al., 1990). This machine can detect most bacteria after 9-12 h of incubation from an initial inocula of 10-100 CFU/mL (McDonald et al., 2002; Larsen et al., 2005; Muller et al., 2008). Although not implemented at CBS, BacT/Alert$^{®}$ culture bottles also exist for detection of anaerobic bacteria. The cascade for bacterial detection and subsequent identification at CBS is shown in Figure 4. As well, several novel proposals for bacterial detection in platelet products exist in various stages of development. A compilation of these techniques is presented in Table 1.

1.3.3 Missed Detection

Although rare, missed detection of contaminant bacteria by sensitive screening platforms has culminated in severe and fatal transfusion reactions (Jacobs et al., 2008; Eder et al., 2009; Palavecino et al., 2010). This is complicated by the fact that recipients of platelet



BacT/ALERT Positive

Micro lab positive

Micro lab negative

False positive

If unit is NOT available

Indeterminate

If unit still available: 2nd culture

Micro lab negative

False positive

Micro lab positive

Same organism

True positive

Different organism

Discordant positive

Figure 4. Cascade for positive identification of bacterial contamination of PCs at CBS. The term “unit” refers to the original PC storage bag plus residual product.

Table 1. New and emerging technologies for detecting bacterial contamination of PCs.

Method	Associated Technology	Principle	Reference
Rapid, Qualitative Immunoassay	Pan Genera Detection Technology (Verax Biomedical, Worcester, MA)	Lateral flow immunoprecipitation is used to detect the presence of conserved bacterial cell wall antigens (lipotechoic acid and LPS on Gram-positive and negative cells, respectively)	Yazer et al., 2010; Vollmer et al., 2010
Multiple pH measurements	N/A	Rate of pH change heralds bacterial growth	Barker et al., 2010
Flow cytometry	BactiFlow® (AES Chemunex, Bruz, FR)	Cleavage-induced activation of fluorochrome by intracellular esterases signals viable cell presence detected by flow cytometry	Dreier et al., 2009
Real-time biosensor (spores)	Label-free exponential signal-amplification system, LEXAS™ (BCR Diagnostics, Jamestown, RI)	*Bacillus cereus* spores inoculated into a platelet sample will germinate in response to metabolic activity of neighbouring bacterial cells, resulting in production of intracellular esterases, and generating more bacterial metabolic substrate. The result is a self-amplifying signal of bacterial presence detected by fluorochrome cleavage	Rotman et al., 2003
Fluorescent bioimaging	µFinder Inspection System (Asahi Breweries, Tokyo, Japan)	PCs and potential contaminant organisms are retained on a metalized filter and stained with a fluorochrome for esterase activity indicative of viable bacteria. Positive fluorescence is discriminated from background signals based on intensity and particle morphology	Motoyama et al., 2008
Differential impedance monitoring detection system	Under development (Biosense technologies, Woburn, MA)	A PC aliquot is withdrawn and subjected to biological stressors (heat-shock, Triton X-100), and the dielectric permittivity of the sample is measured to detect metabolic flux consistent with universal bacterial stress response	Rieder et al., 2008; Rieder et al., 2011
Real-time PCR	ABI 7000 series (Applied Biosystems, Foster City, CA)	Detects eubacterial 16S rDNA	Mohammadi et al., 2003, 2005

Real-time PCR (continued)	“	Primers and probes specific for eubacterial 16S rDNA	Rood et al., 2008
	MX4000™ (Stratagene, La Jolla, CA)	Primers and probes specific for *S. epidermidis divIVA* and *icaA* genes	Mastronardi and Ramirez-Arcos, 2007
Real-time, reverse transcriptase PCR	Lightcycler® (Roche, Indianapolis, IN)	Primers and probes specific for eubacterial 23S ribosomal (r)RNA and *groEL* mRNA (encoding 60-kDa heat-shock protein)	Dreier et al, 2004
	ABI 7000 (Applied Biosystems, Foster City, CA)	Platform from Rood et al. (2008) with additional sensitivity added by detection of 16S rRNA	Rood et al., 2010
Micro-calorimetry	Thermal Activity Monitor (TA Instruments, New Castle, DE)	Heat flow is measured over time as proportional to bacterial metabolism during proliferation	Trampuz et al., 2007
Near Infrared (NIR) Spectroscopy	NIR6500 (Foss NIRSystems, Laurel, MA, USA); NIRGun (Shizuoka Shibuya Seiki, Hamamatsu, Japan)	Fluctuations in NIR spectra based on electromagnetic waves transmitted through the contaminated units are compared to those obtained for sterile reference units	Saranwong et al., 2010

units are often immunocompromised at the time of transfusion, increasing the risk of an ATR. Published reports have demonstrated the validity of this concern. During a study performed by the American Red Cross in 2004, three septic reactions involving platelet units that tested negative by the BacT/ALERT® system were contaminated with CoNS and one of them was identified as *S. epidermidis* (Fang et al., 2005). A study conducted in Norway with outdated PCs that had initially tested negative showed that one out of 1 061 platelet units was contaminated with *S. epidermidis* (Larsen et al., 2005). Platelet units tainted with this bacterium have been also missed when screened with the Pall eBDS system (Holme et al., 2005). Slow growth of *S. epidermidis* in PCs is well-documented (Ezuki et al., 2007; Störmer et al., 2008), and is a tentative explanation for missed detection of this species (Mohr, 2006; Murphy et al., 2008). In terms of Gram-negative species, bacteria of the genus *Serratia* grow more slowly in PCs than other bacteria (Brecher et al., 2000), and have caused severe or fatal reactions following false-negative cultures (Yomtovian et al., 2006; Ramirez-Arcos et al., 2006; Brecher and Hay 2005; Roth et al., 2000).

1.3.4 Pathogen Reduction

An ideal scenario would be one in which all potential bloodborne pathogens could be eliminated from transfusable blood products. While this technology may not render the currently used bacterial and viral detection systems obsolete, it would provide an added barrier to contamination for enhanced blood supply safety. Pathogen reduction technologies (PRTs) have been introduced and may potentially remedy the concern of missed detection of slow-growing bacteria as well as new and emerging pathogens for which no specific assays exist. Two companies currently hold patents on similar strategies of pathogen reduction: Caridian BCT (Mirasol), and Cerus Corporation (Intercept). Both use long wavelength, UVA irradiation in combination with the nucleic acid photosensitizers amotosalen and riboflavin (vitamin B_2), respectively (Knutson et al., 2000; Li et al., 2004). The Intercept blood system received European clearance for use with PCs in 2002 (http://investor.cerus.com/Investors/Press-Releases/Press-Release-Details/2002), and no significant attributable adverse outcomes have yet been reported (Cazenave et al., 2011). It has been proven effective against a wide range of microorganisms, including viruses, Gram-positive and Gram-negative bacteria, aerobes, anaerobes, and spirochetes (Lin et al., 2004). However, *Bacillus cereus* spores are resistant to the treatment (Knutson et al., 2000), and no studies on the impact of PRT on bacterial biofilms have been performed. PRT has still not received approval for use in the US or Canada due to conflicting reports on platelet hemostatic effectiveness subsequent to the PRT treatment process (Galan et al., 2011; Reikvam et al., 2010; Vamvakas, 2011; The Mirasol Clin Eval Study Grp, 2010; Kerkhoffs et al., 2010; Ostrowski et al., 2011). The utility of PRT was highlighted in one recent report in which an ATR was “intercepted” by the application of Intercept prior to transfusion of an apheresis unit which was contaminated with *Sphingomonas paucimobilis* (Jimenez-Marco et al., 2010).

Another proposal has been the use of short-wave ultraviolet light to directly interfere with pathogen nucleic acids (Mohr et al., 2009). The advantage of this technique is the relative lack of impact on the PC preparation procedure; however, it is still under preliminary development.

Prophylactic supplementation of blood products with conventional antibiotics is not feasible due to risk of allergy-induced ATR in addition to rapid evolution of bacterial resistance. However, Mohan et al. (2010) have recently proposed the use of synthetic and natural platelet-derived antimicrobial peptides as a means to reduce pathogen titre in blood products. These small molecules could not, however, completely eliminate all types of bacteria from tainted units, and treatment of PCs was much less effective compared to treatment of plasma for reducing bacterial load, presumably due to sequestration of peptides by the platelets (Mohan et al., 2010). Using the cationic antimicrobial polypeptide ε–poly-L-lysine, Tanaka et al. demonstrated that the growth of three different bacterial species could be inhibited with minimal effect on platelet quality (2010). Ultimately, more research would be needed in order for the latter proposals to become a reality, including more extensive investigation of potential adverse reactions in animal models.

1.4 PLATELET ADDITIVE SOLUTIONS (PASs)

As an alternative to storage in plasma, PASs were created to decrease plasma-related transfusion reactions as well as to conserve plasma for fractionation (Azuma et al., 2009). First generation PASs (eg, T-Sol, Baxter, Deerfield, IL; Intersol®, Fenwal, Lake Zurich, IL) consist of sodium-citrate-acetate chloride solutions. Subsequent formulations were supplemented with potassium and magnesium for the suppression of platelet metabolism to slow progression of the platelet storage lesion (Gulliksson et al., 2003). For improved buffering capacity, SSP+ (MacoPharma, Tourcoing, France), Composol (Fresenius, Bad Homburg, Germany) and PAS-G (Pall Corp., Port Washington, NY) were created by augmenting the earlier formulations with phosphates, gluconate, or glucose (Gulliksson et al., 2000; Gyongyossy-Issa et al., 2009). The influence of PASs and PAS components on the quality of stored platelets is well documented (Zhang et al., 2008a and 2008b; Azuma et al., 2009; Reikvam et al., 2010; Ostrowski et al., 2011; Galan et al., 2011), and PASs are now used routinely at a number of platelet collection centres across Europe (Cazenave et al., 2011; Sandgren et al., 2010; van der Meer et al., 2010). PASs are recommended for use with PRTs which are approved in Europe (Janetzko et al., 2009; Cazenave et al., 2011). As well, the PAS Intersol® has been recently approved in the United States for storage of Amicus apheresis PCs (U. S. Food and Drug Administration Reference NDA BN080041/0). It is anticipated that PRT will be implemented in Canada in the near future which will necessitate the use PAS, since this solution is associated with greater post-PRT treatment platelet quality in comparison to plasma-based platelet suspension (Janetzko et al., 2009).

An advantage of PAS is that it is manufactured under sterile conditions with quality control, reducing the risk of microbial contamination compared to plasma. However, the normal range of opportunity for contamination still exists during both blood collection and component production, and there is evidence that bacteria can grow in PAS-PCs (Dumont et al., 2009 and 2011). Thus, it is valid to study bacterial contamination in either platelet storage media.

1.5 POLYETHYLENE GLYCOL (PEG)

PEG is an aqueous flexible polymer made up of repeating ether subunits of the general structure seen in Figure 5A (Roberts et al., 2002). Different chemistries can be used to

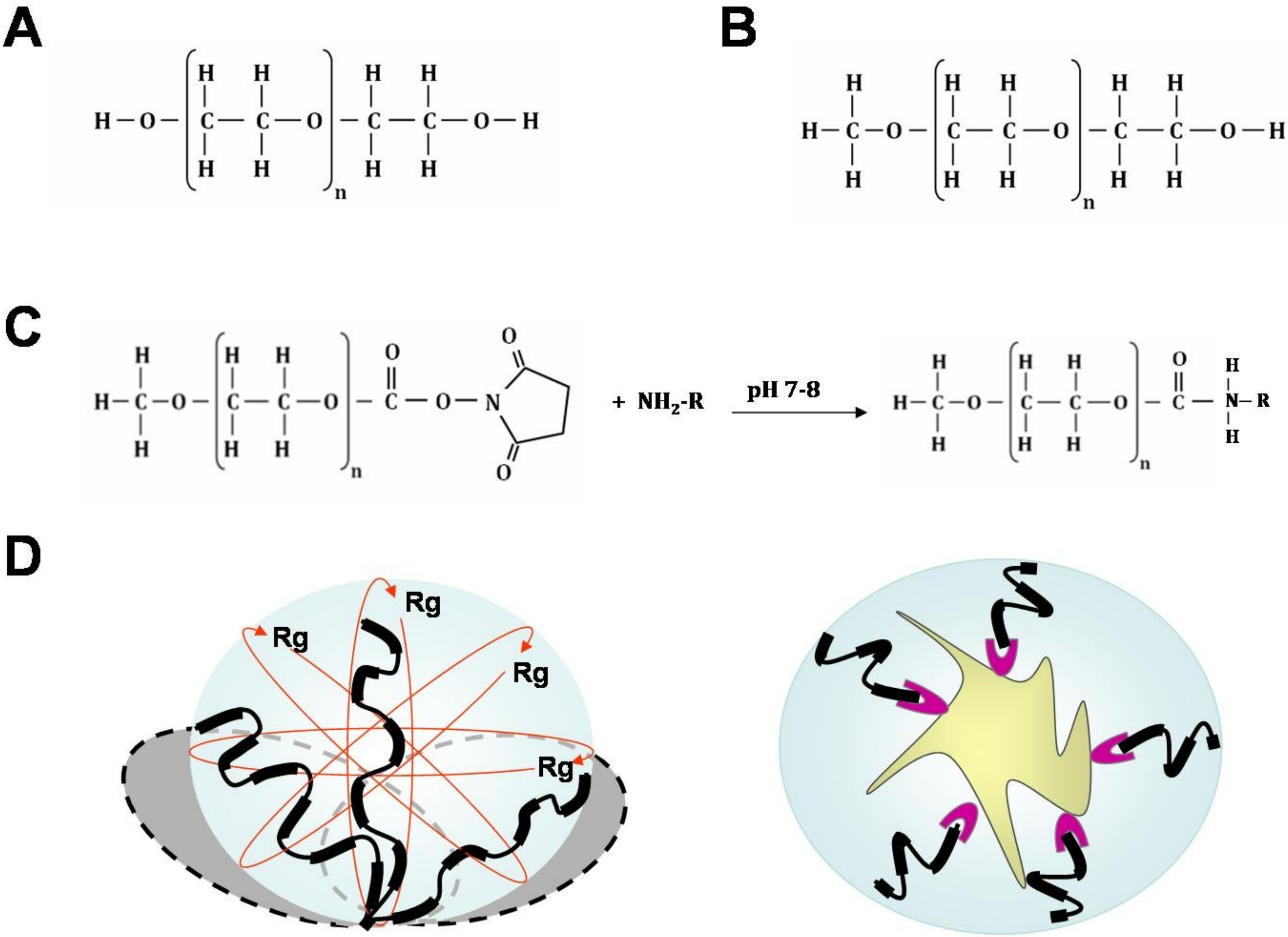

A
B
C
+ NH₂-R
pH 7-8
n
D
Rg
Rg
Rg
Rg

Figure 5. PEG structure and function in molecular shielding. Chemical structures of (A) PEG, (B) methoxy-PEG, and (C) the amine reactive derivative succinimidyl carbonate (SC)-PEG are shown. (D) A large zone of steric hindrance is generated by the mPEG polymer due to high flexibility. Addition of a chemical linker enables binding of mPEG to the cell surface resulting in antigen shielding over the radii of gyration, Rg. Adapted from Scott and Chen (2004).

link this molecule to a desired surface or substrate, as is increasingly attractive for a variety of medical applications.

1.5.1 Uses for PEG in Medicine

An important application of this molecule is its use for half-life extension of protein-based therapeutics. To this end, PEG is typically modified with a methoxide ion to obtain methoxy-PEG (mPEG; Figure 5B). This abrogates the molecule's ability to crosslink neighbouring polypeptides which would otherwise interfere with its therapeutic potential (Roberts et al., 2002). The grafting of PEG onto proteins is easily accomplished by conjugation to reactive N-terminal amino groups via acetylation. As an example, the addition of succinimidyl carbonate (SC) onto the PEG molecule is the basis for derivation of a first-generation 'activated' PEG that reacts preferentially with lysine residues, and to a lesser extent, histidine and tyrosine (Figure 5C; Miron and Wilchek, 1993).

The major mechanisms by which PEGylation prolongs retention of protein-based therapeutics in the human body include decreasing its immunogenicity and delaying its enzymatic degradation in the liver (Roberts et al., 2002). PEG can also be applied to decrease renal clearance of smaller molecules and increase solubility of certain proteins at physiological pH (Kang et al., 2009). PEGylated therapeutics which are currently available on the North American market are reviewed by Kang et al. (2009). Briefly, they include PEGylated interferon α-2b (PegIntron®) and interferon α-2a (Pegasys®) for chronic hepatitis C infection, filgrastim (Neulasta®) for disease prevention in neutropenic patients, ademase (Adagen®) for severe combined immunodeficiency, certolizumab (Cimzia®) for Crohn's disease manifestations, visomant (Somavert®) for acromegaly, asparaginase (Oncaspar®) for acute lymphoblastic leukemia, aptanib (Macugen®) for age-related macular degeneration, and continuous erythropoietin receptor activator (Mircera®) for anemia due to renal failure (Kang et al., 2009). Applications of PEGylated liposomes have been indicated for drug delivery, such as for coagulation factors and doxorubicin (Yatuv et al., 2010; Plosker, 2008), and PEGylation of adenoviral vectors for gene therapy also holds promise for cancer treatment (Yao et al., 2010).

In the same tradition of reducing immunogenicity of exogenous proteins, PEGylation can be applied to shield antigenic moieties on cell surfaces. Viral binding and host cell entry is hampered by PEGylation of traditionally receptive cell culture lines (Sutton and Scott, 2010), and prion infectivity can be similarly suppressed due to host cell PEGylation (Scott, 2006). Medical procedures involving allogenic transplant of donor cells and subsequent adverse reactions due to graft rejection are relevant candidates for PEGylation-based alternatives aimed at reducing host inflammatory response. For example, PEGylated pancreatic islets transplanted in diabetic patients have improved long-term (up to one year) survival in the body as compared to non-PEGylated islets, in the absence of aggressive immunosuppressive regimens (Yun Lee et al, 2007).

1.5.2 Uses for PEG in Transfusion Medicine

Arguably the best-studied medical application of cell PEGylation can be found in transfusion medicine. By shielding antigenic proteins displayed on donor blood cells from the recipient immune system, the risk of alloimmunization due to chronic transfusions may be drastically reduced (Scott et al., 1997; Murad et al., 1999a and 1999b; Chen and Scott, 2003). Importantly, the reduced immunogenicity attributed to

PEG-based molecular shielding of major and minor blood group antigens has been corroborated by four independent research groups (Jeong and Byun, 1996; Hortin et al., 1997; Armstrong et al., 1997; Murad et al., 1999a). Studies in mice have shown normal osmotic fragility, morphology, and survival of PEGylated erythrocytes in circulation, and clearance of sheep erythrocytes from murine peripheral circulation is vastly suppressed by pre-transfusion blood PEGylation without detriment to the host (Scott et al., 1997). Even at high molecular weights, the antigenicity of PEG is negligible, and antibody formation due to immunization with PEG requires covalent attachment to immunogenic carrier molecules in combination with Freund's adjuvant (Roberts et al., 2002).

Although RBCs are a prime target for this application, platelets are also amenable to grafting with mPEG (Rossi et al., 2010). Work of a collaborator suggests that PEGylation of platelets renders them less susceptible to cold-induced microaggregation, which would be advantageous to platelet storage time extension (Maurer-Spurej, unpublished data). Also, PEGylation of platelet-sized latex particles has been shown to neutralize ~ 80% of particle surface charges and > 80% of surface interactions with plasma proteins (Le and Scott, 2010). Other groups have investigated the use of PEG for minimizing bacterial attachment to biomaterials (Norde and Gage, 2004; Nejadnik et al., 2008; Saldarriaga Fernández et al., 2010 and 2011). However, the use of platelet PEGylation as a method of reducing bacterial attachment during platelet storage has not been studied until now. It is reasonable to propose that charge camouflage and prevention of receptor-ligand interactions via PEGylation of platelets or other cell types could similarly be used to abrogate bacterial attachment (Figure 5D).

1.6 BIOFILM DETECTION

Biofilm formation can be detected and analysed in a variety of ways which may vary as a consequence of the unique biofilm characteristics of the microbial species in question, and the goals of the study. Several detection strategies have been applied in the literature to study *S. epidermidis* biofilms at both the phenotype and genotype level. For pure cultures, biofilms are typically grown on slides, multi-well tissue culture plates, test tubes, flow cells or specialized biofilm reactors for downstream applications. Alternatively, naturally-occurring biofilms can be isolated from environmental specimens including plant and animal tissues, soil, and industrial or medical device surfaces.

Direct light microscopic examination may reveal bacterial clustering suggestive of biofilm formation. The use of flow cells may facilitate the study of real-time biofilm growth, and can be outfitted with various substrates conducive to colonization by the species in question. In complex samples, techniques for probing bacterial DNA such as fluorescent *in situ* hybridization (FISH) provide a reliable means to distinguish different microbes and their distribution, while denaturing gradient gel electrophoresis (DGGE) is a current popular method for assessing microbial diversity in environmental biofilms (Wuertz et al., 2004). High resolution imaging by scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM), or atomic force microscopy (AFM) may afford better quality data by providing information on biofilm topology, fluid dynamics and bacterial distribution at various stages of biofilm development (Neu et al., 2010). Unfortunately, the high cost and specialized training required for the use of these machines is a limiting factor for many laboratories.

Biofilm quantification can be rapidly performed by staining the microorganisms being studied. Specifically, dyes such as crystal violet (Christensen et al., 1985), and safranin (Wu et al., 2003) can be used to stain peptidoglycan on surface-attached cells, while alcian blue can be used to stain mucopolysaccharides and PIA (Hussain et al., 1992). Dye intensity can then be measured directly or indirectly (eg, through stain elution) to provide a semi-quantitative estimate of bacterial presence (Christensen et al., 1985; Toledo-Arana et al., 2005). Enumeration of surface-adherent cells by plating on solid media provides better quantitative value; however, a technical challenge resides in the fact that methods used to dislodge adherent bacteria (eg, sonication or mechanical disruption) can result in cell death and under-estimation of viable cell presence (Gagnon and Slawson, 1999). Alternatively, these methods may be inefficient at complete cell removal, which also can result in underestimations and significant data variability (Gagnon and Slawson, 1999).

Biofilm synthesis can be detected with Congo red dye which accumulates at high concentrations in extracellular polysaccharides such as PIA, resulting in hyper-pigmentation seen as black, crusty colonies (Freeman et al., 1989). Proteinase K can be used to deduce proteinaceous biofilm presence, as in the case of *S. epidermidis* Aap overexpression (Banner et al., 2005). However, studies have shown that relatively few *S. epidermidis* strains display this *ica*-independent biofilm phenotype (Kogan et al., 2006).

A more sensitive technique for detection of biofilm synthesis can include an immunoassay specific for a biofilm biomolecule. In particular, rabbit polyclonal IgG against *S. epidermidis* PIA has been previously developed for indirect immune-fluorescence microscopy (Mack et al., 2001). The advantage of this technique is that it can be applied to complex biological specimens, including foodstuffs, tissues, and soil, so that biofilms can be detected in a natural growth environment.

Genes associated with slime production (for example, *icaADBC*) can be amplified to determine whether a strain or species has the genetic capacity for biofilm formation (Mastronardi and Ramirez-Arcos, 2007). However, gene presence does not equate to expression, and gene regulatory mechanisms such as transcriptional repression or transcript degradation could consequently inhibit biofilm production. Therefore, it is recommended that a supportive, phenotypic assay for biofilm detection is used. Still, biofilm gene presence in strains with a biofilm-negative phenotype heralds the potential for biofilm production under alternative circumstances. For instance, *S. epidermidis icaADBC* genes are suppressed under nutrient-limiting conditions due to indirect activation of IcaR expression by σ-factor B (Jäger et al., 2005), but the opposite is true in the presence of ethanol (Conlon et al., 2002). In *S. epidermidis* strains harbouring insertion sequence IS256, translocation of this element into the *icaADBC* locus can quench PIA synthesis, which may be restored after serial passages due to spontaneous sequence excision in a process known as phase variation (Ziebuhr et al., 1999).

Despite multiple existing and evolving strategies used for biofilm assessment, the procedures described above constitute the prevailing methods in the literature for accurately characterising biofilm formation by *S. epidermidis* and other CoNS.

1.7 BIOFILM FORMATION AND MISSED BACTERIAL DETECTION

The isolation of bacterial contaminants from PC storage bags despite failed recovery of the organisms from the corresponding culture bottles used for screening presents intriguing implications for growth within the environment of stored PCs (Figure 6).

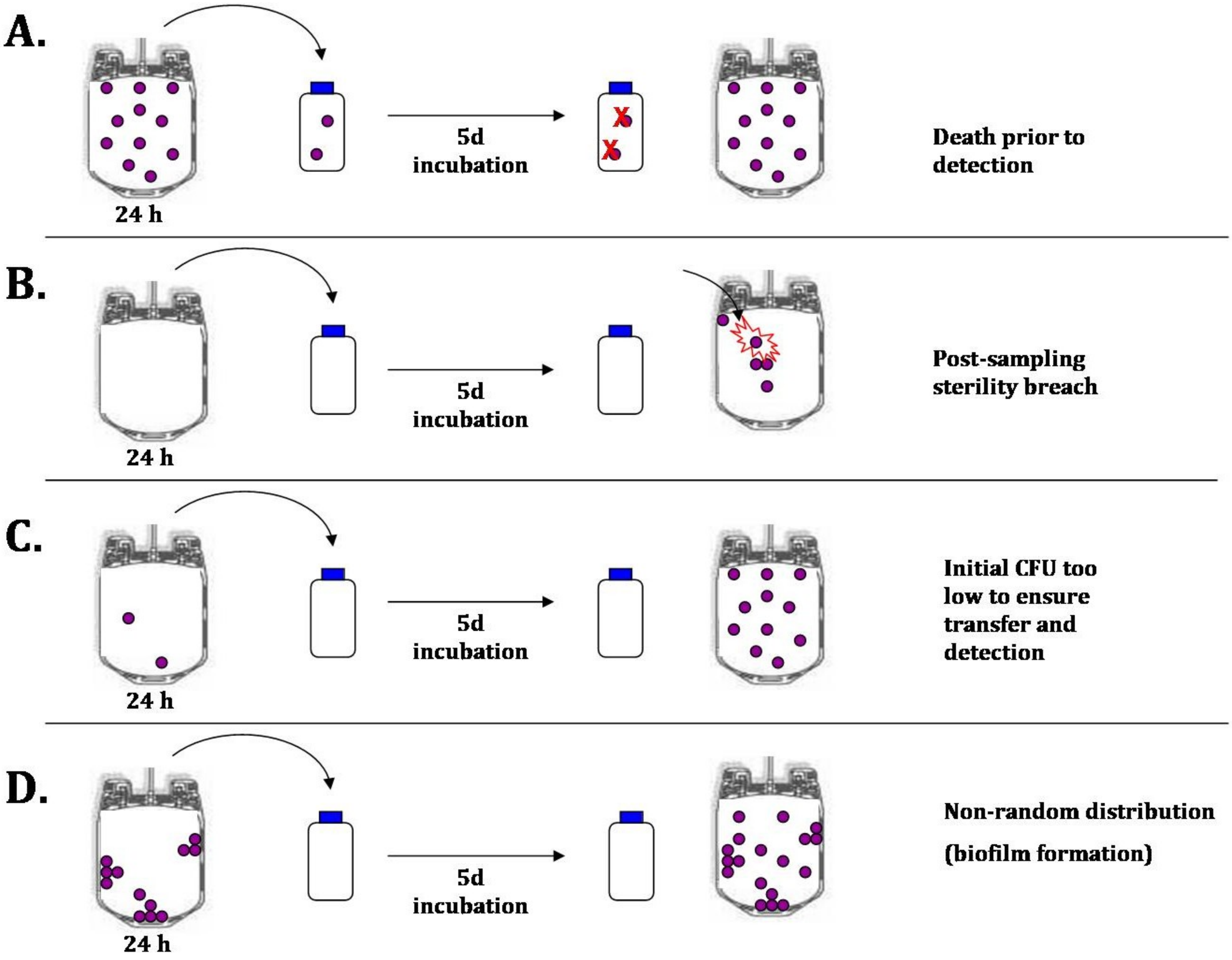

A.
24 h
5d incubation
Death prior to detection
B.
24 h
5d incubation
Post-sampling sterility breach
C.
24 h
5d incubation
Initial CFU too low to ensure transfer and detection
D.
24 h
5d incubation
Non-random distribution
(biofilm formation)

Figure 6. Possible mechanisms for missed bacterial detection despite contamination.

The first possibility is that bacterial contaminants, which are introduced to the culture bottles during sterility testing, are of a concentration representative of the main platelet bag, but do not thrive as well as they do in the PC (Figure 6A). This scenario would require the assumption that the platelet milieu is more supportive than the BPA culture media; however, we know this is not the case since culture bottles are incubated at physiological temperatures and contain nutritive support designed to facilitate bacterial growth. The transfer of the platelet sample to the bottle culture media may induce a growth lag associated with environmental adaptation processes of resident bacteria. However, the sample transmitted to the culture bottle has a generous volume of initial platelet product (~10%), which reduces the likelihood of a poor adaptive response.

A second possibility is that contaminant bacteria are introduced post-sampling via a breach affecting the main PC component (Figure 6B). These contaminants would not have been transferred to the BPA bottle. Although plausible, damage to platelet bag integrity would virtually always result in product leakage and signal prompt disposal.

The third and four most plausible possibilities involve missed collection of the contaminant bacteria upon sampling. In one scenario (Figure 6C), the bacterial load is not sufficient for representation in the collected volume (~4% of the total product) despite the 24 h pre-sampling incubation period. A second scenario proposes that the contaminant bacteria are unequally distributed in the platelet bag at the time of sampling (Figure 6D). These circumstances could result in non-inoculation of viable bacteria, despite bacterial presence in the main component. Unequal distribution of bacteria within the platelet bag despite uniform agitation alludes to the existence of one or more factors which prevent uniformity and promote clustering, and is the major focus of this thesis.

1.8 HYPOTHESES

It is hypothesized that attachment of the predominant platelet contaminant *S. epidermidis* to platelet bag plastic and sequestration by platelets or platelet aggregates constitutes a mechanism by which biofilm formation may occur during PC storage. It follows that at least some CoNS strains isolated from contaminated PCs should possess biofilm-forming potential. It is also hypothesized that biofilm formation in stored PCs will be altered by changes to the platelet storage medium, such as the use of a PAS in place of plasma. Furthermore, it is postulated that bacterial biofilm formation can be inhibited during PC storage by platelet PEGylation which would reduce bacterial aggregation.

1.8.1 Relevance

Despite knowledge that biofilm growth is a persistent problem due to the use of plastics in biomedical devices which are amenable to bacterial colonization, only one study was performed prior to the present work which evaluated bacterial adherence to blood transfusion sets (Parment et al., 1993). This study was presented as a follow-up to a *S. marcescens* outbreak originating from blood bags contaminated at the manufacturing or packaging facility (Heltberg et al., 1993). No further studies have emerged to assess the feasibility, occurrence or impact of biofilm initiation or accumulation during blood product storage until the present thesis research was undertaken (Greco et al., 2007, 2008, 2010, and 2011). This work denotes a need for further research into the pathogenic potential of biofilm-forming *S. epidermidis* in a transfusion setting.

1.9 OBJECTIVES

Objective 1: To Characterize *S. epidermidis* Growth and Biofilm Formation under Simulated Platelet Storage Conditions

In order to assess biofilm formation by *S. epidermidis* in a platelet storage context, biofilm detection methods described in Section 1.6 have been adapted for the present investigations, largely due to past success in multiple external research studies (Christensen et al., 1985; Toledo-Arana et al., 2005; Mack et al., 2001; Freeman et al., 1989; Arciola et al., 2003). In particular, the ability of *S. epidermidis* to form biofilms on platelet bags and platelet aggregates is demonstrated by SEM. Furthermore, a semi-quantitative assay using crystal violet dye for bacterial biofilm detection has been standardized in PCs (Greco et al., 2007).

Objective 2: To Assess the Biofilm-Forming Potential of CoNS Responsible for PC Contamination

Although biofilm formation by *S. epidermidis* is typically associated with hospital-acquired infections, recent studies have shown that a low but significant population of *S. epidermidis* strains, which are capable of forming biofilms, are present as commensal organisms in healthy individuals. This suggests that blood and platelet donors could be carriers of pathogenic *S. epidermidis* strains, since biofilm formation constitutes a pathogenic mechanism for persistent host colonization (Kozitskaya et al., 2004; de Araujo et al., 2006). Therefore, this study was undertaken to characterize the biofilm-forming ability of a collection of CoNS strains that were isolated from contaminated PCs in Canada in order to assess the risk of biofilm formation in the environment of stored

PCs. This has been accomplished via the use of aforementioned biofilm detection strategies (Section 1.6; Greco et al., 2008).

Objective 3: To Examine the Effect of Platelet Additive Solution on Bacterial Growth and Biofilm Formation

The effect of PASs on platelet quality has been previously been assessed (Zhang et al., 2008a and 2008b; Gyongyossy-Issa et al., 2009). However, only one other group has examined the effect of PAS on the growth of contaminant microorganisms (Dumont et al., 2009 and 2011), and the resultant effect of this scenario on platelet quality has never been investigated until now (Greco et al., 2010).

The present study provides a comparison of growth and biofilm formation by the clinically-relevant microorganisms *S. epidermidis* and *S. liquefaciens* in PAS-suspended PCs (PAS-PCs) and plasma-suspended PCs (plasma-PCs). The influence of these bacterial species on platelet quality has been examined using a panel of markers including pH, O_2, CO_2, glucose, lactate, platelet count, MPV, CD62P expression and phosphatidylserine exposure.

Objective 4: To Apply Platelet PEGylation as a Means to Reduce *S. epidermidis* Biofilm Formation in Stored PCs

Since cell surface modification has been previously applied to shield antigenic proteins from binding by exogenous ligands such as antibodies or viral receptors, it has been hypothesized that PEGylation of platelets will similarly mask molecules required for bacterial attachment. Hence, this study purports that PEGylation reduces the ability of *S. epidermidis* to bind to the platelet surface and consequently inhibits bacterial-induced platelet aggregation and biofilm formation (Greco et al., 2011).

Objective 5: To Examine Differential Protein Expression Associated with *S. epidermidis* Cell Division and Biofilm Formation

Several molecular factors have been previously identified which contribute to *S. epidermidis* biofilm formation. As well, biofilm formation is traditionally associated with reduced metabolic processes and cell division (Monds and O'Toole, 2009). In this study, an immunological approach was selected to examine differential expression of growth and biofilm proteins generated by *S. epidermidis*. Specifically, molecular cloning of recombinant genes, expression induction in a heterologous host, protein purification, and rabbit immunization have been applied to generate polyclonal IgG specific to cell division (DivIVA, FtsZ) and biofilm-associated (AtlE, SdrG) proteins, in addition to guanylate methyl kinase, Gmk, which was selected for its potential suitability as a gel loading and assay control.

2. MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

All bacterial strains were stored at -80°C in brain-heart infusion (BHI) broth (BD Biosciences, Franklin Lakes, NJ) containing 15 % glycerol prior to use. Culture purities were confirmed by observation of typical colony morphology after streaking stock cultures on appropriate growth media and overnight incubation prior to experimentation. For all liquid cultures, agitation was 250 rpm.

S. epidermidis strains ATCC 35984 (also known as RP62A) and ATCC 12228, both purchased from the American Type Culture Collection (ATCC, Manassas, VA), were used as biofilm-positive and biofilm-negative controls, respectively. *S. epidermidis* strain O-47 (donated by Professor Friedrich Götz, Universität Tübingen, Germany) was used for experiments involving PAS and for Western blot analysis of *S. epidermidis* protein expression in sessile versus planktonic culture due to ease of suspension and PIA positivity. Trypticase Soy Agar (TSA; BD Biosciences, Franklin Lakes, NJ) was used for plating. Liquid cultures were established in Trypticase Soy Broth (TSB; BD Biosciences, Franklin Lakes, NJ) plus 0.5 % glucose (TSBg). Incubations were performed at 37°C.

The *S. liquefaciens* strain used in PAS experiments was isolated from a contamination event at the CBS component production laboratory involving unexplained agglutination of red blood cells (Martincic et al., 2008). Its ease of suspension in growth media made its use preferable to other available *Serratia* strains. Liquid cultures were established in Luria-Bertani (LB; BD Biosciences, Franklin Lakes, NJ) broth containing 0.5 % casamino acids and 0.2 % glucose, while TSA was used for colony counts and culture purity verification. All *S. liquefaciens* cultures were incubated at 22±2 °C.

E. coli strain DH5α was used for cloning, while *E. coli* strains BL21(DE3), C41(DE3), and C43(DE3) were selected as host strains for *S. epidermidis* recombinant protein expression (Miroux and Walker, 1996). Specifically, BL21(DE3) carries the λ DE3 lysogen which encodes T7 RNA polymerase (T7pol) under control of the isopropyl-1-thio-ß-D-galactopyranoside (IPTG)-inducible lacUV5 promoter (Studier et al., 1990). The strain C41(DE3) is a more stable derivative of BL21(DE3), in that it harbours an uncharacterized mutation which prevents cell death associated with expression of many recombinant toxic proteins (Miroux and Walker, 1996). The subsequently-derived C43(DE3) strain was generated from C41(DE3) to be resistant to additional recombinant, toxic proteins (Miroux and Walker, 1996). *E. coli* liquid cultures were established in LB medium supplemented with 50 μg of kanamycin (Kan)/ml when required. Incubations were performed at 37°C.

2.1.1 CoNS Isolated by Canadian Blood Services (CBS)

Thirteen contaminant CoNS strains were isolated during routine screening of platelet concentrates by CBS between January 2006 and May 2007 (Table 2). The strains were isolated from culture bottle contents which tested positive for contamination with the BacT/ALERT® 3D system (bioMérieux, Marcy L'Etoile, France). Initial speciation was conducted by an external testing laboratory (Nucro-Technics Inc., Scarborough, Canada) using the automated Vitek 2 system (bioMérieux). Confirmatory identification was performed in our laboratory using the API Staph kit (miniaturized biochemical test strips; bioMérieux) and relatedness of some strains was assessed by ribotyping at the Health Canada Bureau of Microbial Hazards (Appendix 11.3.4).

Table 2. CoNS isolated by CBS during routine PC sample screening.

PC type	Strain	Confirmatory Test[*]	API Staph (%ID)[†]	Ribotyping[§]
BC	06010	True positive	*S. epidermidis* (97.3%)	*S. epidermidis*
	06038	True positive	*S. epidermidis* (97.8%)	*S. epidermidis*
	07003	True positive	*S. capitis* (95.4%)	ND
Apheresis	06033	Indeterminate	*S. lugdenensis* (64.6%)	*S. hominis*
	07006	Indeterminate	*S. epidermidis* (98.5%)	*S. epidermidis*
	07009	True positive	*S. warneri* (85.7%)	ND
	07022	True positive	*S. epidermidis* (94.8%)	ND
	07026	True positive	*S. epidermidis* (90.4%)	ND
	07043	False positive	*S. epidermidis* (97.8%)	ND
PRP	06043	True positive	*S. hominis*[¥] (72.5%)	*S. epidermidis*
	07023	Indeterminate	*S. hominis* (81.0%)	ND
	07040	Indeterminate	*S. capitis* (98.3%)	*S. capitis*
	07045	Indeterminate	*S. epidermidis* (94.8%)	ND

BC, buffy coat; PRP, platelet-rich plasma; ND, not done.

[*] Confirmatory testing proceeded according to algorithm shown in Figure 4.

[†] Percent confidence of species identification from initial test using the API Staph database (bioMérieux, Marcy L'Etoile, France).

[§] See Appendix 11.3.4 for additional data.

[¥] Re-identified as *S. epidermidis* based on ribotyping analysis.

2.2 PREPARATION OF PCs

Blood was collected and processed by licensed medical laboratory technologists according to the standard operating procedures of CBS and the Network Centre for Applied Development (netCAD) in Vancouver, Canada. All research using PCs was approved by the CBS Research Ethics Board and the healthy volunteer blood donors provided signed, informed consent prior to donation.

2.2.1 PCs Prepared by the Platelet-Rich-Plasma (PRP) Method

PCs derived by the PRP method were collected at CBS Newfoundland Centre and stored in CLX® platelet storage bags composed of polyvinylchloride (PVC) with tri (2-ethylhexyl) trimellitate plasticizer (Pall Corporation, Mississauga, Ontario, Canada). PC sterility was confirmed upon receipt of each unit by plating 1 mL on LB agar with overnight incubation at 37°C. Once received, the PCs were stored at room temperature on a platform platelet agitator. All experiments using these products were commenced at least 2 days prior to the unit expiration date.

2.2.2 PCs Prepared by the Buffy Coat (BC) Method

PCs derived by the BC method (BC-PCs) were collected by trained phlebotomists at netCAD and subsequently processed by a skilled medical laboratory technologist as per a collaborative experimental design. A diagram of the PC splitting procedure and experimental design is shown in Figure 7. Briefly, five replicates of eight ABO blood group-compatible buffy coats (~50 mL) were pooled and split evenly by weight and mixed with either 300 mL plasma or 300 mL of PAS SSP+ (MacoPharma Canada, Montreal, Quebec, Canada) as

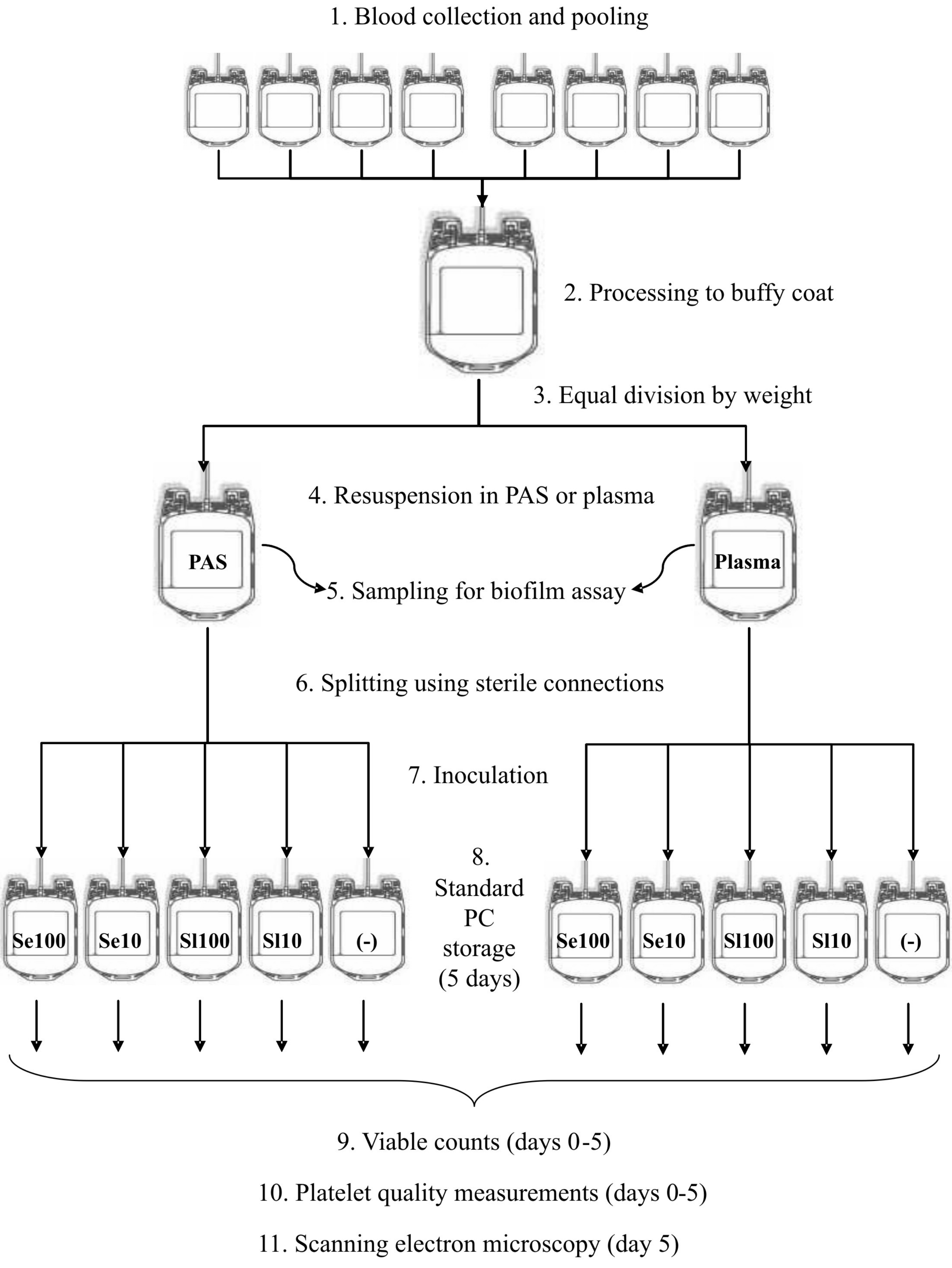

1. Blood collection and pooling
2. Processing to buffy coat
3. Equal division by weight
4. Resuspension in PAS or plasma
PAS
Plasma
5. Sampling for biofilm assay
6. Splitting using sterile connections
7. Inoculation
Se100
Se10
Sl100
Sl10
(-)
8. Standard PC storage (5 days)
Se100
Se10
Sl100
Sl10
(-)
9. Viable counts (days 0-5)
10. Platelet quality measurements (days 0-5)
11. Scanning electron microscopy (day 5)

Figure 7. Flow chart of platelet preparation in PAS and bacterial inoculation.

previously described (Zhang, 2008). A 20 mL sample was removed for biofilm assays, and the remainder of the PC was subdivided into five CLX® platelet storage bags containing final volumes of ~60 mL/bag, into which the bacteria were inoculated. Sterile connections and standard platelet storage conditions were maintained during the entire process, and sterility was confirmed as before (Section 2.2.1).

2.2.3 Filtration of PCs to Obtain Platelet-Poor Plasma (PPP)

PPP was created by passing PRP-derived PCs through sterile 0.45 µm pore filters (Sartorius Corp., Edgewood, NY). Platelet counts were performed at the Ottawa Stem Cell Laboratory (Ottawa, Canada) on an ADVIA® 120 Hematology System (Siemens, Deerfield, IL). For experiments comparing bacterial growth in PAS versus plasma (Objective 3), PPP was supplied by CBS netCAD as a by-product of BC-PC component production.

2.2.4 PEGylation of BC-PCs

The procedural cascade for platelet PEGylation experiments is shown in Figure 8. Succinimidyl carbonate mPEG with a molecular weight of 5 000 Da (Laysan Bio Inc., Arab, AL) was diluted to 0.1 g/ml in PEG buffer (50 mM K_2HPO_4, 105 mM NaCl). The solution was added to BC-PCs at an equal volume ratio via sterile injection into the platelet container over 15 min with gentle agitation, followed by 2 h incubation. PEGylation was deemed successful based on a 90% or greater reduction in detection of the cell surface marker CD9, which served as an indicator of molecular shielding. Although not specific to platelets, CD9 is ubiquitously expressed on the platelet surface, and shielding of this molecule in platelet populations via PEGylation has been standardized as a control at the UBC Centre for Blood

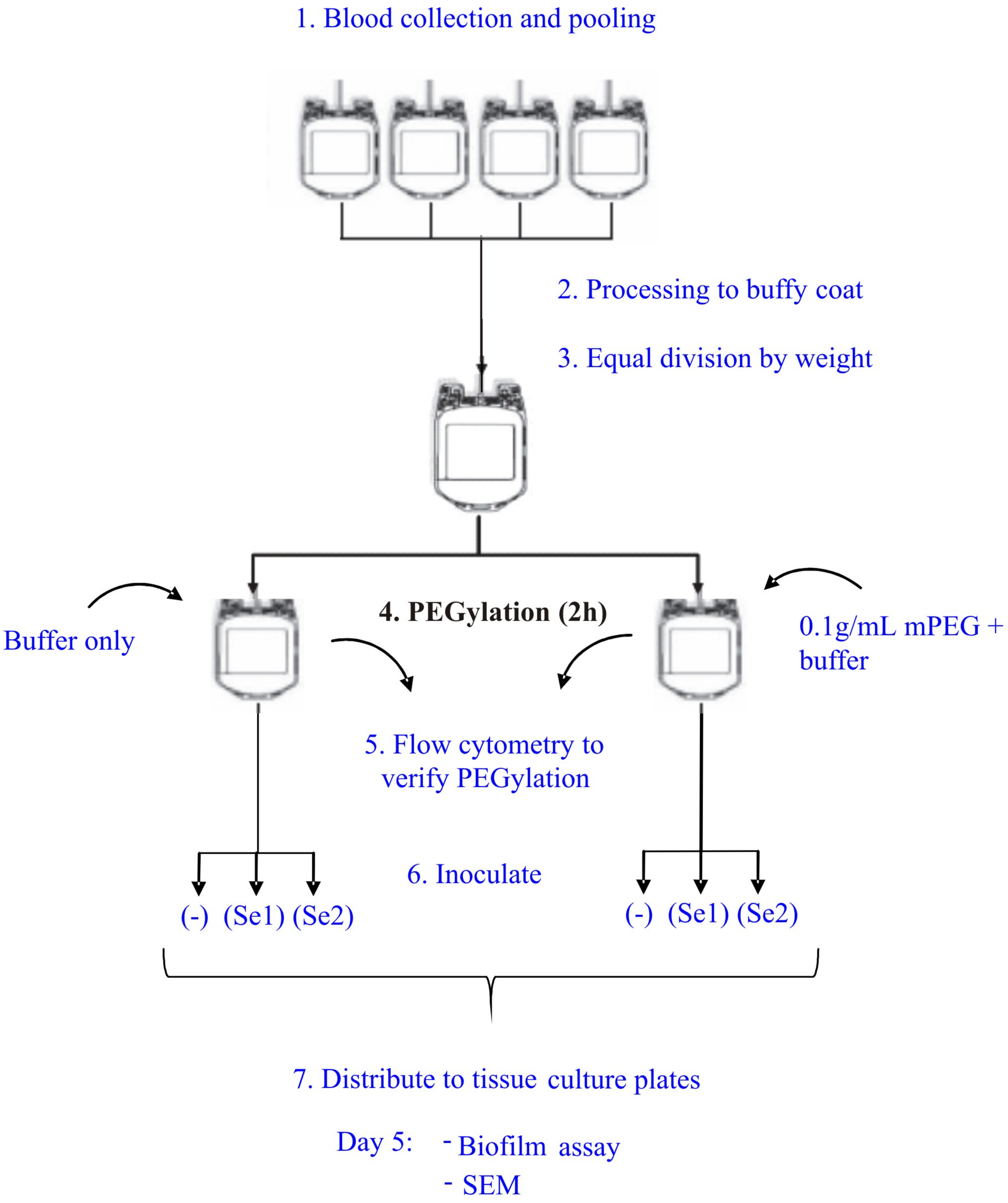

1. Blood collection and pooling
2. Processing to buffy coat
3. Equal division by weight
Buffer only
4. PEGylation (2h)
0.1g/mL mPEG + buffer
5. Flow cytometry to verify PEGylation
6. Inoculate
(-) (Se1) (Se2)
(-) (Se1) (Se2)
7. Distribute to tissue culture plates
Day 5: - Biofilm assay
- SEM

Figure 8. Flow chart of platelet PEGylation and bacterial inoculation.

Research. Briefly, monoclonal anti-human CD9-FITC (eBioscience, San Diego, CA) was used at a concentration of 62.5 ng per 100 µL containing 5×10^6 platelets. The percentage of gated, positive events was measured on a FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA) and compared to non-PEGylated controls.

2.2.5 Platelet Quality Measurement

Blood gas and electrolyte testing was performed directly from the sampling syringe to avoid mixing with air. Partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2), pH, glucose and lactate levels were obtained using a self-calibrated GEM Premier 3000 Blood Gas Analyzer (Instrumentation Laboratory, Orangeburg, NY, USA). For samples with a pH value outside the instrument detection threshold, an Orion 3 Star benchtop pH meter (Thermo Scientific, Waltham, MA, USA) was used. Platelet concentration and mean platelet volume (MPV) were measured on an ADVIA® 120 Hematology System (Siemens, Deerfield, IL). On each day of storage, all PCs were examined for anomalies such as lack of platelet swirling or the presence of visible aggregates, since both phenomena can be used as indicators of bacterial contamination (Wagner and Robinette, 1996). The use of flow cytometric detection of cell surface markers for the assessment of platelet activation and degranulation is described in Section 2.9.

2.3 BACTERIAL GROWTH RATE DETERMINATION

Samples of bacterial cultures were collected and diluted in sterile phosphate buffered saline (PBS) in a 10-fold series, followed by replicate plating of all dilutions on TSA. Colonies were counted the next day and the corresponding dilutions were used to extrapolate values for actual bacterial concentrations. This method of serial dilution was also used to confirm dilution accuracy of samples prepared for initial inoculations.

2.3.1 Preparation of Bacteria for Initial Inoculations

To prepare pre-determined concentrations of bacteria for inoculation of platelet concentrates, overnight cultures were diluted to $OD_{600} = 0.05$ in fresh media followed by incubation until mid-logarithmic phase under ideal conditions, as determined by extrapolating from graphs prepared previously (eg, Appendix 11.4.1). Specifically, growth time points for *S. epidermidis* O-47 and *S. liquefaciens* CBS isolate strains were estimated by comparing optical density readings at a 600nm wavelength to corresponding colony counts of serially-diluted culture samples to establish the logarithmic and stationary phases for those species in regular growth media under optimal incubation conditions. At time points consistent with mid-logarithmic phase, cultures were pelleted at 5 000 rpm at 4^{o}C, resuspended thoroughly in BHI + 15% glycerol, vortexed well, distributed into 1 mL aliquots representing 10^{8} CFU/mL and stored at -80^{o}C prior to use. Bacterial concentrations were verified by plating serial dilutions both before and 24 h after freezing (Appendix 11.4.2). Subsequently, two 1-ml aliquots per batch were thawed each week to confirm bacterial viability and concentration stability. Prior to PC inoculation, bacterial suspensions were thawed for 2 min at 37^{o}C and diluted with phosphate buffered saline (PBS) in a 10-fold series.

2.3.2 Bacterial Growth under Simulated Platelet Storage Conditions

Fresh (indate) PCs were spiked to a final initial concentration of 10-100 colony forming units (CFU)/mL of *S. epidermidis*. Dilution accuracy was verified by plating serial dilutions on

TSA. Samples were distributed into 6-well polystyrene tissue culture plates (Corning, Cat. No. 3516, Acton, MA), incubated at room temperature (22 ± 2^{o}C) on a platelet agitator, and sampled every 24 h for 5 days. Since PC volume per unit was limited, this set of experiments was performed in tissue culture plates in order to establish a high number of replicate assays and negative controls using the same platelet unit for each repetition, so that individual discrepancies between units was not a confounding factor in result variability. Differential gas exchange experienced in the culture plate could be a potential factor affecting bacterial growth rate compared to normal storage in PVC platelet bags; however, additional experiments outlined later address this concern. Parallel growth experiments were done in TSBg as a control. Growth rate assays for bacteria cultured in TSBg were repeated independently 3 times in duplicate for establishing a mean value (n = 3; ± standard deviation, SD) at each sample time point.

2.3.3 Bacterial Growth under Platelet Storage Conditions

PCs were inoculated with approximately 10 or 100 CFU/bag (equivalent to 0.2 or 2 CFU/mL, respectively) of either *S. epidermidis* strain O-47 or *S. liquefaciens* CBS isolate, incubated for 15 min with agitation before initial sampling (day 0), and then incubated under routine PC storage conditions. At 24 h intervals for 5 days, a 2.5 mL sample was withdrawn using aseptic technique in order to measure bacterial concentration and platelet parameters (Refer to cascade in Figure 7). After sampling, the final PC volume always remained within the recommended range for a CLX® bag (40-70 mL) so that an acceptable surface area-to-volume ratio was maintained during the course of the experiment. The accuracy of the initial inocula and daily bacterial concentrations were confirmed by diluting samples in a 10-fold series, plating all dilutions in duplicate onto TSA (BD Biosciences, Franklin Lakes, NJ), and counting colonies after 24 h incubation at either 37 ^{o}C (*S. epidermidis*) or 22 ± 2^{o}C (*S. liquefaciens*). This procedure was repeated five times. Growth rate assays for bacteria cultured in PCs were repeated independently 3 times in duplicate for establishing a mean value at each sample time point (n = 3; ± SD).

2.4 CRYSTAL VIOLET BIOFILM ASSAYS

Bacterial biofilm-forming ability was assessed by a biofilm staining assay. Overnight cultures of *S. epidermidis* reference and CBS strains were diluted to an OD_{600} = 0.1 in TSBg. Three-mL aliquots were transferred into the wells of 6-well tissue culture plates and incubated statically overnight at 37^{o}C, or at room temperature on a platform agitator (60 rpm) for 5 days. At this time, samples were aspirated and the wells were rinsed gently 3 times with 3 mL PBS (pH 7.4). The wells were then stained for 2 h with 0.3% crystal violet dye solution (BD Biosciences, Sparks, MD), rinsed with PBS as before, and the remaining crystal violet was eluted with 2 mL of an 80 % ethanol: 20 % acetone (vol:vol) mixture as previously described (Toledo-Arana et al., 2005). From each well, 200 µL were transferred 6 times into a 96-well microtitre plate and absorbance values at a 492 nm wavelength (A492nm) were measured with an Expert Plus microplate reader (Montreal Biotech, Canada). Final absorbance values were corrected with values obtained for the eluate of non-inoculated control wells, which had been processed in an identical manner to the experimental samples. Corrected means with absorbance values > 0.5 were considered positive. This experiment was repeated at least five times in duplicate for each strain (n = 5; ± SD).

2.4.1 Modified Crystal Violet Assay for Bacteria Grown in PCs

Bacterial concentrations equivalent to ~ 1x10^7 CFU/mL were added to indate PCs followed by incubation on a platelet agitator for 5 days. Biofilm staining was then performed with the 0.3% Gram crystal violet dye solution in the same manner as above (Section 2.4), but with 15 repetitions in duplicate, including 6 readings per well. For objective 1, PC glucose and pH measurements were monitored before inoculation (day 0) and after each experiment (day 5) using Diascreen urinalysis reagent test strips (Hypoguard, Minneapolis, MN). Glucose and pH levels were designated as the mode scores of 15 independent experiments.

To correct for contribution of platelet and/or plastic staining to the measured absorbance values, wells containing non-spiked liquid (TSBg or PCs) were processed in the identical manner as the experimental samples and A492nm readings from these control wells were subtracted from values measured for experimental wells to obtain a final set of adjusted absorbance values. Statistical significance was then determined by Student *t*-test ($P \leq 0.05$). Five of the above experiments were replicated concurrently in duplicate for enumeration of viable, surface-attached bacteria. Specifically, cells were scraped from wells into 4 mL of PBS, serially-diluted and plated on LB agar for bacteria quantification.

The minimum initial inoculum required for biofilm formation to occur in indate PCs was also determined by this method. PCs were inoculated with *S. epidermidis* to a concentration of approximately 1x10^7 CFU/mL, and 10-fold serial dilutions were performed in PCs down to a concentration of 10 CFU/mL. BF assays were performed as described above. These assays were independently repeated twice for both ATCC 12228 and ATCC 35984, and dilution accuracies were verified as before.

For the PAS experiments (Objective 3), each bacterial strain was inoculated into 3 mL PAS- or plasma-PCs in 6-well tissue culture plates (Corning Life Sciences, Cat No. 3156) at a concentration of ~10^7 CFU/well. The plates were incubated for 5 days at 22±2°C on a Thermolyne Vari-Mix rocker (Thermo Scientific, Waltham, MA, USA) in order to establish mature biofilms. Biofilms were then quantified by staining plastic-adherent material with 0.3% Gram crystal violet as described previously (Section 2.4). This time, stain intensity was measured at λ = 490 nm by a SpectraMax190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). All experiments were repeated five times in duplicate, with six readings per sample (n = 5; ± SD).

For experiments involving platelet PEGylation (Objective 4), PCs were inoculated with ~10^7 colony forming units per ml overnight cultures of either *S. epidermidis* ATCC strain 12228 (biofilm-negative) or ATCC strain 35984 (biofilm-positive). After 15 min agitation, samples were transferred to 6-well tissue culture plates (Corning Cat. No. 3516) and incubated for 5 days, at 22±°C with constant, gentle agitation prior to staining, performed as before (Section 2.4), with stain intensity measured at λ = 490 nm by a SpectraMax190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). PEGylation biofilm assays were performed eight times in duplicate, with six readings per sample.

2.5 SCANNING ELECTRON MICROSCOPY

Samples prepared for scanning electron microscopy (SEM) were submitted to Agriculture and Agri-Food Canada Microscopy facilities where final processing and SEM was performed by highly-skilled associate scientists. Specifically, samples were dehydrated in a graded ethanol series (20%, 40%, 60%, 80%, 95% and 100% ethanol), then subjected to critical-point drying (Bozzolla and Russell, 1991). Dried samples were mounted on 13 mm aluminum stubs with silver cement (Ladd Industries, Burlington, VT) and coated with a gold layer of ~ 20 nm thickness in a Hummer V Technics sputter coater.

Samples were examined in a Philips XL30 ESEM (Eindhoven, Netherlands) scanning electron microscope operated at 7.5 kV accelerating voltage. Grayscale micrographs were obtained in digital form in TIFF format (2576 x 1936 pixels) and were further processed for publication using Adobe Photoshop 5 graphics software. Details for initial SEM sample preparation, performed independently, are outlined in Sections 2.5.1 and 2.5.2.

2.5.1 Preparation of Cells in Suspension

To examine the interactions of *S. epidermidis* ATCC 35984 and 12228 with platelets by SEM, PCs were inoculated with bacteria to a concentration of approximately $1x10^7$ CFU/mL and incubated in 6-well tissue culture plates for 3 to 5 days. At this time, the liquid containing visible, suspended aggregates, was collected and fixed in a solution of 4% paraformaldehyde (vol:vol) and 2.5% glutaraldehyde (vol:vol) in PBS for 2 h at room temperature. For experiments involving PEGylation (Objective 4), samples were supplemented with 2.5 volumes of acid citrate dextrose (7.04 mmol/L citric acid monohydrate, 93.0 mmol/L sodium citrate dihydrate, 136 mmol/L D-glucose; pH 6), pelleted at 3 000 × *g* for 5 min, and fixed as before.

2.5.2 Preparation of Platelet Bags

Overnight cultures of *S. epidermidis* ATCC 35984 and 12228 were diluted to an $OD_{600} = 0.1$ in 60 mL TSBg and injected into CLX® platelet storage bags which were either sterile or preconditioned with PCs. Specifically, bags were preconditioned by incubating PCs inside the bags for at least 24 h with complete drainage prior to the injection of the cultures. The bags were incubated at room temperature on a platelet agitator. After 6 days, the bags were drained via the sampling site and washed once with 60 mL of sterile dH_2O for 10 min under gentle agitation to remove nonadherent cells. PVC coupons measuring approximately 1 cm x 1 cm were cut from the bags using sterile scissors and fixed as before, in a paraformaldehyde and glutaraldehyde solution, prior to SEM.

For experiments involving PAS (Objective 3), the PC units that were used for bacterial growth rate determination were simultaneously used for SEM. On the fifth day of storage, coupons measuring 1 cm^2 were excised from the platelet storage containers at a site approximately 3 cm from the sampling port. The coupons were rinsed with PBS, fixed in 2.5 % glutaraldehyde (vol:vol) for 2 h at room temperature, and then stored at 4^oC.

2.6 INDIRECT IMMUNOFLUORESCENCE MICROSCOPY

Production of the *S. epidermidis* biofilm matrix polysaccharide PIA was examined by immunofluorescence using the protocol of Heilmann et al. (1996). A total of 15 µL of overnight cultures of all CoNS strains used in the study were applied to poly-L-lysine-coated immunofluorescence slides (Goal Seal Products, Portsmouth, NH) and allowed to air-dry. Rabbit polyclonal IgG raised against purified *S. epidermidis* PIA was used as the primary antibody (Mack et al., 2001). Anti-PIA IgG diluted 1:100 in PBS was added to the slides which were then incubated at 37^oC for 30 min in a humid chamber. Slides were rinsed gently with a stream of PBS, and a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO) was then added. Slides were again incubated at 37°C for 30 min in a humid chamber, rinsed with PBS and rinsed again briefly with sterile ddH_2O. ProLong® Gold antifade reagent (Invitrogen, Eugene, OR) was used as mounting media. Slides were visualized at a 100 X oil immersion objective with a 0.5 s exposure time using an Olympus BX51 fluorescence microscope (Olympus Corp., Tokyo, Japan). Images were captured with a CoolSNAP™-Pro 1.4 megapixel cooled CCD camera and Image-Pro® Express software version 4.0 (both by Media Cybernetics, Silver Spring, MD). *S. epidermidis* ATCC strains 35984 and 12228 were used as PIA-positive and PIA-negative control strains, respectively. Additionally, ATCC strain 35984 was incubated in PBS without primary IgG to control for non-specific binding of the secondary IgG, and was incubated in PBS without secondary IgG to control for endogenous fluorescence of the bacteria. All strains were tested for PIA production three times independently.

2.7 CONGO RED AGAR TEST

Congo red agar was prepared according to the protocol of Freeman et al. (1989) with slight modifications. Briefly, a concentrated solution of Congo red stain and saccharose (both from Sigma-Aldrich Canada Ltd., Oakville, Canada) in ddH_2O was filter-sterilized and added to heat-sterilized BHI agar to final concentrations of 0.8 g/L and 36 g/L, respectively. After inoculation with each CoNS strain, plates were incubated for 24 h at 37°C, with two repetitions per isolate. Rough, black colonies indicate biofilm production while a biofilm-negative phenotype is characterized by formation of smooth red or pink colonies.

2.8 POLYMERASE CHAIN REACTION (PCR) CONDITIONS

PCR amplification of *divIVA*, a gene implicated in cell division of Gram-positive cocci

(Fadda et al., 2003; Ramirez-Arcos et al., 2001), was used for speciation of *S. epidermidis* since our laboratory has shown that amplification of this gene permits discrimination of *S. epidermidis* from other CoNS (Mastronardi and Ramirez-Arcos, 2007). The primer pair which was used to amplify the *S. epidermidis divIVA* gene was: 5'-GCGCGTCGACAGAGCTCAAATTGTTGAAATAG-3' (Se*divIVA*Fw) and 5'-GCGCGGATCCTTAATTATTTGATGTTGATTG3-' (Se*divIVA*Rev). The 5' end of the *icaD* gene (positions 7 to 246) was PCR-amplified and used to distinguish potential biofilm-forming strains from biofilm-negative isolates. The *icaD* fragment was amplified with primer pair 5'-AAGCCCAGACAGAGGCAATATCCA-3' (Se*icaD*Fw) and 5'-AGTACAAACAAACTCATCCATCCGA-3' (Se*icaD*Rev). Since *icaA* lies upstream of *icaD* with an overlap of 37 nucleotide base pairs, *SeicaD*Fw is also contained within the 3' end of the *icaA* gene. Both primer sets were designed using the genome sequence information of *S. epidermidis* strain ATCC 35984 (Accession no. NC 002976) and were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Amplifications were performed in a Mastercycler ep Thermal Cycler PCR system (Eppendorf, Hamburg, Germany) as follows: 5 min at 95ºC; 40 cycles of denaturation for 30 s at 94ºC, annealing for 30 s at 47ºC, and extension for 56 s (*divIVA*) or 30 s (*icaD* fragment) at 72ºC; 5 min at 72ºC; and hold at 4ºC. All reactions were constituted in a final volume of 100 µL containing the following reagents: 75.5 µL of sterile ddH_2O, 10 µL 10X PCR buffer containing 15 mM $MgCl_2$ (Qiagen Canada, Mississauga, Ontario), 2 µL dNTP mix (0.2 mM of each dNTP; New England Biolabs, Ipswich, MA), 1 µL of each primer (0.2 µg/mL), 0.5 µL HotStarTaq® Plus DNA polymerase (2.5 U/reaction; Qiagen Canada, Mississauga, Ontario), and 10 µL of bacterial cell suspension which provided the chromosomal template DNA. Cell suspensions were prepared by inoculating strains into nuclease-free, sterile ddH_2O to a concentration equivalent to a 0.5 McFarland turbidity standard (Hardy Diagnostics, Santa Maria, CA), or approximately 10^8 CFU/mL. All PCRs were repeated at least twice using freshly prepared template.

2.9 FLOW CYTOMETRY

Platelet CD62P expression was measured as an indicator of platelet degranulation. Triplicate samples were diluted with PBS pH 7.4 to a concentration of ~ 300 x 10^6 platelets/mL and incubated with 5 µL mouse monoclonal anti-hCD62P-phycoerythrin (PE) IgG (Beckman Coulter Canada, Mississauga, ON). An isotype non-reactive IgG-PE (5 µL) was used as a control for non-specific binding and for setting the gates for positive events. As a positive control, platelets were activated with 10 U thrombin after addition of 5 µL 25 µM gly-pro-arg-pro peptide to inhibit fibrin formation (both from Sigma-Aldrich Canada, Oakville, ON). Following a 30 min incubation, 0.5 mL of PBS was added to dilute the samples for analysis on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA).

Annexin V binding was measured by flow cytometry as a rapid method to assess the procoagulant potential of platelets and the loss of phospholipid asymmetry of cell membranes (Dachary-Prigent, 1993; Cookson et al., 2010). PCs were diluted to a concentration of ~100 x 10^6 platelets/mL with HEPES/Ca^{2+} buffer and stained with an Annexin V–FITC conjugate (BD Biosciences, Sparks, MD). A positive control was prepared using 2 µL Ca^{2+} ionophore. This is a mobile ion carrier that transports one divalent calcium ion into the cell in exchange for two H^+ (Lapetina et al., 1978). Eukaryotic cells are impermeable to Ca^{2+} in the absence of active transport; however, use of the calcium ionophore ensures cellular uptake of any external Ca^{2+}, leading to maximum platelet activation (Lapetina et al., 1978). A negative control was prepared using HEPES/EDTA buffer. Specifically, EDTA is a potent anticoagulant that irreversibly binds Ca^{2+} (Best et al., 1982). After a 30 min incubation period, samples were diluted with 1 mL HEPES/Ca^{2+} buffer (or 1 mL HEPES/EDTA buffer for the negative control) and analyzed by flow cytometry.

2.10 MOLECULAR CLONING

The supervised creation of *S. epidermidis* recombinant gene constructs was mediated by undergraduate students. A *S. epidermidis divIVA* pET30a+ expression construct harbouring a C-terminal 6XHis tag and α-DivIVA IgG was created previously in our laboratory. GenScript Corporation (Piscataway, NJ) was commissioned to create a construct encoding the peptide sequence NIDKNTVPSDLTDSC attributed to the *S. epidermidis* fibrinogen binding protein SdrG (GenScript ID No. 33504-1). The resultant peptide was appended to the immunogenic carrier keyhole limpet hemocyanin (KLH) and used to immunize rabbits. Immune sera IgG was then purified by the company, and delivered to our laboratory.

S. epidermidis genes associated with cell division (*ftsZ*), biofilm formation (*atlE*) and housekeeping (*gmk*) genes were cloned separately into the multiple cloning site of the pET30a+ expression vector (Novagen®; Appendix 11.6.1) so that the genes would be expressed with either an N- or C-terminal 6XHis tag. For complete nucleotide sequences of the cloned genes, refer to Appendix 11.6.2. Standard DNA manipulations and cloning procedures were used (Sambrook and Russell, 2001). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR reactions were performed with HotStarTaq® DNA polymerase (Qiagen Canada, Missisauga, Ontario) and cycling conditions were the same as for *S. epidermidis divIVA* and *icaD* amplifications described previously, with an extension time of 60 s per kilobase pair (kbp). DNA samples were electrophoresed at 120 V for 1-2 h in 1% agarose gels made in Tris-Acetate EDTA. A PCR purification kit (Qiagen Canada, Mississauga, Ontario) was used to clean PCR products according to manufacturer recommendations. Restriction digests of PCR products and the pET30a+ vector were performed at 37°C for 2 h using appropriate endonucleases and the compatible buffers (New England Biolabs Canada, Pickering, Ontario). The PCR purification kit was used as before to purify the nucleic acids, and a ligation reaction with 2 000 U T4 DNA ligase in reaction buffer (containing 1 mM ATP; both from New England Biolabs Canada, Pickering, Ontario) was carried out for 2 h at room temperature prior to transformation. Constructs were transformed into chemically-competent, restriction negative *E. coli* DH5α, and positive transformants were selected by plating on 50 µg/mL Kan. Confirmatory sequences for all plasmid inserts and diagnostic PCRs were obtained from the Core DNA Synthesis and Sequencing Facility (University of Ottawa), or the Ontario Genomics Innovation Centre (Ottawa Health Research Institute). Plasmids and corresponding primers generated in this study that were selected for downstream use can be found in Table 3. Anticipated recombinant protein molecular weights were determined using the Sequence Manipulation Suite website (online available: http://www.bioinformatics.org/sms2). Full sequences for cloned peptides and proteins are contained in the Appendix (Section 11.6.3).

2.11 PROTEIN ISOLATION

For protein expression, confirmed plasmids carrying the *divIVA, ftsZ, altE* and *gmk* genes were transformed into the *E. coli* expression strains BL21(DE3), C41(DE3), and/or C43(DE3). When transformed into *E. coli* BL21(DE3) and its derivative strains, IPTG

Table 3. PCR primers and resultant plasmids used for protein overexpression.

Se Gene	Primer Pair	Sequence (restriction endonuclease)	6XHis	Plasmid Generated
divIVA	SepidivpETFW SepidivpETREV	5’-GCGCCATATGCCTTTTACACCAAGTG-3’ (Nde1) 5’-GCGCCTCGAGATTATTTGATGTTGATTG-3’ [*] (XhoI)	CTD	pIMET1
ftsZ	ftsZNFW ftsZNREV	5’-GCGCCATATG**CACCATCATCATCATCAT**TTAGAATTTGAACAAGGATT-3’ (NdeI) 5’-GCGCCTCGAGTTATTAGCGTCTAGTTCTTCTAGAAC-3’ [*] (XhoI)	NTD	pALZ-N
atlE	atlECFW atlECREV	5’-GCGCATTAATATGGTATCTAGTCAAAAAACATCAT C-3’ (AseI)[†] 5’-GCGCCTCGAGTAAATAGTATTTACTAATCC-3’ [*] (XhoI)	CTD	pALE-C
gmk	gmKCFW gmKCREV	5’-GCGCATTAATATGGATAAGGAAAAAGGACT-3’ (AseI)[†] 5’-GCGCCTCGAGTTTTTTGACCTCCAGTA-3’ [*] (XhoI)	CTD	pALK-C

All plasmids were derived from a pET30a+ origin, and all amplifications were performed on a *S. epidermidis* (Se) strain ATCC 35984 template. Restriction sites are underlined, and 6XHis coding sequence is in bold. CTD, C-terminal domain; NTD, N-terminal domain.

[*] Anneals to complementary strand

[†] Compatible with NdeI.

induction at the genomic lacUV5 promoter results in expression of T7pol which then binds the T7 promoter region of the pET vector and mediates transcription of the downstream recombinant gene at the plasmid multiple cloning site (Miroux and Walker, 1996). DivIVA, FtsZ, AtlE, and Gmk expression was induced with 0.4-1 mM IPTG. After 3 h of shaking incubation at 37°C, cultures were sedimented at 4°C for 20 min at 5 000 x *g*, and kept at -20°C overnight. Cells were then thawed on ice in equilibration wash buffer (50 mM sodium phosphate buffer pH 7.3, 0.3M NaCl), supplemented with Complete Mini, EDTA-free protease inhibitor tablets (1X Concentration; Roche, Indianapolis, IN), and lysed by sonication with a Sonic Dismembranator Model 100 (Fisher Scientific, Ottawa, Canada) as follows: 3 X 60 s pulses with 5 min pause on ice between pulses. Cell debris was sedimented at 20 000 x *g* for 20 min at 4°C. Supernatant was recovered and added to Co^{2+} Talon® resin (Clontech, Mountain View, CA) followed by incubation with gentle rocking at room temperature for 20-30 min. Resin was washed 3 X with equilibration wash buffer and a 700 x *g* centrifugation between washes. Resin was then transferred to a polypropylene column and rinsed with equilibration wash buffer (1 bed volume), and increasing concentrations of imidazole (1 bed volume each of 5, 10, 20 mM, respectively) before elution in 500 μL of 250 mM imidazole. Samples isolated at all stages of protein isolation were recovered and assessed by SDS-PAGE to determine the expression construct and strains which produced the greatest recombinant protein yield in the soluble protein fraction to be used for protein purification and antibody production. These were determined to be pIMET1 (DivIVA-CTDHis), pALZ-N (FtsZ-NTDHis), pALE-C (AtlE-CTDHis), pALK-N (GmK-NTDHis), all in a C41(DE3) background (Table 3). The eluted proteins were subjected to Western analysis with α-pentaHis monoclonal IgG (Qiagen Canada, Mississauga, Ontario) as a confirmatory test. Proteins were then dialysed overnight in PBS with Pierce Slide-A-Lyzer Dialysis Cassettes having a 10 kDa molecular weight cut-off (MWCO; 3mL vol; Thermo Scientific, Rockford, IL). In some instances, a Spectra/Por® dialysis membrane was used (6-8 kDa MWCO; Spectrum Laboratories, Rancho Dominguez, CA). Proteins were quantified by Bradford assay (Biorad, Hercules, CA), and concentrated using Microcon centrifugation filter units with MWCOs of 10 kDa or 30 kDa (Millipore, Bedford, MA).

For total soluble protein isolation from staphylococci, overnight cultures were used to inoculate fresh cultures at a 1:100 dilution. Specifically, *S. epidermidis* strain O-47 was selected due to biofilm positivity and ease of suspension which ensured relative uniformity during dilution procedures. Bacteria were grown to stationary phase under static conditions in TSBg overnight at 37^{o}C in 6-well tissue culture plates (Corning, Cat. No. 3516, Acton, MA). Supernatant cells representing the planktonic fraction were removed and pelleted for 5 min at 5 000 x *g*, followed by resuspension in equilibration-wash buffer (50 X dilution). Wells were rinsed 2 X in PBS and adherent cells representing the biofilm cell fraction were scraped from the wells into equilibration-wash buffer, pelleted and resuspended in the same manner as for planktonic cells. *S. epidermidis* total protein was harvested by mechanical lysis in a FastPrep® instrument using the FastProtein^{TM} Blue Matrix (MP Biomedicals, Solon, OH) as follows: 2 X 6.5 m/s x 40 s with a 5 min pause on ice in between. Cell debris was pelleted at 20 000 x *g* at 4^{o}C for 10 min. Supernatants were then collected and used for SDS-PAGE and Western blots. All experiments were repeated at least three times separately.

2.11.1 Western Blot Analysis

Proteins were resolved by 12 % SDS-PAGE using a Mini Protean® electrophoresis/blotting system (BioRad, Hercules, CA) and protein concentrations were standardized prior to membrane transfer by spectrometry and densitometry of a Coomassie blue stain. Proteins were then transferred to a PVDF membrane (Immobilon-P; Millipore, Bedford, MA), again using the Mini Protean® system (Biorad, Hercules, CA). Specifically, blotting was performed at 40 kV overnight at 4°C, or at 90 kV for 1.2 h at room temperature in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol; pH 8.1-8.4) with stirring and ice packs. Subsequently, membranes were rinsed in Tris buffer saline (TBS; 20 mM Tris, 0.5 M NaCl) for 10 min and blocked either overnight at 4°C or for 1 h at room temperature in TBS + 3 % non-fat skim milk powder. Excess blocking solution was removed with three 10 min washes in TBS. Immunoblots were performed in TBS + 0.05% Tween-20 (TBST) using optimized dilutions of IgG as follows: α-DivIVA (1:1 000), α-Gmk (1:500) α-FtsZ (1:1 000) α-AtlE (1:500) α-SdrG (1:500). Membranes were incubated with primary antibodies for 1hr at room temperature with gentle agitation and washed 3 X with TBST for 10 min each time. Membranes were subsequently incubated for 1 h with an alkaline-phosphatase (AP)-conjugated goat-anti-rabbit secondary antibody (diluted 1:3000 in TBST). A fluorescent AP substrate (AttoPhos®; Roche, Indianapolis, IN) was placed on the membranes for 1-5 min prior to UV exposure on an Alpha Imager™ 2200 (Cell Biosciences, Santa Clara, CA). Protein concentrations were compared by densitometry using the imaging system software.

2.11.2 Antibody Production

Ethical approval was obtained from the University of Ottawa Animal Care Committee for all animal-related procedures pertaining to antibody production (Protocol ME-229). Female New Zealand White rabbits were subcutaneously administered 100 μg recombinant *S.*

epidermidis protein in a 1:1 ratio with Gerbu adjuvant (Biotechnik GmbH, Wieblingen, Germany) at 4 injection sites (500 µL/site) with boosts every 14 days until final bleeds on day 56. Immune sera were tested during this period for IgG presence, sensitivity, and specificity by Western blot analysis using the sera in place of a 1^{o} antibody. IgG was then purified by immunoaffinity using fast protein liquid chromatography (FPLC) on a ÄKTA system (GE Healthcare) by SACRI Antibody Services (Calgary, Alberta, Canada).

2.12 STATISTICAL ANALYSES

Growth rate assays for bacteria cultured in TSBg and PCs were repeated independently 3 times in duplicate for establishing a mean value at each sample time point (n = 3; ± SD). Data points at each day were compared with respect to different strains and/or different media (eg, TSBg versus PCs; ATCC 12228 versus ATCC 35984) using a Student *t*-test ($P \leq 0.05$). To determine whether the bacterial concentration had a significant impact on the various platelet parameters studied over time in each medium (Objective 3), a mixed model analysis with compound symmetric series correlation for repeated measures was performed in collaboration with Dr. Qi-Long Yi (CBS senior statistician) using SAS/STAT® Statistical Software (SAS Institute, Cary, NC; Appendix 11.4.4). A P-value of ≤ 0.05 was considered to be significant.

Biofilm assays in TSBg were repeated a minimum of 5 times in duplicate with six repetitions per well (n = 5; ± SD). As part of Objective 1, a novel environment for biofilm formation experiments (eg, in PCs) was introduced, and negative control for biofilms in PCs had not yet been established. To estimate the number of experimental repetitions required for data validity, the following formula was used: $n = [t^2 \text{ x } p(1-p)]/m^2$, where n = required number of repeats (sample size), t = confidence interval at 95% (standard value of 1.96), p =

estimated prevalence of biofilm positivity (0.99; from preliminary experiments in outdated platelets), and m = margin of error at 5 % (standard value of 0.05).

For objectives 3 and 4, biofilm assays in PCs were repeated until a suitable level of significant difference compared to controls ($P \leq 0.05$) was achieved, namely 5 times (Objective 3), and 8 times (Objective 4) respectively, and performed in duplicate with six repeated measures as before (± SD). Values were normalized to those obtained from non-inoculated controls using the respective PC unit, and compared using a Student *t*-test.

The pH and glucose values obtained using reagent strips (Objective 1) constituted interval data, and so the mode scores were determined for analysis.

The Congo red and other biofilm quantification assessments were compared using the Kappa coefficient (κ) for agreement of results (Landis and Koch, 1977). Interpretation of the magnitude of κ was made according to the following proposed standards for strength of agreement: ≤ 0 = poor, 0.01–0.20 = slight, 0.21–0.40 = fair, 0.41–0.60 = moderate, 0.61–0.80 = substantial and 0.81–1.0 = almost perfect (Landis and Koch, 1977).

3. RESULTS: OBJECTIVE 1

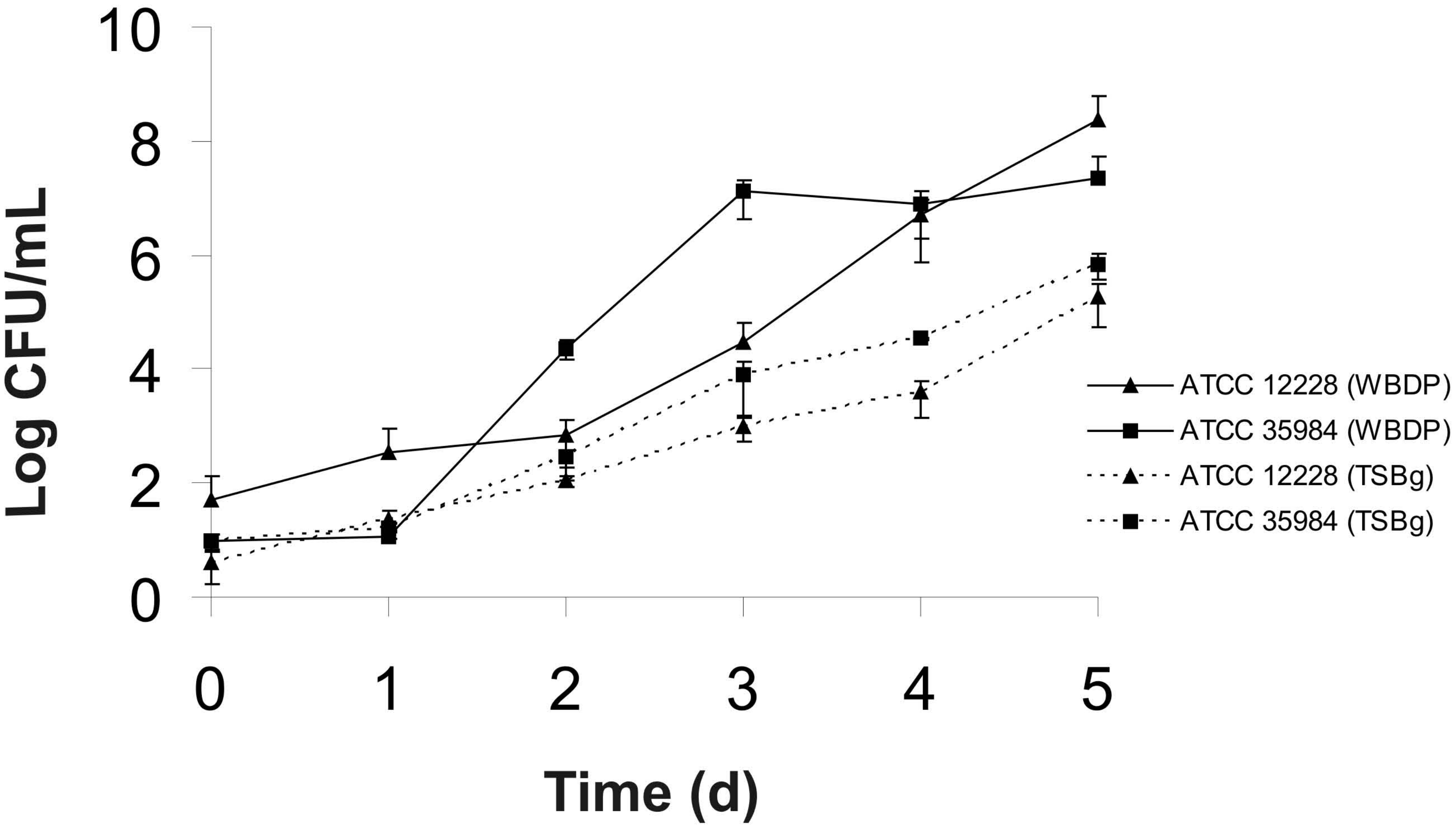
Results of experiments aimed at assessing the biofilm-forming potential of *S. epidermidis* under platelet storage conditions are presented in this section.

3.1 *STAPHYLOCOCCUS EPIDERMIDIS* GROWTH UNDER PLATELET STORAGE CONDITIONS

To determine whether the ability of a particular *S. epidermidis* strain to form biofilms would affect its growth pattern in the presence of platelets, biofilm-positive as well as biofilm-negative strains were used. The growth rates were established in platelet concentrates (PCs) and in normal, glucose-enriched media (TSBg) by viable cell counts performed over 5 days. The results of growth curves in both PCs and TSBg are presented in Figure 9. An initial lag period lasted approximately 24 to 48 h prior to the onset of exponential phase. This is consistent with data published previously which attributes a 48 to 72 h time-span requirement for *S. epidermidis* to reach logarithmic growth in PCs spiked with low initial inocula (Brecher et al., 2000; Hillyer et al., 2003). The growth rates of a biofilm-positive and a biofilm-negative strain of *S. epidermidis* were not substantially different. The only exception was on day 2 of growth in PCs, when the viable cell count for biofilm-forming ATCC 35984 marginally surpassed that of ATCC 12228 ($P = 0.049$).

3.2 *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILM FORMATION UNDER PLATELET STORAGE CONDITIONS

A semi-quantitative crystal violet assay was adapted to determine whether *S. epidermidis* bacteria would form biofilms in PCs under the conditions of platelet storage. Because biofilm formation in the platelet bags would be logistically difficult to quantify, these



Log CFU/mL
10
8
6
4
2
0
0
1
2
3
4
5
Time (d)
ATCC 12228 (WBDP)
ATCC 35984 (WBDP)
ATCC 12228 (TSBg)
ATCC 35984 (TSBg)

Figure 9. Growth rates of biofilm-positive (ATCC 35984) and biofilm-negative (ATCC 12228) *S. epidermidis*. Means ± S D of 3 separate experiments conducted in either Trypticase Soy Broth + 0.5 % glucose (TSBg) or indate, whole blood-derived PCs prepared by the platelet-rich plasma method (WBDPs) are presented.

assays were optimized in 6-well culture plates. Biofilms attached to the plastic material of the plates were stained with crystal violet dye. This dye is retained by bacterial cells and therefore the amount of the stain eluted is proportional to the quantity of adherent bacterial cells present (Toledo-Arana et al., 2005; Christensen et al., 1985). Staining patterns revealed that bacterial adhesion was strongest in the ring in the wells which was consistent with the solid-liquid-air interface during agitation (Figure 10A).

The results of the biofilm staining assay in PCs and in TSBg are presented in Figure 10B. Biofilm formation by *S. epidermidis* ATCC 12228 was significantly lower than that exhibited by the ATCC 35984 strain in both TSBg and PCs ($n = 15$; $P < 0.05$). Biofilm formation by strain ATCC 35984 in TSBg was not significantly greater than in PCs ($P = 0.32$). Traditionally, *S. epidermidis* ATCC 12228 is selected for use as a biofilm-negative control strain for experiments designed to evaluate the biofilm-forming abilities of other strains or clinical isolates grown in standard culture media (Arciola et al., 2004; Fitzgerald et al., 2006). However, there was significantly greater staining of adherent matter (~10-fold, $P < 0.05$) observed after this strain had been inoculated in PCs, as compared to equivalent spiking in TSBg (Figure 10B). This indicates that the environment of platelet storage triggers biofilm formation by biofilm-negative *S. epidermidis* ATCC 12228. Viable cell counts for parallel experiments showed that similar amounts of bacteria were present in the biofilms generated by both strains in PCs ($n = 5$; $P = 0.08$). *S. epidermidis* biofilm formation was measured in PCs spiked with initial inocula ranging from 10 to $1\text{x}10^7$ CFU/mL. Biofilms became apparent at initial inocula as low as 100 CFU/mL for the ATCC 35984 strain and at $1\text{x}10^4$ CFU/mL for the ATCC 12228 strain.

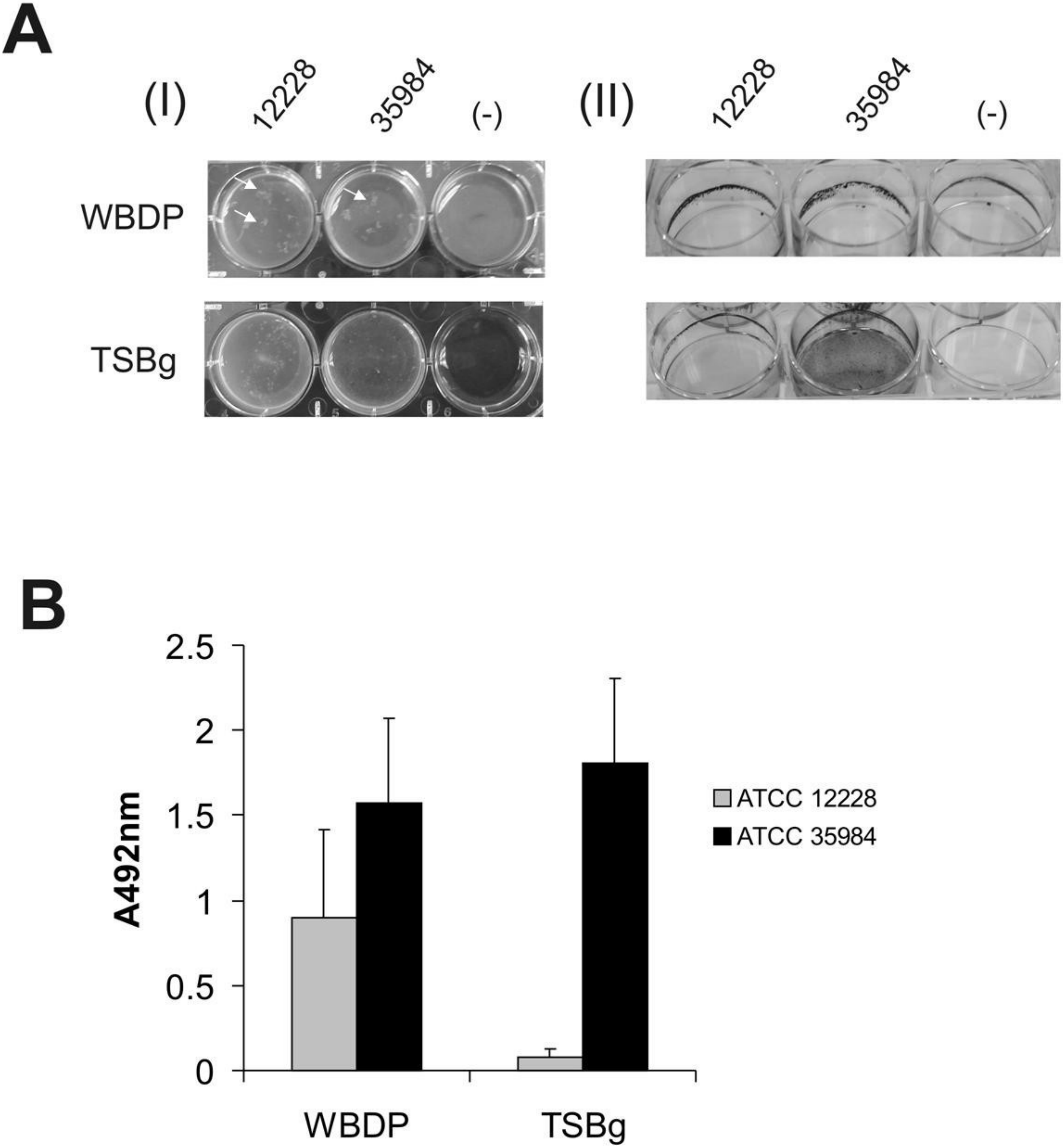

A
(I)
12228
35984
(-)
(II)
12228
35984
(-)
WBDP
TSBg
5
6
B
2.5
2
1.5
1
0.5
0
A492nm
WBDP
TSBg
ATCC 12228
ATCC 35984

Figure 10. Biofilm formation of *S. epidermidis* in whole blood-derived platelets (WBDPs). Images in (A) were captured with a Sony Cybershot digital camera, and show an example of culture plates on Day 5 of a typical biofilm assay (I) before and (II) after crystal violet staining of adherent bacteria. Arrows point to aggregates. Biofilm quantification by microplate reading of crystal violet stain intensity is shown in (B). Bars reflect the means ± SD of 15 separate experiments in PCs and 7 separate experiments in TSBg.

During the growth curve experiments, pre- and post-monitoring of PCs with reagent test strips revealed a consistent drop in glucose concentration in spiked samples only (initial inocula ~10-100 CFU/mL). Glucose levels were depleted 80-90 % from 500 mg/dL on day 0 (day of spiking) to 100 mg/dL on day 5 in samples inoculated with either ATCC 35984 or ATCC 12228 (modes, n = 15). Glucose levels in non-spiked PCs were normally stable at 500 mg/dL after the full 5-day incubation (mode, n = 15). After 5 days, pH values decreased from 6.5 to 6 in samples spiked with either strain (modes, n = 15), while the pH values of non-spiked PCs remained at pH 6.5 (mode, n = 15).

Filtering PCs through a 0.45 μm pore size reduced platelet quantity by 99.3 %, or by an average of 1286 x 10^{9} platelets/L, forming platelet-poor plasma (PPP). Biofilm assays performed in this medium resulted in 50.9 % and 93.1 % decreases in biofilm formation by ATCC 35984 and ATCC 12228 *S. epidermidis* strains, respectively compared to biofilms formed in PCs (Figure 11).

3.2.1 Formation of *S. epidermidis* Biofilms on Platelet Aggregates

It was noted that many aggregates had formed freely in the wells of spiked PCs by the end of the 5-day incubation period (Figure 10A, arrows). Also, many plastic-attached aggregates were only loosely bound and became detached in the rinsing process prior to the bacterial enumeration and biofilm staining procedures. To investigate the composition of these aggregates, additional cultures were established to obtain aggregate samples for SEM analysis. SEM-based examination of aggregates obtained from a culture of *S. epidermidis* ATCC 35984 in PCs revealed a mixture of bacteria and platelets which frequently displayed physical interactions (Figure 12). Micrographs revealed that the dendritic protrusions of the platelets, which are typical of an activated platelet phenotype,

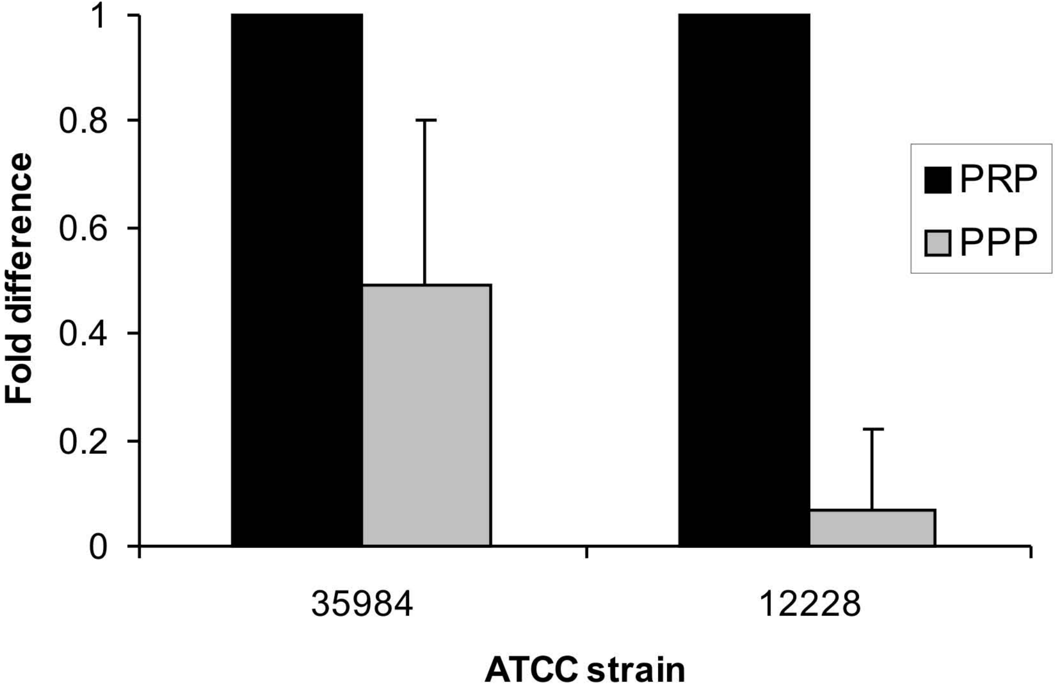

1
0.8
0.6
0.4
0.2
0
Fold difference
35984
12228
ATCC strain
PRP
PPP

Figure 11. *S. epidermidis* biofilm formation is decreased in platelet-poor-plasma (PPP). Whole blood-derived platelets prepared by the platelet-rich-plasma (PRP) method were filtered through a 0.45 μm pore size to create PPP. Means of 11 biofilm assays performed in duplicate are shown ± SD. Biofilm quantifications were achieved by microplate reading of crystal violet stain intensity, and differences in biofilm staining under the two growth conditions are significant for both strains ($P < 0.05$).

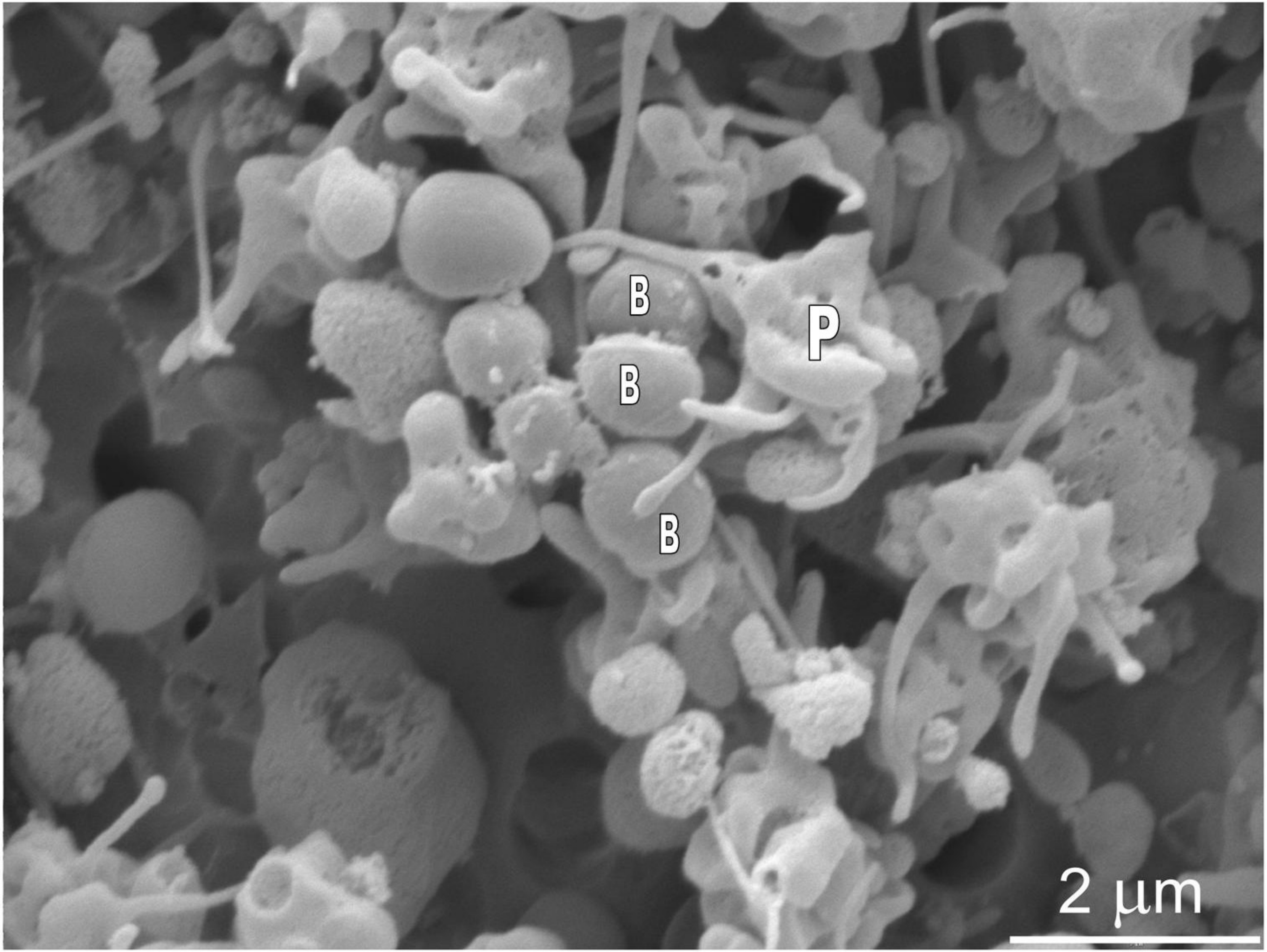

B
P
B
B
2 µm

Figure 12. Scanning electron microscopy image of *S. epidermidis* ATCC 35984 cells associated with platelets. Note that the phenomenon of circumferential adherence of bacteria (B) is in progress by an activated platelet (P).

extend to form contacts with the bacteria. This interaction was also observed with the 'biofilm-negative' strain of *S. epidermidis* (Appendix 11.2.1).

3.2.2 *S. epidermidis* Biofilm Formation on the Inner Surface of Platelet Storage Bags

Sterile CLX® platelet bags and CLX® bags preconditioned with PRP were used to investigate whether this plastic material, comprised of polyvinyl chloride (PVC), would serve as a suitable surface for *S. epidermidis* biofilm formation. The bags were injected with sterile TSBg and inoculated with *S. epidermidis.* After a 5-day incubation at room temperature on a platelet agitator, the walls of the bags were then cut into ~1 cm x 1 cm squares (coupons) and the inner surfaces were examined by SEM. *S. epidermidis* ATCC 35984 biofilms frequently developed on the raised, diamond-shaped ridges on the inner surface of the bags (Figure 13A). These accumulations were widespread multicellular clusters encased in slime (Figure 13B), and plastic attachment of solitary cells was less frequently observed. Slime was seen as darker, featureless areas in large bacterial clusters (Figure 13B and 13C, arrowheads), and was occasionally surrounded by small bacterial groups (Figure 13B). The bacteria, interspersed with slime, exhibited multiple layers of thickness in the large clusters (Figure 13C). These characteristics are attributed to a biofilm phenotype (Monds and O'Toole, 2009), and were therefore classified as such accordingly. The above findings were consistently obtained by thoroughly examining three 1 cm x 1 cm coupons.

It was also observed that the 'biofilm negative' strain *S. epidermidis* ATCC 12228 was able to form biofilms on PRP pre-conditioned CLX® bag material (Figure14A). However the biofilms of the *ica*-positive ATCC 35984 strain were denser with respect to

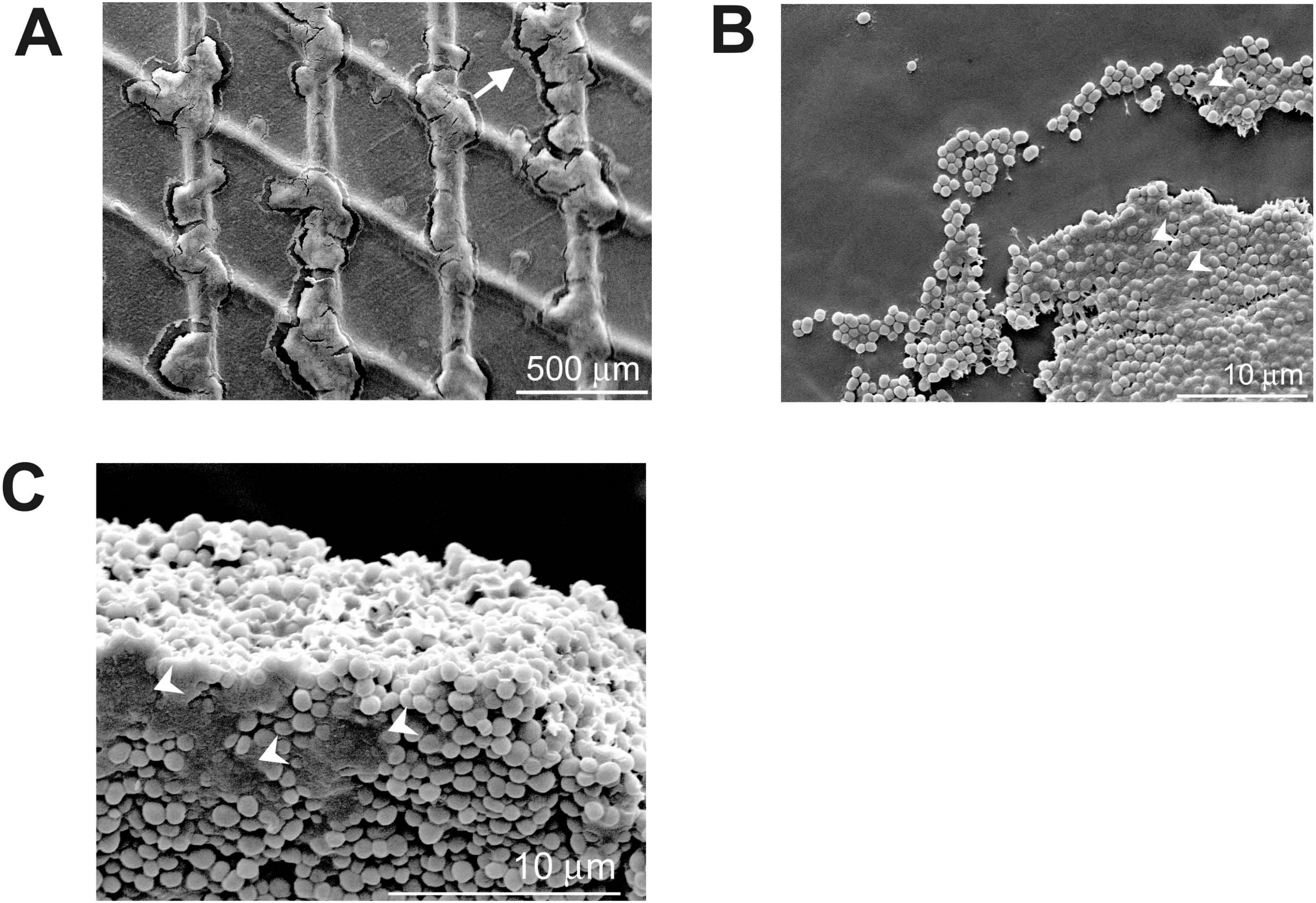
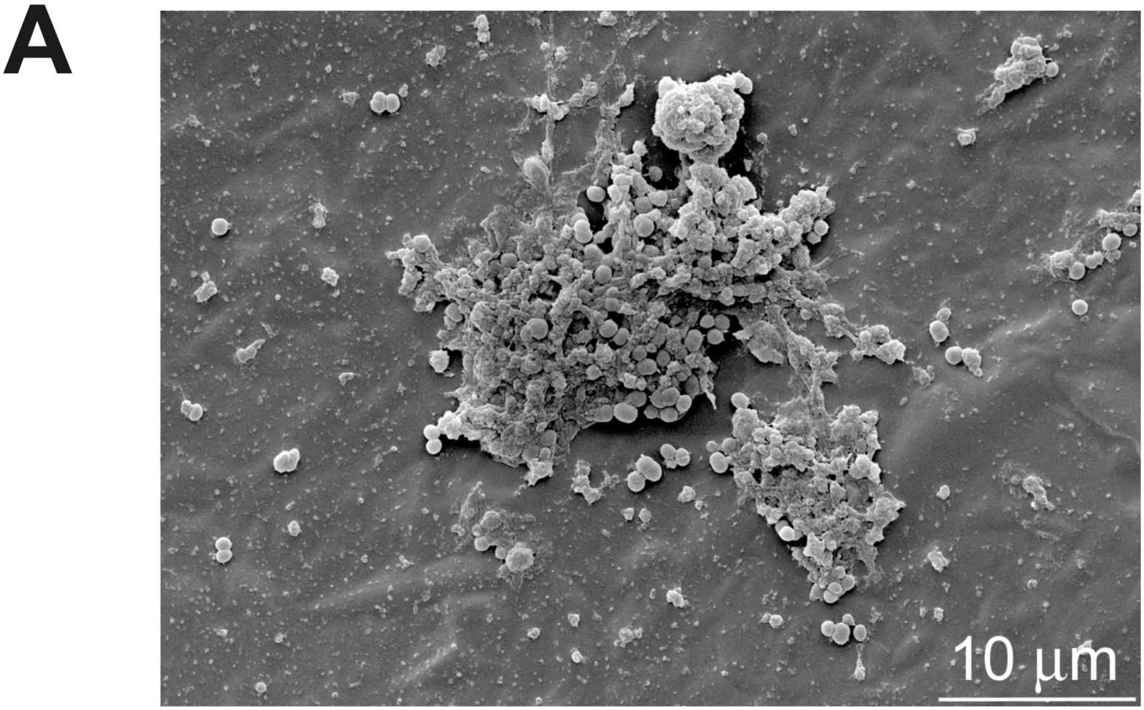

A
500 µm
B
10 µm
C
10 µm

Figure 13. Biofilm formation by *S. epidermidis* ATCC 35984 on CLX® platelet storage bag material. Scanning electron micrographs show samples that were fixed with OsO_4 vapour and subsequently air-dried. (A): Clusters of bacterial cells are associated with the raised, diamond-shaped ridges of the bag (arrow). (B): An enlarged field from (A) shows a multicellular cluster attached to a PVC coupon. (C): A thick cluster of the ATCC 35984 strain is shown. Arrowheads point to biofilm extracellular matrix material in (B) and (C).


A
10 µm

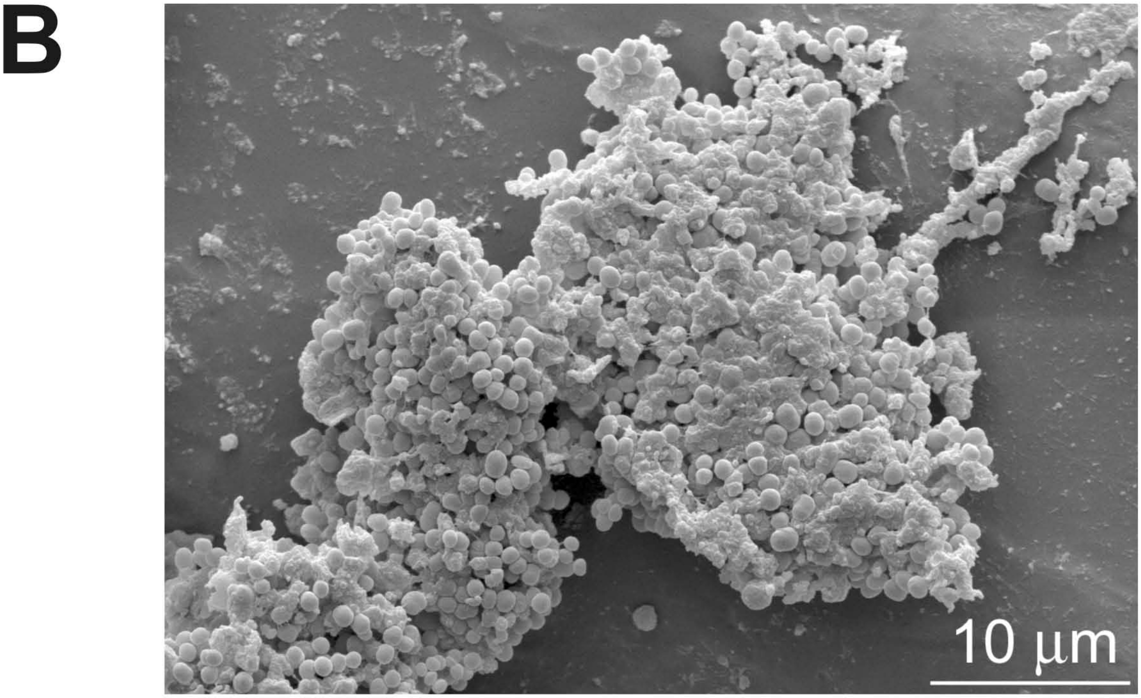

B
10 µm

Figure 14. Scanning electron microscopy images of *S. epidermidis* grown on platelet storage bag plastic pre-conditioned with PCs. (A): Clusters of the ATCC 12228 strain. (B): Compact clusters of the ATCC 35984 strain. Both specimens were fixed with paraformaldehyde and glutaraldehyde, dehydrated in ethanol, and critical-point dried before mounting on aluminum stubs and sputter-coating with gold. Images represent a typical SEM view of 1 cm x 1 cm coupons excised from the storage bags.

bacterial populations and extended over larger surface areas (Figure 14B). ATCC 12228 biofilms occupied less than 20 % of the coupon area covered by ATCC 35984 biofilms, although no precise quantifications were made.

4. RESULTS: OBJECTIVE 2

Results pertaining to the characterization of CoNS isolated from contaminated PCs in Canada are presented in this section. Specifically, 13 strains were examined for biofilm-forming ability by a variety of tests, and it was determined that the strain results for Congo red assay, crystal violet assay, *icaD* gene presence and PIA production were in substantial agreement ($\kappa = 0.64$). The results of these various biofilm assessments are compiled in Table 4. Biofilm formation in PCs was positive ($A492 > 0.5$) for almost all *S. epidermidis* strains (Appendix 11.3.3).

4.1 SPECIATION OF CoNS ISOLATED BY CBS DURING ROUTINE PC SAMPLE SCREENING

Details pertaining to the isolation and identification of CoNS implicated in platelet contamination events at CBS are displayed in Table 2. CoNS isolated from contaminated PCs were classified according to criteria established at CBS, as presented in Figure 4: true positive (if the same microorganism was isolated during initial and confirmatory testing), false positive (if confirmatory testing was negative) or indeterminate (if an initial positive could not be confirmed because either the implicated platelet unit was not available for repeat testing or the confirmatory culture bottle was discarded before bacterial identification). Ribotyping of selected strains was performed by a fellow lab member and collaborators at the Health Canada Bureau of Microbial Hazards to investigate strain relatedness (Appendix 11.3.4).

4.2 AMPLIFICATION OF *divIVA* AND *icaD* GENE TARGETS

PCR was performed to distinguish *S. epidermidis* strains from other CoNS via amplification of the putative cell division gene *divIVA* which has previously been shown

Table 4. Compilation of biofilm assessment results for CoNS isolated by CBS during routine PC sample screening.

Strain	**ID***	***divIVA***	**CRA**	**CV**	***icaD***	**PIA**
35984[§]	*S. epidermidis*	+	+	+	+	+
12228[§]	*S.epidermidis*	+	-	-	-	-
06010	*S. epidermidis*	+	-	-	-	-
06038	*S. epidermidis*	+	-	-	-	-
07003	*S. capitis*	-	+	-	-	-
06033	*S. hominis*[†]	-	-	-	-	-
07006	*S. epidermidis*	+	+	+	+	+
07009	*S. warneri*	-	-	-	-	-
07022	*S. epidermidis*	+	-	-	-	-
07026	*S. epidermidis*	+	+	+	+	+
07043	*S. epidermidis*	+	-	-	-	-
06043	*S. epidermidis*[†]	+	+	+	+	+
07023	*S. hominis*	-	-	-	-	-
07040	*S. capitis*	-	-	-	-	-
07045	*S. epidermidis*	+	-	+	-	-

CRA, Congo red agar test; CV, crystal violet stain; PIA, polysaccharide intercellular adhesion (immunodetection).

* Identification based on API Staph (bioMérieux, Marcy l'Etoile, France).
[§] ATCC control strain
[†] Amended based on ribotyping (Appendix 11.3.4).

to exhibit high sequence conservation within this species, but substantial divergence from other staphylococci (Mastronardi and Ramirez-Arcos, 2007). Both control *S. epidermidis* strains ATCC 12228 and ATCC 35984 tested positive for *divIVA* with a fragment present which was consistent with the expected 777 base pair size (Figure 15A). A comparable fragment was amplified in 8 of the 13 isolates tested (Figure 15A; Table 4). These results were in agreement with the positive identifications of *S. epidermidis* by API Staph, with the exception of isolate 06043 which was initially identified as *S. hominis* but later confirmed to be *S. epidermidis* by ribotyping analysis (Table 2). As expected, *S. epidermidis* control strain ATCC 12228 tested negative for *icaD*, while ATCC 35984 displayed a fragment reflecting amplification of the 239 bp gene fragment which was targeted (Figure 15B). Strains 06043, 07006, and 07026 all yielded amplification products which indicated the presence of the *icaD* gene (Figure 15B; Table 4).

4.3 ASSESSMENT OF SLIME AND BIOFILM FORMATION

Staphylococcal strains were assessed for a slime phenotype on Congo red agar, which was used as an indicator of biofilm production (Freeman et al., 1989). Four of the 13 CoNS strains (31 %), formed rough, black colonies consistent with slime production (Figure 16; Table 4).

All strains were also assessed for biofilm formation in TSBg using a crystal violet staining assay (Christensen et al., 1985). Four of the 13 isolates (31 %) produced biofilms on a polysterene surface, as evidenced by crystal violet stain intensity. All of these were *S. epidermidis* (Figure 17; Table 4). With the exception of strain 07045, these biofilm-producing strains were also slime-positive by the Congo red agar test. The Congo red-positive *S. capitis* strain 07003 did not produce biofilms on polystyrene (Figure 17).

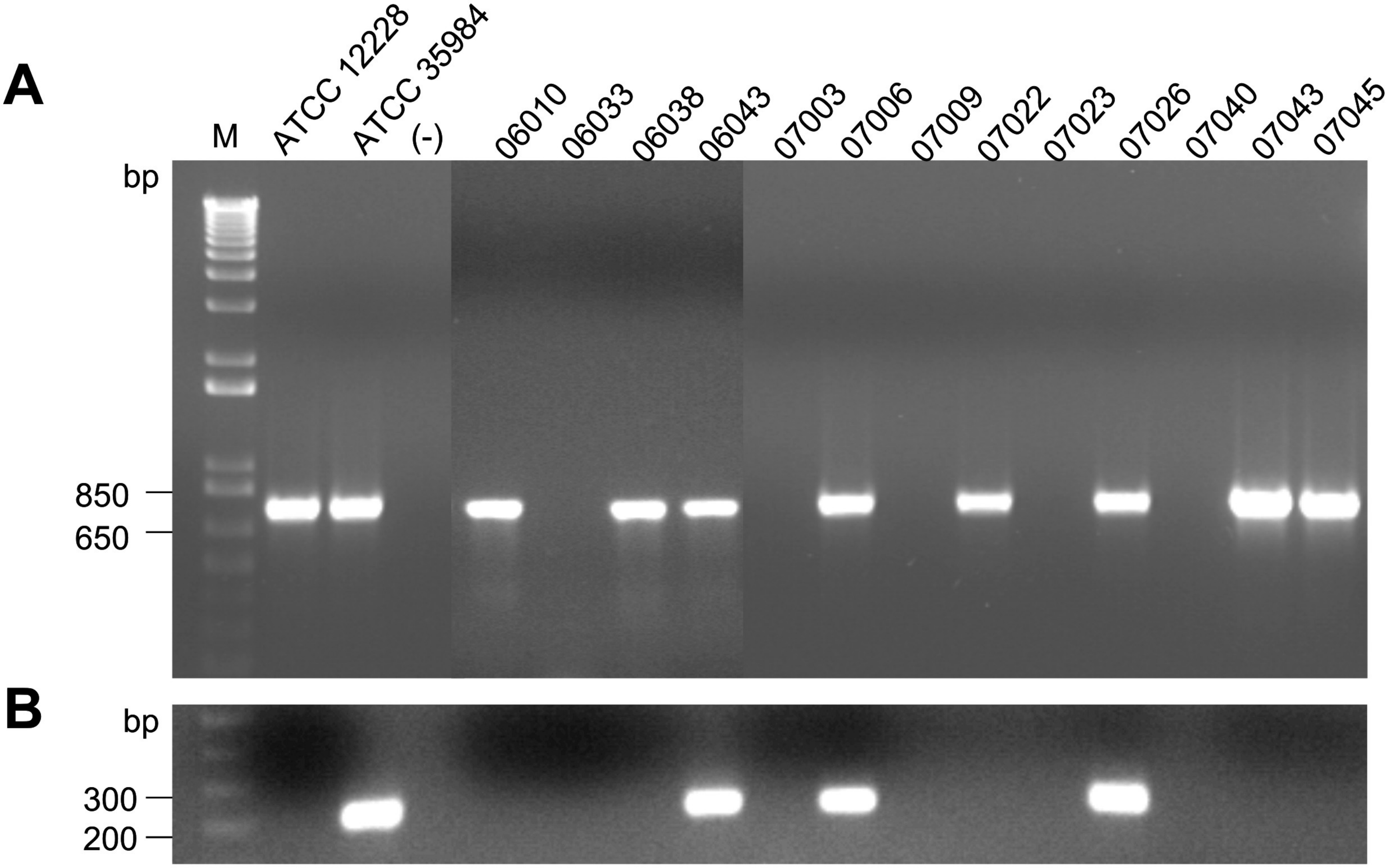

A
M
ATCC 12228
ATCC 35984
(-)
06010
06033
06038
06043
07003
07006
07009
07022
07023
07026
07040
07043
07045
bp
850
650
B
bp
300
200

Figure 15. PCR detection of *S. epidermidis* genes (A) *divIVA* and (B) *icaD* from CoNS PC isolates. M, molecular weight marker; (-) negative control (no DNA template); ATCC 12228, *divIVA*-positive, *icaD*-negative control strain; ATCC 35984, *divIVA*-positive, *icaD*-positive control strain. (A) The expected size of the *divIVA* fragment is 777 bp. (B) The expected *icaD* fragment size is 239 bp.

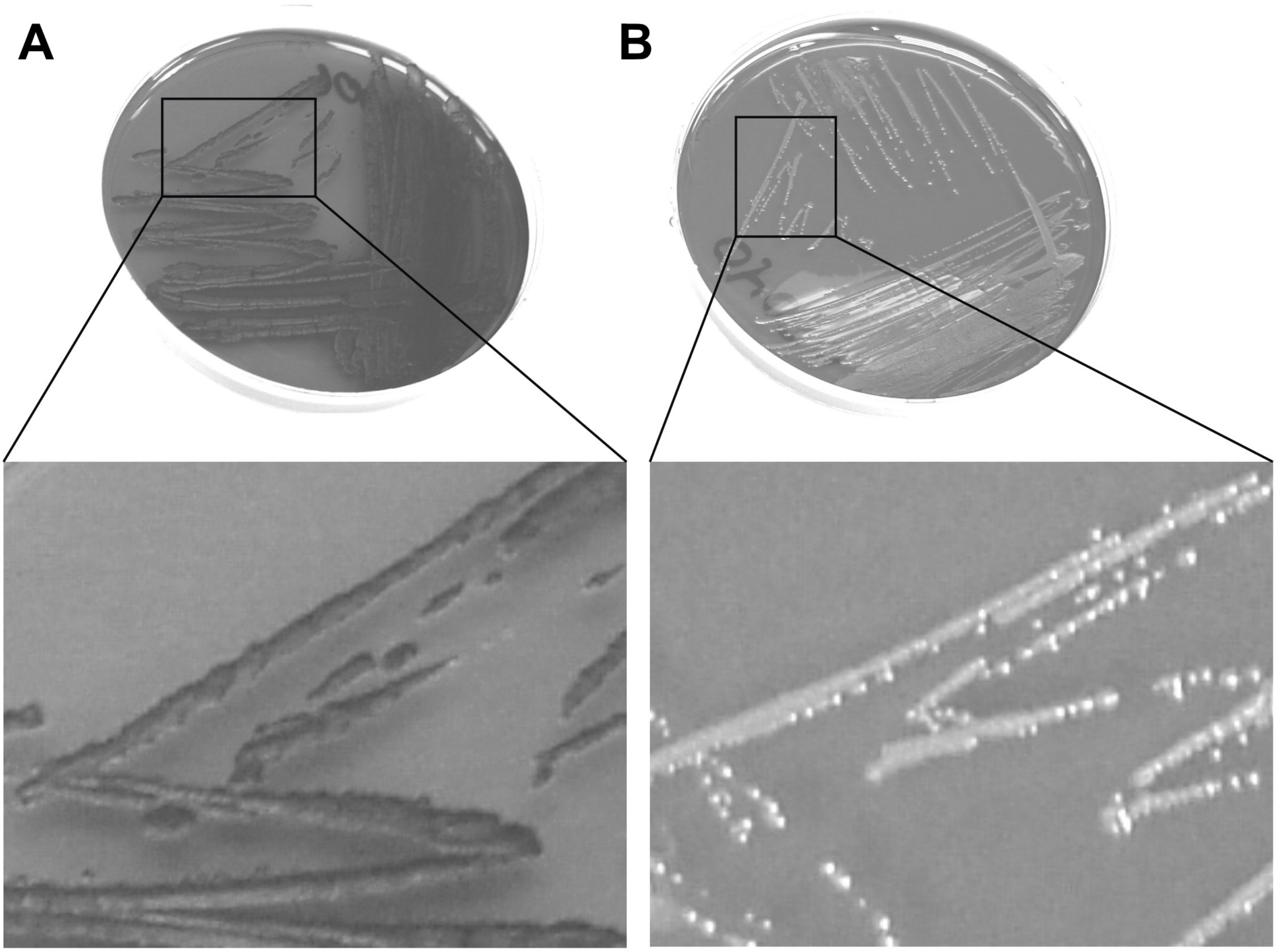

A
B

Figure 16. Biofilm-forming ability of staphylococci is inferred by phenotypic effects when grown on Congo red agar. Slime-producing strains form rough, black colonies such as strain 07006 (A), while the colonies of strains which do not produce slime are observed as pink or red, such as strain 07040 which depicted as light colonies in this photograph (B).

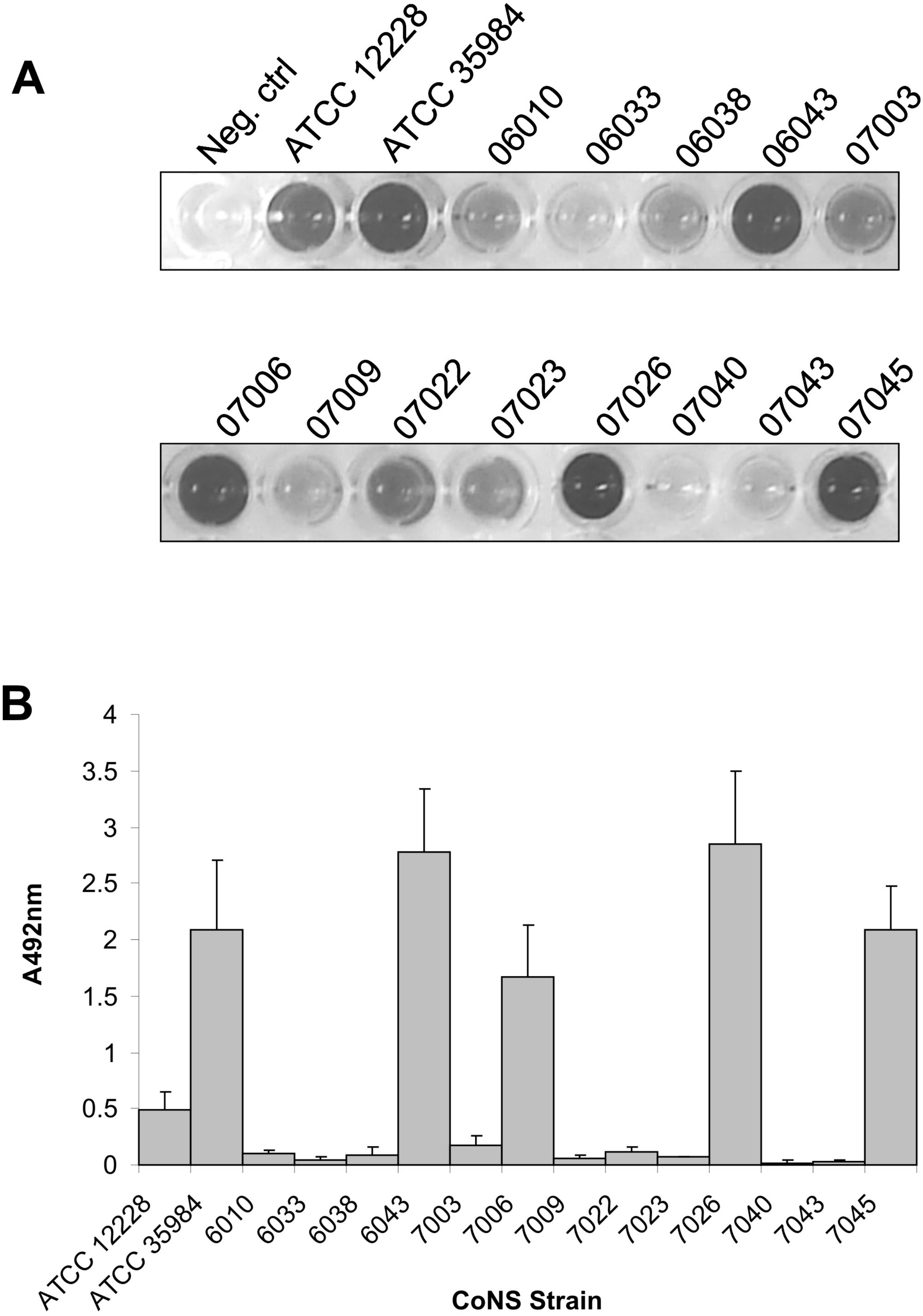

A
Neg. ctrl
ATCC 12228
ATCC 35984
06010
06033
06038
06043
07003
07006
07009
07022
07023
07026
07040
07043
07045
B
A492nm
4
3.5
3
2.5
2
1.5
1
0.5
0
ATCC 12228
ATCC 35984
6010
6033
6038
6043
7003
7006
7009
7022
7023
7026
7040
7043
7045
CoNS Strain

Figure 17. Biofilm formation on polystyrene by CoNS strains isolated from contaminated PCs. (A) Eluates of the crystal violet biofilm stain for all strains are shown. (B) Mean + SD of microplate measurements of crystal violet stain intensity which is directly proportional to biofilm production (n = 3).

4.4 DETECTION OF POLYSACCHARIDE INTERCELLULAR ADHESIN (PIA)

Using polyclonal anti-PIA IgG, an indirect immunofluorescence approach was conducted to determine whether the CoNS isolates were capable of PIA synthesis (Figure 18). The fluorescein-linked secondary IgG was distinctly localized in the intervening spaces between clusters of *S. epidermidis* 06043, 07006 and 07026 strains (Figures 18F, 18H, and 18J, respectively), which is indicative of biofilm formation, and consistent with observations of the PIA-positive *S. epidermidis* strain ATCC 35984 (Figure 18B). Like PIA-negative *S. epidermidis* strain ATCC 12228 (Figure 18D), the remaining isolates displayed no affinity to the anti-PIA IgG (Table 4; Appendix 11.3.2). Interestingly, *S. epidermidis* 07045, which displayed biofilm formation in the crystal violet assay (Figure 17), did not the harbour *icaD* gene, and slime production was negative by Congo red agar and PIA immunoassay (Figure 15B, Appendix 11.3.1 and 11.3.2 respectively).

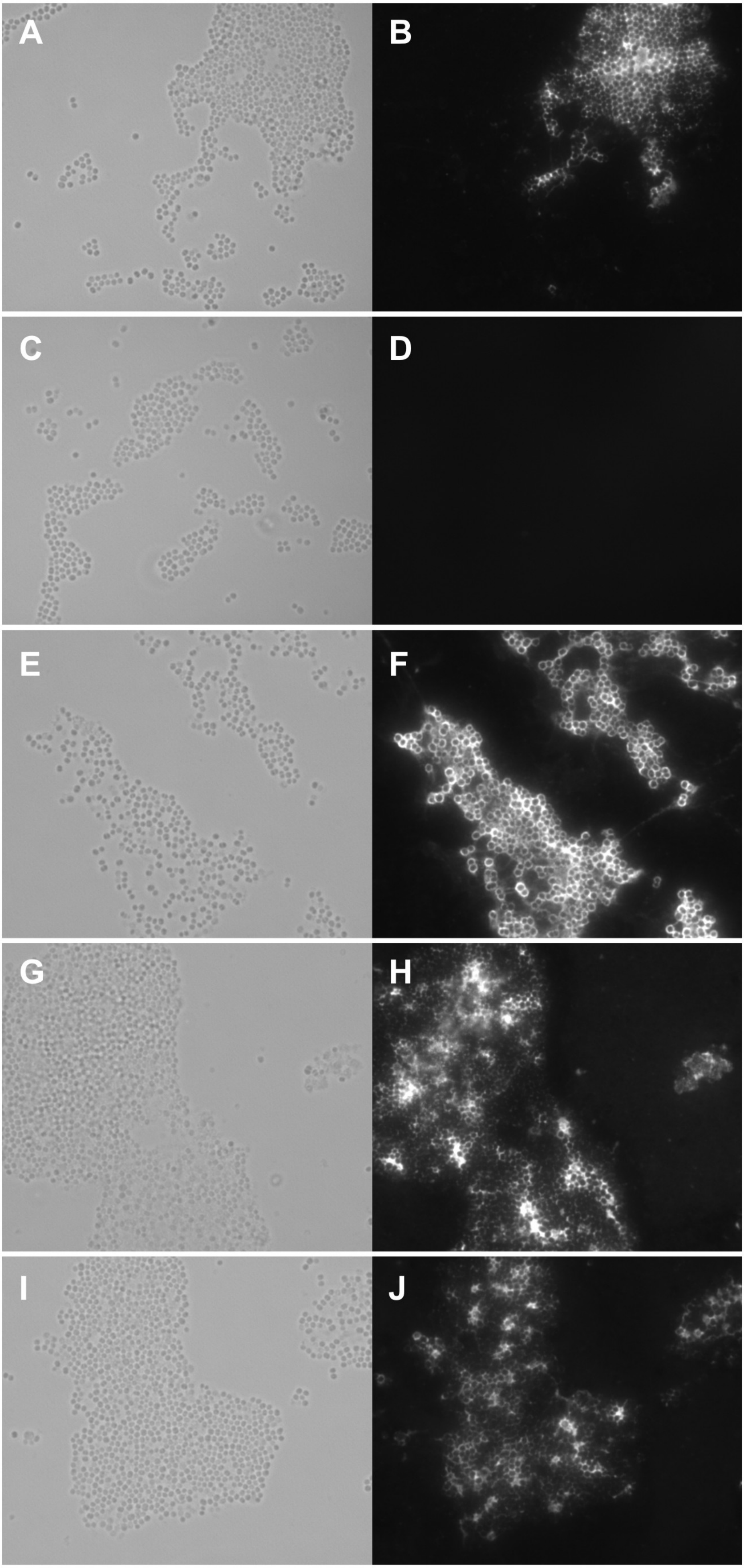

A
B
C
D
E
F
G
H
I
J

Figure 18. Production of PIA by PC contaminant isolates. Indirect immunofluorescence was performed using primary antibody α-PIA and FITC-conjugated secondary antibody. *S. epidermidis* ATCC 35984 (A, B) and ATCC 12228 (C, D) were included as biofilm-positive and biofilm-negative controls, respectively. Contaminant strains which were positive for PIA production are: *S. epidermidis* 06043 (E, F), 07006 (G, H), and 07026 (I, J). Phase contrast images (A, C, E, G, I) correspond to adjacent fluorescence images (B, D, F, H, J, respectively). Magnifications are at 100X objective.

5. RESULTS: OBJECTIVE 3

Results pertaining to bacterial growth in the presence of the PAS SSP+ and the subsequent effects on platelet quality are presented in this section.

5.1 BACTERIAL GROWTH IN PAS VERSUS PLASMA

Contamination of PCs with *Serratia liquefaciens* was evident due to changes in product appearance, and was visible sooner in PAS-suspended PCs than in plasma-suspended PCs (Figure 19). Changes in PC appearance were not obvious in any *S. epidermidis*-tainted PCs. Bacterial growth in PCs is shown in Figure 20. *S. liquefaciens* grown in plasma-PCs exhibited an apparent lag period that extended 24 h longer than did its counterpart in PAS-PCs (Figure 20A). Statistical analysis indicated that the *S. liquefaciens* growth rate in plasma-PCs was significantly reduced compared to the equivalently-spiked PAS-PCs at both the 10 and 100 CFU/bag initial inocula (P = 0.0001, 0.0029, respectively). In contrast, there was no obvious difference in the detected CFU of *S. epidermidis* when it was inoculated into either PAS-PCs or plasma-PCs (Figure 20B). Interestingly, *S. epidermidis* numbers remained essentially static in the absence of platelets (Figure 21). *S. epidermidis* replication in platelet-poor-plasma (PPP) or PAS containing 30% plasma was significantly lower as compared to their doubling time in the same media containing platelets (Figure 20B and Figure 21; P = 0.0369, P < 0.001, respectively). *S. liquefaciens* exhibited similar growth over time, regardless of platelet presence or absence in either plasma or PAS (Figure 20A and Figure 21; P = 0.3175 and 0.2771, respectively).

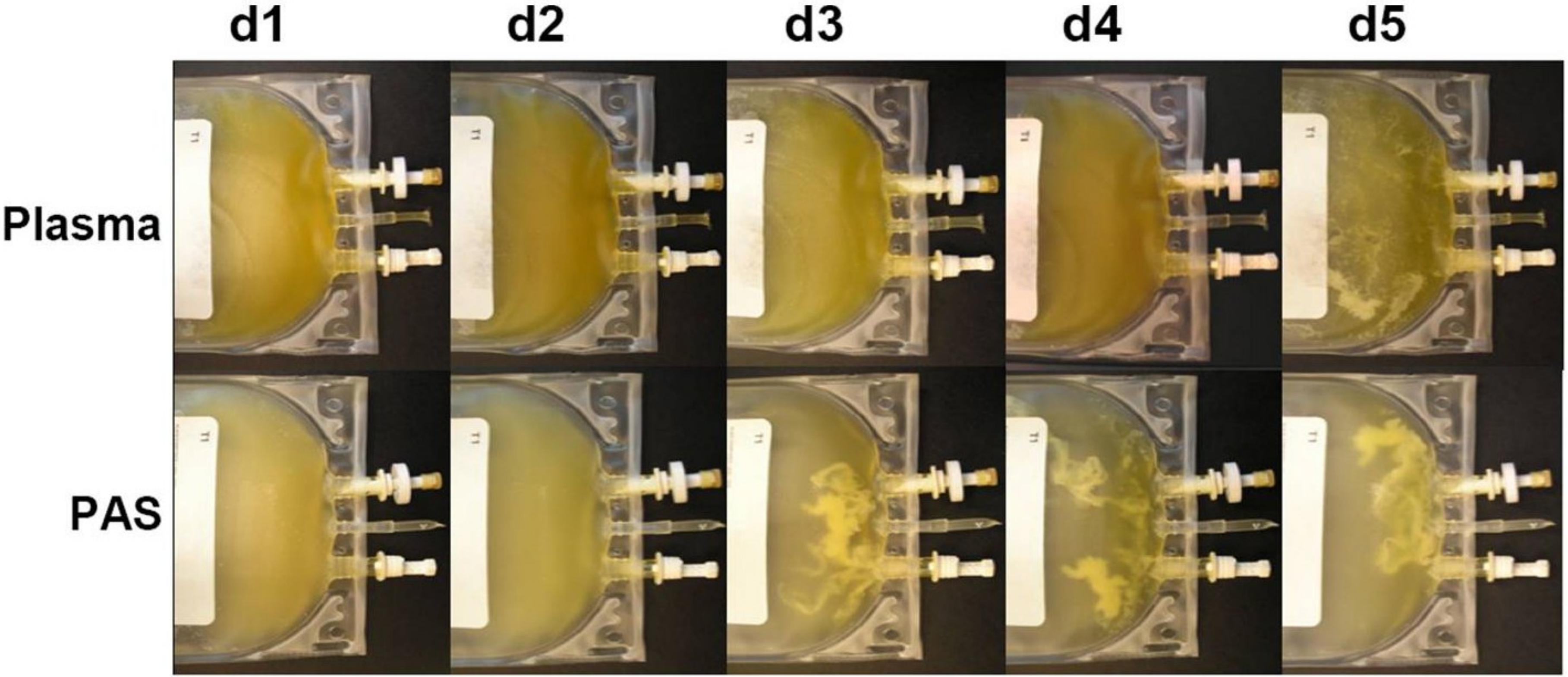

d1
d2
d3
d4
d5
Plasma
PAS

Figure 19. Visible appearance of *S. liquefaciens*-contaminated PAS-PCs (PAS) and plasma-PCs (Plasma) over time. Digital images of PCs inoculated with 100 CFU/bag were taken at approximately 24 hour intervals.

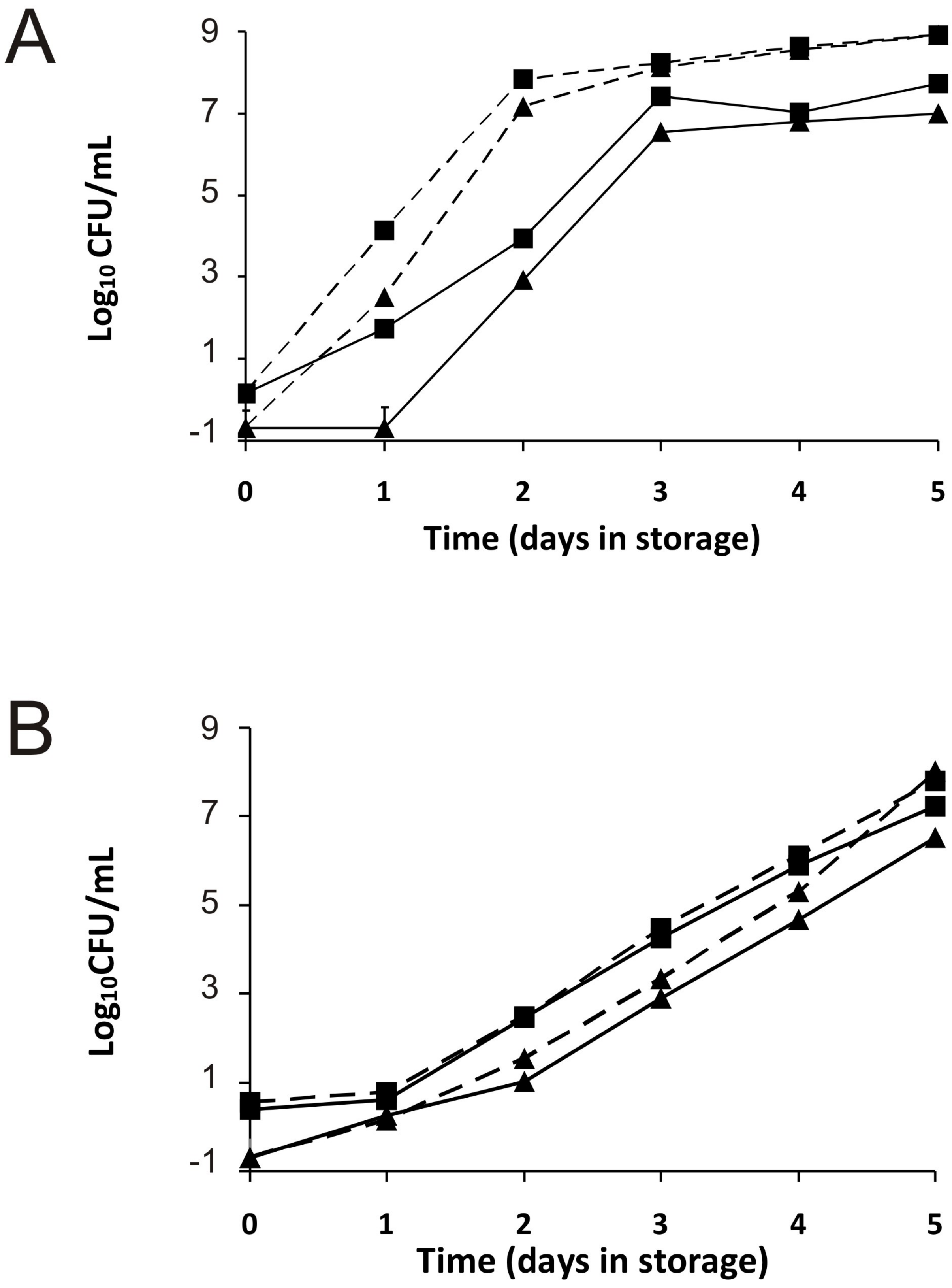

A
9
7
5
3
1
-1
Log₁₀ CFU/mL
0
1
2
3
4
5
Time (days in storage)
B
9
7
5
3
1
-1
Log₁₀CFU/mL
0
1
2
3
4
5
Time (days in storage)

Figure 20. Growth of (A) *S. liquefaciens* and (B) *S. epidermidis* in BC-PCs suspended in either plasma (solid line) or PAS (dashed line). Normal PC storage conditions were used for incubations (22 ± 2°C with agitation). Two initial inocula are represented: 10 CFU/bag (▲) and 100 CFU/bag (■). Means ± SD of five independent experiments are shown.

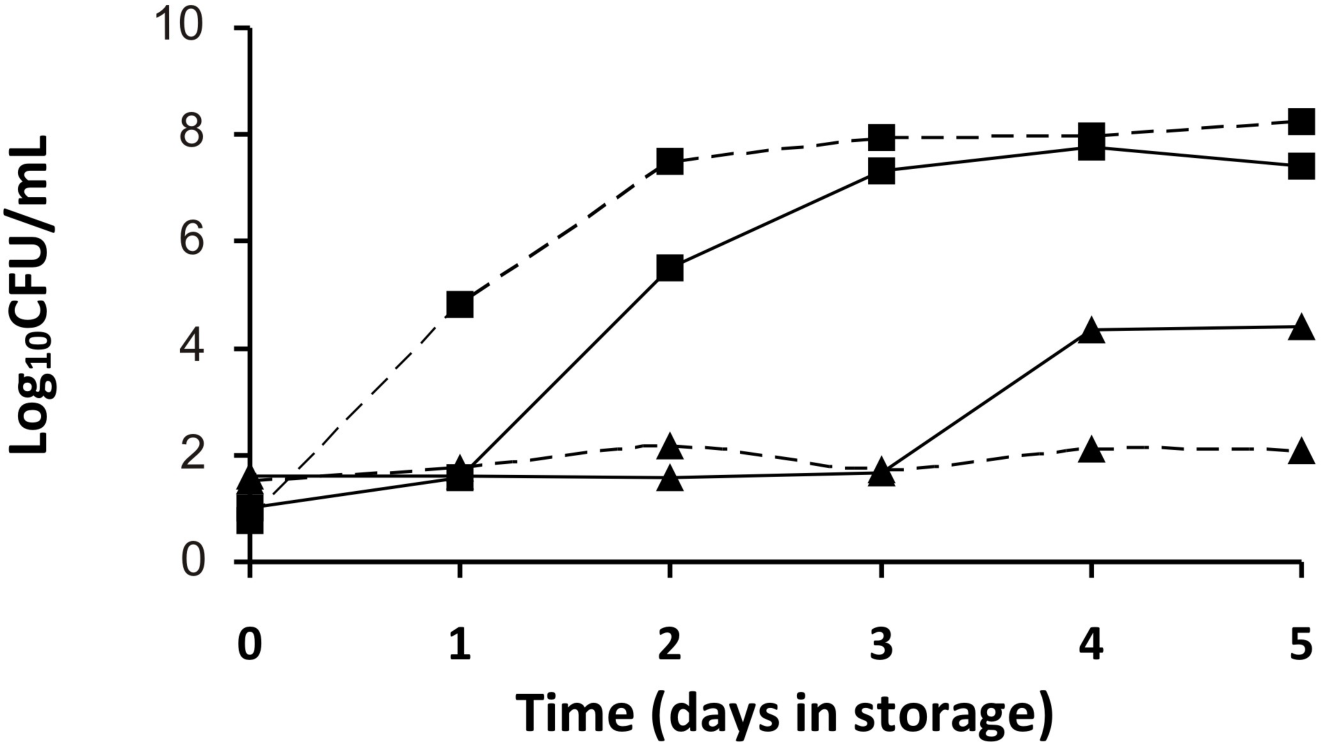

10
8
6
4
2
0
Log_{10}CFU/mL
0
1
2
3
4
5
Time (days in storage)

Figure 21. Growth of *S. liquefaciens* (■) and *S. epidermidis* (▲) in either platelet-poor plasma (solid line) or PAS containing 30% plasma (dashed line). Normal PC storage conditions were used for incubations (22 ± 2^{o}C with agitation). Means ± SD of three independent experiments are shown.

5.2 BIOFILM FORMATION

S. liquefaciens and *S. epidermidis* were tested for their ability to form biofilms in either PAS- or plasma-PCs. At day 5, both bacterial species displayed significantly greater biofilm formation in plasma-PCs than in PAS-PCs (Figure 22A). This was confirmed by quantifying the stain intensity obtained at 490 nm (Figure 22B; P = 0.0004 and 0.04, respectively).

To determine whether bacteria adhered to the inner surface of the platelet storage containers, coupons from the storage bags were fixed for SEM. Micrographs revealed that biofilm material adhered to all the spiked samples, regardless of the suspension medium (Figure 23). *S. epidermidis* biofilms were denser and larger on average in the presence of plasma (Figure 23A) than in PAS (Figure 23B). In PAS-PCs, the adherent *S. epidermidis* cells were dispersed and infrequent (Figure 23B). When day 5 coupons were examined from bags of plasma-PCs inoculated with *S. liquefaciens*, extensive biofilm formation was observed (Figure 23C). On average, *S. liquefaciens* biofilms in plasma-PCs displayed a higher density of bacteria than those from the PAS-PCs (Figure 23D).

5.3 PLATELET QUALITY

PCs spiked with ~10 or ~100 CFU/bag of either *S. epidermidis* or *S. liquefaciens* were assessed for signs of abnormal platelet quality parameters by data collection over a 5 day storage period (Table 5). Platelet concentrations and mean platelet volumes (MPVs) did not change significantly with increasing bacterial titers (Appendix 11.4.3). However, these parameters were not measured after the first indications of clumping due to risk of blockage and resultant damage to the hematology and blood gas analysis equipment. The remaining platelet quality results are summarized in subsections 5.3.1 and 5.3.2.

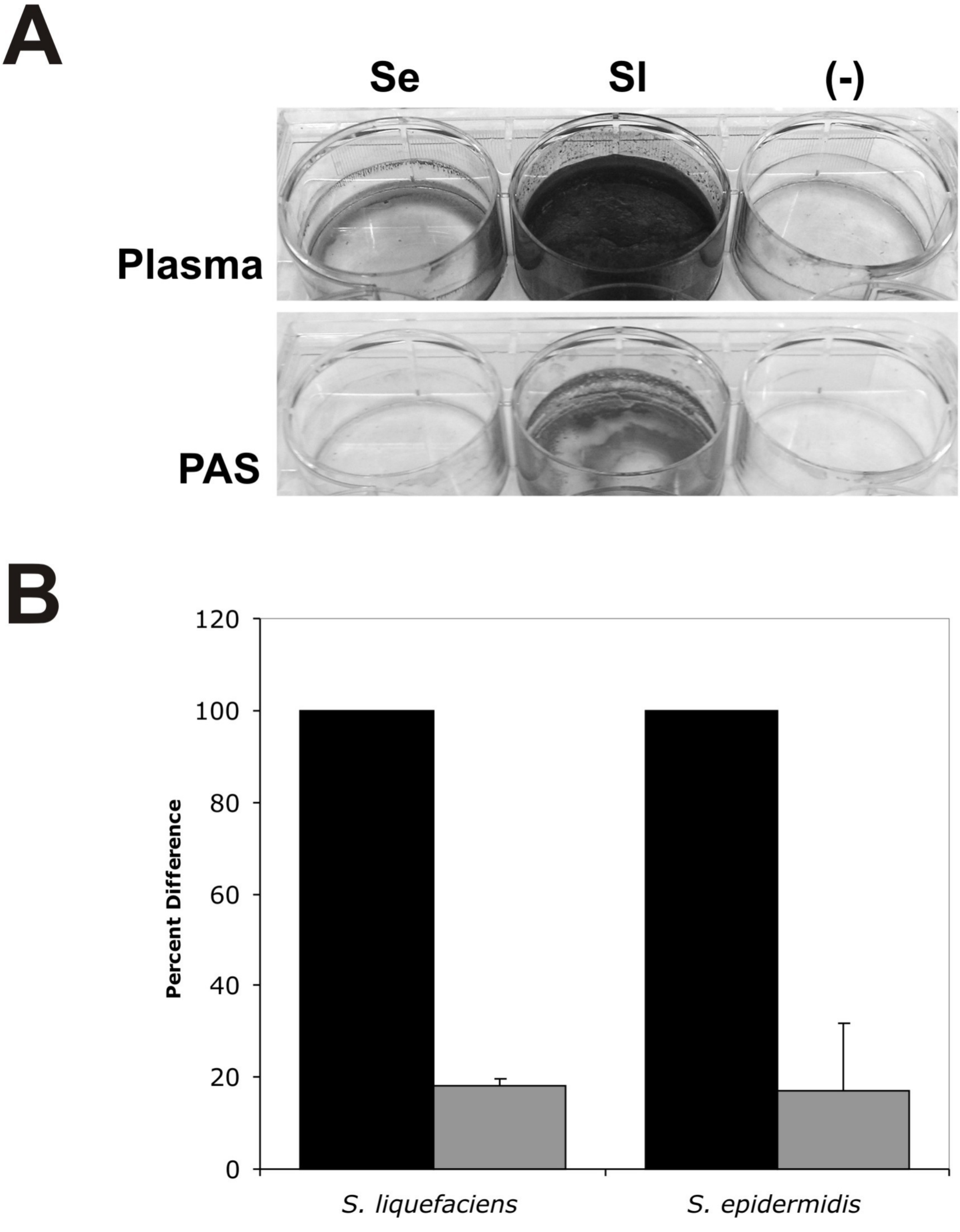

A
Se
Sl
(-)
Plasma
PAS
B
120
100
80
60
40
20
0
Percent Difference
S. liquefaciens
S. epidermidis

Figure 22. Biofilm formation by *S. epidermidis* (Se) and *S. liquefaciens* (Sl) in PCs suspended in either plasma or PAS. (A) Day 5 biofilms were stained with 0.3 % crystal violet. (B) Eluted stain intensity at λ = 492 nm was measured for biofilms in PAS-PCs (n = 4, grey) and normalized to biofilms grown in plasma-PCs (n = 6 , black). Non-inoculated controls are labelled (-).

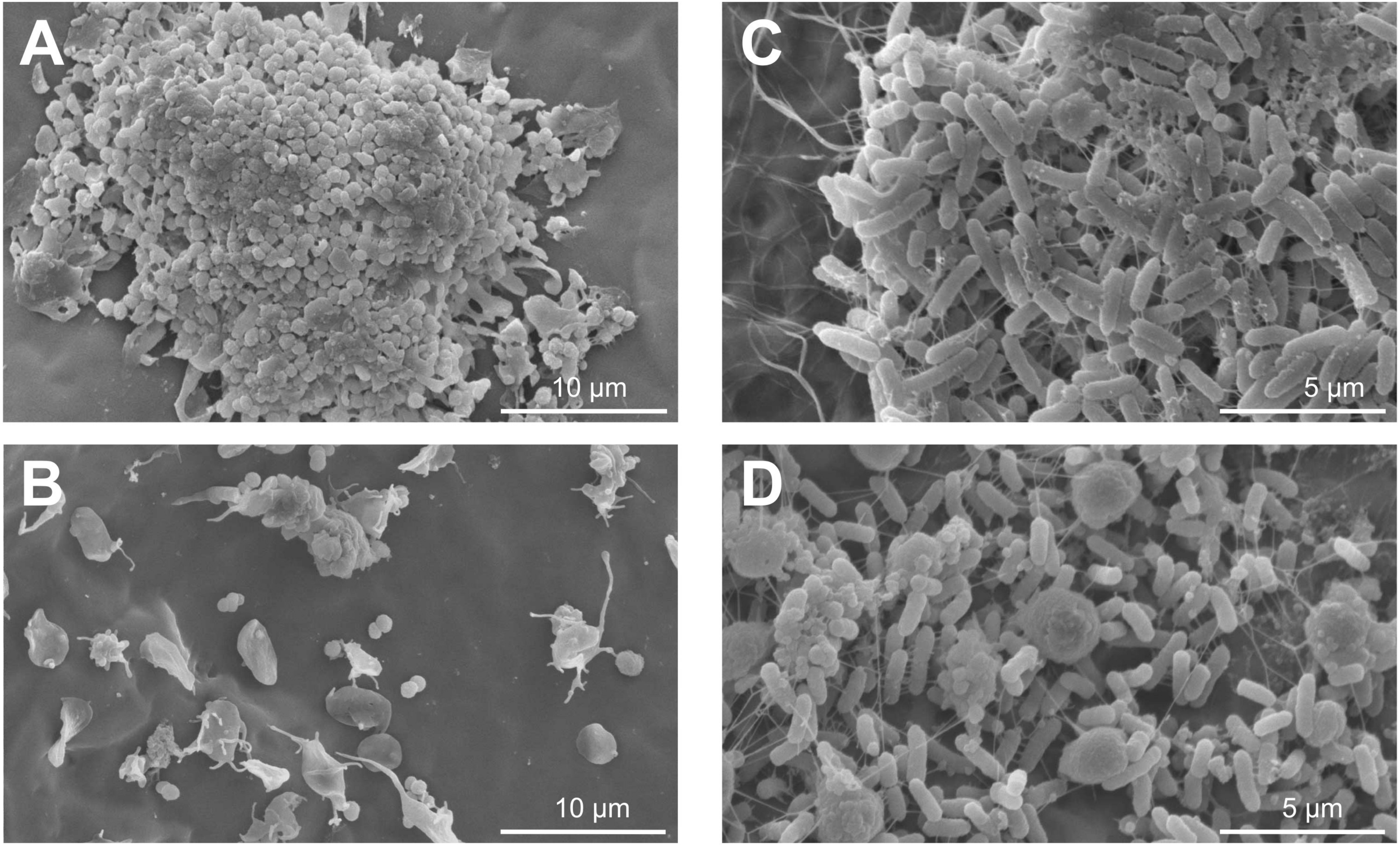

A
10 µm
C
5 µm
B
10 µm
D
5 µm

Figure 23. Scanning electron micrographs of the inner surface of platelet storage bags which held BC-PCs inoculated with either (A, B) *S. epidermidis* or (C, D) *S. liquefaciens*. PCs were suspended in either (A, C) plasma or (B, D) PAS. Fields of view shown represent typical visuals from the scan of 1 c m^2 coupons from three or more separate experiments from the fifth day of storage with *S. epidermidis* (initial inocula = 2 CFU/mL) or *S. liquefaciens* (initial inocula = 0.2 CFU/mL). *S. liquefaciens* fimbriae are visible as hair-like projections in (C) and (D).

Table 5. Quality parameters over time for PCs suspended in PAS or plasma after inoculation with 10 or 100 CFU/bag of *S. liquefaciens* (Sl) or *S. epidermidis* (Se).

Parameter	**Species**	**Medium**	**CFU /bag**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**P-value***
pH	Sl	plasma	100	7.16 ±0.06	7.34±0.02	7.39±0.02	6.97±0.03	ND	0.0008†
			10	7.13±0.03	7.33±0.03	7.38±0.03	7.21±0.27	ND	>0.05†
		PAS	100	7.08±0.02	7.17±0.03	6.84±0.25	ND	ND	<0.0001†
			10	7.09±0.03	7.18±0.02	7.05±0.17	6.63±0.15	ND	<0.0001†
	Se	plasma	100	7.17±0.06	7.35±0.03	7.38±0.04	7.38±0.02	7.34±0.05	>0.05
			10	7.13±0.04	7.34±0.03	7.40±0.02	7.37±0.03	7.36±0.03	>0.05
		PAS	100	7.08 ±0.03	7.17±0.03	7.21±0.02	7.20±0.03	7.19±0.02	>0.05
			10	7.08±0.03	7.17±0.02	7.21±0.02	7.22±0.04	7.21±0.03	>0.05
	Neg	plasma	NA	7.15±0.07	7.34±0.03	7.38±0.03	7.36±0.03	7.35±0.03	NA
		PAS	NA	7.09±0.29	7.18±0.03	7.21±0.03	7.23±0.03	7.21±0.02	NA
pO_2 (mmHg)	Sl	plasma	100	131±22	124±13	137±7.8	62.0±42	ND	0.0047†
			10	135±20	127±2.9	122±32	104±57	ND	>0.05†
		PAS	100	126±30	121±7.5	35.6±53	ND	ND	<0.0001†
			10	129±17	113±19	62.0±46	4.33±7.5	ND	<0.0001†
	Se	plasma	100	134±17	128±6.3	119±29	142±11	124±39	>0.05
			10	127±24	131±4.9	125±32	143±8.8	141±8.8	>0.05

		PAS	100	124±38	118±5.1	119±19	134±16	119±31	>0.05
			10	127±30	115±6.3	110±33	139±13	136±13	>0.05
	Neg	plasma	NA	127±30	129±6.5	126±17	137±16	137±10	NA
		PAS	NA	125±35	124±10	110±33	119±27	136±14	NA
pCO$_2$ (mmHg)	Sl	plasma	100	61±3.0	33±0.6	26±1.4	86±64	ND	0.0297[†]
			10	62±5.0	34±2.0	28±1.2	ND	ND	>0.05[†]
		PAS	100	27±2.3	21±2.0	42±26	ND	ND	0.0527[†]
			10	27±2.6	20±1.5	24±10	93±5.6	ND	>0.05[†]
	Se	plasma	100	59±7.6	31±2.1	27±2.1	45±38	56±45	>0.05
			10	62±7	32±1.7	25±1.2	42±33	60±69	>0.05
		PAS	100	28±1.4	20±1.2	17±1.6	33±29	43±32	>0.05
			10	28±1.7	22±0.58	18±0.58	28±20	48±60	>0.05
	Neg	plasma	NA	62±7.8	35±0.6	29±1.4	40±27	43±35	NA
		PAS	NA	28±2	21±1	18±1	16±0	14±2	NA
Glucose (mmol/L)	Sl	plasma	100	16.9±2.0	15.8±3.5	16.4±1.3	11.7±4.6	ND	>0.05[†]
			10	16.8±2.3	15.9±3.4	16.4±1.1	12.4±5.6	ND	>0.05[†]
		PAS	100	5.74±0.7	5.34±0.7	1.84±2.0	ND	ND	<0.0001[†]
			10	5.52±0.9	5.24±0.8	4.1±0.9	0[§]	ND	0.0002[†]
	Se	plasma	100	16.8±2.3	16.3±2.9	16.2±1.6	14.8±1.4	14.3±0.6	>0.05
			10	16.7±2.4	15.9±3.6	16.0±1.3	15.0±1.4	14.8±1.1	>0.05
		PAS	100	5.62±0.9	5.12±0.8	5.14±0.3	4.02±0.4	3.54±0.3	>0.05

			10	5.62±0.9	5.20±0.8	4.94±0.5	4.20±0.6	3.76±0.5	>0.05
	Neg	plasma	NA	17.0±2.0	16.4±3.1	15.6±1.9	15.0±1.7	14.6±1.0	NA
		PAS	NA	5.62±0.9	5.30±0.7	4.92±0.3	4.38±0.4	3.60±0.6	NA
Lactate (mmol/L)	Sl	plasma	100	7.28±0.9	8.14±0.8	10.15±1.0	11.6±1.4	ND	>0.05[†]
			10	7.36±0.8	8.32±0.8	10.1±0.6	12.0±1.2	ND	>0.05[†]
		PAS	100	3.72±0.7	4.42±0.8	6.62±1.7	ND	ND	>0.05[†]
			10	3.62±0.7	4.46±0.9	5.84±1.2	9.03±6.8	ND	>0.05[†]
	Se	plasma	100	7.32±0.8	8.28±0.9	10.3±1.2	12.0±1.0	13.7±1.5	>0.05
			10	7.28±0.8	8.16±1.0	10.2±0.9	12.2±1.2	12.3±3.3	>0.05
		PAS	100	3.75±0.8	4.36±0.8	5.70±0.9	7.06±1.2	8.18±1.1	>0.05
			10	3.68±0.6	4.42±0.8	5.80±1.0	6.78±1.2	8.54±1.2	>0.05
	Neg	plasma	NA	7.36±0.8	8.20±0.8	9.90±0.9	11.9±0.7	13.6±0.9	NA
		PAS	NA	3.64±0.6	4.38±0.8	5.82±0.8	6.62±1.3	8.58±1.0	NA

Means ± standard deviation for each data set comprised of five independent experiments are shown. Neg, negative (non-spiked control); NA, not applicable.

* From mixed model analyses of the influence of each bacterium on the indicated platelet variables.

[†] Data analyses were performed only until sampling became unfeasible (indicated ND, not determined) due to the presence of aggregates.

[§] Glucose concentrations which caused a machine error message (eg, below detection threshold) were assigned a value of zero.

5.3.1 *S. epidermidis*-Inoculated Units

None of the PCs inoculated with *S. epidermidis* displayed visible signs of contamination, and any reduced swirling noted over time could have been attributed to the normal platelet storage lesion because of its similarity in appearance to the non-inoculated PCs. No statistically significant changes within the panel of platelet parameters were observed in *S. epidermidis*-containing PCs as compared to negative controls (Table 5). By day 4, the PC inoculated with ~100 CFU/bag of *S. epidermidis* displayed a somewhat faster rate of pH decrease, although this variation was not statistically significant. Despite the fact that the initial glucose concentration in plasma-PCs is higher than in PAS, it is evident that the amount of this carbon source was not limiting for *S. epidermidis* in PAS-PCs, because the detectable growth rates were similar in both suspending media (Figure 20B). Differences in CD62P expression and Annexin V–binding were not significant when comparing PAS and plasma-PCs, regardless of *S. epidermidis* presence or absence.

5.3.2 *S. liquefaciens*-Inoculated Units

The swirling phenomenon was diminished or lost in PAS-PCs by day 3 of incubation with either 10 or 100 CFU/bag of *S. liquefaciens*. At this time, the first evidence of clumping became apparent (Figure 19), and corresponded to a detected supernatant bacterial concentration of ~10^8 CFU/mL. This count is consistent with *S. liquefaciens* early stationary phase growth in a similar medium, characterized by a reduced doubling time and similar to the findings of Ezuki et al. (2007). Once aggregates or clumps became apparent, they remained for the duration of storage. The plasma-PCs displayed microaggregates (>1 mm diameter) 4 days after inoculation; however, it was typically not until day 5 of storage that plasma-PC units showed diffuse plastic-adherent material. The platelet parameter values over time for PCs spiked with *S. liquefaciens* are displayed in Table 5. *S. liquefaciens* growth over time significantly influenced the pH, pO_2, and pCO_2 levels in plasma-PCs, but only in the bags inoculated with 100 CFU. However, pH, pO_2, and glucose levels in PAS-PCs were significantly influenced in bags with both initial inocula of 10 and 100 CFU.

Flow cytometry indicating platelet procoagulant status and platelet degranulation are presented in Figure 24A and B, respectively. Phosphatidylserine exposure increased significantly by Day 3 in the PAS-PCs that had been spiked with 100 and 10 CFU/bag *S. liquefaciens* compared to negative controls (P = 0.01 and 0.003, respectively; Figure 24A). As well, surface CD62P expression was significantly higher by Day 2 in PAS-PCs corresponding to the higher initial inoculum of *S. liquefaciens* (P = 0.04) or on Day 3, for samples with an originally lower initial inoculum (P = 0.03; Figure 24B). Due to decreased sample quality, CD62P expression could not be reliably quantified beyond Day 2 from PAS-PCs which were initially inoculated with 100 CFU/bag *S. liquefaciens*. Plasma-PCs containing *S. liquefaciens* only showed significant CD62P surface expression by Day 3 and only in the sample inoculated with the higher initial CFU count (P = 0.05; Figure 24B).

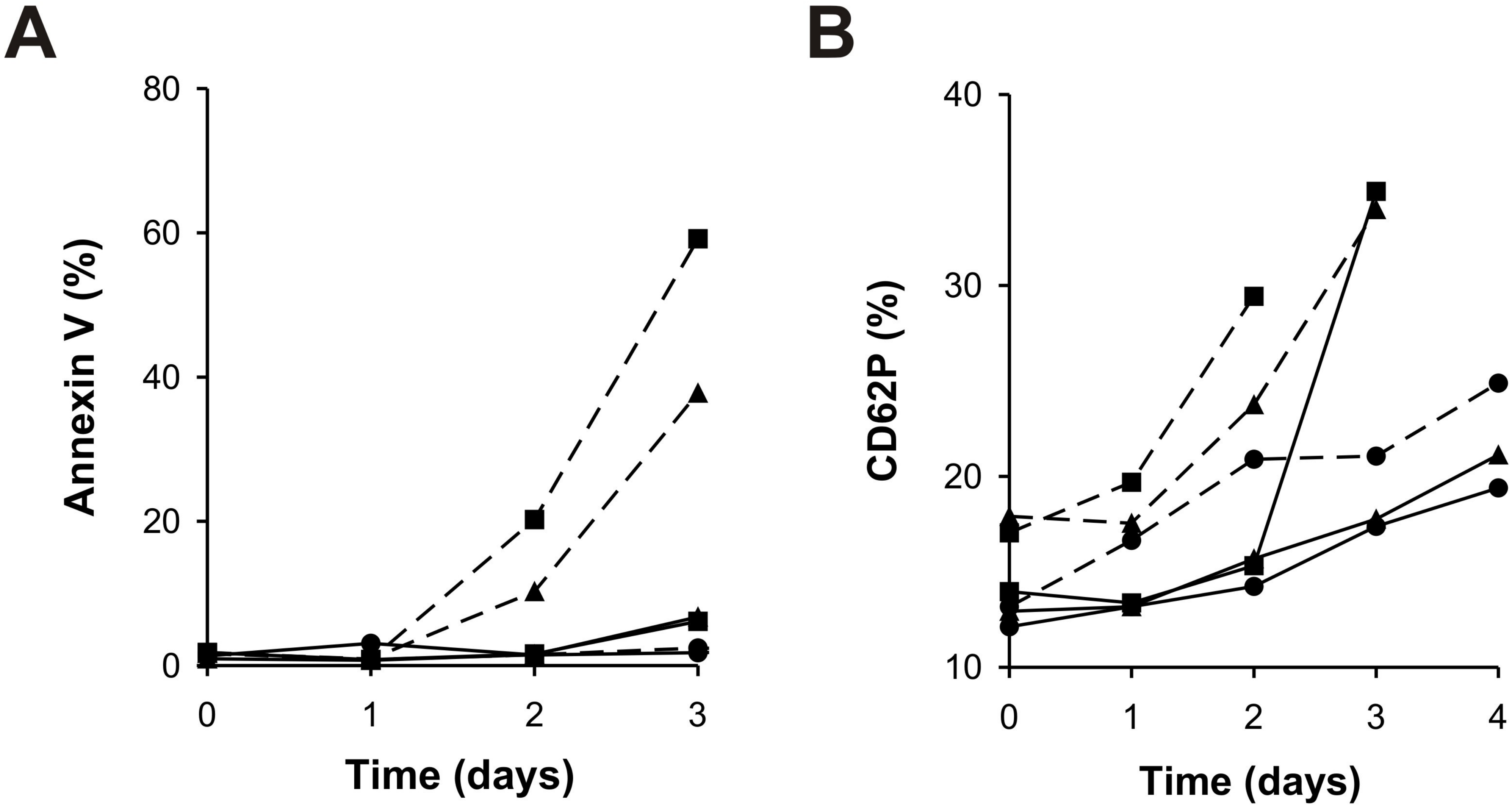

A
B
Annexin V (%)
80
60
40
20
0
0
1
2
3
Time (days)
CD62P (%)
40
30
20
10
0
1
2
3
4
Time (days)

Figure 24. Platelet surface annexin V binding (A) and CD62P externalization (B) induced by incubation with *S. liquefaciens* grown in plasma-PCs (—) or PAS-PCs (– – –). Annexin V binding was used to detect membrane surface phosphatidylserine and platelet surface expression of CD62P served as a degranulation marker. Fluorescence flow cytometric data from five experiments using nonspiked units (●) or units spiked with either 10 CFU/bag (▲) or 100 CFU/bag (■) are shown.

6. RESULTS: OBJECTIVE 4

The effect of platelet PEGylation on bacterial biofilm formation was assessed, and the resultant findings are the subject of this section.

6.1 PLATELET SURFACE MODIFICATION WITH PEG FOR PREVENTION OF *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILM FORMATION

To determine if PEGylation decreases bacteria-platelet attachment, PCs were diluted 1:1 (vol:vol) with either methoxy (m)PEG in mPEG buffer, or mPEG buffer alone and inoculated with biofilm-positive or -negative *S. epidermidis* strains. Prior to inoculation, flow cytometry was used to confirm platelet surface shielding by mPEG, and minimal detection of surface marker CD9 was observed subsequent to grafting (Figure 25). After 3-4 days, small particulates appeared in the non-PEGylated PCs inoculated with either strain. Non-inoculated controls and the inoculated, PEGylated PCs retained uniform appearances over the 5-day duration, although a few replicates containing non-PEGylated, non-inoculated samples showed minute aggregates after 5 days. A high proportion of discoid platelets were still observed in the PEGylated PCs after 5 days (Figure 26A, i), while the non-PEGylated counterpart displayed moderate or extensive platelet activation (Figure 26A, ii). In the PEGylated samples that had been inoculated with *S. epidermidis*, the increase in platelet activation and microaggregation was minimal, and evidence of clumping or biofilm formation was scarce (Figure 26A, iii). By comparison, macroscopic debris was present in the non-PEGylated, but otherwise equivalent samples (Figure 26A, iv). Biofilm formation by *S. epidermidis* was significantly reduced in PEGylated PCs compared to non-PEGylated samples for both biofilm-negative and -positive strains (P = 0.02 and 0.04, respectively; Figure 26B).

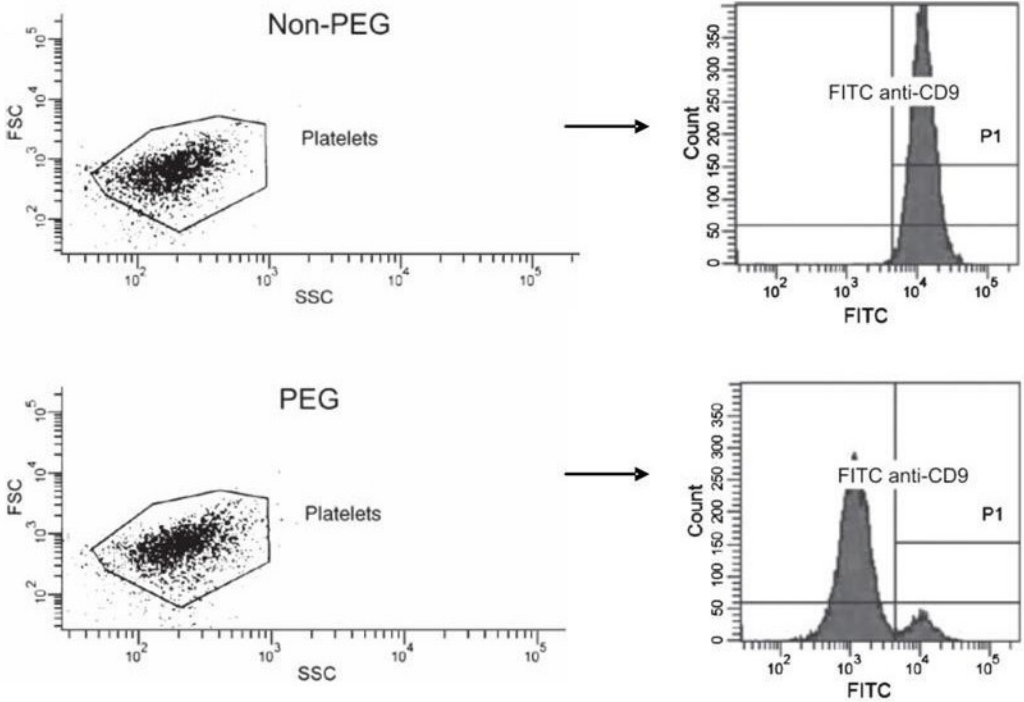

Non-PEG
FSC
SSC
Platelets
FITC anti-CD9
P1
Count
FITC
PEG
FSC
SSC
Platelets
FITC anti-CD9
P1
Count
FITC

Figure 25. FACS analysis of PEGylated (PEG) and non-PEGylated (Non-PEG) platelets. Purified platelets were stained with anti-CD9-FITC-conjugated antibody, then analysed for CD9 shielding using a BD FACS Canto II flow cytometer and accompanying software. (A) Forward scatter (FSC) and side scatter (SSC) identifying the platelet population. (B) Histograms showing FITC log intensity.

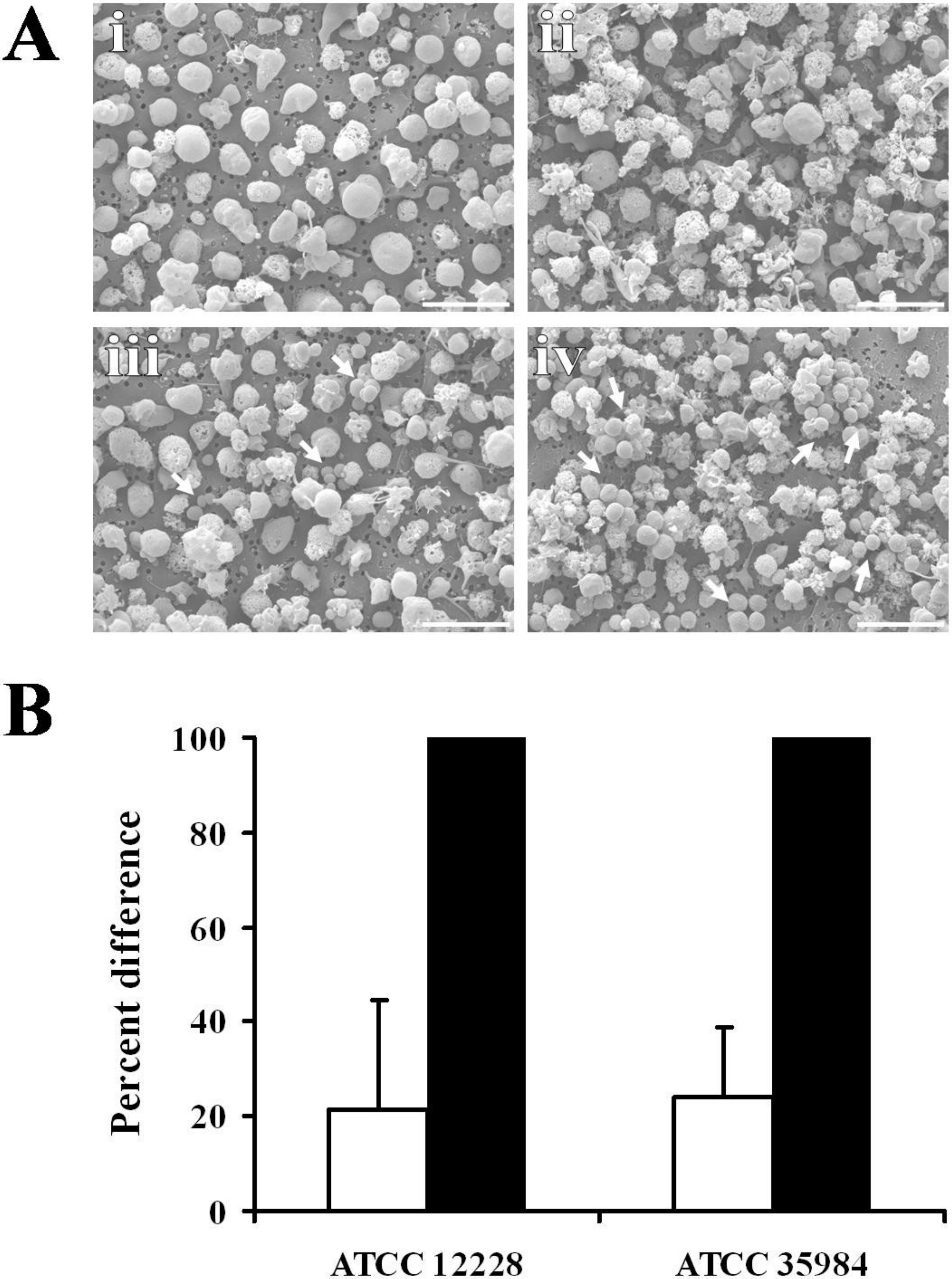

A
i
ii
iii
iv
B
100
80
60
40
20
0
Percent difference
ATCC 12228
ATCC 35984

Figure 26. Effect of platelet PEGylation on platelet aggregation and biofilm formation. Panel (A): Scanning electron micrographs of PEGylated (i, iii) and non-PEGylated (ii, iv) PCs stored for five days. Biofilm-positive *S. epidermidis* strain ATCC 35984 was inoculated into samples shown in (iii, iv) on day 0. Scale bars are all 5 µm. Arrows point to bacterial cells. Panel (B): Day 5 biofilms of *S. epidermidis* ATCC 35984 or *S. epidermidis* ATCC 12228 (biofilm negative) grown in PEGylated PCs were stained with a 0.3% crystal violet solution and quantified by microplate reading of eluted stain at λ = 492 nm (white bars, n = 8), with normalization to biofilms grown in non-PEGylated PCs (black bars, n = 8).

7. RESULTS: OBJECTIVE 5

The development and application of anti-*S. epidermidis* antibodies for distinguishing differential protein expression in biofilm culture versus standard culture is described in this section.

7.1 EXPRESSION OF *STAPHYLOCOCCUS EPIDERMIDIS* PROTEINS ASSOCIATED WITH CELL DIVISION AND BIOFILM FORMATION

Genes encoding *S. epidermidis* proteins AtlE, FtsZ, and Gmk were successfully cloned for the purpose of antibody production with the assistance of undergraduate students. Plasmids carrying these genes and *E. coli* expression strains facilitating optimal protein expression were selected for downstream purification (Table 3). Specifically, *E. coli* C43(DE3) was found to be amenable to high yield expression of all protein types within 2-3 h post-induction with IPTG (Appendix 11.6.4). Recombinant proteins were successfully purified by Cobalt affinity chromatography (Talon®; Appendix 11.6.5) with typical yields of approximately 1 mg AtlE, 3 mg DivIVA, 4 mg FtsZ, and 12 mg Gmk per litre of induced culture, as determined by the Bradford assay. Rabbit immunizations led to development of immune sera rich in polyclonal IgG specific for the corresponding antigen. Immunoaffinity purification of the IgGs from immune sera was commissioned from SACRI Antibody Services (Calgary, AB). This also included purification of IgG against *S. epidermidis* DivIVA from immune sera obtained through previous work in our laboratory. Alternatively, polyclonal IgG specific for *S. epidermidis* SdrG was commissioned separately (Genscript Corp., Piscataway, NJ). The specificity of these antibodies to purified *S. epidermidis* recombinant protein was confirmed. Total soluble protein extracts from *S. epidermidis* were used to determine the optimal working concentrations of IgG for use as primary antibodies in Western blot analyses. These concentrations were determined to be between 1:500 and 1:1000 for DivIVA, AtlE, Gmk, and SdrG, and between 1:1000 and 1:2000 for FtsZ.

Using equal concentrations of total protein from biofilm and planktonic cultures of biofilm-positive *S. epidermidis* strain O-47, expression levels of the aforementioned proteins were compared from three separate experiments (Figure 27). It can be seen that only AtlE exhibited a significant increase in expression during biofilm growth. Differences in expression of the other markers were not significant, as determined by densitometry. The development of these antibodies has resulted in specific and sensitive probes which detect the corresponding protein in *S. epidermidis* planktonic and biofilm cultures in TSBg growth media. Due to excessive human protein presence in the environment of PRP, Western blots could not be used to elucidate *S. epidermidis* proteins when the bacteria were inoculated into PCs.

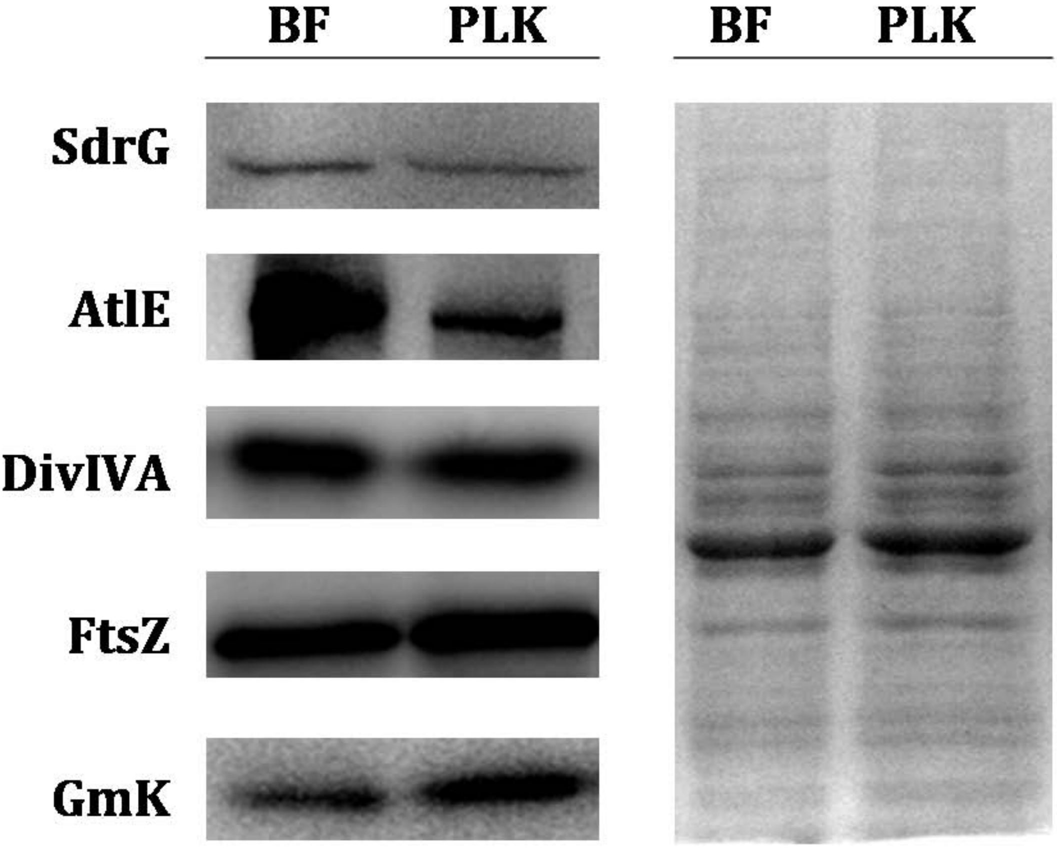

BF
PLK
BF
PLK
SdrG
AtlE
DivIVA
FtsZ
GmK

Figure 27. Comparative expression of SdrG, AtlE, FtsZ, DivIVA and Gmk in biofilm (BF) versus planktonic (PLK) *S. epidermidis* cell lysates. Total soluble protein from strain O-47 was harvested by FastPrep® mechanical lysis. Total protein concentration was spectrophotometrically determined prior to electrophoresis. Immunoblots are representative of at least 3 repetitions per primary IgG. A representative SDS-polyacrylamide gel of total *S. epidermidis* protein is displayed adjacent to the blots for confirmation of gel-loading accuracy.

8. DISCUSSION

Routine sample screening has drastically reduced the incidence of transfusion of contaminated PCs, and resultant ATR events (Benjamin and Mintz, 2005; Palavecino et al., 2010). However, approximately 1 in 100,000 units present sufficient contamination to result in clinical septic reactions, despite the use of sensitive detection measures (Walther-Wenke, 2008; Eder et al., 2009; Ramirez-Arcos, 2010a). More recently, the emergence of chemical and photochemical pathogen inactivation technologies assures that all units are treated as if contaminated and remedies the problem while reducing dependence on automated detection (Pineda et al., 2006; Cazenave et al., 2011). Despite these improvements, transfusion research had yet to consider that biofilm formation during PC storage was a strong possibility, since it is now known to be the default mode of bacterial growth, and free-floating cells exist less frequently in nature (O'Toole et al., 2000). It is hypothesized that biofilm formation results in non-uniform bacterial distribution within a contaminated blood component, likely contributing to missed bacterial detection (Figure 6D). It is important to emphasize that *S. epidermidis* biofilm formation is characterized by a decrease in growth rate (Cramton and Götz, 2003), which might also contribute to the characteristic slow growth displayed by *S. epidermidis* during PC storage. As previously mentioned in the literature, slow growth is a commonly proposed explanation for missed detection (Murphy et al., 2008; Figure 6C).

The work performed in this thesis confirms that biofilm formation in PCs can occur, using *S. epidermidis* as the major model microorganism. Characterization of staphylococcal strains obtained from contaminated PC units revealed inherent biofilm-forming ability in select isolates, confirming relevance to transfusion safety. Furthermore, the applications of platelet PEGylation and supplementation with PAS were presented as two means by which biofilm formation can be at least partially abrogated in a platelet storage environment (Figure 28).

8.1 PROMOTION OF GROWTH AND BIOFILM FORMATION BY THE PC STORAGE ENVIRONMENT

Several pathogenic bacterial species have proven to be adept at forming biofilms on polymeric materials, including *S. epidermidis, S. aureus, Pseudomonas aeruginosa, E. coli* and *S. marcescens* (Cramton and Götz, 2003; Ketyi, 1995; Yu et al., 1996; Yousefi et al., 1998; Jones et al., 2006). This thesis demonstrates that *S. epidermidis* is capable of forming biofilms with bacteria adhering to the plastic of platelet storage bags and to platelet aggregates in an environment representative of platelet storage conditions. *S. liquefaciens* propensity for biofilm formation on platelet bags was also confirmed.

S. epidermidis biofilms were reduced in PPP compared to PRP (Figure 11), and pre-conditioning the platelet storage containers with PCs facilitated adherence of *S. epidermidis* (Figure 14). These results demonstrate that increased availability of bridging materials such as plasma proteins (eg, fibrinogen) and/or platelets is associated with increased biofilm formation (Figure 28A). It is possible that the adequate yet suboptimal conditions for bacterial growth provided in the platelet storage environment could also be important factors for inducing biofilm formation. It has been reported that strains of *S. epidermidis* and *S. marcescens* isolated from clinical samples or involved in transfusion reactions present increased ability to adhere to the tubing of blood bags sets (Parment et al., 1993). Although any plastic-attached bacteria would be subjected to the stress of agitation which is required for platelet storage, a previous study showed that *S.*

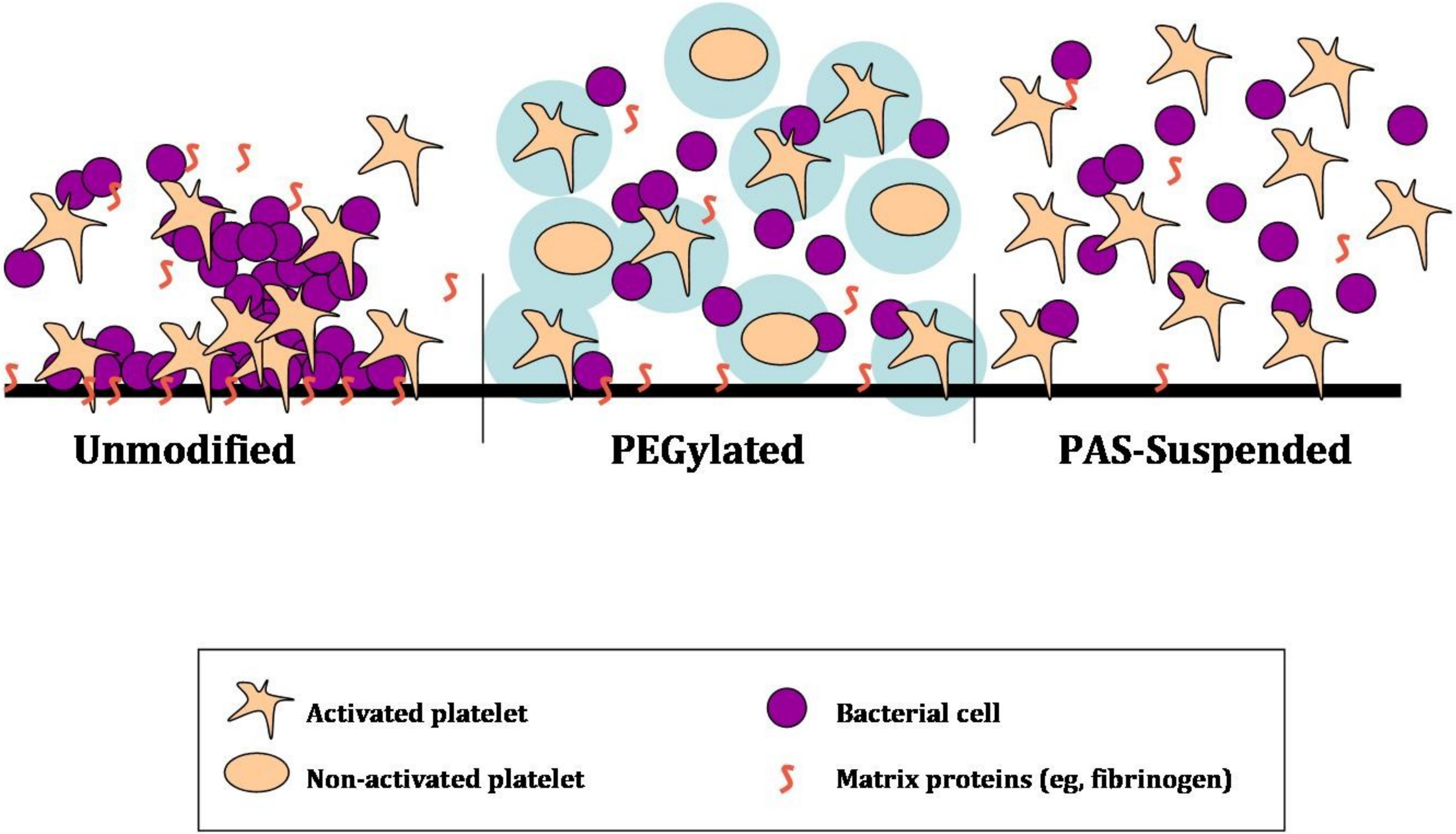

Unmodified
PEGylated
PAS-Suspended
Activated platelet
Bacterial cell
Non-activated platelet
Matrix proteins (eg, fibrinogen)

Figure 28. Model of bacterial biofilm formation in stored PCs, and proposed mechanisms of biofilm reduction by platelet PEGylation, and the use of platelet additive solution (PAS).

epidermidis adhesion to plastic is independent of shear stress in 1% PPP (Post, 2003). It should be noted that platelet bags are often agitated between the hands immediately before transfusion to encourage product uniformity. This process could dislodge biofilm cells and facilitate transmission of the contaminant microorganism.

During platelet storage, *S. epidermidis* grew on plastic situated at the air-liquid interface which is a hallmark of biofilm formation. The ideal pH and sufficient glucose concentration present in a typical, indate platelet unit are sufficient for survival and growth by this species. Glucose is required for the synthesis of *N*-acetylglucosamine, which is a building block of the polysaccharide secreted by biofilm-positive strains to establish an extracellular biofilm matrix (Gerke et al., 1998; Mack et al., 1992). Since glucose has been shown to stimulate *S. epidermidis* adherence to plastic materials and to promote biofilm formation (Mack et al., 1992), glucose present in platelet units may promote biofilm formation of slime-producing contaminants, in addition to being a basic food source for the bacteria.

While initial studies of *S. epidermidis* growth in PCs prepared by the PRP method showed significant fluctuations in glucose and pH (Section 3.2), pH and glucose fluctuations were not significant indicators of *S. epidermidis* presence in the buffy-coat PAS-PCs or plasma-PCs (Table 5). However, the former research was conducted with higher initial inocula (10-100 CFU/mL) compared to the PAS studies (0.2-2 CFU/mL), resulting in more rapid proliferation and concomitant depletion of platelet quality. Furthermore, incubations in the former study were performed in tissue culture plates instead of platelet bags, and the higher O_2 saturation attributed to the first scenario would result in increased platelet metabolism, including more rapid glucose consumption and acidification of the corresponding medium.

The growth of *S. epidermidis* in PAS-PCs is not altered significantly compared to its growth in plasma-PCs. Both environments support bacterial proliferation due to the contribution of platelet metabolism to the maintenance of a low pH. Specifically, a pH of ~ 6.8-7.2 is maintained in PCs due to platelet metabolism, which includes the conversion of glucose to lactic acid, and the evolution of CO_2 *via* the tricarboxylic acid cycle (Baker et al., 2006). The growth of *S. epidermidis* in plasma can only be achieved by the maintenance of pH at typical PC values (Störmer et al., 2008). This explains why *S. epidermidis* numbers were essentially static in the absence of platelets (Figure 21), and is consistent with previous findings using a *S. epidermidis* initial inocula of less than 2 CFU/mL (Störmer et al., 2008). The pH of stored PCs is consistent with human skin pH to which CoNS are adapted. In contrast, *S. liquefaciens* is not necessarily adapted to human skin pH, and so is not hindered by a pH in excess of 7.5.

Furthermore, release of platelet internal iron reserves could contribute to the different growth patterns of *S. epidermidis* in PCs compared to their growth in PPP and TSBg. Once platelets have lysed, ferritin stores are released and can provide an additional source of iron for the bacteria (Infante et al., 2007). As well, release of iron from the carrier molecule transferrin, which is present in plasma, can provide an additional source of this element. Previous studies have shown that citrated anticoagulants encourage staphylococcal growth in platelet concentrates by mobilizing transferrin-bound iron which is then utilized by the bacteria (Matinaho and Parkkinen, 2005). Anticoagulants are essential to prevent premature platelet aggregation, and their use in PC component production cannot be avoided; however, supplementation with sodium bicarbonate at levels greater than 0.5 % has been shown to reverse the process of citrate-induced iron release, effectively inhibiting growth of *S. epidermidis* (Matinaho et al., 2005).

8.1.1 CoNS Characterized for Biofilm-Forming Ability

Three out of thirteen (23%) CoNS strains isolated from contaminated PCs during a period of 16 months have emerged which consistently presented a propensity for biofilm formation. Two of the three strains were true positive platelet contaminants while the third strain could not be confirmed as a true positive, although both the initial and secondary culture bottles were positive. The secondary (confirmatory) culture was discarded before bacterial identification was performed. These three *S. epidermidis* strains comprise the only isolates which were positive for production of slime, the presence of the *icaD* gene marker, production of PIA, and biofilm formation in rich media and platelets as measured by crystal violet staining (Table 4). The Congo red agar test used to assess slime production and the crystal violet biofilm staining assay are standard methods which are applied for qualitative and semi-quantitative assessment of biofilm formation by clinical CoNS isolates, respectively. Specifically, these tests have been used for CoNS implicated in ocular infections (Duggirala et al., 2007), bovine mastitis (Oliveira et al., 2006), catheter-related infections (Rohde et al., 2004; Petrelli et al., 2006; Touati et al., 2007) neonatal septic events (de Silva et al., 2002; Klingenberg et al., 2005), as well as for determination of the biofilm-forming potentials of CoNS commensals obtained from healthy individuals (Arciola et al., 2003; Satorres and Alcaraz, 2007). The present research demonstrates that these techniques are also reliable for classification of biofilm producer *S. epidermidis* strains isolated from contaminated

PCs due to consistency with the alternative biofilm assessment methods employed herein. In particular, the *ica* loci of the *icaD*-positive isolates were verified to be functional by the presence of PIA using indirect immunofluorescence. Production of PIA is often associated with *S. epidermidis* biofilms. However, this species can also exhibit alternative mechanisms of biofilm formation, such as proteinaceous biofilm construction with accumulation-associated proteins (Rohde et al., 2005; Hennig et al., 2007; Banner et al., 2007; Rohde et al., 2007) and extracellular teichoic acids (Kogan et al., 2006). These PIA-independent biofilm formation mechanisms may explain why *S. epidermidis* isolate 07045 was a strong biofilm producer based on the crystal violate assay despite lack of Congo red positivity, PIA production and *icaD* amplification (Table 4).

8.1.2 *S. epidermidis* Expression of Growth and Biofilm-Associated Proteins

Several molecular factors have been implicated in *S. epidermidis* growth and biofilm formation, and in order to investigate this phenomenon further in a platelet storage environment, production of growth and biofilm-associated biomolecules in this environment must be addressed. The indirect immunofluorescence assay which was used for PIA detection may hold promise for distinguishing PIA production directly from contaminated PCs, which would circumvent the need for standard strain culture prior to conducting immunofluorescence-based diagnostics. However, the blocking action of plasma proteins presents a challenge for sensitive, specific detection by this means.

As well, the production of anti-staphylococcal immune sera in rabbits constitutes a valuble tool for downstream investigations of growth and biofilm formation at the molecular level. IgG raised against *S. epidermidis* cell division proteins (DivIVA, FtsZ), biofilm proteins (AtlE, SdrG) and a housekeeping protein (Gmk) were purified and optimized for expression studies in biofilm cultures. Immunodetection of *S. epidermidis* protein expression using the aforementioned IgGs revealed no difference in levels of soluble Gmk, SdrG, FtsZ and DivIVA proteins when grown in biofilm culture compared to standard, planktonic growth (Figure 27). Stable expression of the *gmk* transcript has been well documented (Vandecasteele et al., 2001), and this study provides support for similar stability of expression at the protein level. Although *gmk* has been identified as a control for *S. epidermidis* gene transcript expression in a variety of conditions (Vandecasteele et al., 2001), no documentation appears to exist for its application at the protein expression level prior to this work.

SdrG expression has been shown to increase *in vivo* in the presence of serum (Sellman et al., 2008). Since the present studies employed traditional growth media, no matrix molecules or other human serum factors were present to promote expression of this known fibrinogen binding receptor. While SdrG may facilitate initial attachment in biofilm formation on biotic surfaces, its expression does seem to be influenced by growth on abiotic substrates such as the polystyrene tissue culture plates used in this study. Alternatively, the observed upregulation in AtlE expression during biofilm growth was not surprising, since it is already documented to have a positive role in plastic surface attachment (Heilmann et al., 1997; Qin et al., 2007).

Previous work has indicated that cytosolic levels of the cell division protein FtsZ remain constant despite a change in bacterial doubling rate over time (Weart and Levin, 2003). The present finding that FtsZ levels do not change during biofilm growth is thus highly plausible, and provides further support for the concept that localization-based regulation of cell division supersedes FtsZ concentration dependence. Expression of the other *dcw* protein studied, DivIVA, was also shown to remain constant irrespective of biofilm formation. Investigations of DivIVA expression in the literature are scant; however, work done on *E. faecalis* has demonstrated that overexpression of the protein results in aberrant cell morphology (Ramirez-Arcos et al., 2005). Valbuena et al. (2006) have also demonstrated DivIVA concentration dependence for normal cell division since partial disruption of the cell division protein FstI in *Corynebacterium glutamicum* resulted in significant increased expression of DivIVA. Preliminary work in our laboratory has shown that *S. epidermidis* DivIVA is dispensable for cell division, but the conserved nature of this protein suggests some as-of-yet unidentified functionality, for which there is ongoing exploration.

8.2 RELEVANCE OF THESIS RESEARCH TO TRANSFUSION SAFETY

The observed slow growth of *S. epidermidis* planktonic cells in PCs can explain missed sampling despite a 24-h incubation prior to culture, as suggested in Figure 6C. This is consistent with the data from previous reports (Blajchman et al., 2004; Ezuki et al., 2007; Störmer et al., 2008), and this theory as it pertains to missed detection is not unique (Mohr, 2006; Murphy et al., 2008). However, it is possible that the missed detection of this species can, in some instances, be attributed to biofilm formation on the inner walls of platelet bags (Figure 6D). This theory can be extrapolated from reported instances where catheter tips colonized by staphylococcal biofilms have yielded negative blood cultures (Ruhe and Menon, 2006). Research in the Ramirez laboratory is in progress to verify whether bacterial biofilm formation increases time to detection with the BacT/ALERT® 3D system (bioMérieux).

This thesis research retains novelty as the first detailed analysis of bacterial biofilm formation during the storage of a blood product. Furthermore, it has been the only work to-date to explore the effect of PC modifications on biofilm formation, and the originality of this research is evidenced by peer acceptance and publication (Greco et al., 2007, 2008, 2010, and 2011). Shortly after release of the first two articles, the bio-imaging system µFinder (Asahi Breweries) was applied to empirically analyze *S. epidermidis* growth dynamics in stored PCs (Motoyama et al., 2009). Microcolony formation inherent to early-stage biofilm growth was detected with this instrument, which provides further support for the hypothesis that biofilm formation by bacterial contaminants is relevant to transfusion medicine. The Ramirez laboratory has since been actively involved in assessing the biofilm-forming abilities of additional CoNS and *Serratia* species isolated from contaminated PCs (Ali et al., 2010; Greco-Stewart et al., 2011). Despite the relatively limited work in this arena, the importance of biofilm formation to transfusion medicine has also been underscored in the review of Savini et al. (2010), who propose novel biofilm treatment strategies for transfusion medicine based largely on the published findings accrued during research for this thesis (Greco et al., 2007). The work presented herein was also a key inspiration for an editorial presented in the November 2010 issue of *Transfusion* which highlights the importance of biofilms in consideration of changing transfusion practices (Yomtovian and Jacobs, 2010). In addition to being featured in the *Washington Post*, AABB News Briefs, and providing the basis for the July 2007 cover image for the journal *Tranfusion*, this work has been cited in several other publications (see Citation Index, Appendix 11.8).

8.2.1 Impact of PAS on Bacterial Growth and Biofilm Formation

The forseen implementation of PAS for the reconstitution of PCs instigated this and other studies of bacterial growth dynamics to be able to predict the nature of future contamination scenarios (Dumont et al., 2009 and 2011). In particular, Dumont and colleagues assessed the growth rates of a panel of bacteria when inoculated separately into PCs suspended in the additive solution Intersol® (Fenwal) at clinically-relevant concentrations. The maximum bacterial concentrations achieved were similar in PAS-PCs and plasma-PCs (Dumont et al., 2011), which is consistent with the trends displayed in this thesis using the PAS SSP+ (MacoPharma). It was also found that most bacteria experienced a shorter lag period in PAS-PCs as compared to plasma-PCs (Dumont et al., 2011), akin to the present observations of *S. liquefaciens* growth in comparable media. In the former study, growth rates of *S. epidermidis* varied greatly between two replicate experiments (Dumont et al., 2011), with the results of one trial being similar to those presented herein. In this thesis, the effect of PAS on bacterial biofilm formation was also investigated, and simultaneous measurements of PC quality in PAS were made.

It was found that both *S. epidermidis* and *S. liquefaciens* had a greater propensity for biofilm formation in plasma-PCs than in PAS-PCs. In contrast to plasma, PAS does not contribute to the deposition of matrix proteins which support bacterial surface attachment on the bag walls (Cox, 2009; Brennan et al., 2009). Consequently, a greater concentration of bacteria can multiply in the supernatant where they are accessible for counting. A model for this proposed mechanism is presented in Figure 28C. Accordingly, the PAS-based platelet storage environment enabled quicker visible detection of contamination in *S. liquefaciens*-inoculated PCs compared to plasma-PCs contaminated with the same inocula (Figure 19). The apparent slower growth rate of *S. liquefaciens* in plasma-PCs may be due to greater proliferation in biofilms on the walls of the storage bags, which could not be collected *via* the sampling port. Motoyama et al. (2009) have shown that although colony counts for *S. epidermidis* remain stable during early platelet storage (< 50 h), the bacteria form microcolonies during this "pseudo" lag phase. This supports the hypothesis that biofilms formed during early PC storage may be linked to decreased CFU availability for counting and/or missed detection (Figure 6D). In order to confirm whether the observed significant difference in growth rate of *S. liquefaciens* in PAS-PCs versus plasma-PCs is valid from a scientific standpoint, the sum of the biofilm and free-floating bacteria would need to be quantified. However, this study was primarily designed to target detectable differences in bacterial growth and platelet parameters which would be of value from a clinical perspective for improved bacterial screening. Since *S. liquefaciens* planktonic titers at stationary phase were similar in both PAS-PCs and plasma-PCs, it can be expected that endotoxin levels would be similar as well. Thus, neither medium would be safer than the other when bacterial concentrations are high.

It was previously discussed that pH fluctuations were not significant for PCs contaminated with 2 CFU/mL or less of *S. epidermidis*. The futility of pH measurement for detection of *S. epidermidis* in PCs was recently accentuated by Barker et al. (2010). This group showed that the rate of change of pH in contaminated PCs was a more effective indicator of bacterial contamination than pH change alone. However, from the panel of bacteria tested, *S. epidermidis* was identified as the sole microorganism that still could not be reliably detected by this improved detection method (Barker et al., 2010).

A parallel reduction of pH, pO_2, and glucose levels with *S. liquefaciens* proliferation was observed in PAC-PCs and plasma-PCs. Similarly, CO_2 generation was positively correlated with the early stationary phase of growth of this bacterium. CD62P expression and Annexin V binding were modest indicators of platelet contamination, with aberrations detected approximately 1-2 days before platelet aggregation and biofilm formation caused by *S. liquefaciens* became visible. These indicators were not influenced by the presence of *S. epidermidis*, even at concentrations as high as 10^8 CFU/mL. These findings have ultimately provided insight to the influence of bacteria on platelet quality indicators in the presence of PAS. Ultimately, it was found that culture-based bacterial detection is still more sensitive than monitoring platelet quality parameters to identify bacterial contamination.

8.2.2 Impact of PEG on *S. epidermidis* Biofilm Formation

The effect of platelet PEGylation on bacterial biofilm formation was also investigated. Platelet-bacteria adhesion and biofilm formation by *S. epidermidis* was significantly reduced in PEGylated PCs. In a recent study, platelet-sized latex particles (1.2 μm) were covalently modified with mPEG, resulting in neutralization of ~ 80% of particle surface charges and a reduction of more than 80% of surface interactions with plasma proteins (Le and Scott, 2010). Also, mPEG was proven to effectively camouflage antigens ranging from 2-13 nm in length on the surface of blood cells (Le and Scott, 2010). These results, coupled with the findings of the present study, support the inference that platelet surface modification with mPEG can be applied to reduce bacterial binding to platelet surface molecules so that platelet attachment and biofilm formation in PCs is minimized. A model for PC PEGylation-associated biofilm prevention is presented in Figure 28B.

Platelets bind bacteria via direct and indirect mechanisms, and several platelet binding proteins have been identified in staphylococci which induce platelet aggregation (Brennan, 2009). Of interest, *S. epidermidis* fibrinogen binding protein SdrG mediates platelet attachment indirectly by bridging with fibrinogen to platelet surface glycoprotein GPIIb/IIIa (Brennan et al., 2009). This molecule normally undergoes a conformational change upon platelet activation, leading to fibrinogen binding site exposure which facilitates downstream signalling causing platelet aggregation (Coller and Shattil, 2009). Thus, platelet PEGylation is expected to not only prevent docking of contaminant microorganisms, but also to inhibit bacterial-induced platelet aggregation (Figure 28B).

Prevention of bacteria-platelet binding would ensure a more uniform bacterial distribution within the platelet unit during contamination, which would reduce instances of missed detection. Patents were filed in Canada (CA2615637), the United States (US20080171024) and Europe (EP1935426) in 2007 describing the use of platelet PEGylation for inhibition of bacterial biofilm formation.

8.3 CLINICAL RELEVANCE OF BIOFILM FORMATION IN PCs

S. epidermidis virulence is based on its ability to establish chronic infections through the formation of biofilms which consist of slow-growing antibiotic-resistant cells that are able to evade the immune system (Cramton and Götz, 2003). The ability of *S. epidermidis* to form biofilms on living cells has been demonstrated in manifestations such as otitis media, sinusitis, and lung infections in patients with cystic fibrosis (Hall-Stoodley et al., 2001; Perloff and Palmer, 2005; Costerton, 2001). It has been shown previously that *S. epidermidis* biofilms can form in the presence of 10 % PRP (Lyte et al., 2003); however, biofilm formation within transfusable PCs was an undescribed phenomenon. The data presented here provide evidence that biofilm formation can occur during PC storage, which would increase the risk of transfusing contaminated units.

Biofilm formation by *S. epidermidis* relies heavily on the transcription of the *icaADBC* locus (Mack et al., 2004; Zhang et al., 2003). Gene products of this locus are responsible for the synthesis, transport, and chemical modification of PIA, which is a key component of the biofilm extracellular matrix of this bacterium (Cramton and Götz, 2003; Mack et al., 2004). *S. epidermidis* ATCC 35984 is an *icaADBC*-positive strain which has been classified in the literature as a strong biofilm producer (Christensen et al., 1985), while *S. epidermidis* ATCC 12228, which lacks the *ica* operon, is generally referred to as biofilm-negative (Arciola et al., 2004; Zhang et al., 2003). It has been shown herein that, despite its limited ability to form biofilms in standard culture media, *S. epidermidis* ATTC 12228 forms surface-attached aggregates in the presence of platelets, substantially mimicking the biofilms observed with the biofilm-producing strain (Figure 10). Almost all CoNS isolates examined in this thesis were also experimentally shown to form biofilms in PCs (Appendix 11.3.3). As mentioned previously, *ica*-negative strains may possess alternative mechanisms for biofilm formation (Chokr et al., 2006; Kogan et al., 2006). Further investigation would provide insight as to whether these mechanisms are uniquely employed by some strains during growth in PCs. The concept that a biofilm-negative strain can form biofilms under platelet storage conditions is troublesome, as it indicates that any *S. epidermidis* isolate found as part of the normal skin flora has this potential. Notably, transfusion of *S. epidermidis* may not result in acute transfusion reactions, since these strains do not induce toxic shock syndromes in contrast to *S. aureus* septic reactions (Zhang et al., 2004). However, biofilm-forming *S. epidermidis* could be responsible for chronic infections that would only be evident a few days after a contaminated PC has been transfused. Consequently, a connection to the transfusion event would not be established.

It is well-documented that the pathogenicity of several bacteria can be linked to their platelet-binding ability (Fitzgerald et al., 2006b; Cox, 2009). The SEM micrographs show a direct interaction between *S. epidermidis* and activated platelets (Figure 12). Research conducted more than a decade ago showed that contact-activated platelets which become bound to biomedical polymers provide a scaffold for *S. epidermidis*, substantiating a mechanism for host-factor mediated device-related infection (Wang et al., 1993a and 1993b). Observations such as those seen in Figure 12 corroborate the proposed event known as circumferential adherence whereby platelets act as covercytes by surrounding the bacteria with the ultimate goal of sequestration (White, 2005; White, 2006). Bacteria sequestered by platelets do not lose viability (White, 2005; White, 2006), and therefore could be transported into the bloodstream from a contaminated platelet unit by this route.

8.3.1 Implications of PC Contamination with Biofilm-Positive CoNS

Confirmation that *S. epidermidis* can form biofilms during platelet storage indicates that this environment may trigger the expression of a virulent phenotype which could intensify this species' potential to cause post-transfusion disease (Greco et al., 2007). As well, the investigation of CoNS strains isolated by CBS has emphasized that *ica*-positive *S. epidermidis* strains are distributed in a healthy community of donors. Fortunately, the

PCs contaminated with *ica*-positive *S. epidermidis* strains tested positive for contamination during bacterial screening and were not transfused. Thus, we can only speculate as to whether transfusion of an *ica*-positive isolate would cause increased ATR severity compared to transfusion of an *ica*-negative isolate at a comparable titre.

It has been shown that carriage of the *icaADBC* locus actually reduces fitness of *S. epidermidis* found on human skin compared to an isogenic *icaADBC* mutant (Rogers et al., 2008). The authors posit that energy expense associated with PIA production is responsible for the decreased bacterial persistence in this niche, although it remains to be determined whether stunted growth or metabolism was responsible for the observed phenomenon. Since PC contaminants are typically thought to originate from donor skin, the possibility exists that more *ica*-positive isolates are able to avoid disinfection than the *ica*-negative counterparts, although this would need to be confirmed experimentally. Alternatively, the *ica*-positive strains isolated from the contaminated PCs may have only transiently colonized the implicated donors, instead of being part of the normal skin flora. Periodic sampling of donor skin could reveal changes in colonizing populations and clarify whether the biofilm-forming strains are persistent or transient commensals.

If PIA production conferred a beneficial advantage toward proliferation in the PC environment, a difference in growth rate between *S. epidermidis* strains ATCC 12228 and ATCC 35984 would be expected; however, this was not the case (Figure 9). Still, a real contamination scenario is likely characterized by initial bacterial concentrations lower than 10-100 CFU/mL. At lower concentrations, a biofilm phenotype may be more important for increased protection from PC self-sterilization by residual phagocytes and polymorphonuclear leukocytes. The protective effect of PIA against these immune cells has already been documented (Vuong et al., 2004a and 2004b).

8.4 FUTURE DIRECTIONS

The laboratory of Dr. Ramirez continues to screen CoNS for biofilm attributes based on the present work to acquire further evidence for the inherent risk of PC contamination with biofilm positive strains (Ali et al., 2010). Ongoing studies with collaborators include subtyping of CoNS strains isolated from contaminated PCs using technologies such as mass spectrometry and amplified fragment length polymorphisms (Rood et al., 2011). Investigation of the molecular signatures of blood product contaminants provides a useful framework for novel approaches toward detection and inhibition of these microbes prior to transfusion. Future efforts focused on biofilm formation within the environment of blood product storage should be extended to include other relevant microorganisms. In particular, *Yersinia enterocolitica* and *Serratia* species are noted for their ability to proliferate, not only in room temperature-stored PCs, but also at refrigeration temperatures due to their psychrotrophic nature, as indicated in events of whole blood contamination (Ramírez-Arcos et al., 2007a). Considerable knowledge pertaining to biofilm formation by *Yersinia* and *Serratia* species already exists (Atkinson et al., 2006; Van Houdt et al., 2007). However, the relevance of Gram-negative biofilms to transfusion medicine is only now being investigated (Greco et al., 2010). The Ramírez laboratory is currently exploring conditions and factors conducive to *Serratia marcescens* biofilm formation during blood product contamination (Greco-Stewart et al., 2011).

The incidence of *ica*-positive *S. epidermidis* strains present as commensal organisms on healthy individuals is documented to be relatively low (Kozitskaya et al., 2004; de Araujo et al., 2006). Nevertheless, 3 of the 8 *S. epidermidis* contaminants isolated from PCs and assessed in this thesis were shown to carry *icaD* and synthesize PIA. To clarify whether PC contamination by biofilm-positive strains is favoured, an evaluation of the bactericidal efficacy of arm disinfectants chlorhexidine and isopropyl alcohol could be performed for an *ica*-positive *S. epidermidis* strain and its isogenic mutant. This would confirm the protective effect of PIA against disinfection as demonstrated by Adams et al. (2005). Additionally, post-disinfection recovery rates for each strain after platelet inoculation would confirm whether biofilm positivity protects against PC self-sterilization. This proposal comprises the foundation for an upcoming project in the Ramírez laboratory, and will complement current knowledge on the efficacy of skin disinfectant concentrations for removal of biofilm- and non-biofilm-forming isolates present in the community. Importantly, skin disinfectant evaluations often test a broad panel of bacterial species, but most do not consider intraspecies diversity of resistance profiles, often favouring a single laboratory strain with questionable clinical relevance. Clarifying the role of skin disinfection on *ica*-positive strain selection and survival during PC contamination is a major step toward improving the overall safety of PC transfusion.

The findings of this thesis encourage the consideration of steps and materials involved in platelet production which can be altered to reduce instances of biofilm formation during platelet storage. For example, examination of bacterial attachment to different platelet bag plastics currently on the market would help to identify the most suitable polymer for reducing bacterial attachment, similar to investigations performed for hospital materials associated with biofilm-based infection. In terms of platelet storage solutions, SSP+ by MacoPharma was the PAS selected for this study due to product availability to CBS; however, alternative PASs (eg, Composol, Fresenius; PAS-G, Pall Corp.) could be similarly tested for influence on bacterial growth and biofilm formation. As previously discussed, bacterial doubling times in Intersol® (Fenwal)-suspended PCs have already been studied (Dumont et al., 2009 and 2011).

Furthermore, platelet PEGylation presents a novel strategy for reducing biofilm growth during platelet storage. Succinimidyl carbonate (SC) is an effective means for covalently linking peptides to PEG via acylation. SC was favoured over benzotriazole carbonate as an acylating agent due to its longer half-life *in vivo* resulting from greater resistance to hydrolysis (Roberts et al., 2002). However, numerous other linker chemistries exist which may be equally effective for retention of PEG on cell surface molecules, including active esters of PEG carboxylic acids, and aldehyde-mediated coupling via Schiff base chemistry (Roberts et al., 2002). Many alternative PEG derivations exist, and experimentation with different polymer lengths and degree of side-chain cross-linking may improve receptor shielding. Also, detection of shielding of specific moieties involved in bacteria-platelet binding (eg, GPIIbIIIa) by flow cytometry would assist in characterizing the molecular mechanisms enabling PEG-based shielding.

This work appears to be the first published evidence that grafting PEG to a eukaryotic cell surface can be used to reduce bacterial binding (Greco et al., 2011). The use of PEG in this context can have numerous applications in future research pertaining to host-pathogen interactions. Consideration of factors such as molecular stability, shielding efficacy, *in vivo* half-life and economy could be investigated by external laboratories with greater production capacity and resources based on these preliminary results. PEG is FDA-approved and deemed non-toxic and safe for human use in a variety of functions, but has not been validated for use with PCs (Kang et al., 2009). The normal, sustained functioning of platelets would have to be confirmed by *in vivo* studies, first in a mouse model and then in clinical trials, before becoming a reality in transfusion medicine. Studies of platelet hemostatic function and half-life could be modelled after procedures for investigations of platelet function post-PRT treatment (Ostrowski et al., 2011; Cazenave et al., 2011).

An appropriate extension of this work would be to examine PEGylation of platelet bag plastic to reduce biofilm formation during PC storage. This approach is highly plausible given heightened interest and accumulating knowledge pertaining to the design of anti-biofouling coatings for medical devices. PEG-based coatings have already been shown to reduce microbial binding to biomaterials (Norde and Gage, 2004; Nejadnik et al., 2008), and the use of the patented PEG-based coating OptiChem® has been proven effective at minimizing staphylococcal biofilm formation on implantable materials *in vitro* and *in vivo* in a mouse model (Saldarriaga Fernández et al., 2010 and 2011).

Furthermore, the production of antibodies in this thesis has presented a novel tool for studying *S. epidermidis* protein expression in numerous contexts. Since this work was performed using stationary cultures, it would be informative to perform additional assays at various time-points in biofilm growth, including phases of initial attachment and accumulation, to gain a broader understanding of biofilm gene induction and to corroborate the findings in the literature. Flow cell chambers would be a useful platform for standardizing biofilm proliferation over time, but was beyond the scope of this present work. As well, the use of these antibodies was limited to analyzing expression in standard culture media; however, it is hoped the IgGs may be applied to generate an assay for examination of *S. epidermidis* protein regulation within the platelet storage environment. Preliminary research efforts proved that isolation of sufficient detectable quantities of bacterial protein from an environment with high levels of background human protein is a formidable challenge. Methods applied to extract bacteria from PCs prior to protein isolation could address this problem, such as the use of immunomagnetic beads (eg, BUGS'n BEADS^{TM} Technology, NorDiag Inc.). However, consideration would need to be made for the impact of sample processing time prior to lysis on observed protein expression levels.

9. CONCLUSIONS

A summary of the major findings of this thesis is presented in Table 6. Importantly, theoretical risk of biofilm formation during platelet storage was confirmed (Greco et al., 2007), and the actual instance of PC contamination by biofilm-positive *S. epidermidis* strains was determined (Greco et al., 2008). This significantly shifts the paradigm of how bacterial contamination issues are approached in transfusion medicine by emphasizing that studies of bacterial contaminants at the strain level may be more informative to clinical treatment, as relayed in the review by Savini et al. (2010) and the editorial by Yomtovian and Jacobs (2010).

Any future changes to platelet storage protocols should be rigorously evaluated with consideration of issues pertaining to biofilm contamination. The advent of platelet additive solutions (PASs) as an alternative to plasma for PC storage has provided a timely example of how a seemingly routine modification can have a dramatic effect on the growth dynamics of contaminant microorganisms (Greco et al., 2010).

This thesis presents a novel mechanism for minimizing biofilm occurrence during platelet storage: platelet PEGylation (Greco et al., 2011). Since PAS seems to greatly minimize biofilm formation in PCs, it is quite possible that PEG will never be applied to platelet therapy for the sole purpose of biofilm inhibition. However, the former and latter findings of this thesis prove that biofilm formation during platelet storage is relevant, and biofilm abrogation is possible by more than one avenue.

Finally, the IgGs generated in this study have equipped our laboratory with tools that can be applied to clarify bacterial growth and biofilm processes at the molecular level in future research, including studies within the *ex vivo* environment of stored PCs.

Table 6. Summary of major findings.

Hypothesis	Result (Y/N; correlation)	Index
S. epidermidis biofilms formed in the presence of 100% PCs can be quantified by crystal violet staining of plastic adherent cells	Y	Figure 10
S. epidermidis biofilms can be formed:		
• on platelet aggregates	Y	Figure 12
• on platelet bags	Y	Figure 13
• on platelet bags preconditioned with platelets	Y	Figure 14
• on platelet bags containing PCs	Y	Figure 23A
• by a traditionally biofilm-negative strain	Y	Figure 10B
S. epidermidis biofilm formation in 100% PCs is dependent on platelet concentration	Y(+)	Figure 11
CoNS isolates derived from contaminated PCs exist which demonstrate biofilm-forming potential with respect to:		
• Genotype (*icaD* gene presence)	Y	Figure 15B
• Phenotype		
- Crystal violet-based biofilm staining	Y	Figure 17
- Congo red dye accumulation	Y	Section 4.3
- PIA production	Y	Figure 18
The results of this study are in statistical agreement	Y	Section 4.3
The use of PAS as an alternative to plasma as a PC storage medium will influence:		
• Growth of contaminant *S. liquefaciens*	Y(+)	Figure 20A
• Growth of contaminant *S. epidermidis*	N	Figure 20B
• Biofilm formation by contaminant *S. liquefaciens*	Y (-)	Figure 22
• Biofilm formation by contaminant *S. epidermidis*	Y (-)	Figure 22
Platelet quality parameters (O_2, CO_2, glucose, lactate, pH, MPV,		

platelet count, activation, degranulation) will fluctuate significantly in PAS as a result of contamination by:		
• *Staphylococcus epidermidis*		
- 10 CFU/bag initial inocula	N	Section 5.3.1 Table 5
- 100 CFU/bag initial inocula	N	
• *Serratia liquifaciens*		
- 10 CFU/bag initial inocula	Y *	Section 5.3.2 Table 5
- 100 CFU/bag initial inocula	Y *	
Platelet PEGylation effectively reduces:		
• *S. epidermidis*-platelet aggregation	Y	Figure 26A
• *S. epidermidis* biofilm formation	Y	Figure 26B
The following *S. epidermidis* cell division and biofilm proteins are differentially regulated when cells are grown in biofilm culture:		
• DivIVA	N	Figure 27
• FtsZ	N	“
• AtlE	Y	“
• SdrG	N	“
The *S. epidermidis* housekeeping enzyme Gmk is constitutively expressed at similar levels in biofilm and planktonic culture.	Y	Figure 27

Y, yes; N, No

* Excluding MPV, platelet count, lactate and CO_2

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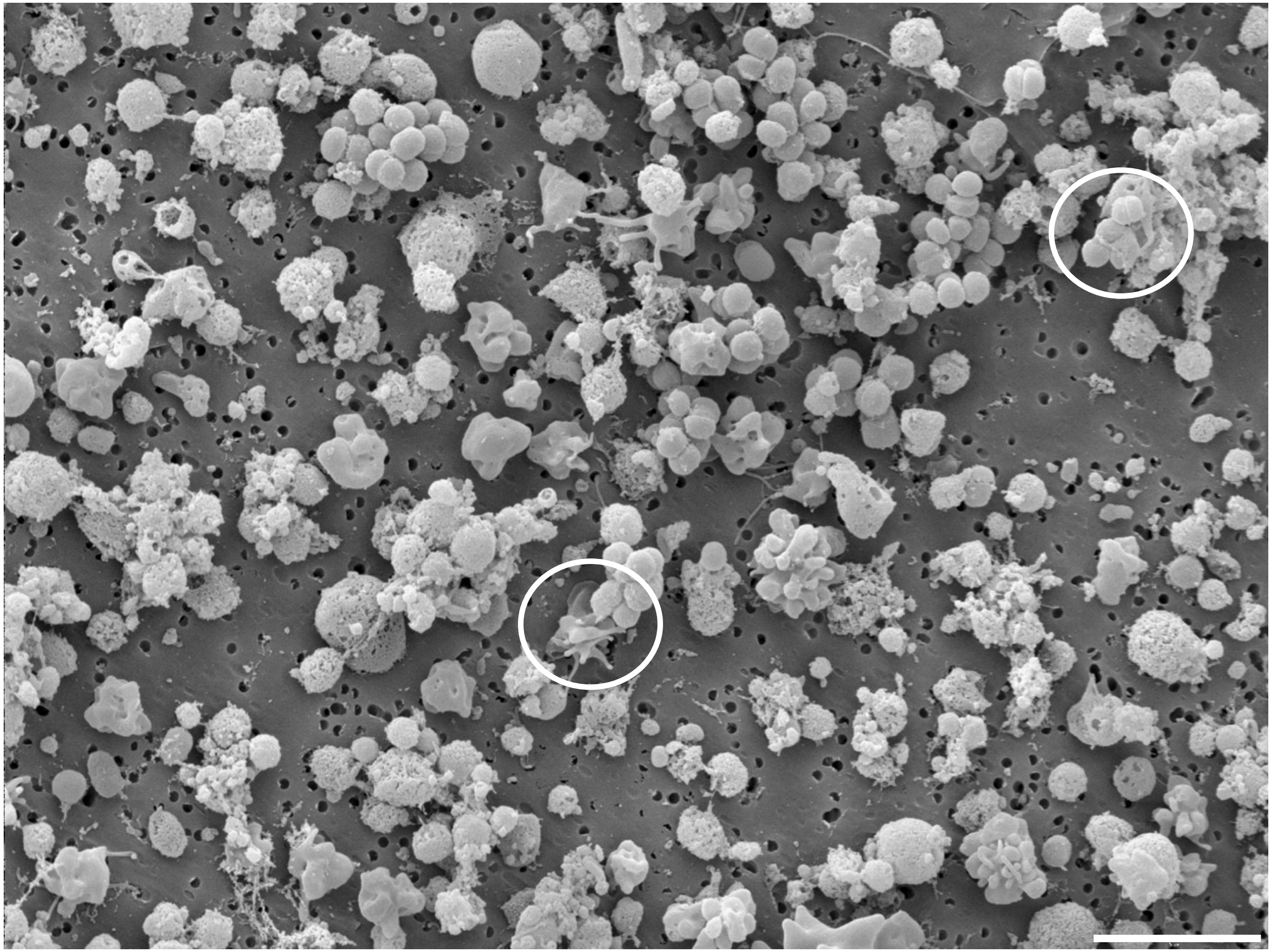
11. APPENDIX

Appendix 11.1.1

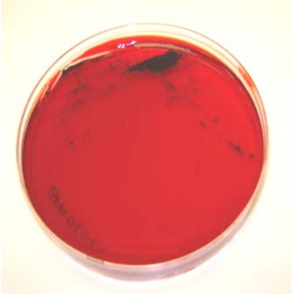


Evidence of *S. epidermidis* ATCC 12228 interaction with platelets (contained in white circles). 3500X magnification. Scale bar is 5 microns.

Appendix 11.2.1

06033

06038

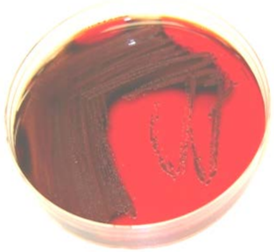


06043

07003

07006

07009



07022

07023

07026

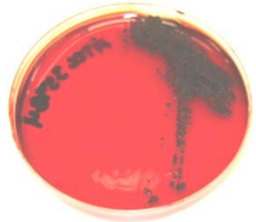
07040

07043

07045

06010

ATCC 12228 (neg)



ATCC 35984 (pos)

Appendix 11.2.2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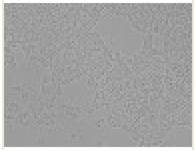
06010_100XFL


06033_100XBF


06033_100XFL


06038_100XBF


06038_100XFL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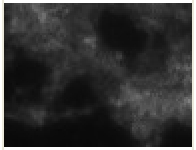
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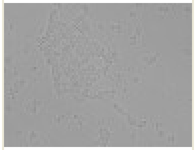
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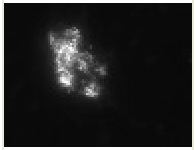
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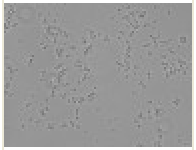
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07009_100XFL


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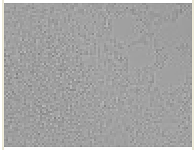
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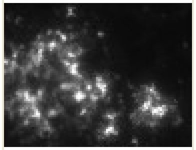
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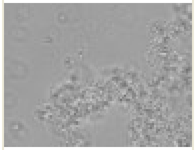
07040_100XBF


07040_100XFL


07043_100XBF


07043_100XFL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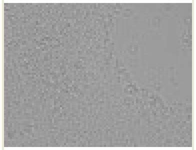
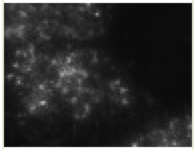
07045_100XFL


12228_100XBF


12228_100XFL

neg


35984_100XBF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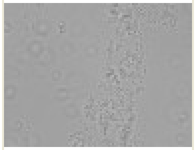
pos

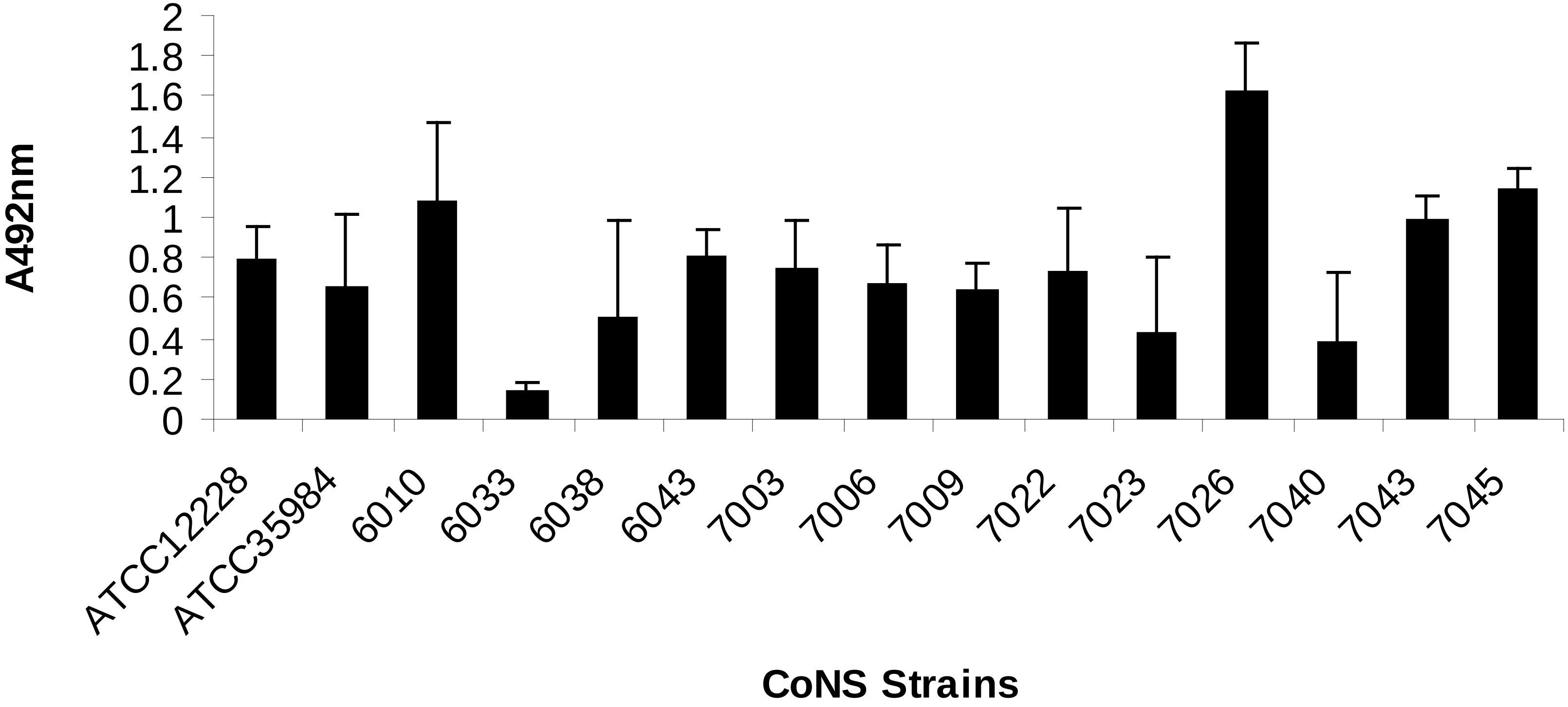

35984_100XBFno1oab


35984_100XFLno1oab

mock

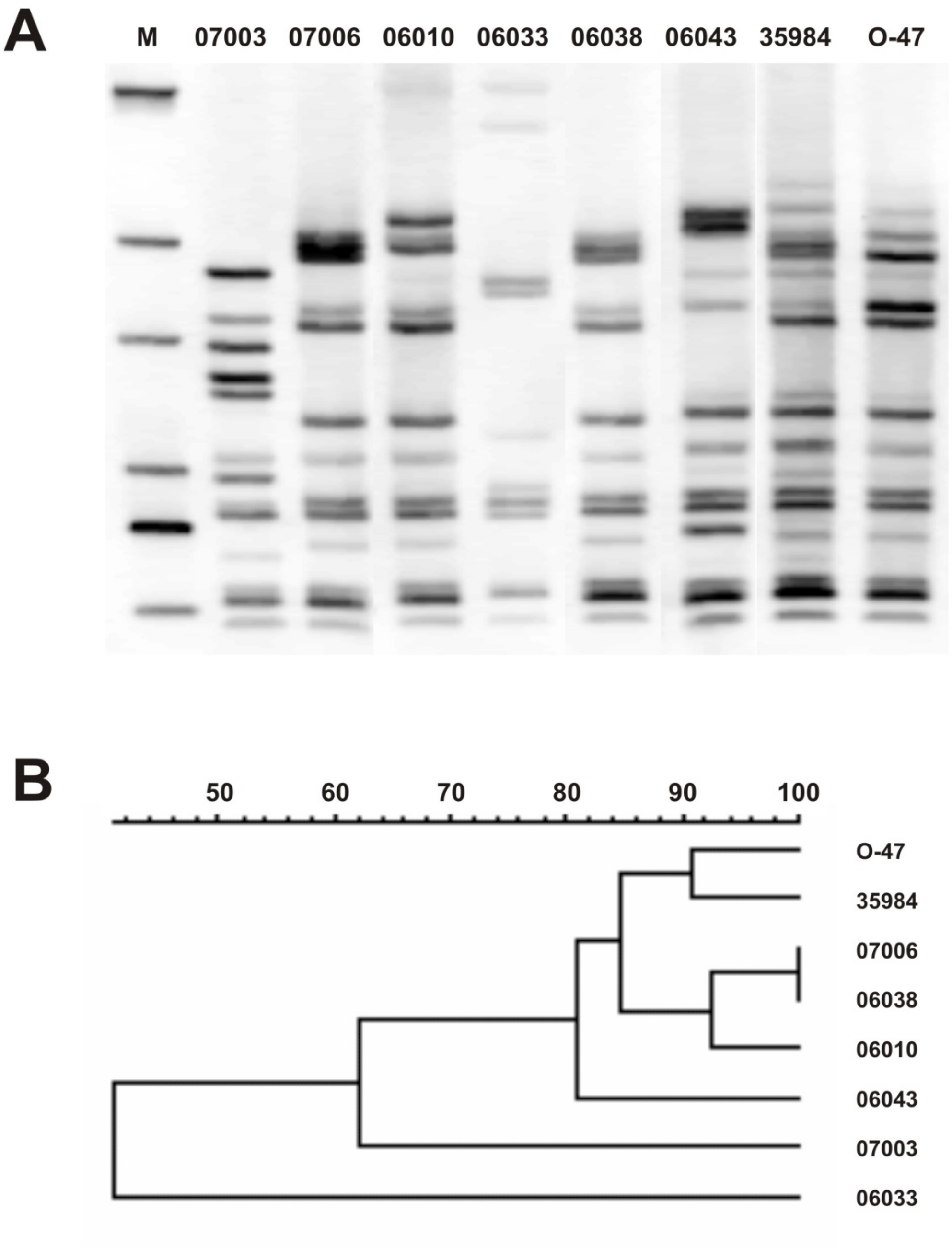
Appendix 11.2.3

Biofilm formation in platelets by coagulase negative staphylococci isolated from contaminated platelet concentrates by CBS.



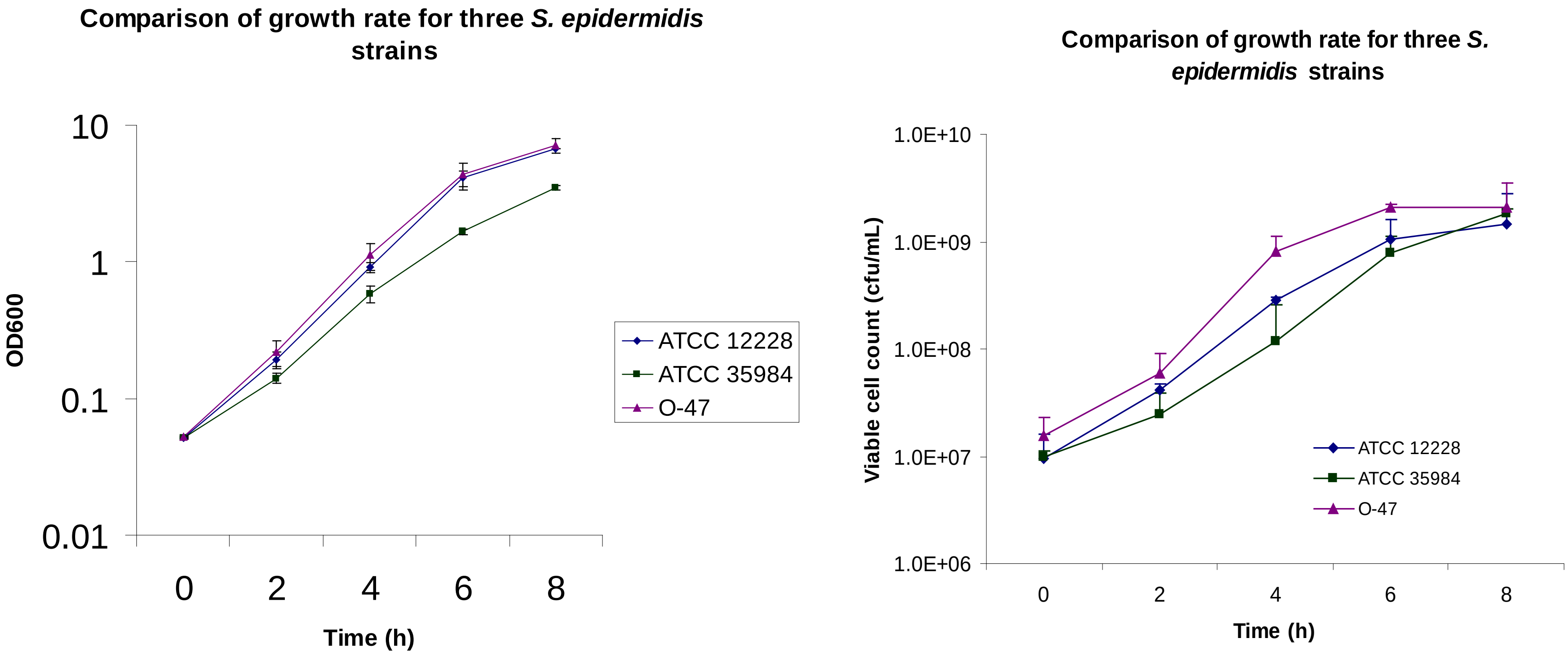
Note: Data was obtained by application of crystal violet staining assay

Appendix 11.2.4



DNA fingerprinting of selected CoNS implicated in contamination of platelet preparations. (A) Profiles were created by automated ribotyping using EcoRI-digested 16S rDNA. M, marker; control strains O-47 and ATCC 35984 are also shown. (B) Phylogenetic tree showing strain relatedness.

Appendix 11.3.1



Comparison of growth rate for three *S. epidermidis* strains

Comparison of growth rate for three *S. epidermidis* strains

Example of growth rate comparison, OD vs viable cell count

Appendix 11.3.2

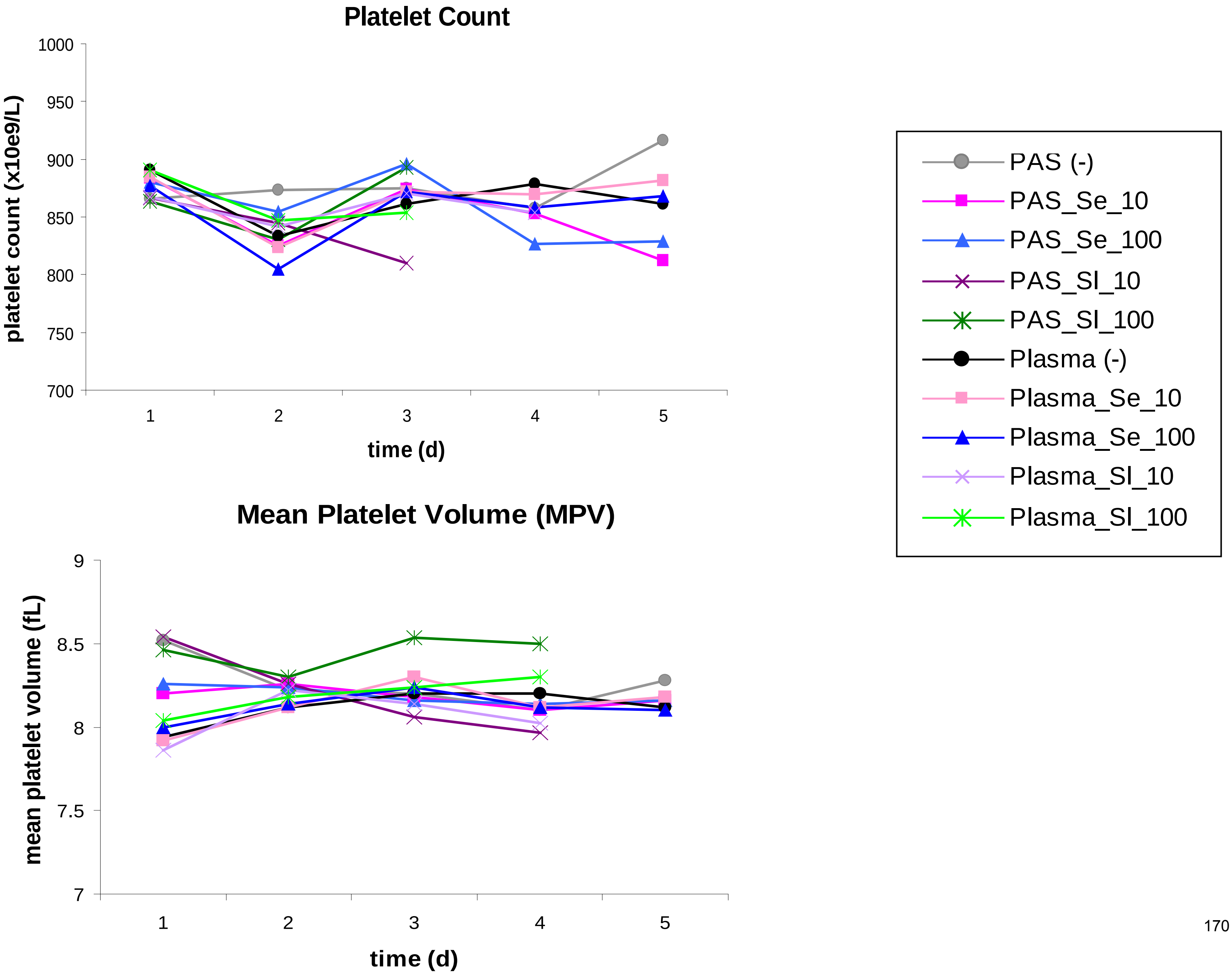
Bacterial Strain	**Standardization of Frozen Stocks for UBC Collaboration** **2009-06-24**						
	Plate A		Plate B		Plate C		Average (CFU/mL)
	Count (vol in ul)	Dilution factor	Count (vol in ul)	Dilution factor	Count (vol in ul)	Dilution factor	
S. liquefaciens CBS strain	>300	10	>300	10	N/A	10	
	>300	100	>300	100	N/A	100	
	>300	1000	>300	1000	N/A	1000	
	>300	10e4	>300	10e4	N/A	10e4	
	87 (10)	10e5	102 (10)	10e5	N/A	10e5	9450
	12 (10)	10e6	10 (10)	10e6	N/A	10e6	1100
	2 (10)*	10e7	0 (10)	10e7	14 (100)	10e7	140
	0 (10)	10e8	0 (10)	10e8	2 (100)*	10e8	0
S. epidermidis strain O-47	>300	10	>300	10	N/A	10	
	>300	100	>300	100	N/A	100	
	>300	1000	>300	1000	N/A	1000	
	>300	10e4	>300	10e4	N/A	10e4	
	56	10e5	54	10e5	N/A	10e5	5500
	4 (10)	10e6	8 (10)	10e6	N/A	10e6	600
	0 (10)	10e7	0 (10)	10e7	6 (100)	10e7	60
	1 (10)*	10e8	0 (10)	10e8	2 (100)*	10e8	0

Example: frozen stock bacterial concentration confirmation (A)

Bacterial Strain	**Standardization of Frozen Stocks for UBC Collaboration 2009-06-24**						
	Plate A		Plate B		Plate C		Average (CFU/mL)
	Count (vol in ul)	Dilution factor	Count (vol in ul)	Dilution factor	Count (vol in ul)	Dilution factor	
S. liquefaciens CBS strain	>300	10	>300	10	N/A	10	
	>300	100	>300	100	N/A	100	
	>300	1000	>300	1000	N/A	1000	
	>300	10e4	>300	10e4	N/A	10e4	
	106 (10)	10e5	98 (10)	10e5	>300 (100)	10e5	10200
	10 (10)	10e6	17 (10)	10e6	117	10e6	1290
	0* (10)	10e7	2* (10)	10e7	N/A	10e7	
	0* (10)	10e8	0* (10)	10e8	N/A	10e8	
S. epidermidis strain O-47	>300	10	>300	10	N/A	10	
	>300	100	>300	100	N/A	100	
	>300	1000	>300	1000	N/A	1000	
	222 (10)	10e4	246 (10)	10e4	N/A	10e4	23400
	13 (10)	10e5	21 (10)	10e5	214	10e5	1847
	2* (10)	10e6	4 (10)	10e6	15	10e6	275
	0* (10)	10e7	0* (10)	10e7	N/A	10e7	
	0* (10)	10e8	0* (10)	10e8	N/A	10e8	

Example: frozen stock bacterial concentration confirmation (B)

Appendix 11.3.3



PAS(-)	Day	**Plt Count (x10e9 cells/L)**	**MPV (fL)**
	1	865.6	8.52
	2	873	8.22
	3	875.2	8.2
	4	857.8	8.1
	5	916.6	8.28
PAS_Se_10			
	1	883.8	8.2
	2	824.8	8.26
	3	874.2	8.18
	4	852.8	8.1
	5	812.2	8.16
PAS_Se_100			
	1	880.4	8.26
	2	854.2	8.24
	3	896.2	8.16
	4	827	8.14
	5	828.8	8.16
PAS_Sl_10			
	1	866.6	8.54
	2	844.4	8.26
	3	810	8.06
	4	712	7.96666667
	5	N/A	N/A

Appendix 11.3.4

Table 1. Results from mixed model analysis comparison with medium for each parameter (p-values)

Medium	*Parameter*	*time trend (p-value)*	*Time square (p-value)*	*Bac type (p-value)*	*Time and Bac type and time interaction (p-value)*
PAS	pH	0.0044	0.0002	0.0013	<.0001
	O_2 (mmHg)	0.019	0.0012	0.1218	0.0003
	CO_2 (mmHg)	<.0001	.	0.0027	<.0001
	Glucose (mmol/L)	<.0001	.	0.0094	<.0001
	Lactate (mmol/L)	<.0001	.	0.4511	0.0187
	Plt_Count	<.0001	.	0.1219	<.0001
	mpv	0.0072	0.0587	0.4077	0.0927
Plasma:	pH	<.0001	<.0001	0.0429	0.0001
	O_2 (mmHg)	0.1026	.	0.3128	0.0164
	CO_2 (mmHg)	<.0001	<.0001	0.5957	0.106
	Glucose (mmol/L)	<.0001	.	0.9471	0.441
	Lactate (mmol/L)	<.0001	.	0.909	0.4266
	Plt_Count	0.0079	0.0025	0.9617	0.4286
	mpv	<.0001	0.0003	0.907	0.9083

Notes:

In PAS medium, there are significant time trend with all pvalue<0.01, except for O2 (>0.1<p<0.05). For PH and O2, the trend in PH and O2 is not only linear but also quadratic (square term significant, p<0.001). All the interactions between time and Bac-type/dose (Se100, Se10, Sl100, Sl10, neg) are significant , except for MPV, suggesting that time trends vary with the bac-type or doses.

In Plasma medium, there are significant time trend with all p-values<0.01, except for CO2 (p>0.05). PH and O2 also have quadratic trend. Most of the interactions between time and Bac-type/dose (Se100, Se10, Sl100, Sl10, neg) are not significant , except for PH (p=0.0001) and CO2(p=0.0164), suggesting that time trends vary with the bac-type or doses in PH, and CO2, but not in all the other parameters.

Table 2. Results from mixed model analysis comparison by medium and bac-type for each parameter (p-values)

Medium: Bac-type	*Parameter*	*time trend (p-value)*	*Time square (p-value)*	*Dose (p-value)*	*Time and Bac type and time interaction (p-value)*
PAS : SL	pH	0.082	0.0072	0.0183	<.0001
	O_2 (mmHg)	<.0001	.	0.0185	<.0001
	CO_2 (mmHg)^A	0.8925		0.4345	0.1289
	Glucose (mmol/L)	<.0001	.	0.0512	<.0001
	Lactate (mmol/L)	<.0001	.	0.331	0.0427
	Plt_Count	0.0002	.	0.1262	0.0002
PAS : SE	pH	<.0001	<.0001	0.7894	0.2727
	O_2 (mmHg)	0.27	.	0.8816	0.7663
	CO_2 (mmHg)	0.06	0.0358	0.6557	0.1432
	Glucose (mmol/L)	<.0001	.	0.964	0.8685
	Lactate (mmol/L)	<.0001	.	0.9673	0.8163
	Plt_Count	0.3112	.	0.7078	0.1704
Plasma:SL	pH	<.0001	<.0001	0.0775	0.0031
	O_2 (mmHg)	0.0203	.	0.241	0.0162
	CO_2 (mmHg)	0.0018	0.0026	0.2449	0.0206
	Glucose (mmol/L)	<.0001		0.8867	0.4186

A: Only data on Day1-3 were used in the analysis. Models with day 4-5 data didn't converge (no outcome)

Notes:

Only those parameters and bac-types which have significant differences in Table 1 were further examine.

For PAS medium, the significant differences shown in Table 1 were mainly due to SE bacteria, but not in Sl type. There is no significant differences seen in PAS: SE type for all the parameters, though significant time trend may still there, but no differences between doses. All the p-values >0.05 for both main effect and interaction. However, in PAS: Sl type, significant differences could be seen between doses for all the parameters.

In Plasma medium, there are significant time trend and interactions seen in SL types in pH, O2 and CO2, but for other parameters and not for the SE type for any parameters (results no shown).

Table 3. Results from mixed model analysis in selected PAS: SL and Plasma SL in selected parameters.(p-values)

Medium: Bac-type	*Effect*	***PAStype***	*Estimate*	*P-value*	*Notes:*
PAS:SL	day		0.2615	0.0036	
pH	Day-square		-0.03874	0.0072	Sl-10 vs SL-neg (p<0.0001); SL-100 vs SL-neg (p<0.0001); SL10 vs Sl 100 (p=0.4709)
	PAStype	PAS_Sl_10	0.3165	0.0082	
	PAStype	PAS_Sl_100	0.2539	0.0294	
	PAStype	pAS(-)	0	.	
	day*PAStype	PAS_Sl_10	-0.2022	<.0001	
	day*PAStype	PAS_Sl_100	-0.1903	<.0001	
	day*PAStype	pAS(-)	0	.	
O2:	dayn		1.722	0.7038	
	PAStype	PAS_Sl_10	62.4584	0.016	Sl-10 vs SL-neg (p<0.0001); SL-100 vs SL-neg (p<0.0001); SL10 vs Sl 100 (p=0.7635)
	PAStype	PAS_Sl_100	64.0526	0.0157	
	PAStype	pAS(-)	0	.	
	dayn*PAStype	PAS_Sl_10	-42.3918	<.0001	
	dayn*PAStype	PAS_Sl_100	-45.5084	<.0001	
	dayn*PAStype	pAS(-)	0	.	
Glucose	dayn		-0.498	0.0013	
	PAStype	PAS_Sl_10	1.5661	0.0699	Sl-10 vs SL-neg (p<0.0001); SL-100 vs SL-neg (p<0.0001); SL10 vs Sl 100 (p=0.2249)
	PAStype	PAS_Sl_100	2.0636	0.0246	
	PAStype	pAS(-)	0	.	
	dayn*PAStype	PAS_Sl_10	-1.0777	0.0002	
	dayn*PAStype	PAS_Sl_100	-1.5162	<.0001	
	dayn*PAStype	pAS(-)	0	.	
Lactate	dayn		1.2238	<.0001	
	PAStype	PAS_Sl_10	-1.486	0.2788	Sl-10 vs SL-neg (p<0.0628); SL-100 vs SL-neg (p<0.8523); SL10 vs Sl 100 (p=0.0811)
	PAStype	PAS_Sl_100	0.6477	0.6449	
	PAStype	pAS(-)	0	.	
	dayn*PAStype	PAS_Sl_10	0.859	0.0282	
	dayn*PAStype	PAS_Sl_100	-0.2465	0.5766	
	dayn*PAStype	pAS(-)	0	.	
Plt_Count	dayn		8.68	0.6505	Sl-10 vs SL-neg (p=0.1749); SL-100 vs SL-neg
	PAStype	PAS_Sl_10	80.9005	0.4814	
	PAStype	PAS_Sl_100	246.32	0.0471	

	PAStype	pAS(-)	0	.	(p<0.0001); SL10 vs Sl 100 (p<0.0411)
	dayn*PAStype	PAS_Sl_10	-58.3203	0.1119	
	dayn*PAStype	PAS_Sl_100	-161.17	<.0001	
	dayn*PAStype	pAS(-)	0	.	
$CO2^A$	dayn		-5	0.2525	Sl-10 vs SL-neg (p=0.8061); SL-100 vs SL-neg (p=0.0381); SL10 vs Sl 100 (p=0.1164)
	PAStype	PAS_Sl_10	-5.7778	0.6617	
	PAStype	PAS_Sl_100	-17.1111	0.222	
	PAStype	pAS(-)	0	.	
	dayn*PAStype	PAS_Sl_10	3.5	0.5645	
	dayn*PAStype	PAS_Sl_100	12.5	0.0527	
	dayn*PAStype	pAS(-)	0	.	
Plasma:SL					
pH	dayn		0.3307	<.0001	Sl-10 vs SL-neg (p=0.1738); SL-100 vs SL-neg (p=0.0003); SL10 vs Sl 100 (p<0.0124)
	dayn2		-0.04798	<.0001	
	PAStype	Pls_Sl_10	0.03175	0.7125	
	PAStype	Pls_Sl_100	0.2265	0.0328	
	PAStype	plasma (-)	0	.	
	dayn*PAStype	Pls_Sl_10	-0.02871	0.3102	
	dayn*PAStype	Pls_Sl_100	-0.1295	0.0008	
	dayn*PAStype	plasma (-)	0	.	
O_2 (mmHg)	dayn		2.86	0.4161	Sl-10 vs SL-neg (p=0.2532); SL-100 vs SL-neg (p=0.0060); SL10 vs Sl 100 (p=0.1626)
	PAStype	Pls_Sl_10	13.89	0.4446	
	PAStype	Pls_Sl_100	33.7538	0.0983	
	PAStype	plasma (-)	0	.	
	dayn*PAStype	Pls_Sl_10	-7.7787	0.1624	
	dayn*PAStype	Pls_Sl_100	-19.7016	0.0047	
	dayn*PAStype	plasma (-)	0	.	
CO_2 (mmHg)	dayn		-42.4447	0.0018	
	dayn2		6.5241	0.0026	Sl-10 vs SL-neg (p=0.4332); SL-100 vs SL-neg (p=0.0556); SL10 vs Sl 100 (p=0.009)
	PAStype	Pls_Sl_10	7.4048	0.6739	
	PAStype	Pls_Sl_100	-27.1656	0.1952	
	PAStype	plasma (-)	0	.	
	dayn*PAStype	Pls_Sl_10	-5.1117	0.3207	
	dayn*PAStype	Pls_Sl_100	15.3263	0.0297	
	dayn*PAStype	plasma (-)	0	.	
Glucose:					
	dayn		-0.614	0.0209	

PAStype	Pls_Sl_10	0.2163	0.9091	Sl-10 vs SL-neg (p=0.7759); SL-100 vs SL-neg (p=0.3884); SL10 vs Sl 100 (p=0.7484)
PAStype	Pls_Sl_100	0.9191	0.6411	
PAStype	plasma (-)	0	.	
dayn*PAStype	Pls_Sl_10	-0.2575	0.532	
dayn*PAStype	Pls_Sl_100	-0.656	0.1953	
dayn*PAStype	plasma (-)	0	.	

A: Only data on Day1-3 were used in the analysis. Models with day 4-5 data didn't converge (no outcome)

Table 4. Bacteria growth comparison between medium :

Bac-type	*Effect*	*Estimate*	*P-value*	*Notes:*
Se100	Intercept	-0.3683	0.8236	There are significant time square trend, and The trend marginally different with the medium
	Medium: Pas vs Plasma	0.3217	0.7488	
	Time	0.2607	0.5208	
	Time-square	0.1856	0.0003	
	Medium*TIME	-0.2731	0.0577	
Sl100	Intercept	-6.2605	0.0094	Very Significant time trend, and Medium influence the bac-growth significantly.
	Medium: Pas vs Plasma	1.4286	0.255	
	Time	7.8693	<.0001	
	Time-square	-0.8757	<.0001	
	Medium*TIME	-2.4275	0.0029	
	Medium*TIME2	0.3401	0.0029	
Se10	Intercept	0.4577	0.803	There is significant time-square trend, not no different between mediums.
	Medium: Pas vs Plasma	0.1061	0.9223	
	Time	-0.6697	0.2274	
	Time-square	0.2474	0.0004	
	Medium*TIME	-0.08278	0.6662	
Sl10	Intercept	-8.8387	0.0115	T Very Significant time trend, and Medium influence the bac-growth significantly.
	Medium: Pas vs Plasma	3.73	0.0612	
	Time	9.4207	<.0001	
	Time-square	-1.0012	<.0001	
	Medium*TIME	-4.2694	0.0001	
	Medium*TIME2	0.5276	0.0006	

Table 4-2. Bacteria growth comparison between with and without platelet:

Bac-type	*Effect*	*Estimate*	*P-value*	*Notes:*
PAS: Se100	Intercept	1.9989	0.0282	There are significant time square trend, and The trend varies with platelets.
	Medium: With vs without Platelet	-2.7284	0.0258	
	Time	-0.4905	0.0688	
	Time-square	0.1125	0.0225	
	Medium*TIME	0.9903	<.0001	
PAS: Sl100	Intercept	2.6942	0.0037	Significant time trend, and lower bac. count with platelet in medium. Time trend has not difference with platelets.
	Medium: With vs without Platelet	-2.5266	0.0256	
	Time	1.3772	<.0001	
	Medium*TIME	0.3149	0.2771	
Plasma: Se100	Intercept	1.7482	0.0591	There is significant time-square trend, not no different . time trend change with platelets.
	Medium: With vs without Platelet	-1.7938	0.1256	
	Time	-0.4669	0.1259	
	Time-square	0.1513	0.0079	
	Medium*TIME	0.4219	0.0369	
Plasma: Sl100	Intercept	1.1036	0.0749	Significant time trend, and bac-counts is lower with platelets, but time trend is independent of the platelets.
	Medium: With vs without Platelet	-2.6813	0.0113	
	Time	1.4	<.0001	
	Medium*TIME	0.2451	0.3175	

Table 5. Bacteria growth associated with other parameter

Bac-type	*Effect*	*Estimate*	*P-value*
Se100	CO2	0.0253	0.0036
	Lactate	-1.0772	<0.0001
Sl100	pH	-2.5897	0.0177
	CO2	0.0304	0.0549
	Glucose	-0.1618	0.0564
Se10	CO2	0.02334	<0.0001
Sl10	pH	-5.3629	0.0074
	Glucose	-0.2797	0.0043

Notes: Parameters were added into established time-medium model one by one.

Appendix 11.4.1

Nucleotide sequences for selected *S. epidermidis* genes (from ATCC 35984 genome project)

divIVA (657nt)
ATGCCTTTTACACCAAGTGAGATTAAGAATAAAGAATTTACACGAGTAAAAAATGGTTTAGAACCTACAGA
AGTTGCAAATTACTTAGAGCAACTAAGTAATGAAATCGAACGTTTAAAAGAAGATAAAAAGCAACTTGAAA
AAGTCATTGAAGATAGAGATTCTAATATTAAATCATATAAAGATGTTCACCAATCAGTAAGCGATGCACTT
ATTCAAGCACAAAAAGTAGGAGAAGAGACAAAACAAGCAGCAACTAAAGAAGCTGAAGCCGTTTTATCAAA
AGCACAAGTACAAGCTGACAGTATTGTGAATGATGCAATTGAAAAAGCGCGTCGTCTAGCTTTCCAAACTG
AAGACATGAAACGACAATCTAAAGTTTTCCGCTCTCGTTTCCGTATGCTTGTTGAAGCACAACTTGACTTA
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CGGCTAATCAGCCTTCTACATCAAGTTCAGAATCTGTGAGTCAATCAAATGCAAATAACGAATCATCACAA
TCAACATCAAATAATTAA

ftsZ (1185nt)
ATGTTAGAATTTGAACAAGGATTTAATCATTTAGCCACATTGAAAGTCATCGGTGTAGGTGGTGGCGGAAA
TAACGCCGTTAACCGTATGATTGACCATGGTATGAATAATGTAGAATTTATTGCCATCAACACTGATGGAC
AAGCTTTAAACTTATCAAAAGCTGAATCAAAAATTCAAATTGGTGAGAAATTAACACGTGGATTAGGTGCT
GGTGCTAATCCTGAAATTGGGAAGAAAGCAGCAGAAGAATCAAGAGAACAAATTGAAGATGCTATCCAAGG
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GTTCAAGTGTAAATAGTAGTTCTAATCATCAATCTGGTGCTAGTGCTAAAGAAGATTCATTCTCTGCACAC
ACTTCTCATTCACAATCAAGTGAAAGTGTAAATGAAAGAAGCCACACTACTAAAGATGATGATATTCCTAG
CTTTATTAGAAATAGAGAAGAAAGACGTTCTAGAAGAACTAGACGCTAA

gmK (624nt)
ATGGATAAGGAAAAAGGACTGTTAATTGTTCTTTCAGGCCCTTCAGGTGTTGGAAAGGGAACTGTTAGAAA
AAAGATATTTGAAGACCCAACTACTTCATATAAGTATTCTATATCAATGACAACACGTCATATGCGTGAAG
GTGAAATTGATGGTGTAGATTACTTCTTCAAAACAAAGGAAGAATTTGAGGCGTTAATTAAAGATGACCAG
TTTATTGAGTATGCACAATATGTAGGTAATTATTATGGTACACCTGTACAATATGTAAAGGATACTATGGA
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CGTTGTAGTTAACGACGAGGTTGAACTCGCTAAGAATCGAATTCAGTCAATTGTTGAAGCTGAGCATTTAA
AAAGAGAGCGAATCGAAGCTAAATATAGAAAAATGTTACTGGAGGTCAAAAAATAA

atlE (4008nt; highlighted sequence used = 1629nt)
ATGGCGAAAAAATTCAATTACAAATTACCGTCTATGGTTGCTTTAACGTTATTTGGCACAGCTTTTACTGC
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CCCTCAAACAAGCAGAAAAAGCTAAAAGCGAAGTTACACAATCAACTACAAATGTATCTGGTACACAAACA
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ACTGATACAAATCAATTACAGGAGACACAATCTGTAGCAAAAGAAAATGAGAAAGATTTAGGAGCTAACGC
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AACTGATATTAATTGGGCAAATGTCAACGCACAAGTATTAAAACAATTTTATGATAAAATTGGTGAAGTCG
GTAAGTACTTCGAAATTCCAACATACAAATAA

Appendix 11.4.2

Amino Acid sequences for *S. epidermidis* proteins (Not including 0.84 kDa for 6XHis). Obtained from ATCC 35984 Genome project

DivIVA: 24.92 kDa
MPFTPSEIKNKEFTRVKNGLEPTEVANYLEQLSNEIERLKEDKKQLEKVIEDRDSNIKSYKDVHQSV
SDALIQAQKVGEETKQAATKEAEAVLSKAQVQADSIVNDAIEKARRLAFQTEDMKRQSKVFRSRF
RMLVEAQLDLLKSEDWDYLLNYDLDAEQVTLEDIHHLHDNDLTPEERAMKQKQNQEISANQPST
SSSESVSQSNANNESSQSTSNN

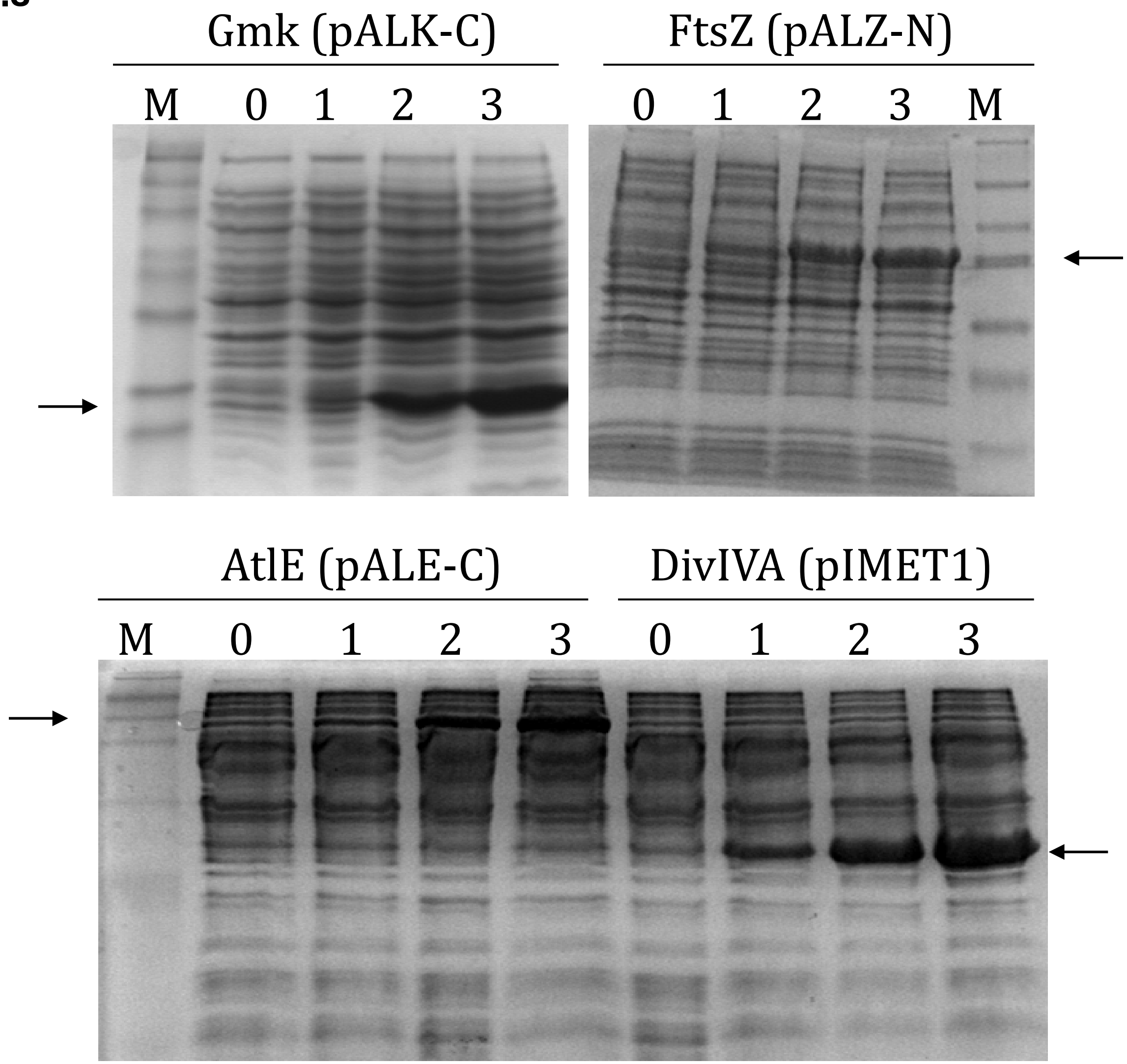
AtlE: 60.32 kDa*
VSSQKTSSLPKYTPKVNSSINNYIRKKNMKAPRIEEDYTSYFPKYGYRNGVGRPEGIVVHDTANDN
STIDGEIAFMKRNYTNAFVHAFVDGNRIIETAPTDYLSWGAGPYGNQRFINVEIVHTHDYDSFARS
MNNYADYAATQLQYYNLKPDSAENDGRGTVWTHAAISNFLGGTDHADPHQYLRSHNYSYAELY
DLIYEKYLIKTKQVAPWGTTSTKPSQPSKPSGGTNNKLTVSANRGVAQIKPTNNGLYTTVYDSKG
HKTDQVQKTLSVTKTATLGNNKFYLVEDYNSGKKYGWVKQGDVVYNTAKAPVKVNQTYNVKA
GSTLYTVPWGTPKQVASKVSGTGNQTFKATKQQQIDKATYLYGTVNGKSGWISKYYLTTASKPS
NPTKPSTNNQLTVTNNSGVAQINAKNSGLYTTVYDTKGKTTNQIQRTLSVTKAATLGDKKFYLVG
DYNTGTNYGWVKQDEVIYNTAKSPVKINQTYNVKPGVKLHTVPWGTYNQVAGTVSGKGDQTFK
ATKQQQIDKATYLYGTVNGKSGWISKYYL

GmK: 24.1 kDa
MDKEKGLLIVLSGPSGVGKGTVRKKIFEDPTTSYKYSISMTTRHMREGEIDGVDYFFKTKEEFEALI
KDDQFIEYAQYVGNYYGTPVQYVKDTMEEGHDVFLEIEVEGAKQVRKKFPDALFIFLAPPSLDDL
RERLVGRGTESDEKIQSRVNEARKEVEMMNLYDYVVVNDEVELAKNRIQSIVEAEHLKRERIEAK
YRKMLLEVKK*

FtsZ: 41.42 kDa
MLEFEQGFNHLATLKVIGVGGGGNNAVNRMIDHGMNNVEFIAINTDGQALNLSKAESKIQIGEKL
TRGLGAGANPEIGKKAAEESREQIEDAIQGADMVFVTAGMGGGTGTGAAPVVAKIAKEMGALTV
GVVTRPFGFEGRKRQTQAAAGVESMKAAVDTLIVIPNDRLLDIVDKSTPMMEAFKEADNVLRQG
VQGISDLIAVSGEVNLDFADVKTIMSNQGSALMGIGVSSGENRAVEAAKKAISSPLLETSIVGAQG
VLMNITGGESLSLFEAQEAADIVQDAADEDVNMIFGTVINPELQDEIVVTVIATGFEDKPSSQGRKA
TSTGFGSSVNSSSNHQSGASAKEDSFSAHTSHSQSSESVSERSHTTKDDDIPSFIRNREERRSRRTRR

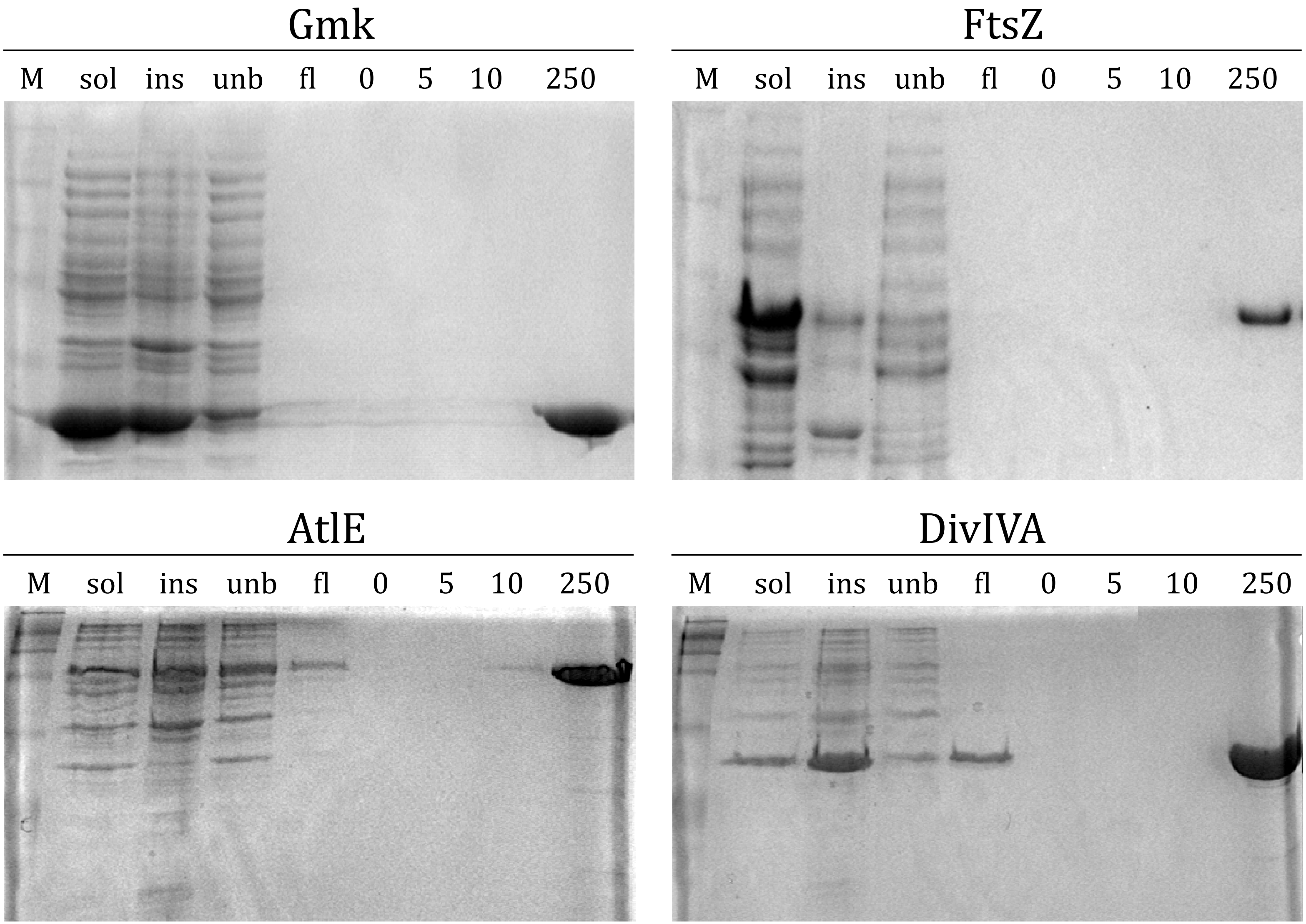
***aa 303-845 of full length sequence**

Appendix 11.4.3



Induction of recombinant *S. epidermidis* protein expression in *E. coli* strain C43(DE3). Total soluble protein extracts are shown at 0, 1, 2, and 3 hr post-supplementation with 0.4-1mM IPTG. Arrows point to expected location of respective recombinant protein based on migration of the protein marker (M). Plasmid source is give in parentheses (Refer to Table 3 for plasmid details).

Appendix 11.4.4



Purification of recombinant *S. epidermidis* proteins by Talon® metal affinity chromatography. M, Marker; sol, soluble fraction; ins, insoluble fraction; unb, unbound fraction; fl, column flow-through; 0, 5, 10, 250 = mM imidazole in wash buffer

Appendix 11.5

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5. Dumont LJ, Wood TA, Housman M, Herschel L, Brantigan B, Heber C, and Houghton H. Validation of a microbial detection system for use with ACD-A platelets with PAS III p latelet additive solution. Transfusion. 2011; DOI: 10.1111/j.1537-2995.2011.03149.x

6. Greco-Stewart VS, Brown EE, Parr C, Kalab M, Jacobs MR, Yomtovian RA, and Ramirez-Arcos SM. *Serratia marcescens* strains implicated in adverse transfusion reactions form biofilms in platelet concentrates and demonstrate reduced detection by automated culture. Vox Sang. 2011. In press.

Citations for Greco et al., 2011

1. Greco-Stewart VS, Brown EE, Parr C, Kalab M, Jacobs MR, Yomtovian RA, and Ramirez-Arcos SM. *Serratia marcescens* strains implicated in adverse transfusion reactions form biofilms in platelet concentrates and demonstrate reduced detection by automated culture. Vox Sang. 2011. In press.

CAREY A. GRECO

Curriculum Vitae

Education

- 07-2011-Present: Project Management Program. Algonquin College, Ottawa. Part-time.
- 09/2005-08/2011: Ph.D., Microbiology & Immunology. University of Ottawa. Thesis Title: Biofilm formation by the coagulase negative bacterium *Staphylococcus epidermidis* during platelet storage. Supervisor: Dr. Sandra Ramírez-Arcos.
- 09/2001-06/2005: B.Sc., Biology (Honours, Biotechnology), *Magna Cum Laude* Distinction. University of Ottawa. Thesis Title: Effect of lipofuscin on proteasome and lysosome function in mammalian cells. Supervisor: Dr. Douglas A. Gray.

Scholarships and Awards

- 09/2009-02/2011: CIHR Banting & Best Canadian Graduate Scholarship ($35,000/year)
- 09/2005-05/2011: University of Ottawa National Excellence Award ($5,535/year)
- 09/2005-09/2009: Canadian Blood Services (CBS) Graduate Fellowship Program ($22,000/year)
- 12/2008: Fisher Scientific Award of Excellence in Graduate Studies ($555)
- 02/2007: Corporate Activities Student Travel Grant, American Society of Microbiology ($500)
- 03/2006: Faculty of Graduate and Postdoctoral Studies travel grant, University of Ottawa ($500)
- 09/2004: Dean's Merit Scholarship, University of Ottawa ($500)
- 05/2004-09/2004: CBS Summer Internship Program ($6,000)
- 09/2001: University of Ottawa Entrance Scholarship ($2,500)

Work History

- 02/2011-Present: Search Analyst, OneMatch Stem Cell and Marrow Network, CBS.
- 01/2010-05/2011: Teaching Assistant (Pathogenic Bacteria; 4000-level), University of Ottawa.
- 10/2006-05/2009: Teaching Assistant (Biochemistry; 2000-level), University of Ottawa.

Committee Experience

- 09/2007-03/2011: Equity, Diversity & Gender Committee, University of Ottawa.
- 09/2007-03/2011: Health Sciences Library Advisory Committee, University of Ottawa.
- 05/2006-02/2009: Graduate Student Council, Biochemistry, Microbiology & Immunology Department, University of Ottawa. Elected VP of Internal Affairs (02/2008).

Volunteer Experience

- 05/2009-07/2011: Intensive Care Unit Volunteer (The Ottawa Hospital)
- 08/2005-02/2011: Canadian Blood Services Hospitality Volunteer (Ottawa Area)

CAREY A. GRECO

Curriculum Vitae

Skills

- Molecular biology: DNA, RNA, protein work (extraction/purifications and electrophoreses); Polymerase Chain Reaction (PCR); gene cloning, yeast-two-hybrid, immunoblots.
- Microbiology: Culture media preparation, prokaryotic/eukaryotic cell culture, Gram staining, competent cell preparation and transformation, antimicrobial susceptibility, bacterial growth rate analysis, sterile technique (BSL-2).
- Microscopy: Light, phase-contrast, and fluorescence microscopy.
- Computer: Windows programs (Microsoft Word, PowerPoint, Excel), Corel Draw, internet-based bioinformatics tools.
- Training: WHIMIS, Occupational Health & Safety, Bloodborne Pathogens Safety, National Institutional Animal User Training (NIAUT), Standard First Aid with level C CPR (St. John's Ambulance; 04/2009), Ontario Class G Driver's license.

Peer-Reviewed Manuscripts

1. **Greco CA**, Maurer-Spurej E, Scott MD, Kalab M, Nekane N, and Ramírez-Arcos SM. PEGylation prevents bacteria-induced platelet activation and biofilm formation in platelet concentrates. Vox Sanguinis. 2011;100(1):336-339.
2. **Greco CA**, Zhang JG, Kalab M, Yi QL, Ramírez-Arcos SM, and Gyongyossy-Issa MIC. Bacterial contamination of platelets stored in additive solution: Effects on platelet quality and bacterial growth dynamics. Transfusion. 2010; 50(11):2344-2352.
3. **Greco C**, Mastronardi C, Pagotto F, Mack D, Ramírez-Arcos S. Assessment of biofilm-forming ability of coagulase-negative staphylococci isolated from contaminated platelet preparations in Canada. Transfusion. 2008; 48(5):969-977.
4. **Greco C**, Martincic I, Gusinjac A, Kalab M, Yang AF, and Ramírez-Arcos S. *Staphylococcus epidermidis* forms biofilms under simulated platelet storage conditions. Transfusion. 2007;47(7):1143-1153.

Abstracts

1. **Greco C**, Zhang JG, Kalab M, Ramírez-Arcos SM, and Gyongyossy-Issa MIC (2010) Effect of platelet additive solution on bacterial dynamics and platelet quality in stored platelet concentrates. Canadian Society for Transfusion Medicine, Vancouver, Canada. May 13-16. Poster (presented by co-author Dr. Gyongyossy-Issa).
2. **Greco C**, Brown E, Zhang J, Gyongyossy-Issa M, and Ramírez-Arcos S. (2009) Insights into the effect of platelet additive solution on biofilm formation by *Staphylococcus epidermidis* and *Serratia liquefaciens* during storage of platelet concentrates. 5th ASM Biofilms Conference, Cancun, Mexico. November 15-19. Poster (presented).
3. **Greco C**, Maurer-Spurej E, and Ramírez-Arcos S. (2008) Platelet PEGylation prevents bacterial attachment and biofilm formation during platelet storage. Annual Meeting of the American

Association of Blood Banks, Montreal, Canada. October 4-7. Poster (presented). Published in Transfusion: 48(s2):SP224.

4. Mastronardi C, **Greco C**, and Ramírez-Arcos S. (2007) Detection of the predominant blood-borne pathogen *Staphylococcus epidermidis* in platelet preparations using *divIVA* and *icaA* as target genes. 107th ASM General Meeting, Toronto, Canada. May 21-25. Poster (presented).
5. **Greco C**, Martincic I, and Ramírez-Arcos S. (2007) Biofilm formation by *Staphylococcus epidermidis* and other coagulase negative staphylococci in platelet preparations is influenced by platelet concentration. 4th ASM Biofilms Conference, Quebec City, Canada. March 25-29. Poster (presented).
6. **Greco CA**, and Ramírez-Arcos SM. (2006) *Staphylococcus epidermidis* biofilms are formed during platelet storage. AMMI Canada-CACMID Annual Conference, Victoria, Canada. March 15-19. Poster (presented). Published in Can J Infect Dis Med Microbiol: 17(1):SP.15.
7. **Greco C**, Martincic I, and Ramírez-Arcos S. (2005) Is the DivIVA protein from *Staphylococcus epidermidis* involved in cell division and cell growth? Joint Meeting of the Three Divisions of the International Union of Microbiological Societies, San Francisco, USA. July 23-28. Poster.
8. Ramírez-Arcos S, Martincic I, and **Greco C.** (2004) Development of molecular assays for the detection and identification of bloodborne bacteria in apheresis platelets in Canada. Annual Meeting of the American Association of Blood Banks, Baltimore, USA. October 23-26. Poster. Published in Transfusion. 44(s1):SP45.

Organizations

- Canadian Society of Microbiologists
- American Society for Microbiology
- Golden Key International Honour Society