Assessing the Impact of Collection, Production, and Storage of Platelet Concentrates on Bacterial Contamination and Product Safety

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Abstract

The bacterial contamination of platelet concentrates (PCs) poses a safety risk to transfusion patients. Despite the implementation of mitigation strategies like donor skin disinfection and bacterial screening of PCs using culture methods, the anaerobic, slow growing bacterial contaminant *Cutibacterium acnes* is frequently transfused into patients. Fortunately, since this bacterium is incapable of proliferating in the aerobic PC storage environment, it has only been associated with mild adverse reactions. However, very little is known about the long-term impact of these transfusion events. This is particularly pertinent, since *C. acnes* is known to harbor a host of virulence factors that it can harness to cause slow developing, chronic infections, and the PC environment has been demonstrated to elicit the enhanced expression of virulence genes in other transfusion relevant bacteria. Furthermore, certain non-infectious transfusion reactions are driven by proinflammatory factors that accumulate in PCs during storage, and interestingly, bacterial interactions with platelets can elicit the release of these factors. It is therefore important to elucidate whether the transfusion of *C. acnes* contaminated PCs enhances the risk of such non-infectious adverse reactions.

My thesis aims to elucidate the role that *C. acnes* contamination plays in the PC manufacturing process from blood collection to storage, and its impact on blood product safety. To that end, I hypothesized that *C. acnes* evades elimination during skin disinfection, contributing to its dominance as a bacterial contaminant of PCs. I further hypothesized that the virulence of *C. acnes* is heightened in the PC storage environment and *C. acnes* contamination results in an enhanced pro-inflammatory profile of PCs. The four objectives designed to test the hypotheses were to 1) investigate the ability of *C. acnes* to resist eradication by donor skin disinfectants, 2) enhance *C. acnes* detection through supplementation of culture media, 3) determine whether the PC storage

environment enhances the virulence of *C. acnes*, and 4) evaluate whether PCs contaminated with *C. acnes* have an elevated pro-inflammatory profile. The data obtained in this study demonstrated that sebum components dampen the efficacy of the current blood donor skin disinfectant against *C. acnes* and may contribute to its dominance as a PC contaminant. I have also shown that the use of a commercially available supplement can reduce the detection time of *C. acnes* during PC screening with culture methods, the use of which has the potential to prevent the transfusion of *C. acnes* contaminated PCs. Furthermore, my data indicated that antigen shielding in PC derived *C. acnes* samples may reduce the acute immune response in a novel silkworm model, thereby dampening the virulence observed. However, the ability of *C. acnes* to adhere to mammalian epithelial cells and the expression of virulence genes involved in tissue invasion and persistence are enhanced in PCs, suggesting that the potential to cause chronic infections by *C. acnes* is augmented in this environment. Finally, I demonstrated that *C. acnes* contamination does not enhance the pro-inflammatory profile of PCs and therefore does not increase the risk of non-infectious adverse reactions.

The research presented in this thesis has helped identify areas of improvement in the blood collection process and provided evidence for the use of supplements to enhance culture based bacterial screening for *C. acnes*. Notably, my work fills a void in our current understanding of the potential risks involved in transfusing *C. acnes* contaminated PCs. In conclusion, I have contributed to the advancement of knowledge in the field of transfusion medicine and provided insight into enhancing the safety of transfusion patients.

Dedication

I dedicate this work to my mother Celine Kumaran,

the strongest and most selfless person I know.

Dada, I pray that you are surrounded by eternal love and peace,

and that you are proud of your little girl.

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Abbreviations

ATR: Adverse Transfusion Reaction

BHI: Brain Heart Infusion media

BPN: Anaerobic culture media used in the BACT/ALERT system.

BPNt: BPN media supplemented with Tween 80

CP+: Clinisept +

FNHTR: Febrile Non-hemolytic Transfusion Reaction

LPS: Lipopolysaccharide

LVDS: Large Volume Delayed Sampling

PAS: Platelet Additive Solution

PBS: Phosphate Buffered Saline

PCs: Platelet Concentrates

PI: Pathogen Inactivation

PR: Pathogen Reduction

RBC: Red Blood Cell

RCC: Red Blood Cell Concentrates

sCD40L: Soluble CD40L

SD: Standard Donor Skin Disinfectant

SL: Sebum-Like

TRALI: Transfusion Related Acute Lung Injury

UV: Ultraviolet

WB: Whole Blood

Chapter 1 Introduction

1.1 General Introduction

Canadian Blood Services is a manufacturer of therapeutic blood products and is responsible for the distribution of safe and effective life essentials required for transfusion and transplantation in Canada excluding the province of Québec. Volunteer whole blood donations are processed into therapeutic components and include platelet concentrates (PCs), red blood cell concentrates (RCCs) and plasma. PCs are used to treat patients experiencing low platelet counts, platelet dysfunction, or acute trauma, and are infused prophylactically in instances where the risk of bleeding is high as in the case of cardiovascular surgery [1]. Unfortunately, PCs have been associated with severe septic transfusion reactions caused by bacterial contamination. Therefore, strategies like the donor screening questionnaire, donor skin disinfection, first aliquot diversion, and bacterial screening of PCs or PC treatment with pathogen inactivation technologies have been adopted by blood suppliers to mitigate the safety risk to transfusion patients [2]. The implementation of PC screening and bacterial identification at Canadian Blood Services has facilitated the acquisition of hemovigilance data [3] which indicate that between August 2017 and December 2023, approximately 0.1% of all PCs manufactured were contaminated with bacteria. Notably, the anaerobic aerotolerant skin commensal *Cutibacterium acnes* was identified as the most represented bacterial contaminant of PCs (Canadian Blood Services, Quality Control Data August 2017- December 2023), and is disproportionately transfused into patients due to late detection [3]. Given that PCs are an essential therapeutic required for the treatment and management of vulnerable patients, the work described herein aims to identify factors that contribute to the over representation of this bacterium as a PC contaminant, provide

recommendations to enhance established risk mitigation strategies, and assess the potential risk associated with the transfusion of *C. acnes* contaminated PCs.

1.2 Platelets

1.2.1 Structure

Platelets are discoid cellular fragments derived from megakaryocytes that are produced in the bone marrow at a rate of approximately 10^{11} platelets/ day. Once released into the blood stream, platelets remain in circulation for 7-10 days after which they are cleared by the liver [4]. Platelets contain bioactive molecules in alpha granules (coagulation factors, cytokines, chemokines, selectin P, etc.), dense bodies (calcium, ADP, ATP, serotonin, etc.) and lysosomes [5]. The contents of these granules are released upon platelet activation following interaction with vascular damage markers, pathogens, and other activated platelets, and facilitate platelet hemostatic and immunomodulatory functions [5]. The role of platelets within the context of maintaining hemostasis and the immune response are described below.

1.2.2 Platelet hemostatic function

Hemostasis is a well-controlled process that is initiated following vascular damage to staunch bleeding and maintain the integrity of the circulatory system. The process can be broadly classified into four stages namely: a) vasoconstriction, b) platelet plug formation, c) coagulation cascade activation, and d) fibrin clot formation [6]. Platelets play an integral role in establishing hemostasis by binding to exposed collagen and von Willebrand factors at the site of damage, initiating the formation of the platelet plug [7, 8]. Bound platelets activate to release coagulation factors and enhance platelet aggregation. Additionally, the membrane of activated platelets serves as a

catalytic site for the assembly of complexes involved in the coagulation cascade and regulate the formation and retraction of the fibrin clot [9, 10].

1.2.3 Platelet immune function

Platelets express multiple pattern recognition receptors on their surface including toll like receptors (TLRs), glycoprotein receptors, and complement receptors which enable the direct or indirect interaction with pathogens like bacteria found in circulation [11]. TLR-2 and TLR-4 are two receptors that have been studied in the context of platelet interaction with bacterial pathogens. Gram positive bacterial cell wall components like peptidoglycan and lipoproteins have been shown to serve as ligands for TLR-2. The activation of TLR-2 in association with TLR-1 or TLR-6 results in platelet activation and elicits the release of α and dense granule content. On the other hand, lipopolysaccharides (LPS) derived from Gram negative bacteria activate TLR-4, and these receptors have been described to be able discriminate between different isoforms of LPS and are able to regulate the immunomodulatory response [12, 13]. Furthermore, platelets express chemokine receptors that can recognize all four classes of chemokines produced at sites of infection which facilitate platelet aggregation [14]. Once activated, platelets degranulate releasing over 300 factors which include a host of platelet microbicidal proteins (PMPs) [15]. Cationic chemokines called kinocidins, antimicrobial peptides (human defensin β), and thymosin β are among the classes of peptides released and have been shown to have either direct or indirect bactericidal activity [16]. Furthermore, platelets can internalize and eradicate invading bacterial and viral pathogens [17], surround clusters of bacteria reducing growth rates [18], and can elicit the formation of neutrophil extracellular traps (NET) [18]. In addition to its ability to identify and eradicate pathogens, platelets play an immunomodulatory role in the adaptive immune response. CD40 ligand (CD40L) released from activated platelets binds to the CD40 receptor found on other

platelets, endothelial cells, and immune cells (lymphocytes and dendritic cells), elicits a pro-inflammatory response, and promotes the activation and maturation of immune cells like macrophages and neutrophils [19]. Dendritic cells are professional antigen presenting cells (APCs) and serve as a bridge linking the innate and adaptive immune response. APC activation and maturation is required to activate effector T cell function and can be induced by CD40L [20]. Furthermore, studies have demonstrated that the activity of T cells like cytotoxic CD8+ T cells and CD4+ helper T cells are enhanced by platelet derived CD40L and the cytokine environment thereby promoting adaptive immunity [21, 22]. Platelets therefore play a vital role in the human body and deficiencies (e.g., thrombocytopenia) can lead to life threatening conditions, which can be treated with platelet transfusion therapy [1].

1.3 Platelet concentrates

Platelet concentrates consists of a concentrated fraction of platelets suspended in plasma or a combination of plasma and platelet additive solution (PAS). At Canadian Blood Services, this therapeutic is primarily manufactured using two methods. The buffy coat method requires either four or seven whole blood donations from which buffy coat fractions are extracted and resuspended in 100% plasma (derived from one of the donors) or a mix of plasma (40%) and PAS SSP+ (60%) respectively. These products are often referred to as pooled PCs or buffy coat derived PCs (Figure 1). The second method involves connecting a donor to an apheresis machine that extracts platelets and plasma and returns the rest of the blood components to the donor in a closed system (Figure 2). If PCs are to be prepared in plasma (40%) and PAS SSP+ (60%), PAS is added post collection. These platelet products are referred to as apheresis PCs. To maintain the quality and functionality of platelets, PCs are stored in plastic gas permeable bags, at room temperature (20-24°C), under

gentle agitation, in the presence of glucose (324mg/dL in 100% plasma, 144mg/dL in plasma and PAS) and neutral pH for 7 days. Platelets are exposed to stressful stimuli during the PC production process and over storage (7 days, constant agitation), which can result in structural, biochemical, and functional changes in platelets [23, 24]. These changes are collectively referred to as the platelet storage lesion (PSL), which result in reduced platelet counts and recovery post transfusion and may promote adverse clinical outcomes [25]. Therefore, investigations into different storage bags and platelet additive solutions that minimize PSL continue to be an area of interest.

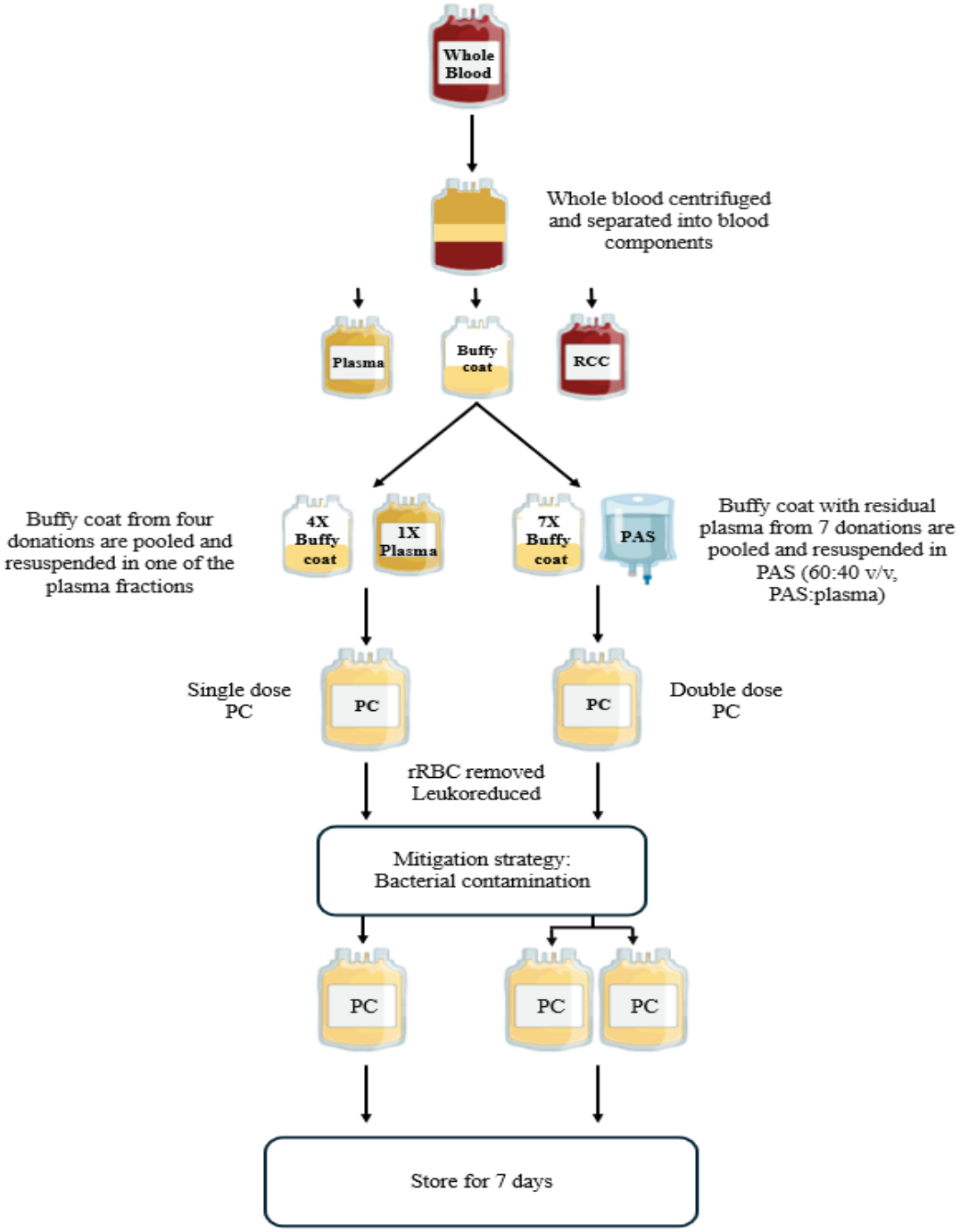


Figure 1. Production of buffy coat derived PCs at Canadian Blood Services.
Buffy coat derived PCs are produced by either resuspending four buffy coats in a single plasma fraction or by resuspending seven buffy coats in plasma (40%) and PAS SSP+ (60%). The PC units are centrifuged to remove residual red blood cells (rRBCs) and filtered to reduce white blood cell content (leukoreduced) and stored for 7 days. Strategies to mitigate risk associated with bacterial contamination are implemented prior to storage Image prepared using Biorender.

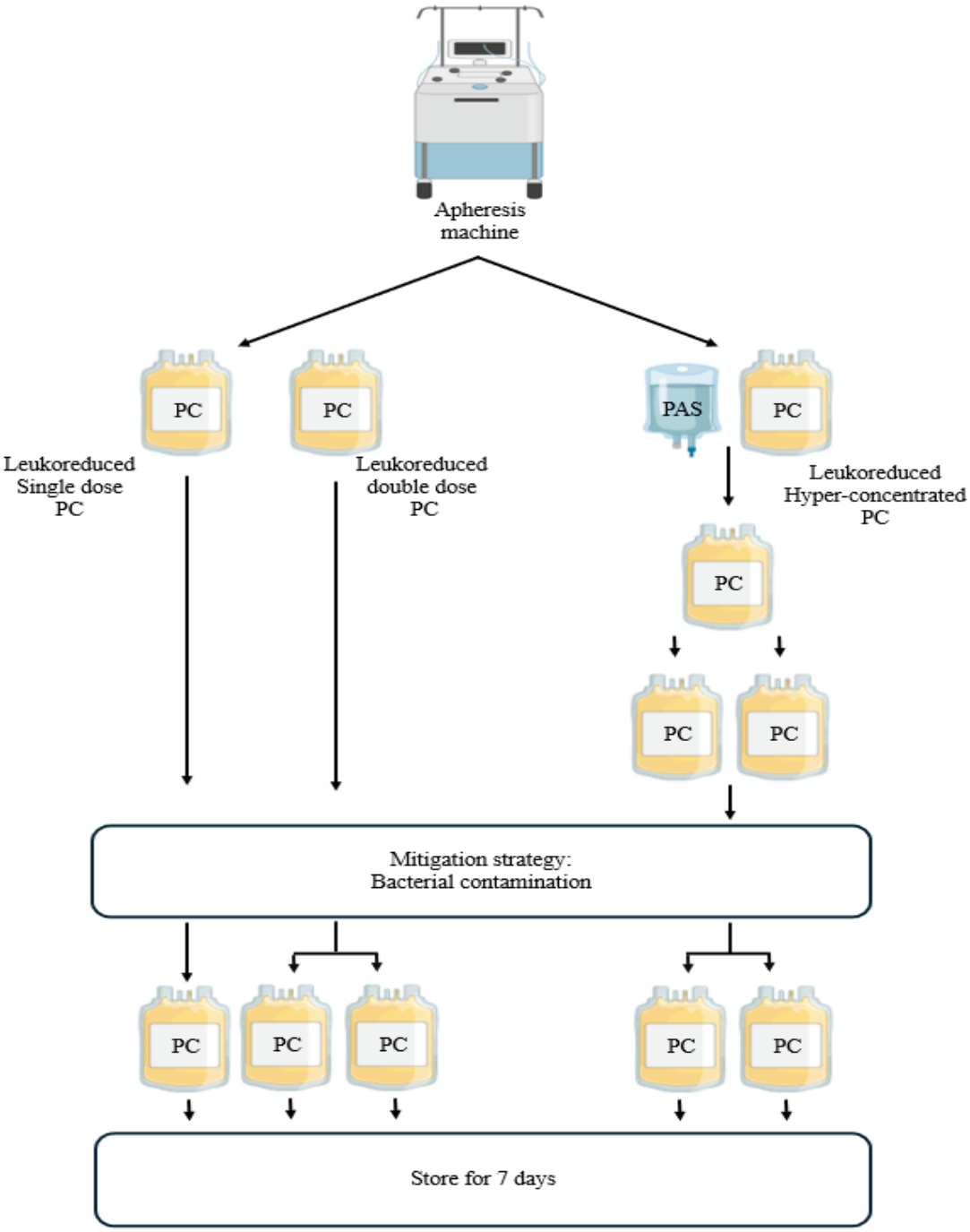


Figure 2. Production of apheresis PCs at Canadian Blood Services.
Apheresis PCs are produced by connecting a donor to an apheresis machine where either a single, double or a hyper concentrated (low plasma volume) PC unit is obtained. The hyper-concentrated PCs are resuspended in PAS SSP+ and stored for 7 days. Strategies to mitigate risk associated with bacterial contamination are implemented prior to storage. Image prepared using Biorender.

1.4 Adverse transfusion reactions

The transfusion of blood products is an important lifesaving tool used to treat individuals at their most vulnerable. The landscape of transfusion medicine has evolved over the years in pursuit of developing safer and more effective transfusion products. Though transfusions are now considered generally safe, adverse transfusion reactions (ATR) do occur [26, 27]. These reactions can be broadly classified into noninfectious and infectious transfusion reactions and can be further categorized into immediate/acute or delayed reactions based on when the onset of symptoms occurs [26]. These adverse events will be briefly discussed below.

1.4.1 Non-infectious adverse transfusion reactions

Examples of non-infectious ATRs include allergic transfusion reactions, febrile non-hemolytic transfusion reactions (FNHTR), and transfusion related acute lung injury (TRALI) [28]. FNHTRs are the most encountered adverse reactions and have a higher incidence with the transfusion of PCs (4.6% of all transfusions) [29]. They are characterized by fever, chills, and tachycardia, and are reported to occur through two pathways namely the immune and non-immune pathway [30]. The immune pathway is mediated by anti-human leukocyte antigen (HLA) antibodies produced in the recipient which bind to HLA on residual leukocytes and platelets in PCs resulting in the release of pyrogens [30]. Anti-HLA antibodies present in the donor's blood have also been reported to elicit FNHTRs [31]. On the other hand, the non-immune pathway is mediated by the accumulation of leukocyte and platelet derived proinflammatory factors like cytokines and sCD40L in PC units during storage resulting in the observed inflammatory response in FNHTRs [32, 33]. The removal of leukocytes using selective filters from blood components (leukoreduction) like PCs has been shown to significantly reduce the occurrence of FNHTRs, yet the risk has not been fully eliminated [34]. TRALI on the other hand is a rare complication (0.02% of transfused components) [35] but

has been associated with high mortality rates (24% of all transfusion related deaths reported) [36], though the exact mechanism involved is not clear, the two-hit model is generally used to explain the progression of TRALI [37]. The first hit in the model refers to the recipient's underlying health condition [38], such as experiencing systemic inflammation. The second hit is the infusion of the transfusion product containing anti-HLA antibodies [39] and/or other immunomodulatory components like soluble CD40L (sCD40L) resulting in an inflammatory response [40, 41].

1.4.2 Infectious adverse transfusion reactions

The inadvertent introduction of microbial pathogens like viruses, bacteria, and parasites via the transfusion of blood products results in infectious transfusion reactions. These reactions can manifest during or soon after (≤ 4 hrs of transfusion) the transfusion event as in most cases of bacterial contamination or can result in delayed infections as in the case of Hepatitis C virus [42]. The implementation of rigorous screening processes, serological testing, and the removal of leukocytes (leukoreduced) from blood products have significantly reduced the risk of infectious transfusion reactions [43, 44], however the risk associated with bacterial contamination prevails [3].

1.5 Bacterial contamination of PCs

Bacterial contaminants encountered in the transfusion setting are mostly introduced at the time of venipuncture from the donor's skin [45]. Donors experiencing bacteremia [46] and breaches in sterility during manufacturing and storage of blood products have also been reported to contribute to the bacterial contamination of blood products [47], albeit to a lesser extent. The PC storage conditions used to maintain the quality and functionality of platelets (20-24°C under agitation) promotes the proliferation of most bacterial contaminants. As a result, clinically significant bacterial concentrations ($\geq 10^5$ colony forming units (CFU)/mL) can be reached resulting in severe

reactions upon transfusion of these products [48]. Both Gram positive (coagulase negative *staphylococci* (CoNS), *Staphylococcus aureus, Clostridium perfringens, Cutibacterium acnes, Bacillus cereus*, and others) [49, 50, 51, 52] and Gram negative (*Serratia marcescens, Serratia liquefaciens, Klebsiella pneumoniae, Escherichia coli,* etc.) [53, 54, 55] bacteria have been implicated in septic transfusion events involving contaminated PCs. Consequently, blood suppliers like the Canadian Blood Services have implemented various strategies to mitigate the safety risk of transfusing contaminated PCs to vulnerable patients (Figure 3). An overview of the different mitigation strategies used by blood suppliers to minimize the risk of infectious transfusion reactions will be discussed below.

1.6 Mitigation strategies

1.6.1 Screening blood donors

Donor screening allows blood suppliers to avoid collecting potentially contaminated blood components that may pose an infectious transfusion risk to recipients. Therefore, individuals interested in donating whole blood, apheresis PCs or apheresis plasma complete a questionnaire that allows Canadian Blood Services to determine donor eligibility. These questions are aimed at gaining an understanding of the donor's general wellbeing, lifestyle choices, and their medical and travel history, to assess and minimize the risk of an infectious transfusion reaction [56].

1.6.2 Donor skin disinfection

Hemovigilance data obtained by multiple blood suppliers indicate that bacteria that form a part of the skin flora are often identified as bacterial contaminants of blood products like PCs and red blood cell concentrates (RCCs) [3, 52, 57, 58]. As a result, the disinfection of the skin at the site

of venipuncture presents a unique opportunity to prevent such contamination events. Canadian Blood Services (Figure 3) together with other blood suppliers like the National Health Service Blood and Transplant in the UK [59], employ an antiseptic solution consisting of 2% chlorhexidine gluconate and 70% isopropyl alcohol (one-step). This combination of disinfectants is considered the gold standard [60] and is used to disinfect the skin of the donor's antecubital region for 30 seconds followed by a 30 second drying time prior to venipuncture. On the rare occasion that a donor reports chlorhexidine sensitivity (3% of Canadian donors), a disinfectant procedure that employs 70% isopropyl alcohol followed by the application of a 2% iodine tincture (2-minute contact time) is prescribed as an alternate and has been shown to be as effective as the standard disinfectant [59]. Bacterial contamination rates assessed at Canadian Blood Services following the implementation of the one-step procedure indicated that there was no significant reduction in rates compared to the two-step procedure (70% alcohol followed by iodine tincture) that was in use prior to 2009 [61, 62].

1.6.3 First aliquot diversion

The insertion of the needle into the antecubital region results in the formation of a skin plug which is subsequently introduced into the donated product [63]. This skin plug can contain bacteria found on and in the different layers of the skin and can serve as a source of bacterial contamination [63]. As a result, the Canadian Blood Services implemented the diversion of the first 30-40 mL of the donation (Figure 3) into a pouch to sequester the skin plug away from the rest of the donation collected in a different bag [62]. de Korte and colleagues were the first to demonstrate that the diversion of the first 10 mL of the whole blood donation could significantly reduce bacterial contamination rates from 0.35% to 0.021%, with a striking reduction in contamination caused by *Staphylococcal* species (0.14% to 0.03%) [64]. Similarly, Japan reported a reduction in bacterial

contamination from 0.17% to 0.05% with the implementation of this measure [65]. Furthermore, reductions of bacterial contamination rates of up to 77% have been reported following the collective implementation of an improved skin disinfection protocol and first aliquot diversion [66].

1.6.4 Bacterial screening of PCs

The implementation of strategies like donor screening, donor skin disinfection and first aliquot diversion have been shown to reduce the risk of adverse reactions caused by bacterial contamination [62, 66]. However, silent donor bacteremia and inadequate disinfection of the donor's skin at the venipuncture site can continue to serve as a source of contamination. Since PCs are stored at temperatures that are more amenable to bacterial proliferation, many blood suppliers proactively screen this blood product prior to transfusion. Some factors that impact the efficiency of the screening method used include the time that PCs are sampled (early or late sampling) and the volume utilized in the screening method [67]. These considerations mainly stem from the fact that the bacterial concentration in PCs soon after collection and production is relatively low (<10 CFU/ PC unit) [67], however during storage most bacteria can proliferate to clinically significant concentrations [48]. Therefore, screening methods requiring large volumes with high sensitivity like culture-based systems are more effectively utilized soon after collection, while screening methods that are less sensitive like rapid tests would be more effective later in the shelf-life of the PC product [67]. The commonly used culture and rapid methods used by blood suppliers will be discussed below.

1.6.5 Automated culture methods

There are three automated culture-based systems that are used by blood suppliers to screen PCs for bacterial contamination. The BACT/ALERT 3D and the VIRTUO systems manufactured by

bioMérieux utilize culture bottles containing growth media that support microbial growth and a pH sensitive colorimetric gel sensor [68]. Microbial proliferation results in an increase in carbon dioxide concentration and a reduction in pH, which causes the gel sensor to irreversibly change colour from grey to yellow. This change in colour is detected by the BACT/ALERT system and it alerts the user of a culture positive bottle [68]. The third culture system is the BACTEC system (Becton Dickinson) with a similar principle as that of the BACT/ALERT systems as it also detects carbon dioxide as a signal of bacterial growth, however, changes in carbon dioxide concentrations are detected by a fluorescent dye sensor [69].

In 2004, Canadian Blood Services began screening apheresis PCs for bacterial contamination using the automated BACT/ALERT 3D culture system [62]. This culture system is widely used by blood suppliers and initially bacterial screening was performed by inoculating a 4-6 mL PC aliquot into an aerobic culture bottle (BPA) 24 hrs post collection, which was incubated in the BACT/ALERT system for 6 days or until a positive result was obtained [62]. The volume used to inoculate the bottles was subsequently increased to 8-10 mL in 2007 following a change to the package insert of the BPA bottles. In both these scenarios, PCs were released into inventory with a negative to date culture status and were stored for 5 days [62]. Though this protocol enabled the retrieval of contaminated PCs and potentially prevented serious adverse reactions [70, 62], it did have some limitations. For instance, this testing algorithm did not account for anaerobic bacterial contaminants, and though rare, fatal septic transfusion reactions involving anaerobic bacteria have been reported [51]. Furthermore, since bacterial contaminants are generally found at very low concentrations in PCs, contaminants can be missed when low volume sampling is performed [71]. Allowing contaminating bacteria to proliferate and using larger sampling volumes has been demonstrated to improve the detection rates of PC bacterial contaminants [72, 73].

These findings supported the transition to a large volume delayed sampling (LVDS) strategy by blood suppliers including Canadian Blood Services to improve bacterial detection, however the algorithms implemented differ between suppliers. For instance, in 2017, a LVDS testing algorithm was adopted at Canadian Blood Services [3] (Figure 3) that stipulates that the PC product be tested at least 36 hrs post collection and that 8-10 mL PC samples be used to inoculate an aerobic (BPA) and anaerobic (BPN) culture bottle for pooled and single apheresis PCs. On the other hand, double apheresis units were to be sampled from the mother bag (prior to the split into two units) and initially tested using three BPA and one BPN bottle. This was later changed to sampling from each of the split units to inoculate one BPA and one BPN bottle. Additionally, the adoption of a LVDS algorithm required the PC units to be held in quarantine following sampling for a minimum of 6 hrs before being released into inventory and the bottle incubation period in the BACT/ALERT system to be extended from 6 to 7 days [3]. Following a rigorous validation of the new algorithm, the shelf life of PC units was also extended from 5 to 7 days at Canadian Blood Services [3]. However, this algorithm differed from guidelines established by the Federal Drug Administration later in 2019 (Table 1), namely in the minimum requirement for sampling to be performed ≥48 hrs post collection, and a 12 hr quarantine period post PC sampling to be implemented to establish a 7-day PC shelf-life, alternatively secondary testing either using culture based or rapid testing methods to extend PC shelf-life from 5 to 7 days was also recommended [74]. In the UK, testing is performed between 24 and 48 hrs, and PCs are only quarantined for 6 hrs before being released into inventory with a 7-day shelf life [75]. The LVDS algorithm implemented at Héma-Québec (2015) aligns more closely with FDA guidelines, as bacterial screening is performed 48 hrs following collection followed by a 12 hr hold prior to release of PCs stored for 7 days [73]. More recently, the Australian Red Cross implemented a LVDS testing algorithm (2021) in conjunction

with the next generation BACT/ALERT VIRTUO system. The algorithm includes sampling PC units 36 hrs post collection and inoculating 9 mL of the sample into one BPA and one BPN culture bottle followed by incubation in the BACT/ALERT VIRTUO system for 7 days. This system automates bottle loading and has demonstrated shorter detection times especially for pathogenic bacterial isolates. Unlike the other algorithms described, the Australian algorithm does not include a post testing quarantine period prior to release into inventory (7-day shelf life), the authors indicated that the early detection of pathogenic bacterial contaminants in the VIRTUO system allows for timely recall of products before they are delivered to hospitals [76].

Despite these differences, all sites that implemented the LVDS bacterial screening algorithm reported on the prevention of the transfusion of bacterially contaminated PCs [3, 73, 75, 76]. Data collected at Canadian Blood between 2017 and 2023 following the implementation of the new algorithm, indicated that the confirmed positive bacterial detection rate increased by 11-fold from 0.01% to 0.11% (Canadian Blood Services, Quality Control Data August 2017- December 2023). This increase was driven mainly by the detection of anaerobic bacterial contaminants (0.09%) (Figure 3). Furthermore, a comprehensive guide was adopted to categorize bacterial screening results to determine whether companion products (RCCs, and plasma) could be released, a simplified table of the categorization strategy can be found in Table 2. The extension of the shelf life of PCs at Canadian Blood Services reduced the outdate rate from 18.9% to 13.1% (2017-2019) with a cost benefit of approximately 1.9 million dollars over 27 months [3]. In addition to these savings, between March 2018 and December 2023, approximately 3635 RCCs and 616 plasma units were re-introduced into inventory bolstering the supply by reducing waste (Canadian Blood Services, Quality Control Data August 2017- December 2023).

Table 1. FDA guidelines for blood manufacturers. LVDS algorithm recommendations for the bacterial screening of apheresis and pooled PCs [74]

Product type	Sampling time of primary culture	Volume sampled	Growth conditions	Minimum quarantine period	Shelf-life	Secondary culture/ rapid testing recommended	Secondary testing method, testing details	Shelf-life extended
Apheresis and pooled PCs	≥ 36 hr	≥ 16 mL	Aerobic and anerobic	12 hr	5 days	Yes	Culture: Apheresis and pooled PC- 8 mL sampled from pool or each split unit @ ≥ 3 days, at least aerobic	No, 5 days
							Culture: Apheresis – 16 mL sampled from each split unit @ ≥ 4 days, both aerobically and anaerobically, 12 hr quarantine	Yes, 7 days
							Rapid: Apheresis and pooled PC sampled as per manufacturer instructions	Yes, up to 7 days
	≥ 48 hr	≥ 16 mL	Aerobic and anerobic	12 hr	7 days	No	N/A	N/A

Table 2. Simplified categorization strategy of bacterial screening results following a positive signal in the BACT/ALERT system. The disposition of companion components (RCCs and plasma) following confirmatory testing and categorization has been listed.

	Initial Bottle/s	Confirmatory bottle/s	Categorization	Disposition of co-components
Bacteria Isolated	No	N/A: BACT/ALERT test negative	False positive	Release
	Yes	Yes: Same species as initial bottle	Confirmed positive	Discard
	Yes	No	Unconfirmed	Discard
	Yes	Not available for testing	Indeterminate	Discard
	Yes	Yes: Different species than in initial bottle	Discordant	Discard

1.6.6 Rapid tests

Though bacterial screening using culture-based methods are generally considered the gold standard, rapid tests provide a means to quickly and proactively screen for bacterial contamination. Rapid methods that have been utilized for bacterial screening include PCR based assays [77], qualitative immunoassays [78, 79], and flow cytometry-based assays [80]. This section will focus on methods that are commonly used by blood suppliers. Nucleic amplification testing (NAT) targeting ribosomal genes and housekeeping genes have been described [81, 82] and German blood

suppliers routinely implement NAT to detect bacterial contamination prior to the release of PCs with demonstrated detection limits of ~10 CFU/mL [83]. Unfortunately, there are no commercially available NAT kits that can be used in the transfusion setting, as a result blood suppliers need to perform rigorous optimization and validation of NAT protocols so they can be used in house. On the other hand, two immunoassays are commercially available, namely the pan genera detection (PDG) assay manufactured by Verax that is based on the detection of Gram-negative lipopolysaccharides and Gram positive lipoteichoic acids [84], and the BacTx assay (Immunetics) based on the detection of bacterial peptidoglycan [79]. The detection limit for PDG and BacTx has been demonstrated to be approximately 10^3-10^5 CFU/mL [85, 86], this highlights the benefits of its utility as a point of care screening strategy. The PDG and the BacTX assays have been approved by the FDA and can be used in conjunction with LVDS as a secondary rapid testing method to release PCs or to extend their shelf-life. All the rapid assays described require a relatively high concentration of bacteria to be detected, which indicates that the time that PCs are tested during storage would be crucial for the success of these methods.

1.6.7 Pathogen reduction/ inactivation

Technologies that proactively inactivate or reduce the bacterial burden of contaminated blood products are a promising tool to enhance the safety of blood products. Three pathogen inactivation (PI) technologies are available, namely: INTERCEPT (Cerus Corp. USA) [87], Mirasol (Terumo BCT Inc, USA) [88] and THERAFLEX (MacoPharma, France) [89]. These technologies inactivate microbial contaminants by targeting and disrupting their nucleic acids [90] and differ in their mechanism of action (Table 3). Hemovigilance data obtained from countries (France (2006-2015), Belgium (2009-2015), and Switzerland (2011-2015)) that implemented INTERCEPT reported no incidences of septic transfusion reaction in the time periods indicated, reflecting the infusion of

over 600,000 PR PCs [91]. These reports suggest that the move toward PI technologies was in the right direction towards enhancing product safety, especially since PI technologies do not only inactivate microbial contaminants, but also residual white blood cells thereby minimizing the risk of non-infectious transfusion reactions [92]. Despite the numerous documented benefits of PI technologies, no system is perfect, and it does have limitations. Notably, the time at which PR technology is applied to blood components is quite important as high bacterial titers prior to inactivation can result in breakthrough contamination events and accumulation of toxins, and these breakthrough events can be species dependent [93]. Furthermore, since PI technologies like INTERCEPT require the penetration of a photosensitizer into the bacterial cell followed by UV radiation [87], it is not clear whether it would be effective against bacterial biofilms that are three dimensional structures [94, 95]. It is also known that PI technologies are not efficient at eradicating bacterial spores [87]. Therefore, PI-treated PCs are considered pathogen reduced (PR) and not pathogen inactivated products.

In an effort to enhance product safety, Canadian Blood Services began the phased implementation of the licensed INTERCEPT PI technology in 2022, which was completed in May of 2024 (Figure 3). The implementation of this technology required modifications to the PC production process as PCs needed to be prepared in a mix of plasma (42%) and PAS SSP+ (58%) (Figure 1 and 2). Specifically for pooled buffy coat PCs, the process was changed from pooling four buffy coat pools in one autologous plasma to pooling seven buffy coat pools in a mix of plasma-PAS as described in Figure 1. Notably, since implementation, no ATRs associated with PR- PCs have been documented in Canada. However, there have been a few reports of breakthroughs of bacterial contamination in PI-treated PCs. Four septic reactions were reported in the US involving the transfusion of PR PCs in 2018 and 2021 which resulted in two fatalities [96]. These cases

originated from different states in the US and the source of bacterial contamination was identified as the manufacturing facility of the blood collection sets, which suggests that implemented PI technologies were ineffective at eliminating these contaminants [96]. However, in some cases, it has been demonstrated that contamination occurred post PI treatment due to damaged PC containers, highlighting the importance of strict quality control throughout PC production, transportation, and storage [97]. Furthermore, in 2023, France reported on a septic adverse reaction following the transfusion of a PR PC involving a sporulating bacterium (*Bacillus cereus*), and three near misses involving *B. cereus* and the skin isolates *S. epidermidis* and *S. aureus* [98], followed by a recent report of ATR involving the sporulating bacterium *Bacillus mobilis* [99]. Another potential residual risk associated with PR PCs stems from the fact that though contaminating bacteria are inactivated it is possible endotoxins or other pyrogens may still be present in the product which could result in adverse reactions [100]. A report published by Benjamin and colleagues suggest that this may be the case, as a patient who received a PR PC experienced a mild FNHTR even though the contaminating Gram negative bacteria was successfully inactivated [91].

As described above, Canadian hemovigilance data indicate that 0.11% of pooled PCs are bacterially contaminated which suggests that it is highly likely that one or more companion products like RCCs are contaminated as well, and this was demonstrated when approximately 42% of *C. acnes* contaminations were confirmed following bacterial testing of RCCs (Canadian Blood Services, Quality Control Data August 2017- December 2023). Using the LVDS algorithm such units would be discarded and prevented from being transfused, however with the implementation of INTERCEPT, and the lack of licensed PI technology to treat RCCs, these contaminated products would be available for transfusion. This highlights the residual risk to transfusion patients.

Table 3. Comparison of the three available pathogen reduction technologies, showing differences in the photosensitizer used and the mechanism of action employed for each method. Table modified from Levy et al. [101]

	INTERCEPT	**Mirasol**	**THERAFLEX**
Photosensitizer	Amotosalen	Riboflavin	NA
UV wavelength	UVA, 320–400 nm	UVB/UVA, 265–370 nm	UVC, 254 nm
UV dose	3 J/cm2	6.2 J/mL	0.2–0.3 J/cm2
Mechanism	Amotosalen intercalates in nucleotide helices, covalently cross-links bases, locking helix preventing replication, transcription and translation	Riboflavin non specifically binds to nucleotides and upon UV exposure irreversibly modifies nucleic acids through oxidation of guanine residues preventing replication	UVC together with rigorous agitation selectively targets nucleotides modifying DNA preventing transcription
Targets	Gram positive and negative bacteria, enveloped viruses and some non-enveloped viruses and parasites [87]	Gram positive and negative bacteria, enveloped viruses and some non-enveloped viruses and parasites [102]	Gram positive and negative bacteria, enveloped viruses and some non-enveloped viruses and parasites [103]

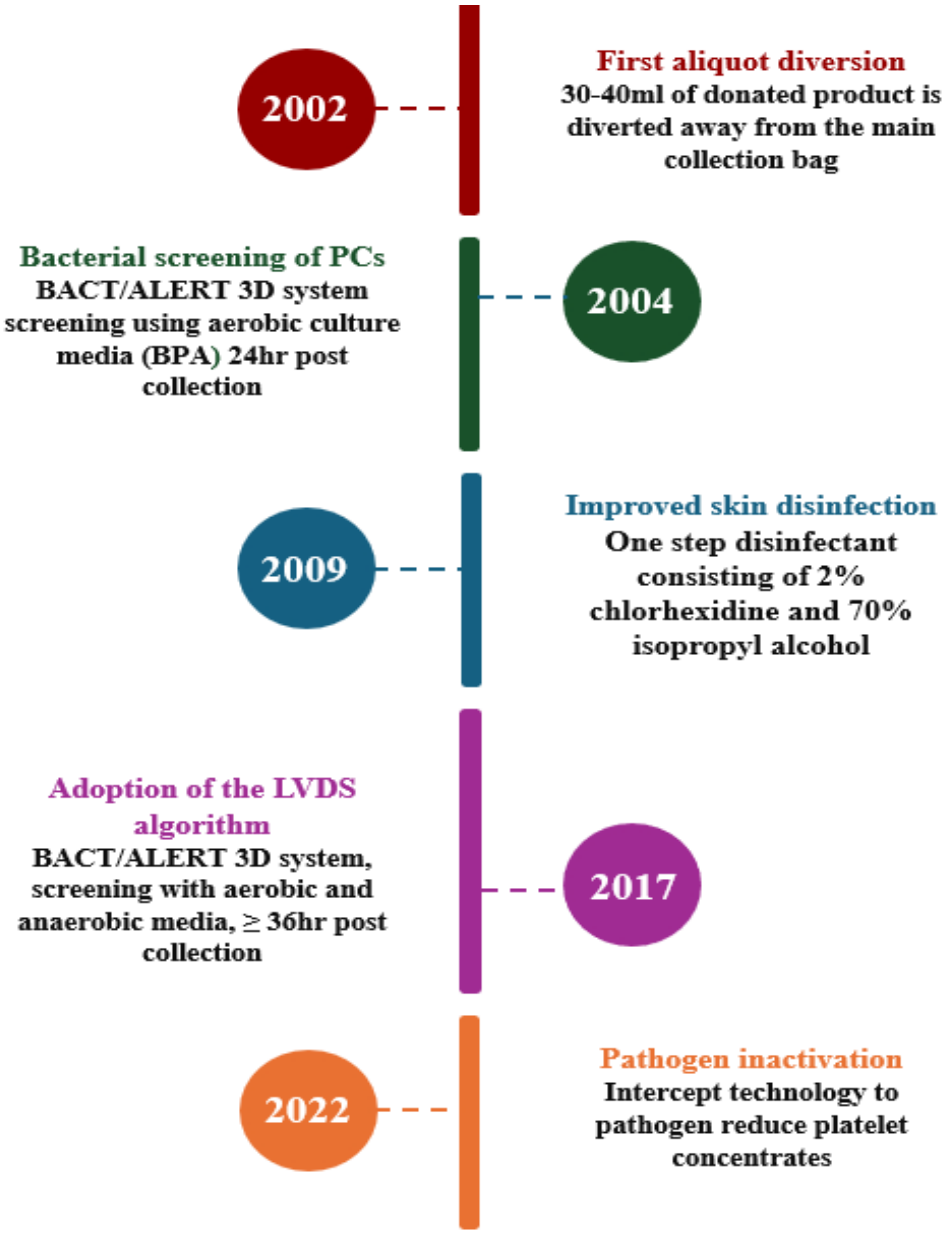
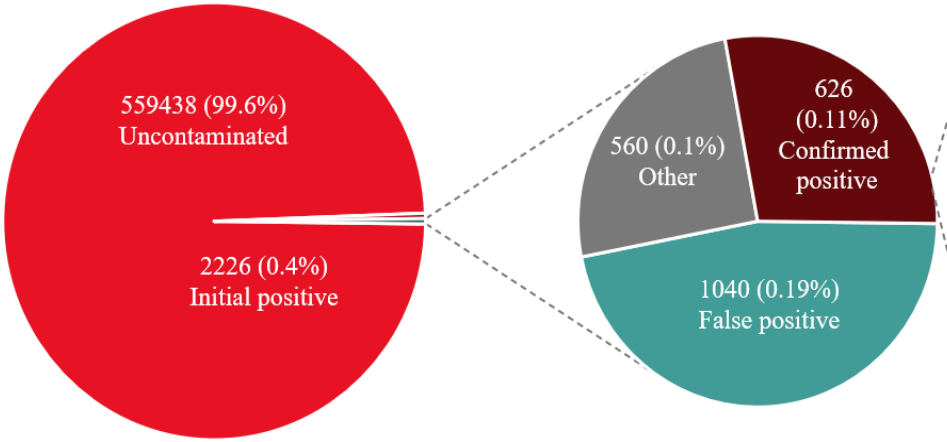


Figure 3. Timeline of the implementation of mitigation strategies to reduce the risk of adverse transfusion reactions caused by bacterial contamination at Canadian Blood Services.
Mitigation strategies include first aliquot diversion of the first 30-40 mL of the donation, bacterial screening using the automated BACT/ALERT culture system, the introduction of an improved one-step disinfectant protocol using 2% chlorhexidine and 70% isopropyl alcohol, the adoption of a large volume delayed sampling (LVDS) algorithm that screened for both aerobic and anaerobic contaminants, and finally the implementation of the INTERCEPT pathogen inactivation technology to produce pathogen reduced PCs.

1.7 Canadian hemovigilance data following the implementation of the LVDS algorithm

Despite employing various mitigation strategies, the bacterial contamination of PCs continues to occur, and these contaminated units can be transfused into patients due to delayed or missed detection by the BACT/ALERT system [50, 3]. Though the LVDS algorithm improved detection rates, bacteria may not be detected during routine screening, but may be captured during quality control testing of outdated units, or during the investigation of septic transfusion reactions and are categorized as false negatives. [3]. Of note, three septic ATRs categorized as false negatives were reported in Canada (rate of ~1/165000), following the implementation of the LVDS algorithm involving *S. aureus* and two CoNS which fortunately did not lead to fatalities [50]. Interestingly, *C. acnes* was the most isolated bacterial contaminant (72%) (Figure 4) and similarly high contamination rates with this bacterium have been reported by other blood centers including Héma-Québec (50%) [73], the German Red Cross Blood Service (54%) [52] and the Netherlands (54%) [58]. Notably, around 42% of confirmatory testing of pooled PCs at Canadian Blood Services were performed on companion RCCs, as the associated PC pools had already been issued to hospitals and transfused, of these units ~ 98% were contaminated with *C. acnes* (Canadian Blood Services, Quality Control Data August 2017- December 2023). This data is consistent with hemovigilance reports that have underscored the high frequency at which *C. acnes* contaminated PCs are transfused into patients at other blood centers [73, 52]. The detection times observed in the BACT/ALERT system for *C. acnes* from contaminated PCs can range from 3-7 days owing to the slow growing nature of this bacterium in culture media [3, 52, 73]. As a result, the window of opportunity to retrieve *C. acnes* contaminated product prior to transfusion is missed. Mild adverse reactions have been identified and associated with the transfusion of *C. acnes* contaminated PCs in retrospective studies performed by German and Dutch blood suppliers [52, 58]. These reactions

are generally missed at the time of transfusion and often associated with underlying conditions, and therefore it is believed that adverse reactions caused by *C. acnes* are potentially underrecognized and under reported [52]. Notably, adverse reactions associated with the transfusion of *C. acnes* contaminated PCs have yet to be reported in Canada [3, 73]. Therefore, this bacterium is often dismissed as a cause for concern, despite there being no studies assessing the long-term impact of these transfusion events on patient health. Taken together the hemovigilance data demonstrates the benefits of the LVDS testing algorithm, however, it also highlights the limitations of the mitigation strategies described and the residual safety risk associated with bacterial contamination of PCs.



		Bacterial species	#
Anaerobes		***Cutibacterium acnes***	**453**
		Staphylococcus saccharolyticus	53
		Atopobium vaginae	3
		Diphtheroid bacilli	2
		Peptoniphilus asaccharolyticus	2
		Bacteriodes fragilis	1
		Clostridium clostridioforme	1
		Eggerthella lenta	1
		Finegoldia magna	1
		Cutibacterium spp.	1
		Anaerobic non-spore forming Gram positive bacilli	1
Facultative anaerobes	Gram Positive	*Staphylococcus epidermidis*	37
		Coagulase Negative Staphylococcus	28
		Streptococcus spp.	26
		Staphylococcus aureus	5
		Bacillus spp.	4
		Bacillus spp. Lactococcus garvieae	2
	Gram Negative	*Klebsiella oxytoca*	1
		Pseudomonas aeruginosa	1
		Serratia marcescens	1
		Proteus mirabilis	1
		Klebsiella pneumoniae ssp pneumoniae	1

Figure 4. Canadian hemovigilance data (2017-2023).

The total number of initial positive bottles obtained following the adoption of the LVDS algorithm, of which 0.11% were further confirmed to be contaminated with bacteria, 0.19% were found to be free of bacterial contamination and categorized as false positives and 0.1% were categorized as “Other” which include indeterminate, discordant, and unconfirmed positive results. A list of bacterial contaminants isolated from confirmed positive PC units indicating that anaerobic bacterial contaminants belonging to the skin microflora are the most isolated bacterial contaminants, with C acnes topping the list at 72%.

1.8 *Cutibacterium acnes*

1.8.1 Taxonomy

Human skin harbors multispecies communities that include bacteria, fungi, and viruses [104]. The harmonious co-existence of these microbes is essential to maintain healthy skin, prime the immune system, and prevent colonization of the skin by more pathogenic microbes [105, 106]. The three bacterial genera isolated from skin that contribute to up to 80% of the total skin microbiome, include *Cutibacterium*, *Staphylococcus* and *Corynebacterium* [107]. Microbiome studies have identified *C. acnes* as the most represented bacterial commensal species found on the skin [108]. It thrives in sebum rich regions with concentrations ranging from 10^2-10^6 CFU/ cm^2 [109] and is known to form aggregates (biofilms) on the surface of the skin and within the deeper layers of the hair follicle [110]. This Gram positive, anaerobic aerotolerant bacterium was first identified in 1894 by Unna and Hodara in acne comedones [111] and was later isolated and cultured by Sabouraud in 1897 [112]. Since its isolation, *C. acnes* has been classified and reclassified into different genera based on its morphology [113, 114], growth requirements and fermentation products [115], and core genetic makeup [116] (Table 4). *C. acnes* isolates can be further categorized based on cell wall composition [117, 118], sequence comparisons of housekeeping genes [119, 120] and ribotyping [121]. The significance of different phylotypes in the clinical setting will be discussed below.

Table 4. Characteristics that supported changes in *C. acnes* nomenclature.

Nomenclature	Supporting Characteristics	Year
Bacillus acnes	Microscopic morphology rod shaped, isolated from acne comedones	1900
Corynebacterium acnes	Morphological similarities to *Corynebacterium*	1923
Propionibacterium acnes	Requirement for anaerobic growth environment, production of propionic acid	1946
Cutibacterium acnes	Difference in core genome compared to dairy derived *Propionibacterium* species	2016

1.8.2 Clinical significance of *C. acnes*

C. acnes has long been associated with the pathogenesis of acne; excessive sebum production together with hyper keratinization results in blocked ducts where *C. acnes* is able to proliferate. These lipid rich niches allow *C. acnes* to utilize sebum derived triglycerides to give rise to fatty acids [122] and to express Christie–Atkins–Munch-Peterson (CAMP) factors and porphyrins which elicit a pro-inflammatory response and promotes tissue damage [123]. Though initially the overgrowth of *C. acnes* was thought to contribute to the progression of acne, recent studies have demonstrated that the dysbiosis of *C. acnes* populations favoring phylotype IA is more likely responsible for the progression of the disease [121].

In general, *C. acnes* is mostly associated with dermatological illnesses and is often dismissed as a skin contaminant when obtained in cultures in the clinical setting [124]. However, in recent years, several studies have identified *C. acnes* as the causative agent of chronic, slow developing infections that can result in severe morbidity and in some cases death [125]. Though there is some debate about the role phylotypes play in the manifestation of disease, the consensus is that phylotypes IB and II are involved in deep seated infections while phylotype IA is associated more with acneic lesions [126, 127]. In a recent study assessing the role of *C. acnes* phylotypes in disease, all phylotypes except phylotype III were isolated from sites of infection [128]. The recalcitrance and severity of most of infections like infective endocarditis of artificial valves [124], prosthetic joint infections [129], spinal disc disinfections [130], and cerebral abscesses [131] have been directly linked to the ability of *C. acnes* to form biofilms and elicit proinflammatory responses [132]. The growing number of reports implicating *C. acnes* in clinically significant infections underscore the fact that this bacterium may not be as harmless as previously believed.

1.9 Clinical significance of biofilms

Biofilms, as described above, are bacterial aggregates encased in a self-produced matrix which is composed of a combination of proteins, extracellular DNA, lipids, and polysaccharides [94]. The ability to form biofilms is considered an important virulence factor as bacteria that reside within them tend to display heightened antimicrobial tolerance [133], can resist environmental [134] and mechanical stressors [135] and are better able to evade the immune response [136]. The matrix that surrounds the biofilm contributes heavily to antimicrobial [95] and immune resistance [136], and its composition can be influenced by its environment [137]. The metabolic stratification in the biofilm [138] and the presence of cells that temporarily display heightened resistance to antimicrobials called persister cells [139] further enhance the resistance observed and contributes

to the recurrence of infections. The presence of biofilms at the site of infection has also been shown to enhance the concentration of inflammatory mediators resulting in chronic inflammation and inhibiting timely wound healing [140].

1.10 Impact of the PC environment on transfusion relevant bacteria

The ability of bacteria to modulate gene expression in response to diverse environmental cues is essential for survival [141]. The Ramirez lab has demonstrated that transfusion relevant *S. epidermidis* and *S. aureus* modulate their gene expression profile in the PC milieu resulting in the upregulation of virulence genes that code for capsule proteins, toxins, antibiotic resistance, and biofilm formation [142, 143, 144]. Notably, these findings are consistent with earlier reports from the Ramirez lab which demonstrated that transfusion relevant bacteria are able to form biofilms in the PC environment thus contributing to sampling errors and missed detection in the BACT/ALERT system [53]. Furthermore, we have also shown that *S. epidermidis* displays a phenotypic switch from a weak biofilm former in media to a strong one in PCs [145]. More conclusively, the Ramirez lab reported that PC derived *S. epidermidis* displays heightened virulence in the invertebrate *Caenorhabditis elegans* model when compared to the same isolates derived from media [146]. Taken together, these studies indicate that the PC environment heightens the virulence expression profile of transfusion relevant bacteria, thereby potentially influencing clinical outcomes in transfusion patients.

1.11 Rationale

As the saying goes, prevention is better than cure, and one of the first points of intervention to prevent the bacterial contamination of blood products is the disinfection of the donor's skin prior to venipuncture. The fact that *C. acnes* is routinely isolated from PCs indicates that the current disinfectant protocol may not be effective at completely eradicating this bacterium. Since biofilms are impacted by their environment and display heightened resistance to antimicrobials, it is plausible that the biofilms formed by *C. acnes* in the skin may be influenced by its sebaceous environment, resulting in enhanced resistance to disinfection and therefore contributing to its dominance as a PC contaminant. Understanding the factors that negatively impact the efficacy of the donor skin disinfectant protocol will aid in investigating more effective alternatives. A retrospective look at the hemovigilance data indicates that a large number of *C. acnes* contaminated PCs are transfused into vulnerable patients due to late detection in the automated BACT/ALERT culture system. This information highlights an opportunity to improve the current detection of this slow growing bacterium, thereby enhancing the safety of the blood supply. Furthermore, since we have previously demonstrated that the PC environment elicits the heightened expression of virulence genes in transfusion related bacterial contaminants, it is essential that we understand the potential of the PC environment to prime *C. acnes* to cause chronic delayed infections by eliciting the heightened expression of virulence genes. Finally, PCs are involved in non-infectious adverse reactions driven by the accumulation of pro-inflammatory factors during storage, notably *C. acnes* is known to display pro-inflammatory characteristics. It is therefore important to ascertain if *C. acnes* contamination in PCs can increase the risk of non-infectious reactions like FNHTR by eliciting the release and accumulation of proinflammatory factors in the PC unit.

1.12 Hypotheses

The transfusion relevant bacterium *C. acnes* resists eradication by standard donor skin disinfection thus contributing to its over representation as a contaminant of PCs.

Furthermore, the PC environment can elicit the expression of *C. acnes* virulence genes and contamination with even low concentrations of *C. acnes* can cause an increase in the pro-inflammatory profile of the PC unit during storage.

1.13 Objectives

The four objectives designed to test the hypothesis have been outlined in Figure 5, which demonstrates how each objective assesses a different aspect of the PC production process.

The objectives have been listed below:

1. To determine if *C. acnes* can evade eradication during blood collection.
2. To enhance the detection of *C. acnes* in the BACT/ALERT system.
3. To investigate if *C. acnes* displays heightened virulence in PCs during storage.
4. To evaluate if *C. acnes* contamination can lead to the accumulation of pro-inflammatory factors in the PC unit during storage.

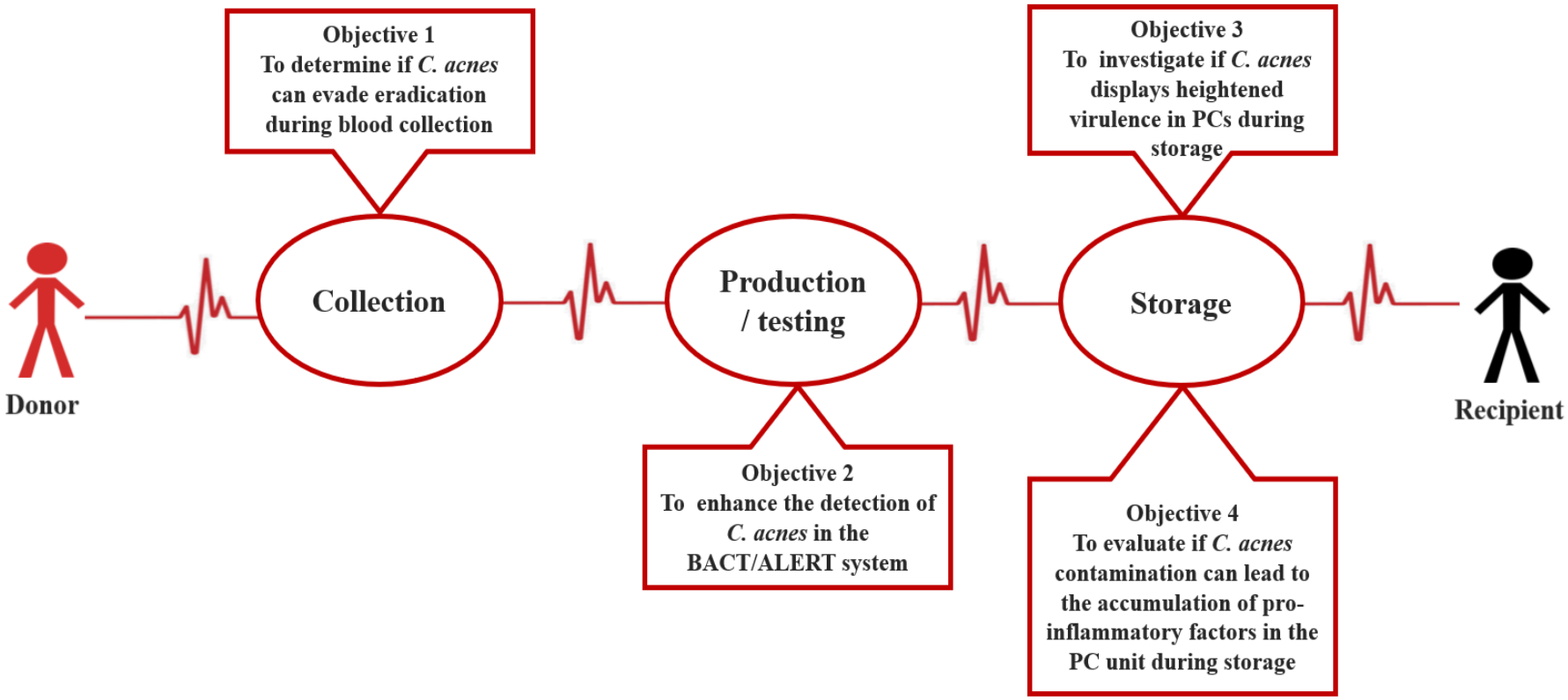


Figure 5. The objectives designed to test the stated hypothesis.

The objectives have been designed to span the PC production process, from collection to production and storage with a focus on improving the safety of PCs.

Chapter 2 Sebum Components Dampen the Efficacy of Skin Disinfectants against *Cutibacterium acnes* Biofilms

2.1 Statement of contribution and status of manuscript

The manuscript titled “Sebum components dampen the efficacy of skin disinfectants against *Cutibacterium acnes* biofilms” has been published in Microorganisms, 2024 Jan 27;12(2):271. doi: 10.3390/microorganisms12020271. PMID: 38399675; PMCID: PMC10891977. Please find licenses and permissions in Appendix B. The numbering of sections, tables and figures have been modified (minor) as per the requirements of the Faculty of Medicine for an article-based thesis.

The conceptualization of the study, the development, optimization, and execution of the methodology, as well as data curation and statistical analyses were performed by Dilini Kumaran. The project was supervised by Dr. Ramirez-Arcos who provided guidance with study design, trouble shooting, and resources to execute the study. The original and final draft of this manuscript was written by Dilini Kumaran and was reviewed and edited by Dr. Ramirez-Arcos.

2.2 Abstract

At Canadian Blood Services, despite the use of 2% chlorhexidine and 70% isopropyl alcohol (standard disinfectant, SD) prior to venipuncture, *Cutibacterium acnes* evades eradication and is a major contaminant of platelet concentrates (PCs). Since *C. acnes* forms bacterial aggregates known as biofilms in the sebaceous niches of the skin, this study aimed to assess whether sebum-like components impact disinfectant efficacy against *C. acnes* leading to its dominance as a PC contaminant. *C. acnes* mono-species and dual-species biofilms (*C. acnes* and a transfusion-relevant *Staphylococcus aureus* isolate) were formed in the presence and absence of sebum-like components and exposed to SD, a hypochlorous acid-based disinfectant (Clinisept+, CP), or a combination of both disinfectants to assess disinfectant efficacy. Our data indicate that sebum-like components significantly reduce the disinfectant efficacy of all disinfectant strategies tested against *C. acnes* in both biofilm models. Furthermore, though none of the disinfectants led to bacterial eradication, the susceptibility of *C. acnes* to disinfectants was heightened in an isolate-dependent manner when grown in the presence of *S. aureus.* The reduction of skin disinfection efficacy in the presence of sebum may contribute to the overrepresentation of *C. acnes* as a PC contaminant and highlights the need for improved disinfection strategies.

2.3 Introduction

Human skin is rich in microbial diversity, and the composition of these bacterial populations can vary by gender [147], site on the skin [108], gut health [148], and environmental factors [149]. Multiple studies have demonstrated that bacterial skin isolates can form aggregates called biofilms [110, 150]. The ability to form biofilms is an important virulence factor conferring to bacteria that dwell within these communities, a heightened ability to resist the action of antimicrobials and the ability to evade host immune responses [151]. It is therefore unsurprising that biofilms have been associated with disease states on the skin [152] and in recalcitrant, deep seated chronic infections such as those encountered in periprosthetic joint infections [153], urinary tract infections [154], and in the lungs of cystic fibrosis patients [155] to name a few. Consequently, skin disinfection prior to invasive procedures, is an important strategy to prevent infection by diminishing the risk of the inadvertent introduction of bacteria from the surface of the skin into the patient.

Cutibacterium acnes is an anaerobic aerotolerant member of the skin flora and is one of the most represented bacterial species on the skin [108]. *C. acnes* thrives in regions rich in sebum where it can form biofilms that can extend into the deep layers of the terminal hair follicle [110]. Generally considered a harmless commensal, *C. acnes* has often been dismissed as a contaminant when isolated from clinical samples [156]. However, in recent years, there has been a growing awareness of the association of this bacterium with chronic slow developing infections such as infectious endocarditis [157], bone infections [158], and prosthetic device infections [153].

Canadian Blood Servies manufactures platelet concentrates (PCs), a blood product used to treat patients with platelet deficiencies. PCs consist of platelets suspended in 100% plasma or in plasma diluted in platelet additive solution and are stored in gas permeable plastic containers at 20–24 °C under agitation to maintain platelet functionality. However, these storage conditions can promote

the proliferation of bacterial contaminants introduced during blood collection. To mitigate the risk of transfusing contaminated PCs, this blood product is routinely screened for the presence of bacteria with the automated BACT/ALERT 3D culture system (bioMérieux, Montreal, QC, Canada) [3]. Hemovigilance studies at Canadian Blood Services have revealed that *C. acnes* is the most isolated bacterial contaminant of PCs accounting for ~70% of all bacteria isolated from positive cultures [3]. Due to the slow growing nature of *C. acnes* in vitro, it is often detected after contaminated units are transfused into vulnerable patients [3]. This occurs despite the regimented use of an aqueous disinfectant solution (chlorhexidine gluconate (2%, v/v) and isopropyl alcohol (70%, v/v)) to disinfect the donor's skin (30 s) prior to venipuncture [61]. Though only a handful of mild transfusion reactions have been attributed to *C. acnes* contaminated blood products [52, 58], it is difficult to ascertain the long-term impacts of these transfusion events.

This study aimed to investigate factors that contribute to the over representation of *C. acnes* as a PC contaminant, by assessing the impact that sebum-like components have on biofilm formation and disinfectant efficacy in mono-species and dual-species biofilm models established with *C. acnes* isolates derived from PCs and a transfusion-relevant *Staphylococcus aureus* isolate.

2.4 Materials and methods

2.4.1 Bacterial strains

Twenty *C. acnes* isolates obtained from PCs that were transfused to patients originating from different geographical regions of Canada (2018–2019) were assessed in this study and have been listed in Table 5. The ATCC 6919 *C. acnes* strain was used as a control for multiplex PCR and for preliminary biofilm assays, and a transfusion-relevant *S. aureus* isolate (CBS 2016-05) [159] was used for dual biofilm assays.

Table 5. Bacterial identifiers of *C. acnes* isolates derived from contaminated PCs and the geographical region of component production.

***C. acnes* ID**	**Geographical location (Canadian Province)**
BPNBT-18005 BPNBT-18120 BPNBT-19210 BPNBT-19223 BPNBT-19269 BPNBT-19329	Alberta
BPNBT-18313 BPNBT-19119 BPNBT-19195 BPNBT-19280	British Columbia/ Yukon
BPNBT-19054 BPNBT-19356	Manitoba
BPNBT-18290 BPNBT-18317 BPNBT-19227 BPNBT-19295 BPNBT-19352 BPNBT-19354 BPNBT-19217 BPNBT-19355	Ontario

2.4.2 Media and Reagents

All bacterial isolates were cultured on tryptic soy agar supplemented with 5% sheep blood (blood agar, BA, Fisher Scientific, Hampton, NH, USA) unless stated otherwise. Bacterial suspensions for biofilm assays were prepared in Brain Heart Infusion media (BHI). Sebum-like components including triolein, squalene, and olive oil (source of oleic acid) were purchased from Sigma Aldrich (Oakville, ON, Canada) while pure jojoba oil was sourced from Now Solutions (Toronto, ON, Canada). These components were either used individually or in combination in previously described ratios [160] to produce a sebum-like emulsion referred to as "SL" in biofilm assays. A neutralizing solution consisting of 10% (v/v) Tween 80 (Sigma Aldrich, Oakville, ON, Canada), 3% (m/v) lecithin (Fisher Scientific, Ottawa, ON, Canada) and 0.3% (m/v) sodium thiosulfate (Sigma Aldrich, Oakville, ON, Canada) was assessed as per ASTM E1054-08 (Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents) [161] and employed in disinfectant efficacy assays. A 20% stock solution of chlorhexidine gluconate (Sigma Aldrich, Oakville, ON, Canada) and pure isopropyl alcohol (Fisher Scientific, Ottawa, ON, Canada) were diluted in sterile water to prepare an aqueous disinfectant solution consisting of 2% chlorhexidine gluconate and 70% isopropyl alcohol (standard disinfectant, SD) and used immediately in disinfectant efficacy assays. Clinisept + (CP) (Clinical Health Technologies, Peterborough, UK) was used as an alternate disinfectant in this study.

2.4.3 Phylotyping *C. acnes* PC isolates

C. acnes PC isolates were sub-cultured on BA and incubated anaerobically at 37 °C for 72 h. Bacterial colonies were collected using a sterile cotton swab and resuspended in Tris EDTA buffer (pH 7.4) and heated to 80°C for 15 min. The bacterial suspension was then treated with 1×10^6 units of lysozyme and RNase A (final concentration 100 µg/mL) and incubated at 37°C for 2 h.

Proteinase K (final concentration of 0.1 µg/µL) and 6% SDS solution at 0.1× of the total volume were added and incubated at 37 °C with agitation (160 rpm) for 2 h. The samples were then placed at 55 °C for 30 min, and genomic DNA was extracted using an equal volume of chloroform: phenol: isoamyl alcohol (25:24:1) and precipitated with sodium acetate and ethanol. A touchdown-multiplex PCR was performed, and the isolates were categorized into different phylotypes based on the banding patterns obtained on a 1.2% agarose gel as previously described [162].

2.4.4 Preliminary assessment of *C. acnes* biofilms with oleic acid supplementation

The twenty *C. acnes* PC isolates and the ATCC 6919 isolate that served as a positive control were sub-cultured on BA for 72 h at 37 °C under anaerobic conditions. Bacterial suspensions corresponding to ~10^7 colony forming units (CFU)/mL were prepared in BHI using a Densimat machine (reading of ~0.15) (bioMérieux, Montreal, QC, Canada). This bacterial suspension was used to seed the wells of 48-well plates that were either uncoated or coated with olive oil. Coated and uncoated wells seeded with media in the absence of bacteria served as the background control. The plates were incubated statically under anaerobic conditions for 7 days at 37 °C. Planktonic cells were removed, adhered cells were washed with sterile water, and the biofilms were fixed for 30 min with 100% methanol. The fixed biofilms were stained with a crystal violet solution for 30 min, following which biofilms were washed to remove excess stain. An aqueous solution of 20% (v/v) methanol and 5% (v/v) acetic acid was used to solubilize the adhered stain, which was quantified using a spectrophotometer at 492 nm. Each condition was tested in technical duplicates and in biological triplicates.

2.4.5 Impact of sebum-like components on *C. acnes* biofilm formation

Four *C. acnes* PC isolates (BPNBT-19269, 19195, 19329, and 19227) representing the different phylotypes identified were chosen for successive experiments based on preliminary biofilm data. Biofilm formation was assessed as described above; however, wells either remained uncoated (control), or were coated with individual sebum-like components (triolein, squalene, jojoba oil, and olive oil) or SL. Each condition was tested in technical duplicates and biological triplicates.

2.4.6 Confocal microscopy

C. acnes biofilms of the four representative PC isolates were established over 7 days at 37 °C under anaerobic conditions in 8-chambered permanox microscope slides (Thermofisher) coated with SL. Biofilms formed in uncoated chambers served as a control. Biofilms were washed and fixed with 2.5% glutaraldehyde for 2 h at room temperature. The fixative was then removed, biofilms washed with phosphate-buffered saline (PBS), and Fluoroshield (Sigma Aldrich, Oakville, ON, Canada) a mounting medium containing DAPI was added to each chamber and was visualized using the LSM 800 microscope and Zeiss EC Plan-Neofluar 10×/0.3 objective lens with excitation by a 405 nm solid state laser.

2.4.7 *C. acnes* and *S. aureus* dual-species biofilm formation in the presence of sebum-like components

The wells of 48-well plates were coated with SL and were seeded with the bacterial suspensions of the four representative *C. acnes* PC isolates (BPNTBT-19269, 19195, 19329, and 19227). The plates were incubated statically for 6 days at 37 °C under anaerobic conditions, at which point 0.25 mL of the planktonic cells were removed and replaced with 0.25 mL of *S. aureus* (CBS 2016-05) bacterial suspension prepared in BHI (10^7 CFU/mL). The plates were re-incubated statically for an additional 24 h under anaerobic conditions at 37 °C. Wells containing mono-species biofilms

served as biofilm controls, while wells containing media alone served as background control. Once biofilms were established, the biofilms were either stained as described above or enumerated by dislodging the biofilms and plating serial dilutions on BA plates to determine bacterial counts of each species (anaerobic incubation, 37 °C, up to 96 h). Each condition was tested using technical duplicates and in biological triplicates.

2.4.8 Disinfectant efficacy assays

2.4.8.1 Mono-species biofilms

Washed preformed biofilms of BPNTBT-19269, 19195, 19329, and 19227 established in either individual sebum-like components or SL coated wells or uncoated control wells were exposed to either 0.5 mL of (a) SD for 30 s as is performed on the skin during donor skin disinfection prior to venipuncture, (b) CP for 2 min as recommended by the manufacturer, (c) to the two disinfectants successively with exposure to SD first followed by CP or (d) to CP first followed by SD. After the appropriate contact time had elapsed, the disinfectant was removed, 0.5 mL of neutralizing solution was immediately added per well, and the biofilm was dislodged. Bacterial suspensions of duplicate wells were immediately pooled and added to 4 mL of neutralizing solution (total 5 mL) and vortexed. The suspension was incubated at 20–24 °C for 5 min to ensure complete neutralization of residual disinfectant. The suspension was vortexed vigorously, serially diluted, plated on BA plates, and incubated anaerobically at 37 °C for 96 h. Biofilms not exposed to the disinfectant provided baseline bacterial counts and served as a control. Each condition was tested in biological triplicates.

2.4.8.2 Dual-species biofilms

The efficacy of SD and CP was assessed on pre-washed mono-species (*C. acnes* or *S. aureus*) and dual-species (*C. acnes* and *S. aureus*) biofilms established in wells coated with SL by exposing biofilms to either SD, CP or successively to SD followed by CP as described above. Biofilms not exposed to the disinfectant provided baseline bacterial counts and served as a control. Each condition was tested using technical duplicates and in biological triplicates.

2.4.9 Biofilm matrix disruption assay

Biofilms of the four *C. acnes* isolates were established in 6-well plates that were either uncoated or coated with SL as described above with the bacterial seed volume modified to 3 mL. Biofilms were washed with PBS, and the test wells were treated with either proteinase K (100 µg/mL) in 20 mM Tris buffer (pH 7.5) and 100 mM NaCl, DNAse 1 in PBS (30 ng/mL), or 10 mM sodium metaperiodate in 50 mM sodium acetate buffer pH 4.5. Biofilms formed in coated and uncoated wells treated with the diluent buffers alone served as a control. Plates were incubated at 37 °C for 1 h, after which the supernatant was removed, and biofilms were fixed, stained, and quantified as described above. The percent composition of each component was obtained by subtracting the readings obtained for test wells from the control wells and dividing that value by the test well readings. Each condition was tested in biological triplicates.

2.4.10 Statistical analyses

A two-way ANOVA analysis was used to compare the differences in disinfectant efficacy against *C. acnes* biofilms established in different sebum-like components. Statistical differences in experiments comparing coated and uncoated wells were performed using either one-way ANOVA or t-test (Student and Welch) as indicated.

2.5 Results

2.5.1 *C. acnes* PC isolates predominantly belong to phylotypes IB and II

Multiplex PCR indicated that 50% of the 20 PC isolates tested belong to phylotype II, followed by phylotype IB (30%), while phylotypes III and IA accounted for 15% and 5% of the isolates tested, respectively (Table 6). Control strain ATCC 6919 isolate was categorized as phylotype IA.

Table 6. Categorization of the 20 *C. acnes* PC isolates based on phylotyping touch down multiplex PCR [162].

***C. acnes* ID**	**Phylotype**	**% (n=20)**
BPNBT-19269	IA	5
BPNBT-19119, 19195, 19210, 19313, 19352, 19356	IB	30
BPNBT-18290, 18317, 19005, 19054, 19120, 19217, 19280, 19295, 19329, 19354	II	50
BPNBT-19223, 19227, and 19355	III	15

2.5.2 The addition of olive oil as a source of oleic acid does not enhance the biofilm formation of all *C. acnes* isolates

All *C. acnes* isolates tested were able to form biofilms in both olive oil coated and uncoated wells (Figure 6). Isolates BPNBT-19269, BPNBT-19295, and BPNBT-19352 formed significantly more biofilms in olive oil coated wells ($p \leq 0.05$), while isolates BPNBT-19119, BPNBT-19005, BPNBT-19054, BPNBT-19354, and BPNBT-19355 formed significantly more biofilms in uncoated wells ($p \leq 0.05$). All other PC isolates tested exhibited similar biofilm formation in both conditions (Figure 6). Four *C. acnes* isolates were chosen for successive experiments representing

the four *C. acnes* phylotypes identified (Table 6), three of these isolates BPNBT-19195 (phylotype IB), 19329 (phylotype II), and 19227 (phylotype III) display similar biofilm formation in coated and uncoated wells, while isolate BPNBT-19269 was chosen since it was the only PC isolate tested that was categorized as phylotype IA.

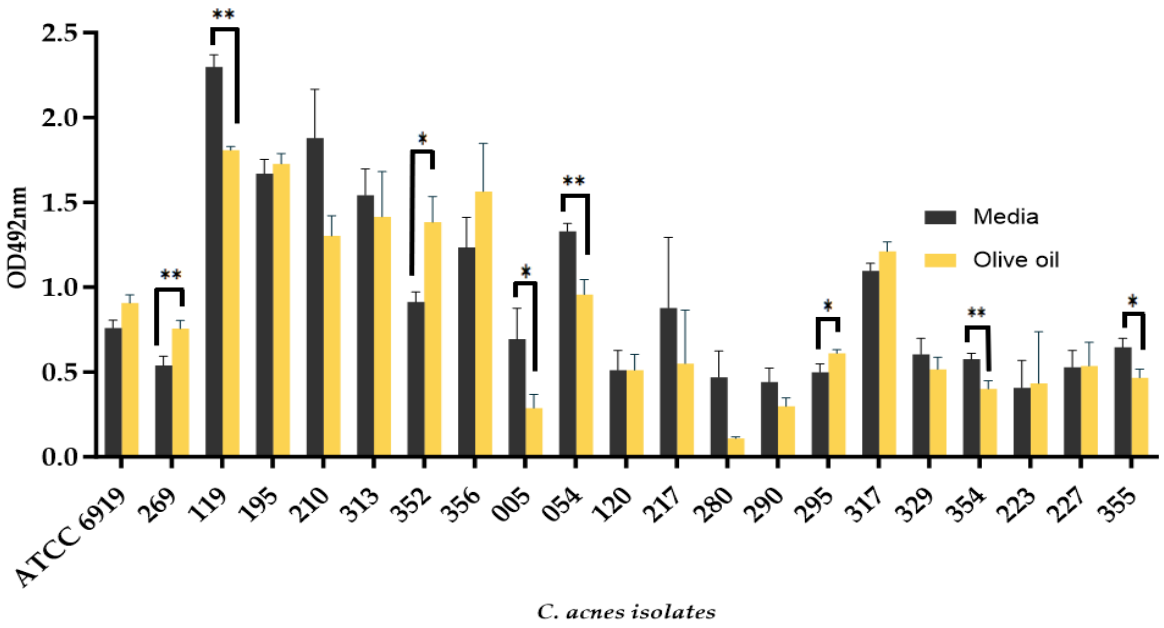


Figure 6. Biofilm formation of *C. acnes* PC isolates.
Biofilm formation of *C. acnes* PC isolates in uncoated wells (black bars) and wells coated with olive oil a source of oleic acid (yellow bars). Student T-test analysis, $p \leq 0.05$ (*), $p \leq 0.01$ (**). N=3

2.5.3 *C. acnes* biofilm formation is impacted by different sebum-like components in an isolate dependent manner

The biofilm formation of the four *C. acnes* PC isolates was generally unchanged in the presence of sebum-like components compared to the uncoated controls (Figure 7). Isolate BPNBT-19269 displayed a significant increase in biofilm formation in wells coated with triolein and SL ($p \leq 0.05$) compared to control uncoated wells. It should be noted; however, that the biofilms formed in uncoated wells were more easily dislodged during the staining and de-staining process and

therefore observed reductions reflect the loss during processing. Squalene on the other hand significantly reduced the biofilm formation of isolate BPNBT-19195 compared to the uncoated control; however, no differences were observed in biofilm formation in the presence of all the other coatings tested. Significantly higher biofilm formation was observed for isolate BPNBT-19227 in wells coated with triolein ($p \leq 0.05$) compared to uncoated wells, with similar biofilm formation observed in all other coated wells. Interestingly, no significant differences in biofilm formation between coated and uncoated wells were observed for isolate BPNBT-19329 (Figure 7). Since SL reflects the pilosebaceous niche more closely, successive experiments were performed using this coating.

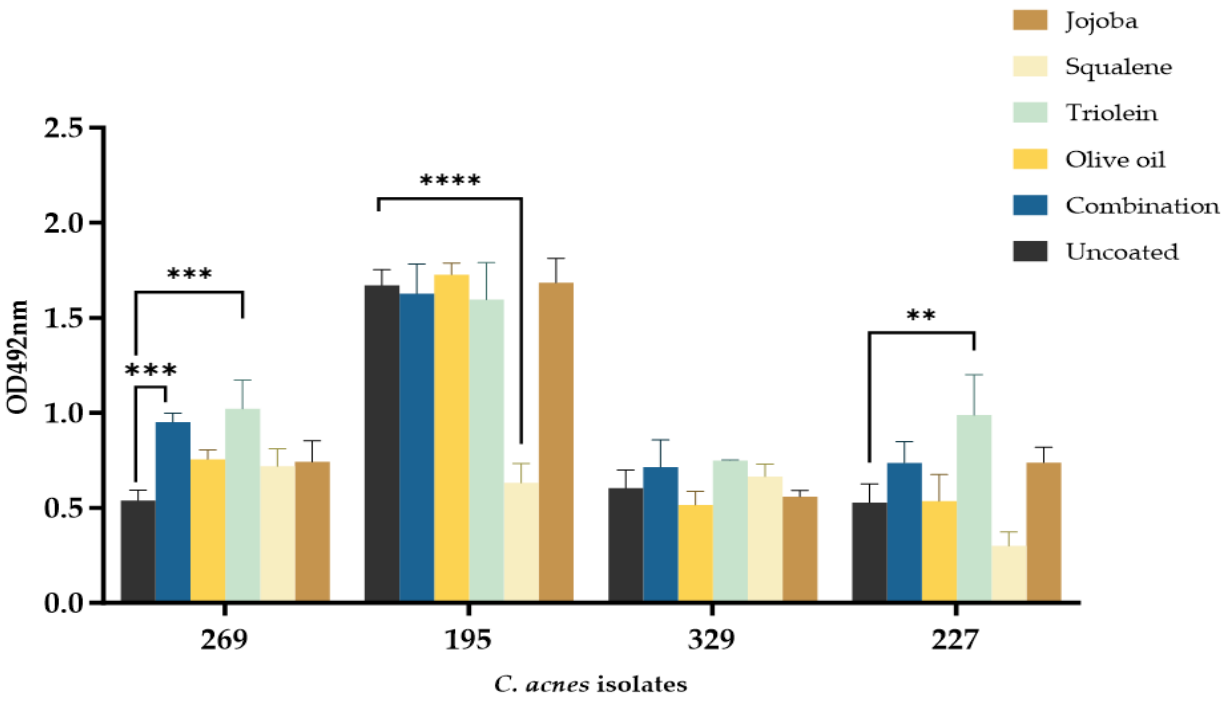


Figure 7. Biofilm formation of *C. acnes* PC isolates in the presence of sebum-like components. Biofilm formation of *C. acnes* PC isolates in uncoated wells and wells coated with individual sebum-like components or the combination (i.e. SL) as assessed by the crystal violet biofilm assay following incubation at 37°C for 7 days under anaerobic conditions. N=3. Significance assessed by One-Way ANOVA analysis $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****).

2.5.4 Denser *C. acnes* biofilms are formed in the presence of sebum-like components

The confocal microscopy images of the biofilms produced in wells coated with SL indicate that the biofilms are denser (visualized by increased fluorescence intensity) than biofilms established in uncoated wells (Figure 8A). Biofilms produced by three of the four isolates BPNTBT-19195, 19329, and 19227 appear to be denser and uniform in wells coated with SL, except for isolate BPNBT-19269 which appears to produce irregular aggregates across the biofilm forming surface consistent with the rough appearance of these biofilms visible to the naked eye. Total bacterial counts obtained for biofilms established in 48-well plates in coated and uncoated wells indicate slight increases in biofilm load (Figure 8B) in wells coated with SL.

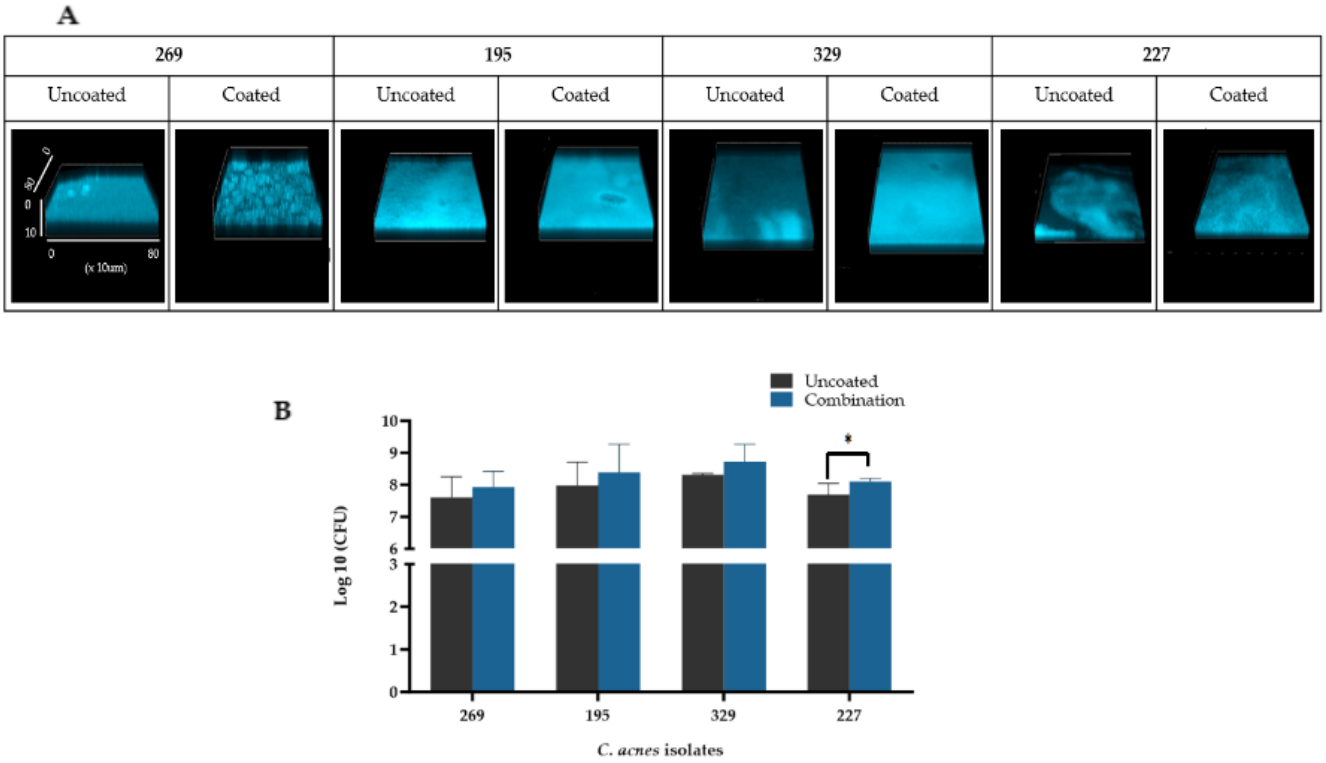


Figure 8. Qualitative and quantitative comparison of *C. acnes* biofilms produced in the absence and presence of sebum-like components.
(A) Confocal microscopy images of *C. acnes* biofilms of the four PC isolates established in uncoated or wells coated with a combination of sebum-like components (i.e. SL) for 7 days at 37°C under anaerobic conditions stained with Fluoroshield. Fluorescence intensity is proportional to bacterial density. The scale in the first image (269 uncoated) applies to all images. (B) Total bacterial counts of *C. acnes* derived from biofilms established in 48-well plates in the absence and

presence of a combination of sebum-like components (i.e. SL) represented as the Log 10 value of colony forming units (CFU) (n=6). Student's t-test analysis, $p \leq 0.05$ (*).

2.5.5 The presence of *S. aureus* in the dual-species biofilms affect *C. acnes* growth in a strain dependent manner

The readings obtained from the crystal violet biofilm assays for the dual-species biofilms of *C. acnes* isolates BPNBT-19269 and 19329 with *S. aureus* CBS2016 did not differ significantly from the combined readings of the mono-species biofilms obtained for the two isolates (Figure 9A). However, the readings of the dual-species biofilms formed with BPNBT-19195 and 19227 with *S. aureus* were significantly higher ($p \leq 0.05$) than the combined readings of the mono-species biofilms (Figure 9A). A comparison of the bacterial concentration in dual-species and mono-species biofilms indicates that three of the four *C. acnes* isolates (BPNBT-19269, 19329, and 19227) have a significantly higher bacterial concentration in the dual-species model ($p \leq 0.05$) when compared to bacterial concentrations obtained in the mono-species model (Figure 9B). On the other hand, *S. aureus* concentrations in the dual-species model were significantly elevated ($p \leq 0.05$) in only two (BPNBT-19195, and 19329) of the four isolates tested, while a significant reduction ($p \leq 0.05$) in the *S. aureus* bacterial load was observed when grown in the biofilm model with the *C. acnes* BPNBT-19269 isolate (Figure 9C).

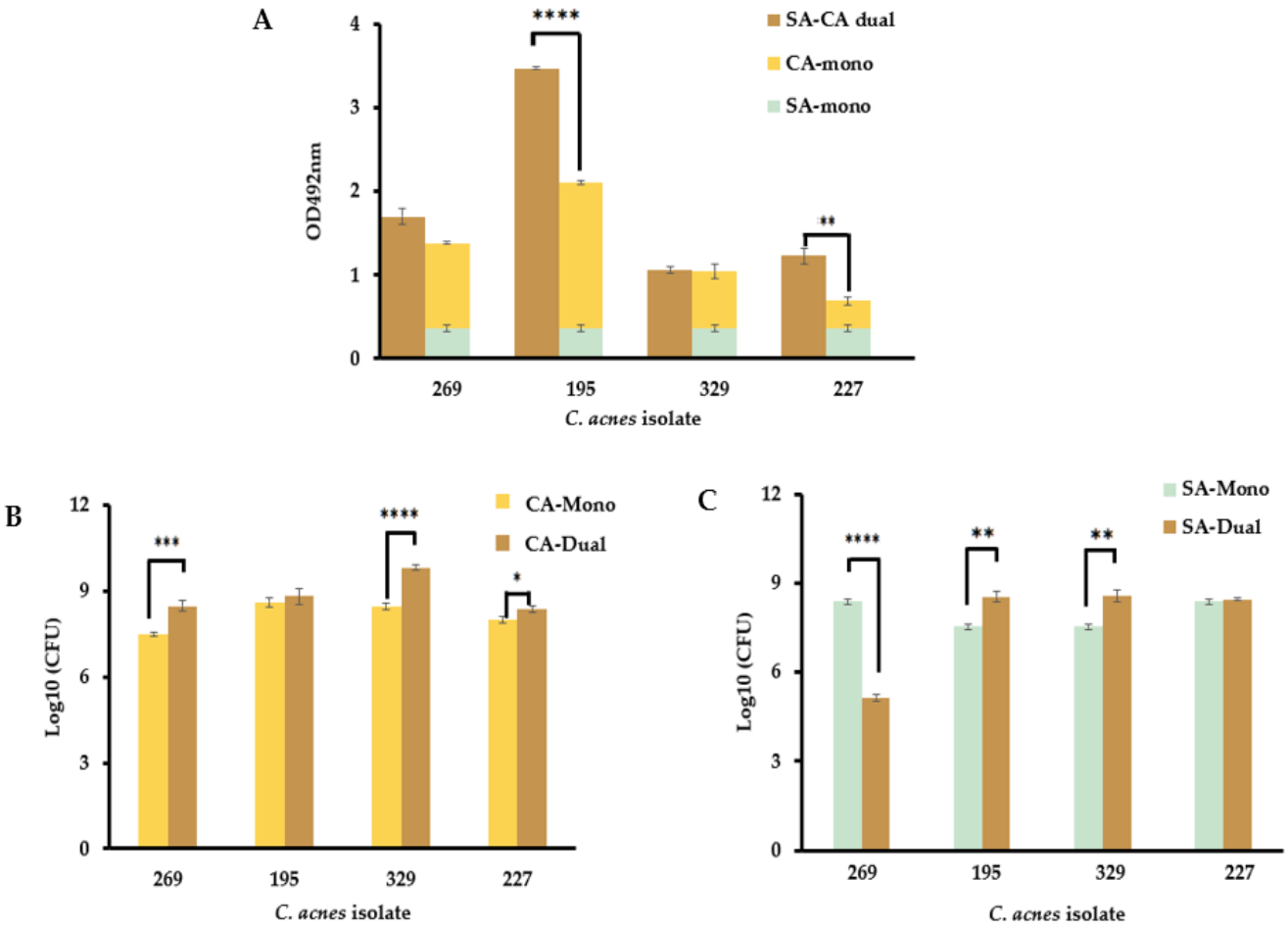


Figure 9. Dual species biofilms of *S. aureus* with four PC *C. acnes* isolates.

A-comparison of the optical density of *S. aureus* and *C. acnes* biofilm formation in mono and dual species biofilm models in the presence of SL, B- bacterial load of the four *C. acnes* PC isolates in the mono and dual species biofilm models established in the presence of SL, C- bacterial load of *S. aureus* in the mono and dual species biofilm models established in the presence of SL. N=3, Student t-test analysis, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****).

2.5.6 Sebum-like components negatively impact the efficacy of the standard donor skin disinfectant and a hypochlorous acid-based skin disinfectant

Mono-species *C. acnes* biofilms resist disinfection in the presence of sebum-like components. The total bacterial counts obtained from the disinfectant efficacy assays performed on all four *C. acnes* mono-species biofilms displayed a significant reduction ($p \leq 0.01$) in the efficacy of SD against biofilms established in wells coated with individual sebum-like components and SL when compared to uncoated wells (Figure 10A). A comparison of the resistance to disinfection displayed by biofilms formed in the presence of the different sebum-like components indicated that there were no significant differences in the inhibition of disinfectant efficacy as a result of the coating. Further assessment of the efficacy of SD and CP used individually on *C. acnes* biofilms established in wells coated with SL indicates that the efficacy of both disinfectants was reduced significantly ($p \leq 0.05$) (Figure 10B). Furthermore, using a combination of the two disinfectants did not increase the disinfectant efficacy against biofilms formed in coated wells to that observed in uncoated wells, and was significantly lower than the efficacy observed in uncoated wells (Figure 10B). A comparison of the efficacy of the two disinfectants used successively indicated that there was no significant difference in the efficacy irrespective of the order in which the disinfectants were applied to the biofilms prepared in wells coated with SL (Figure 10B).

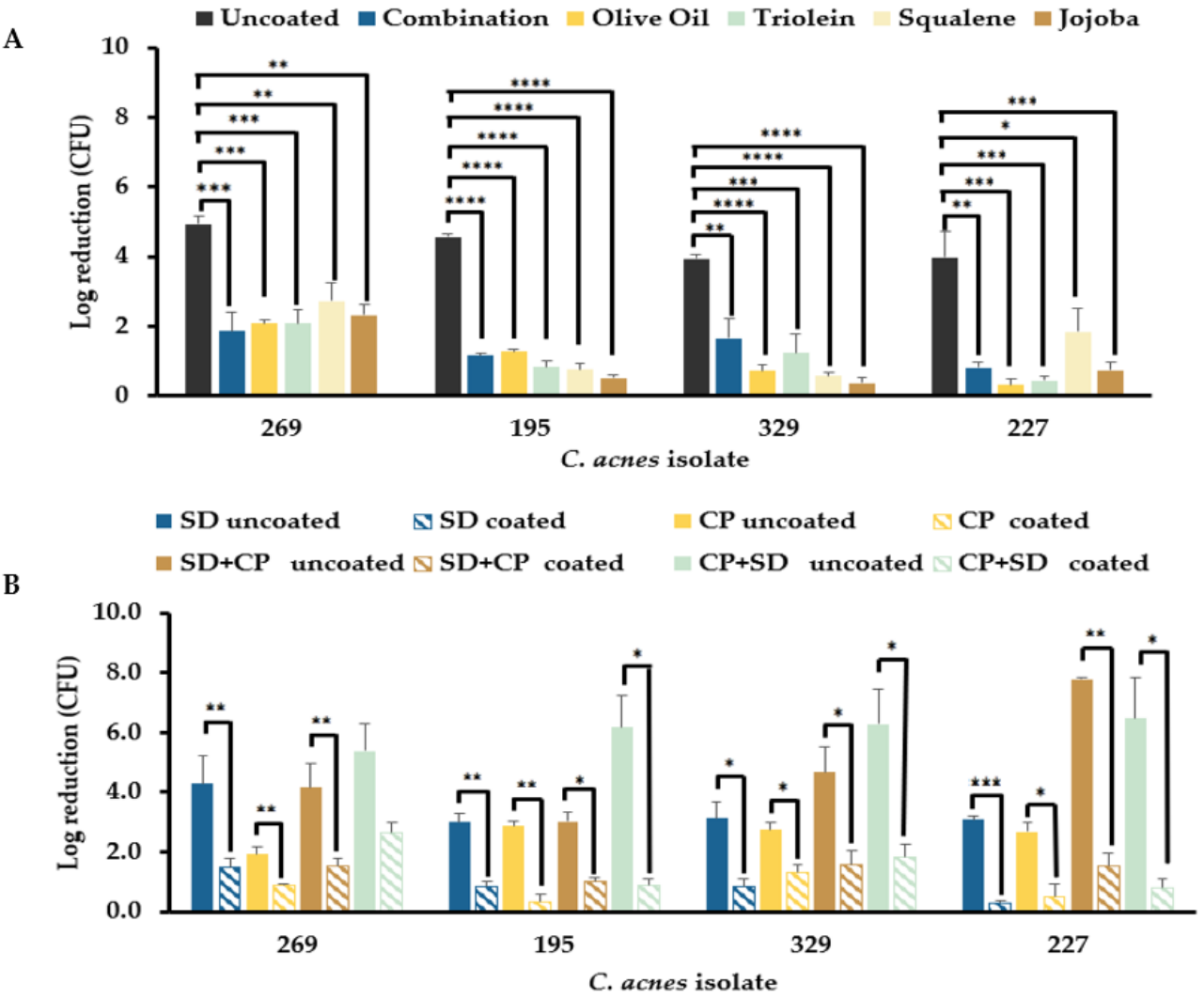


Figure 10. Disinfectant efficacy of skin disinfectants against *C. acnes* biofilms.

A- Reduction of biofilm bacterial load of the four *C. acnes* PC isolates established in the presence of individual or a combination of sebum-like components (i.e., SL) compared to the control (uncoated) following treatment with SD disinfectant (2% chlorhexidine, 70% isopropyl alcohol). B- reduction of biofilm bacterial load of biofilms established in SL compared to the control (uncoated) following treatment with either SD, Clinisept+ (CP) or successive treatment with the two disinfectants. N=3, $p \leq 0.05$ (*), statistical analysis A- One-Way ANOVA, B- Welch's t-test, $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****).

2.5.7 *S. aureus* in mono and dual species biofilm models resist elimination by skin disinfectants in the presence of sebum-like components

S. aureus was not eliminated in either mono-species or dual-species biofilms when SD, CP, or a combination of the two disinfectants was used (Figure 11). A significant increase ($p \leq 0.05$) in CP disinfectant efficacy was observed against *S. aureus* and *C. acnes* BPNBT-19269 (Figure 11A) in the dual-species biofilm model compared to the respective efficacies observed in the mono-species models. A similar trend was observed for *C. acnes* BPNBT-19195 in the dual-species biofilm model (Figure 11B). The disinfectant efficacy of the three treatments against isolate BPNBT-19329 was significantly increased ($p \leq 0.05$) in the dual-species biofilm model when compared to the mono-species biofilm model; however, an increase in disinfectant efficacy against the *S. aureus* isolate in the dual-species model was only observed when a combination of SD and CP was used (Figure 11C). A significant reduction in disinfectant efficacy against *S. aureus* in the dual-species model with *C. acnes* BPNBT-19227 was only observed when treated with SD (Figure 11D).

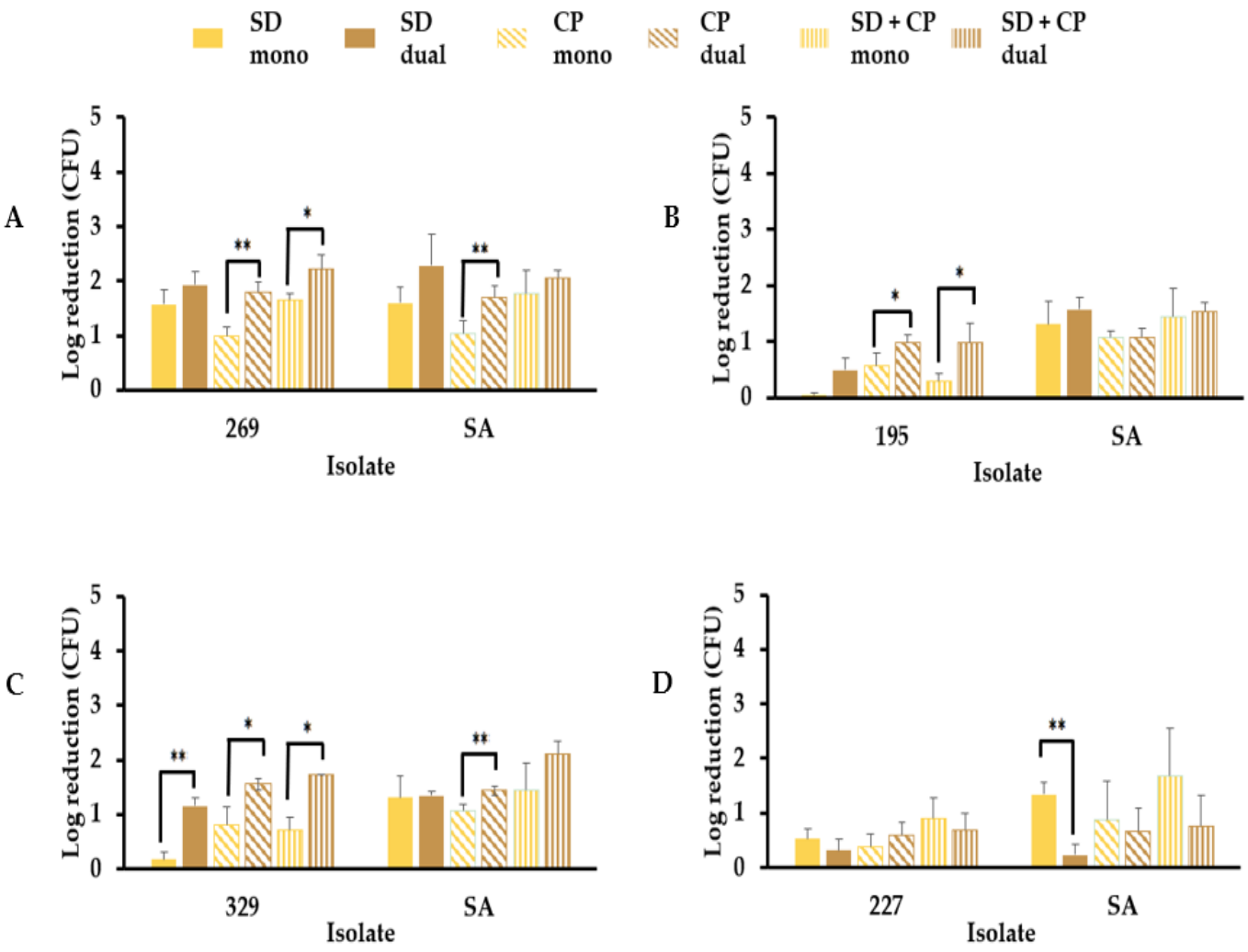


Figure 121. Disinfectant efficacy of skin disinfectants against *C. acnes* and *S. aureus* dual species biofilms.

Reduction of *C. acnes* and *S. aureus* bacterial load in dual-species biofilms established in the presence of SL compared to reductions observed in mono-species biofilms following treatment with SD, CP or SD and CP combined. Comparison in dual species model consisting of A- *C. acnes* isolate BPNBT-19269 and *S. aureus* , B- *C. acnes* isolate BPNBT-19195 and *S. aureus* , C- *C. acnes* isolate BPNBT-19329 and *S. aureus* , and D - *C. acnes* isolate BPNBT-19227 and *S. aureus* N=3, $p \leq 0.05$ (*), Welch's t-test, $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

2.5.8 Sebum-like components can impact the composition of *C. acnes* biofilm matrix

In all four *C. acnes* isolates biofilms tested, the extracellular matrix was found to be mainly composed of protein and extracellular DNA in both uncoated and SL coated wells (Figure 12). The polysaccharide composition of biofilm matrices was almost negligible in BPNBT-19269, 19195, and 19227, and was low in isolate BPNBT-19329 (8-18%). There was a significant reduction in the protein and eDNA composition of the extracellular biofilm matrix of two of the four *C. acnes* isolates (BPNBT-19269, BPNBT-19195) established in SL coated wells compared to the uncoated control, accompanied by no changes to the polysaccharide composition. In isolates BPNBT-19329 and BPNBT-19227 no significant changes to biofilm matrix composition were observed when biofilms established in SL coated wells were compared to uncoated wells (Figure 12).

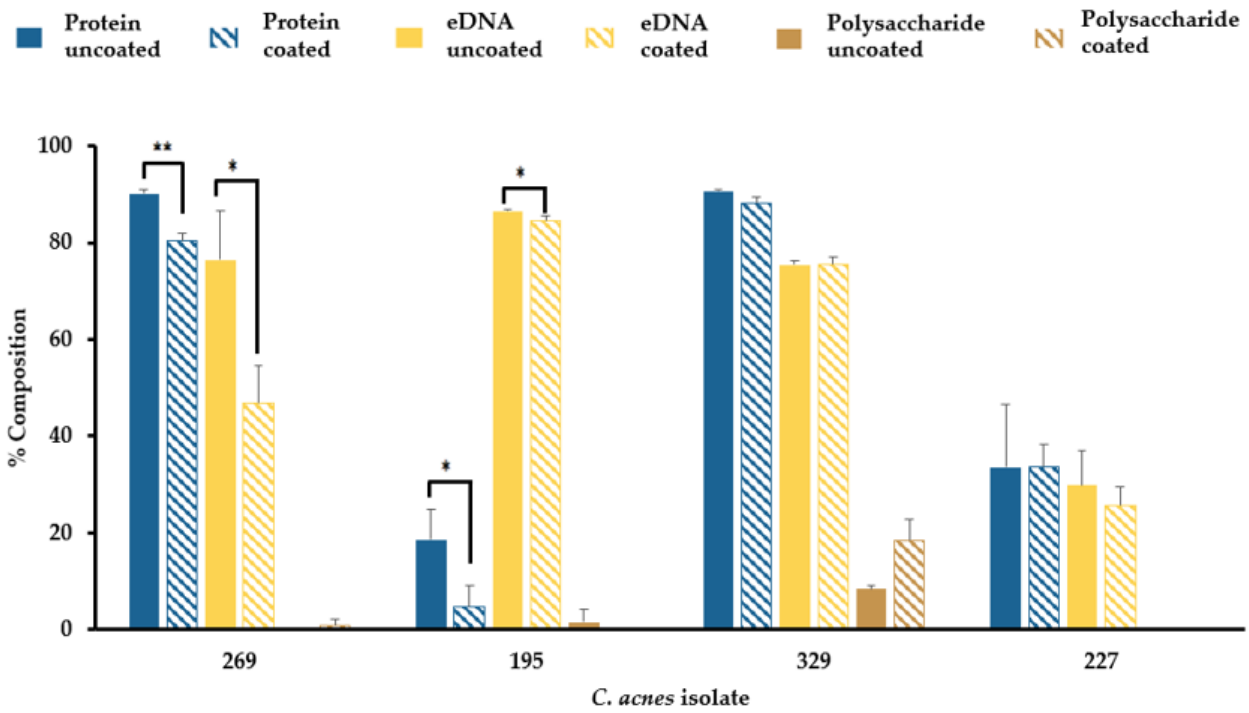


Figure 12. Composition of *C. acnes* biofilm extracellular matrix.
Comparison of extracellular matrix composition of *C. acnes* biofilms established in the absence or presence of SL as assessed by crystal violet staining following matrix disruption. N=3, $p \leq 0.05$ (*), Welch's t-test, and $p \leq 0.01$ (**).

2.6 Discussion

The disinfection of donor skin prior to blood collection serves as a key intervention to prevent bacterial contamination of blood products. Disinfectant solutions consisting of chlorhexidine gluconate and isopropyl alcohol are considered the gold standard of skin disinfectants [60] and are used at Canadian Blood Services and by other blood suppliers [59, 61, 163]. However, bacterial contamination of blood products continues to occur, highlighting the limitations of skin disinfection [3, 52, 58]. The lipophilic bacterium *C. acnes* thrives in the sebaceous niches of human skin as biofilms [110] and is the predominant species isolated during PC screening with culture methods [3, 52]. This is despite the fact that the antecubital region, the site of blood donor venipuncture, is mostly populated with *Corynebacterium* spp. and coagulase negative staphylococcal species [108]. In this study, the efficacy of the standard disinfectant (chlorhexidine and isopropyl alcohol) and an alternate hypochlorous acid-based disinfectant (Clinisept+) was tested against *C. acnes* biofilms in the presence and absence sebum-like components in mono- and dual-species models. Our data demonstrate that the presence of sebum-like components can significantly reduce the disinfectant efficacy of both disinfectants tested. The dampened efficacy of the standard disinfectant coupled with the poor penetration properties of chlorhexidine gluconate in alcohol [164], may contribute to the ability of *C. acnes* to escape donor skin disinfection resulting in this species being overrepresented as a blood product contaminant.

In this study, twenty *C. acnes* isolates obtained from contaminated PCs were assessed and 80% were found to belong to phylotypes IB and II. Notably, these phylotypes have been associated with deep seated infections which are often associated with biofilm formation in the clinical setting [165]. It is therefore important to understand the factors contributing to the ineffective disinfection of donor skin if the risk to transfusion patients is to be mitigated. Bacteria associated with biofilms

display a heightened resistance to antimicrobials and the environmental conditions the biofilms are established in may impact these observed characteristics. Sebum components like squalene, oleic acid, and palmitic acid (jojoba oil) have been shown to inhibit or diminish biofilm formation and possess antimicrobial properties [166, 167, 168]. However, the work presented herein demonstrates that the presence of sebum-like components (individual or in combination) does not significantly impact the overall biofilm formation of *C. acnes* but can result in a slight increase in bacterial load. Studies that assess the impact of sebum components on biofilm formation of a larger numbers of *C. acnes* isolates could be performed to confirm these findings.

Of note, tween 80 (polyethylene sorbitol ester of oleic acid) and the sodium salt of oleic acid are important components of neutralizers of chlorhexidine gluconate and isopropyl alcohol [169]. Since oleic acid is an important component of sebum, it is possible that the mere presence of sebum may act as a neutralizing shield against donor skin disinfection, particularly in niches of the skin where it is found in high concentrations, leading to reduced efficacy against bacteria like *C. acnes* that reside there. The extracellular matrix (ECM) of biofilms plays various roles that impact the unique properties displayed by biofilms. In the context of antimicrobial activity, the ECM may prevent or retard the diffusion of antimicrobials or sequester these agents preventing contact with their bacterial targets [170, 171]. The ECM is generally composed of proteins, extra-cellular DNA, polysaccharides, and lipids [94] and the composition of these components can vary depending on the environment in which the biofilm is formed [172]. In our study since the biofilms were established directly on lipid coating, it was not feasible to assess the ECM fraction of the biofilms without inadvertently contaminating samples with the coating, instead we aimed to assess the composition of other components to determine if any significant changes could infer the incorporation of lipid. Our data indicate that there is a significant reduction in the protein and e-

DNA content of the biofilms produced by two isolates (BPNBT-19269 and 19195) in SL coated wells that is not offset with a corresponding increase in polysaccharide content. This could imply that lipids found in the environment (coating) could potentially be incorporated into the ECM. Since all isolates displayed heightened resistance to disinfection, it is not possible to elucidate if and to what extent the potential incorporation of lipid into the ECM has on the observed resistance. Additionally, changes in the lipid composition of the bacterial membrane have been reported to modulate the fluidity of the membrane in the biofilm state thereby changing the susceptibility of bacteria to antimicrobials [173]. In our study, an assessment of bacterial membrane composition was not performed due to technical difficulties in preventing the contamination of coating into the membrane sample. As a result, the incorporation of exogenous lipids from the coating into the bacterial membrane in *C. acnes* biofilms cannot be dismissed as a contributing factor for the increased resistance to disinfection observed. Future studies using a modified sebum biofilm model allowing for reduced direct contact with the coating could facilitate an in-depth analysis of both the ECM and biofilm associated bacterial membrane.

In nature, biofilms are often found in multi-species communities, and members of these communities may exert influence on each other thereby impacting growth and antimicrobial susceptibility [174, 175]. Abbott et al. demonstrated that *C. acnes* belonging to phylotype IA can inhibit the growth and/or delay the maturation of *S. aureus* biofilms leading to reduced biofilm mass [174]. The data obtained in our dual-species model that included *S. aureus* and *C. acnes* isolate BPNBT-19269 (phylotype IA) was consistent with these findings since a significant reduction in *S. aureus* concentration was observed. However, this reduction was not observed when dual-species biofilms were established with *C. acnes* isolates belonging to other phylotypes, indicating that the inhibitory properties may be *C. acnes* phylotype dependent. The susceptibility

of *S. aureus* to antimicrobials has been shown to vary in the presence of other species, for instance, *C. acnes* derived factors significantly enhance the sensitivity of *S. aureus* biofilms to antimicrobials [174], while dual-species biofilms consisting of *S. aureus* and *C. albicans* increased the resistance of the former to vancomycin a hundred-fold [175]. Our data indicate that *C. acnes* does not enhance the sensitivity of *S. aureus* to SD in three out of the four dual-species models assessed. However, the sensitivity of three *C. acnes* isolates to oxidant damage by CP was enhanced when grown in a dual-species biofilm with *S. aureus*. The mechanisms engaged in antioxidant responses are not fully elucidated in *C. acnes*; however, our data show that the presence of *S. aureus* disrupts these mechanisms when grown together in a dual-species biofilm model. Previous work has shown that the biosynthesis of staphlyoxanthin, an antioxidant produced by *S. aureus*, is inhibited by exposure to squalene thereby increasing its susceptibility to oxidants [176]. It is therefore plausible that in the dual-species model, *S. aureus* or its secreted factors may directly inhibit the synthesis of antioxidants or may sensitize *C. acnes* to sebum components which in turn interfere with the antioxidant response. Taken together our results indicate that though the sebum environment affords protection against disinfection, this resistance can be modulated in multi-species communities, and these variations may be phylotype dependent.

Though the in vitro model used in this study mimics a sebum-like environment, there are components of the pilosebaceous unit that are not represented. For instance, sapienic acid, a fatty acid unique to human sebum that is known to have antibacterial properties [177], was not included. Furthermore, the cellular structures forming the different layers of skin and the complex crosstalk between bacteria and the immune system required to maintain homeostasis on the skin [178] is not reflected in our model. Nonetheless, it should be noted that the quantitative suspension test [179] and antimicrobial dilution assays [180] are the standard methods employed to assess the efficacy

of disinfectants. These standard methods do not assess the impact of biofilm formation or other components that may be present in the natural site of disinfection on disinfectant efficacy. Our data indicate that the omission of these components and biofilm formation may lead to the overestimation of the efficacy of disinfectants.

2.7 Conclusions

The presence of sebum components on the skin may dampen the efficacy of the gold standard disinfectant consisting of 2% chlorhexidine and 70% isopropyl alcohol. This study highlights how the low penetration properties of this disinfectant together with the inhibitory effects of sebum components could contribute to the overrepresentation of *C. acnes* as a PC contaminant. Furthermore, this study emphasizes the need for biofilm models that better reflect the natural niche that a bacterium inhabits in order to have a more accurate understanding of how they respond to different stressors, including antimicrobials and disinfectants. Finally, understanding the shortcomings of current disinfectant strategies provides a platform on which future innovation aimed at developing more effective disinfectants that are less susceptible to the inhibitory properties of sebum can be built.

2.8 Acknowledgements

Volunteer blood donors and staff at the Canadian Blood Services Blood4Research netCAD facility (Vancouver, BC, Canada) for whole blood collection and PC production for this study. Peter Ochalski (Cellular Imaging Facility, Faculty of Science, University of Ottawa) for technical support in acquiring confocal microscopy images.

Please refer to Appendix A1 for supplementary information.

Chapter 3 Nutrient Supplementation of Culture Media Improves the Detection of *Cutibacterium acnes* in Platelet Components by an Automated Culture System

3.1 Statement of contribution and status of manuscript

The manuscript titled “Nutrient supplementation of culture media improves the detection of *Cutibacterium acnes* in platelet components by an automated culture system” has been published in Vox Sanguinis. 2023 Nov;118(11):930-937. doi: 10.1111/vox.13529. Epub 2023 Sep 25. PMID: 37749879. Licensing for reprinting can be found in Appendix B. The numbering of sections, tables and figures have been modified (minor) as per Faculty of Medicine requirements for an article-based thesis.

The conceptualization of the study, the development, optimization, and execution of the methodology, as well as data curation and statistical analyses were performed by Dilini Kumaran. The project was supervised by Dr. Ramirez-Arcos who provided guidance with conceptualization and study design, trouble shooting, and resources to execute the study. The original and final draft of this manuscript was written by Dilini Kumaran and was reviewed and edited by Dr. Ramirez-Arcos.

3.2 Abstract

Background and Objectives: Platelet concentrates (PCs) contaminated with *Cutibacterium acnes* are often transfused prior to detection by the BACT/ALERT system. Though *C. acnes* is implicated in mild transfusion reactions, delayed clinical effects are unknown. This study assessed the ability to enhance *C. acnes* detection by supplementing culture media with Tween 80 (T80, an oleic acid source) and a commercial nutrient supplement.

Materials and Methods: Anaerobic culture bottles (BPN) were supplemented with T80 or oleic acid. T80-supplemented BPN bottles were inoculated with four *C. acnes* isolates (10 or 100 colony-forming units [CFU]/bottle) or other transfusion-relevant bacteria (10 CFU/bottle). Samples of plasma containing SSP+ (platelet additive solution [PAS]) (PAS-plasma) at different concentrations, plasma-PCs, and PAS-PCs, spiked with two *C. acnes* isolates (10 CFU/bottle), were inoculated into T80-supplemented BPN bottles. Furthermore, plasma-PCs were spiked with *C. acnes* and tested in BPN bottles supplemented with the BD Difco Supplement VX (BDVx). Bottles were incubated in the BACT/ALERT system and times to detection (TtoD) were compared (N = 3)

Results: A reduction in T to D of ≤3.5 days was observed for *C. acnes* in T80-supplemented BPN, while other species did not show the same effect. However, false positives were observed when T80-supplemented BPN was inoculated with PAS-plasma (except for 70% PAS:30% plasma), plasma-PCs or PAS-PCs. Oleic acid supplementation also resulted in false positives. Interestingly, BDVx-supplemented BPN reduced the T to D of *C. acnes* in PCs by ≤1.2 days ($p < 0.05$), with no false-positive results.

Conclusion: BDVx supplementation for detection of *C. acnes* from PCs could result in timely unit retrieval, preventing the transfusion of contaminated products. In clinical settings, T80 supplementation could significantly enhance *C. acnes* detection from non-blood-derived samples.

Keywords: Platelet concentrates, *Cutibacterium acnes*, automated culture system, culture media supplementation

3.3 Introduction

Platelet concentrates (PCs) consist of platelets that are either suspended in plasma or a combination of plasma and platelet additive solution (PAS) and are used to treat individuals experiencing low platelet counts or acute trauma [181]. Bacteria are mainly introduced into donated blood from the donor's skin at the time of venipuncture [182], while donor bacteremia [183] or sterility breaches during manufacturing contribute to a lesser extent [184]. To mitigate the risk associated with the transfusion of contaminated blood products, Canadian Blood Services have implemented several strategies, which include donor screening, donor skin disinfection, first aliquot diversion, and bacterial screening of PCs with both aerobic (BPA) and anaerobic (BPN) culture bottles using the automated BACT/ALERT 3D culture system (bioMérieux, Saint-Laurent, QC, Canada) [3]. Despite these measures, bacterial contamination continues to occur, with *Cutibacterium acnes,* an anaerobic, aerotolerant member of the skin microflora, accounting for approximately 70% of all bacteria isolated from contaminated PCs [3].

Canadian Blood Services use a large volume, delayed sampling testing algorithm that stipulates that bacterial screening takes place at least 36 h after collection, followed by a minimum 6-h post-sampling quarantine period prior to release into inventory [3]. Briefly, sampling is performed by inoculating an 8–10 mL PC sample into a BPA bottle and a BPN bottle respectively, after which the bottles are incubated in the BACT/ALERT 3D system for 7 days at 36 ± 1°C. Data gathered at Canadian Blood Services indicate that approximately 36% of contaminated PCs are issued to hospitals and presumably transfused, and that *C. acnes* contamination accounts for 95% of these units [3]. One of the major challenges attributed to this trend is the inherent slow-growing nature of *C. acnes* in culture media, which results in observed detection times ranging from 3 to 7 days in the BACT/ALERT system and the inability to retrieve contaminated PCs in a timely manner

[3]. Fortunately, as this anaerobe is incapable of proliferating in the aerobic PC environment [185], *C. acnes* has only been implicated in a few mild transfusion reactions [52, 58]. It should be noted, however, that very little is known about the long-term impacts of transfusing *C. acnes*-contaminated PCs into vulnerable patients. This is especially pertinent since *C. acnes* has been implicated in serious, slow-developing, chronic infections [157]. Therefore, the early detection of *C. acnes* in the BACT/ALERT system could potentially prevent the transfusion of contaminated PCs into susceptible patients.

Several studies have investigated the nutritional requirements of *C. acnes* in vitro and have shown that its growth requires a host of amino acids and vitamins supplemented into culture media [186], and that it can be enhanced with the addition of oleic acid, a skin sebum component [187]. Furthermore, there have been multiple reports that have used Tween 80 (T80) as a source of oleic acid to enhance the growth of other bacterial species in vitro [188, 189]. In this study, we evaluated the ability of pure oleic acid, T80 and the BD Difco Supplement VX (BDVx) to improve *C. acnes* detection. The BDVx supplement consists of essential growth factors including vitamins and amino acids used to promote the growth of fastidious bacteria. Enhancing the detection of *C. acnes* may prevent the release and transfusion of PCs contaminated with this bacterium.

3.4 Materials and methods

3.4.1 PC and plasma units

Leukocyte-reduced PC pools were prepared by the buffy coat method and suspended in 100% plasma. PC and plasma units were manufactured according to standard procedures at the Canadian Blood Services netCAD Blood4Research Facility (netCAD, Vancouver, Canada) and were shipped to the Canadian Blood Services Microbiology laboratory in Ottawa, Canada. For experiments where PCs were resuspended in platelet additive solution (PAS), double dose apheresis units were collected according to standard procedures at Canadian Blood Services, and then diluted with PAS SSP+ (Macopharma, Tourcoing, France) to obtain PC samples containing approximately 1.5×10^9 platelets/mL corresponding to the minimum platelet yield requirement for apheresis units. Ethical approval for this study was granted by the Canadian Blood Services Research Ethical Board.

3.4.2 Bacterial strains

Four *C. acnes* strains BPNBT-19195, BPNBT-19153, BPNBT-19322, and BPNBT-19422, and one isolate each of *Staphylococcus epidermidis* (BPNBT-18090), *Serratia marcescens* (CBS 07/05), and *Staphylococcus saccharolyticus* (BPNBT-20617) isolated from positive culture bottles obtained during routine bacterial screening of PCs at Canadian Blood Services were used for this study. Enumerated bacterial suspensions corresponding to approximately 1×10^8 colony forming units (CFU)/mL were prepared in Brain Heart Infusion (BHI) supplemented with 15% (v/v) glycerol and verified by plating serial dilutions on tryptic soy agar supplemented with 5% sheep blood (BA).

3.4.3 Supplements

Pure oleic acid and T80 (Sigma-Aldrich, Oakville, ON, Canada) were assessed in this study. A stock solution consisting of T80 (20% (v/v)) in BPN media extricated from BPN bottles was prepared (BPNt). The solution was autoclaved and mixed when warm to produce a homogenous solution and stored at room temperature until use. The BD DifcoTM Supplement VX (BDVx) (VWR, Mont-Royal, Québec) was reconstituted and employed as per manufacturer's recommendations.

3.4.4 Effect of the direct supplementation of oleic acid in BPN media bottles

Pure oleic acid (Sigma-Aldrich, Oakville, ON, Canada) was used to supplement BPN bottles to different final concentrations 100, 200, 300 and 400µg/ mL. Each concentration was tested two independent times in the absence of bacterial inoculum or PCs. Briefly, oleic acid was added to 1-mL aliquots of BPN media, vortexed and quickly aspirated into 1 mL syringes and was used to inoculate BPN bottles. An additional 9 mL of BPN media was then added to the BPN bottles to obtain a total volume inoculum volume of 10 mL. The bottles were incubated in the BACT/ALERT 3D system (36 ± 1°C) for 7 days or until a positive flag was obtained.

3.4.5 Assessment of T80 concentrations used for BPN media supplementation in the BACT/ALERT 3D system

Two bacterial loads of *C. acnes* and five T80 concentrations derived from the serial dilution of BPNt in BPN media were tested in BPN bottles as described in Figure 13. Enumerated stocks of C. acnes isolates were serially diluted in unsupplemented BPN media and the spiking suspensions were prepared in the appropriate T80 supplemented BPN or unsupplemented BPN media. BPN culture bottles were then inoculated with 9 mL of the appropriate media and 1 mL of the spiking suspension. Bottles inoculated with 9 mL of BPN media and 1 mL of spiking suspension prepared

in BPN media served as the unsupplemented control. Bottles inoculated with 10 mL of supplemented or BPN alone served as the un-spiked controls. The bottles were incubated in the BACT/ALERT 3D (36 ± 1°C) system until a positive flag was obtained for spiked bottles or for a maximum of 10 days. An extended incubation period of 10 days was used since the isolate BPNBT-19195 is a slow grower in media and may not have triggered a positive flag within the 7-day incubation period used for routine bacterial screening of PCs. Each concentration was tested at least three independent times per bacterial isolate and T80 concentration tested.

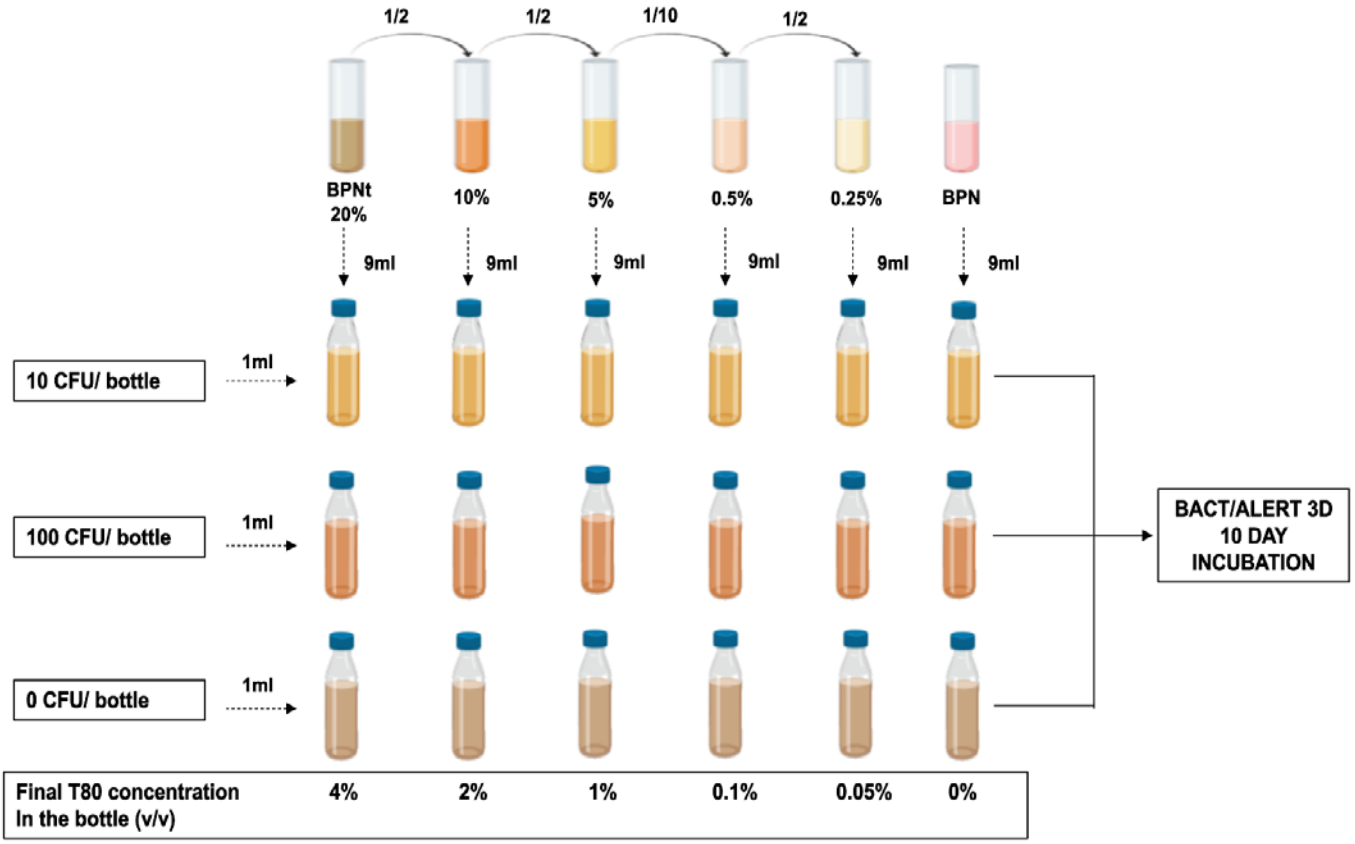


Figure 13. Supplementation of BPN media bottles with Tween 80 (T80).

BPN bottles were supplemented with T80 to varying final concentrations (0.05%–4%, v/v) and were assessed for their ability to enhance the detection of four *Cutibacterium acnes* platelet concentrate isolates tested at two bacterial loads (10 and 100 colony forming units [CFU]/bottle). Controls included media and media supplemented with T80 in the absence of bacteria, and the comparative control included unsupplemented media inoculated with bacteria. Bottles were incubated for 10 days in the BACT/ALERT 3D system and experiments were performed three independent times. Images created on Biorender.com.

3.4.6 Impact of T80 supplementation on the growth of *C. acnes* in media

T80 supplemented (4%, v/v) or unsupplemented BPN media bottles were inoculated with 10 CFU/mL of *C. acnes* BPNBT-19195 and BPNBT-19153 as previously described. Bottles were incubated for up to 7 days (36 ± 1oC) in the BACT/ALERT 3D system. Bottles were sampled every 24h and immediately returned to the BACT/ALERT system. The samples were serially diluted in BPN media and plated on BA to determine bacterial counts. Experiments were repeated three independent times per species.

3.4.7 Effect of T80 supplementation on the detection of other transfusion relevant bacteria in media

Spiking bacterial suspension of *S. saccharolyticus, S. marcescens, and S. epidermidis* corresponding to 10 CFU/ mL were prepared in BPNt or BPN as previously described. BPN media bottles were inoculated with 1 mL of bacterial suspension (~10 CFU/ mL) and 9 mL of either BPN or BPNt. BPN bottles inoculated with media (supplemented and unsupplemented) alone served as controls. Bottles were incubated (36 ± 1°C) in the BACT/ALERT 3D system until a positive flag was obtained for a maximum of 10 days. Each species was tested three independent times.

3.4.8 Effect of supplementation on the detection of *C. acnes* in PCs

BPN media bottles were supplemented to a final concentration of 4% (v/v) of T80 and *C. acnes* isolates BPNBT-19153 and BPNBT-19195 were chosen for further testing in PCs. These two isolates were selected due to their characteristic slow growth in the BACT/ALERT system making them ideal model organisms for worst case scenarios of detection of *C. acnes*. Before BPN bottles were supplemented with T80, 10 mL of BPN media were aseptically extricated from the bottles, after which 10 mL of PCs containing approximately 10CFU of bacteria, and 10 mL of BPNt were

inoculated into the bottles. BDVx supplementation was assessed by inoculating BPN bottles with 9.5 mL of PCs containing 10CFU of bacteria and 500µL of reconstituted BDVx. BPN bottles inoculated with an unspiked sample of the same PC unit served as the sterility control, bottles inoculated with PCs containing bacteria served as the unsupplemented control, and supplemented bottles inoculated with PCs in the absence of bacteria served as the unspiked controls. The bottles were then incubated in the BACT/ALERT 3D system (36 ± 1oC) until a positive flag was obtained or for maximum of 10 days. Each supplement was tested with at least three PC units. Furthermore, 10 mL of PC samples containing 10CFU of BPNBT-19195 were inoculated into BPN bottles with or without supplementation with T80 and incubated for 7 days in the BACT/ALERT system at 36 ± 1^{o}C. The bottles were sampled every 24h and returned to the BACT/ALERT system. Samples were serially diluted and plated for bacterial enumeration. The experiments were repeated three times.

3.4.9 Impact of reducing plasma content in the detection of *C. acnes* in T80 supplemented bottles

Plasma samples were diluted in SSP+ platelet additive solution (PAS) (Macopharma, Tourcoing, France) to obtain a range of suspensions consisting of 100% plasma to 30% plasma in PAS (v/v). BPN media was extricated as described previously, and 10 mL of the plasma suspensions and 10 mL of BPNt were used to inoculate the bottles. BPN bottles inoculated with plasma suspension alone served as a sterility control. Plasma dilutions that did not give rise to false positive with T80 supplementation were used in successive experiments. Bottles were inoculated with BPNt and the appropriate plasma suspensions or PCs prepared in PAS in the predetermined plasma concentrations containing ~10 CFU of *C. acnes* BPNBT-19153 or BPNBT-19195. Bottles inoculated with plasma suspensions or PCs in PAS containing bacteria alone served as

unsupplemented controls, while bottles inoculated with plasma suspensions or PCs in PAS alone served as sterility controls, and bottles inoculated with plasma suspensions or PCs in PAS and BPNt served as unspiked controls. The bottles were then incubated in the BACT/ALERT 3D system until a positive flag was obtained or for maximum of 10 days. Experiments were performed three independent times.

3.4.10 Statistical analyses

Statistical tests were performed to determine the difference of time to detection between supplemented and unsupplemented samples. The time to detection across technical replicates was used to calculate the standard deviation. For growth curve comparisons, the differences in bacterial count were assessed for each time point tested. P-values were determined using the Student T-test (unpaired, two tailed, unequal variance) using Excel. Differences were deemed significant if the p-value obtained was ≤ 0.05 (95% confidence interval).

3.5 Results

3.5.1 T80 supplementation of BACT/ALERT BPN media enhances the growth and decreases the time to detection of *C. acnes*

The direct addition of oleic acid to BPN bottles resulted in false positive flags in the BACT/ALERT system (data not shown), however, supplementation with BPNt [stock solution of T80 (20% (v/v) in BPN media] did not yield false positive results and was used in further assessments. Figures 14A and 14B show that the slowest detection times in media were observed for isolates BPNBT-19195 (100 CFU/bottle: 6.52 ± 0.19 days, 10 CFU/bottle: 9.15 ± 0.63 days), and BPNBT-19153 (100 CFU/ bottle: 5.44 ± 0.49 days, 10 CFU/bottle: 5.79 ± 0.50 days). Two concentrations of T80 (4%, and 2%, v/v) reduced detection times in all four isolates at both bacterial loads tested. However, T80 concentrations lower than 1% resulted in delayed detection of isolate BPNBT-19322 at both bacterial loads assessed (Figures 14A and 14B). Though all concentrations of T80 tested reduced time to detection of isolates BPNBT-19153, BPNBT-195, and BPNBT-19422, significant reductions were observed for *C. acnes* BPNBT-19195 tested at 10 CFU/ bottle with T80 4% ($p=0.015$) and 2% ($p=0.049$) (Figure 14B), and at 100 CFU/ bottle with T80 2% ($p=0.030$). Similarly, significantly lower times to detection were observed for isolate BPNBT-19422 tested at 10 CFU/ bottle with T80 4% ($p=0.044$), 0.1% ($p=0.007$), and 0.05% ($p=0.044$) (Figure 14B). All bottles that were not inoculated with bacteria remained negative during the 10-day incubation period in the BACT/ALERT system. Notably, T80 supplementation (4%, v/v) did not significantly change the time to detection of the other transfusion relevant bacteria tested, *S. saccharolyticus, S. marcescens, and S. epidermidis* (Figure 14C).

Enhanced time to detection in the BACT/ALERT system was directly related to growth promotion in BPNt media as demonstrated for isolates BPNBT-19195 and BPNBT-19153 (Figure 14D).

Increased bacterial loads in supplemented bottles were observed with a maximum difference of ~ 2.8 ± 0.23 Log at day 4 and ~ 2.8 ± 0.23 Log at day 3 detected for isolates BPNBT-19195 and BPNBT-19153 respectively, after which differences in bacterial load between supplemented and unsupplemented bottles decrease, until on day 7 similar bacterial loads were obtained. At all timepoints, bacterial counts in BPNt media were significantly higher than those obtained in unsupplemented media ($p<0.05$), except on day 7 for BPNBT-19153, when the difference was not significant.

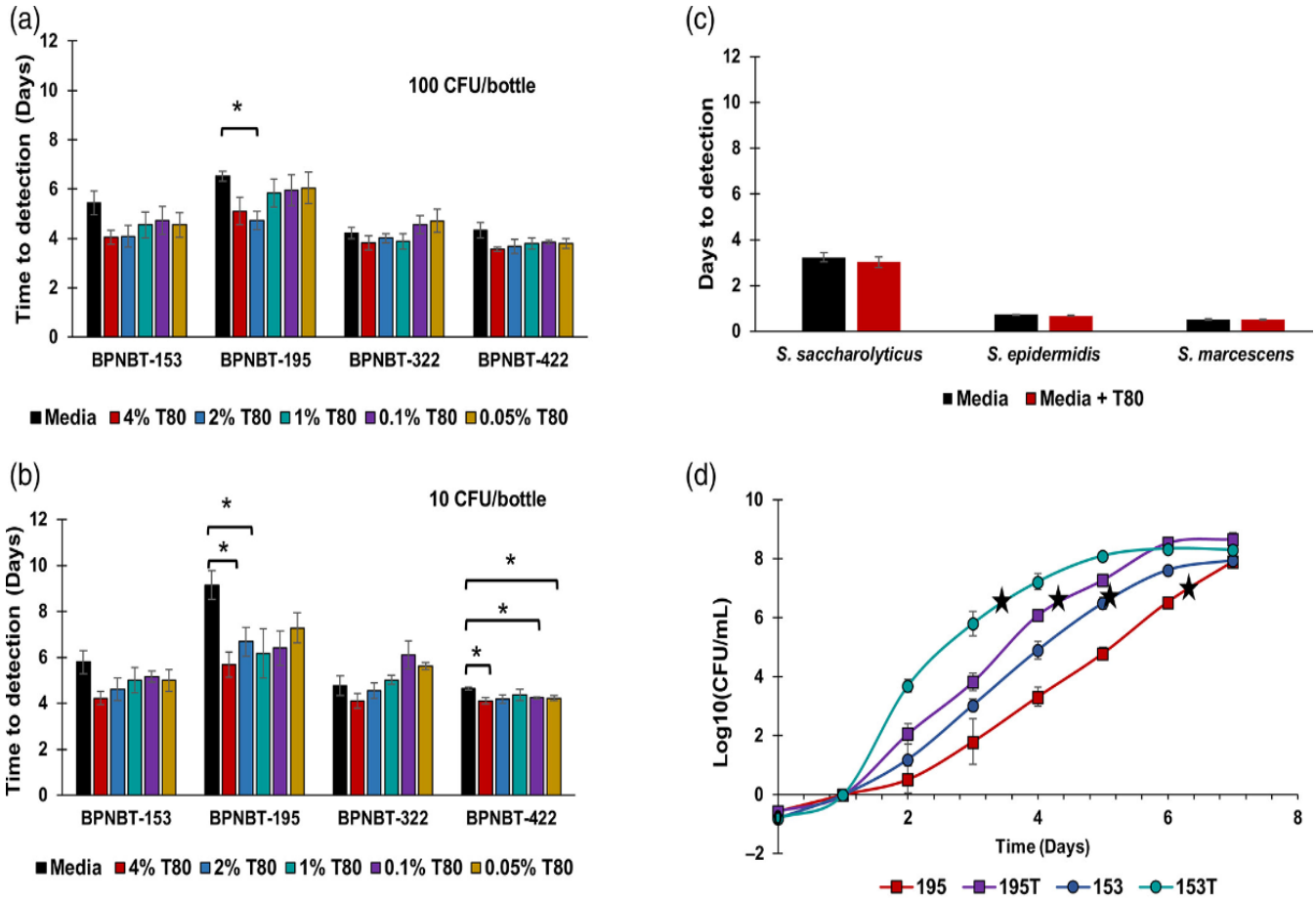


Figure 14. Time to detection of transfusion-relevant bacteria from media in the BACT/ALERT system with Tween 80 (T80)-supplemented BPN bottles

Time to detection of *Cutibacterium acnes* platelet concentrate isolates with varying concentrations of T80 supplementation, compared with unsupplemented BPN media when inoculated with (a) 100 colony-forming units [CFU]/bottle and (b) 10 CFU/bottle. (c) The time to detection of *Staphylococcus saccharolyticus*, *Staphylococcus epidermidis* and *Serratia marcescens* with (red bars), and without (black bars) T80 supplementation (4%, v/v). (d) Comparison of growth curves of *C. acnes* isolates BPNBT-19195 and BPNBT-19153 in the BACT/ALERT system with T80-supplemented (195—purple square, 153—green circle) and -unsupplemented (195—red square, 153—blue circle) BPN bottles. Time to detection in the BACT/ALERT 3D system has been indicated by black stars. Student's t-test analysis, *p value ≤0.05. N = 3.

3.5.2 Plasma and platelet derived factors contribute to the false positive results obtained with T80 supplementation of BPN bottles

T80 supplementation gave rise to false-positive flags in the BACT/ALERT 3D system in every instance where PCs or plasma were inoculated into BPN bottles in the absence of bacteria. Times to detection of false-positive results were 1.10 ± 0.28 days and 1.24 ± 0.22 days for unspiked PCs and plasma, respectively. Importantly, supplementation of BPN bottles with T80 did not inhibit the growth of *C. acnes* from samples of spiked PCs as exemplified with isolate BPNBT-19195 (Figure 15). Furthermore, all but one dilution of plasma in PAS (70% PAS + 30% plasma) resulted in false-positive flags. Significant reduction in times to detection was observed for BPNBT-153 of 2.23 days ($p < 0.005$) and BPNBT-195 of 1.26 days ($p < 0.005$) from diluted plasma (70% PAS + 30% plasma) in T80-supplemented BPN bottles (Figure 16a). However, when PCs were prepared in the same plasma to PAS ratios, it led to false positives in the BACT/ALERT system with times to detection of 0.83 ± 0.05 days.

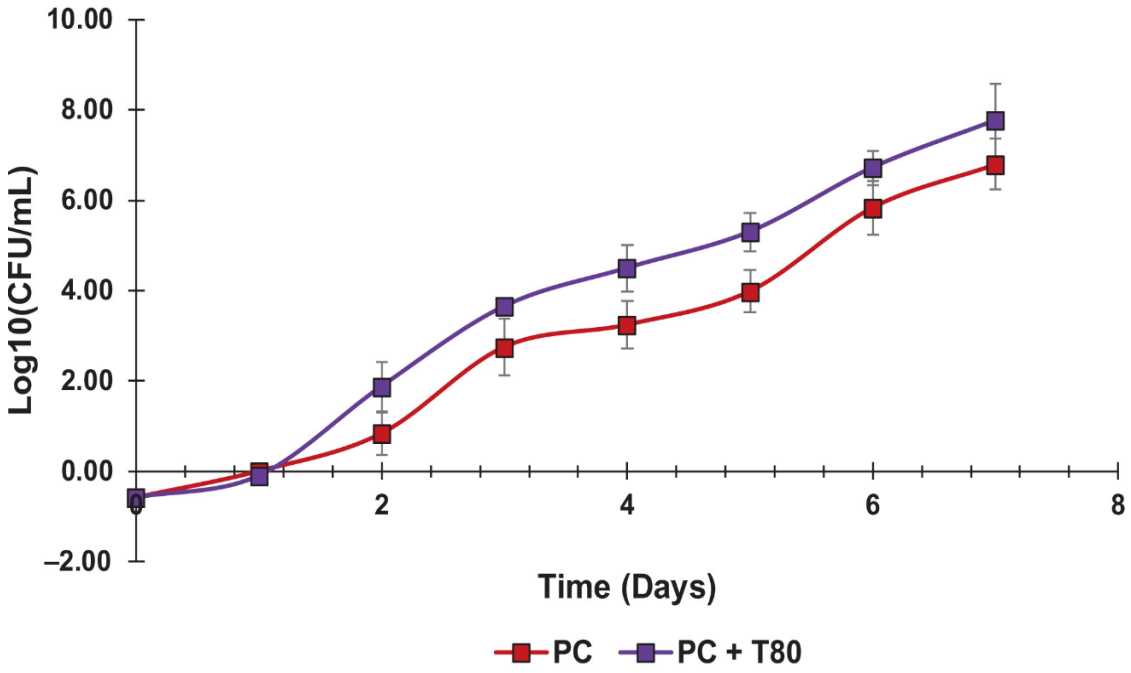


Figure 15. Growth curves of *Cutibacterium acnes* in platelet concentrates (PCs) in the BACT/ALERT system.
Comparison of growth curves of *C. acnes* BPNBT-19195 inoculated into PCs in the BACT/ALERT system with Tween 80 (T80) supplemented (purple square) and unsupplemented (195—red square) BPN bottles. N = 3. CFU, colony-forming units.

3.5.3 BDVx supplementation reduces the time to detection of *C. acnes* in PCs

Supplementation of BPN media with BDVx supplement resulted in a reduction in the time to detection of the two *C. acnes* isolates tested BPNBT-19153 (0.62 ± 0.26 days, $p = 0.048$) and BPNBT-19195 (1.15 ± 0.29 days, $p = 0.030$) in PCs suspended in 100% plasma (Figure 16b) with no false-positives results.

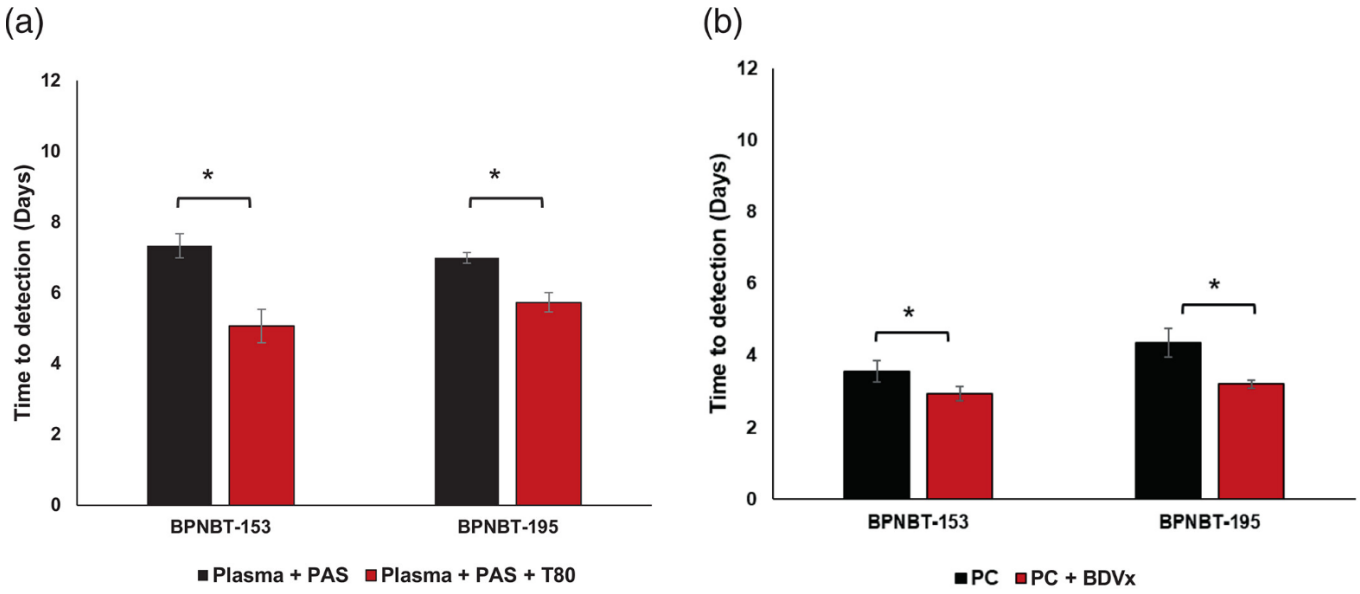


Figure 16. Time to detection of *Cutibacterium acnes* in platelet concentrates (PCs) in the BACT/ALERT system with Tween 80 (T80) and nutrient supplemented BPN bottles.

C. acnes isolates BPNBT-19153 and BPNBT-19195 were inoculated into BPN bottles at a concentration of 10 colony-forming units/bottle, (a) combination of platelet additive solution (PAS) and plasma (70% PAS, 30% plasma) with T80 (4%, v/v) supplementation; (b) from PCs with BD Difco Supplement VX (BDVx) supplementation. Student's t-test analysis, *p value ≤0.05. N = 3.

3.6 Discussion

A major obstacle faced in the transfusion setting that culminates in the transfusion of *C. acnes*-contaminated PCs is the inability to detect *C. acnes* early during bacterial screening. This study aimed at assessing two supplements, T80 and BDVx, to enhance the growth and therefore the detection of *C. acnes* in BPN bottles by the BACT/ALERT system used at Canadian Blood Services.

T80 has been used to enhance the growth of other bacteria like *Lactobacillus casei* [188], *S. aureus* [189] and *Corynebacterium accolens* [190] in vitro. It has been suggested that T80 enhances growth in a species-dependent manner by providing essential nutrients needed for growth, by changing membrane permeability and by enhancing nutrient availability [189]. The work described in this study demonstrates that supplementation of BPN media with T80 (4%, v/v) can also significantly enhance the detection of *C. acnes* from media in the BACT/ALERT system. Furthermore, our growth curve analysis indicated that T80 supplementation promoted the growth of *C. acnes*, resulting in bacterial loads that trigger an earlier positive flag in the BACT/ALERT. Unfortunately, T80 supplementation triggered false-positive flags when PCs prepared in plasma or PAS were tested. These results indicate that the false-positive triggers are caused by plasma and platelet factors such as plasma- and platelet-derived lipases [191, 192]. The lipases likely degrade T80, releasing oleic acid, which causes changes in the gel of the bottle sensor leading to positive flags. Though this could hinder the detection of *C. acnes* in blood products, supplementation of BPN media with T80 could potentially be used in the clinical setting for non-blood samples where incubation periods can be long, allowing for timely intervention of *C. acnes* infections [193]. However, since clinical samples can be derived from various sources, this strategy should be tested to ensure that these samples do not contain factors that may lead to false positives in the presence

of T80 supplementation. Importantly, our data indicate that T80 supplementation (4%, v/v) did not hinder the detection of other bacterial species from media as evidenced by similar detection times in supplemented and unsupplemented bottles. Therefore, T80 supplementation (4%, v/v) will not inadvertently prevent or delay the detection of other bacterial contaminants.

Interestingly, the addition of the commercial supplement BDVx resulted in the early detection of *C. acnes* from contaminated PCs by up to 24 h in the BACT/ALERT system when supplemented at the vendor's recommended concentrations. The BDVx supplement is used in clinical laboratories to enhance the growth and isolation of *Haemophilus* and *Neisseria* species by providing growth-promoting factors including vitamins and cofactors like nicotinamide adenine dinucleotide essential for bacterial metabolism and enzymatic processes [194]. It is possible that *C. acnes* growth is promoted in a similar fashion although the exact component of the BDVx supplement involved in the observed enhanced detection of *C. acnes* requires further investigation.

Our study involved supplementation of commercial BPN bottles, providing proof of principle, that could serve as a basis for the development of enhanced media by vendors for application in blood product screening with automated culture systems. Although our study could be complemented by testing more *C. acnes* isolates, we have provided strong evidence that growth and detection of *C. acnes* can be improved during PC screening with the BACT/ALERT system. Reduction in detection times by 24 h could provide blood suppliers with the ability to recall *C. acnes*-contaminated products before they are transfused into vulnerable patients, thereby improving the safety of the blood supply.

3.7 Acknowledgements

The authors thank volunteer blood donors and staff at the netCAD Blood4Research Facility in Vancouver (British Columbia, Canada) for PC production. Dr. Lori Daane (Sr. Director Scientific

Affairs, bioMérieux) provided insights for the experimental design of the study. This work was supported by Canadian Blood Services and Health Canada.

Please refer to Appendix A2 for supplementary information.

Chapter 4 The Platelet Concentrate Storage Environment Heightens *Cutibacterium acnes* Ability to Adhere to Mammalian Cells and Enhances Potential for Tissue Invasiveness

4.1 Statement of contribution and status of manuscript

The manuscript titled “The platelet concentrate storage environment heightens *Cutibacterium acnes* ability to adhere to mammalian cells and enhances potential for tissue invasiveness” has been prepared for submission to the peer-reviewed journal *Transfusion*.

The conceptualization of the study, the development, optimization, and execution of the methodology, as well as data curation and statistical analyses were performed by Dilini Kumaran. The project was supervised by Dr. Ramirez-Arcos who provided guidance with conceptualization and study design, trouble shooting, and resources to execute the study. The original and final draft of this manuscript was written by Dilini Kumaran and was reviewed and edited by Dr. Ramirez-Arcos.

4.2 Abstract

Background: The anaerobic *Cutibacterium acnes* is a common bacterial contaminant of platelet concentrates (PCs) and is frequently identified by the BACT/ALERT culture system only after contaminated PCs are transfused into vulnerable patients. Though these transfusion events do not cause severe acute transfusion reactions, not much is known about the long-term impacts to the patient. Of note, the PC environment has been shown to enhance the virulence in transfusion relevant bacteria like *Staphylococcus epidermidis*. Since *C. acnes* harbors a host of virulence genes and is known to cause severe chronic infections, this study aimed to assess the impact of the PC milieu on the virulence of *C. acnes* PC isolates using a silkworm (*Bombyx mori*) animal model.

Study design/Methods: Buffy coat PCs and Brain Heart Infusion (BHI) broth were inoculated with either *Staphylococcus aureus* (CBS 2016-05, positive control for virulence and adherence studies) or two *C. acnes* (BPNBT-19195 and BPNBT-19329) isolates and maintained under PC storage conditions for 5 days. Samples from these cultures were used to compare BHI and PC derived bacteria by 1) inoculating silkworm larvae (10/group) and assessing mortality over 72 hrs, 2) evaluating adherence to mammalian epithelial cells (HEK 293T) in aerobic and anaerobic conditions, and 3) assessing the differential expression of virulence genes involved in tissue invasion and persistence. Furthermore, melanization and superoxide dismutase (SOD) activity of the silkworm hemolymph inoculated with *C. acnes* were evaluated to assess the activation of the innate immune response.

Results: Inoculation of PC derived *C. acnes* samples resulted in significantly higher silkworm survival compared to BHI derived counterparts. In contrast, PC derived *S. aureus* caused significantly reduced silkworm survival compared to BHI cultures. Significantly lower

melanization and SOD activity were observed in the hemolymph of larvae inoculated with *C. acnes* derived from PCs compared to BHI. All isolates displayed higher adherence to HEK 293T cells when derived from PCs compared to BHI cultures. *C. acnes* BPNBT-19195 and BPNBT-19329 cultured in PCs displayed higher expression of genes encoding for hyaluronate lyase (*hyl*, tissue invasion) and radical oxygenase (*roxP*, adaptation and persistence) compared to BHI derived samples. On the other hand, the triacylglycerol lipase gene (PPA2105, persistence) was upregulated only in BPNBT-19195.

Discussion: The PC storage milieu modulates the virulence of bacterial contaminants in a species dependent manner. The reduction in hemolymph melanization and SOD activity indicate that PC components may shield *C. acnes* antigenic factors thereby dampening its virulence in silkworms. These findings may explain the low incidence of acute septic transfusion reactions despite the high level of transfusions with *C. acnes* contaminated PCs. Importantly, the data suggest that PCs may prime *C. acnes* to cause chronic infections by enhancing its ability to adhere, invade and persist in host tissue, which warrants further investigation.

4.3 Introduction

Platelet concentrates (PCs) are therapeutic blood products that are used to treat patients with platelet disorders [1]. These blood products have been associated with severe adverse reactions caused by bacterial contamination, and this risk is exasperated by the storage conditions used to maintain PC quality and function [2, 48]. The implementation of strategies like routine culture based bacterial screening of PCs has facilitated the timely retrieval of bacterially contaminated PCs, thereby preventing serious adverse reactions [2]. Despite these advances in blood product safety, there exists a residual risk associated with missed or late detection of slow growing bacterial contaminants [3].

Several reports from blood suppliers in Australia, Canada, Germany, and the Netherlands have indicated that the anaerobic slow growing bacterium *Cutibacterium acnes* is the most isolated bacterial contaminant of PCs and is detected late in culture based screening systems, resulting in the transfusion of these contaminated units [57, 3, 52, 58]. Canadian Blood Services data indicate that 98% of contaminated PC that were issued to hospitals and transfused were contaminated with *C. acnes*, however, no transfusion reactions have been documented to date (Canadian Blood Services, Quality Control Data August 2017- December 2023). Notably, only a few probable cases of mild adverse reactions were retrospectively identified by the German Red Cross and the Sanquin Blood Supply foundation [52, 58]. Consequently, *C. acnes* is often dismissed as a cause for concern in the transfusion setting, even though very little is known about the long-term impacts of transfusing this bacterium into vulnerable patients.

Similarly, in clinical settings, *C. acnes* was routinely written off as a harmless contaminant in positive cultures [116], however several studies have demonstrated that this bacterium harbors a

number of virulence genes and produces enzymes such as triacylglycerol lipases (*PPA2105*) that play a role in nutrient acquisition and immune evasion [195, 196, 197], hyaluronate lyase (*hyl*) that degrades the extracellular matrix, facilitating bacterial tissue invasion and spread [198], and radical oxygenase (*roxP*) that aids in the adaptation to oxygen rich environments allowing *C. acnes* to colonize aerobic niches [199]. Notably, recent reports have associated *C. acnes* with chronic infections such as infectious endocarditis [124], prosthetic joint infections [129], brain abscesses [131], and it has even been linked to the progression of prostate cancer [127]. This growing body of evidence suggests that this bacterium may not be as innocuous as previously believed, especially in the context of chronic infections.

The PC milieu has been shown to elicit expression of virulence genes in transfusion relevant bacteria like *Staphylococcus epidermidis* and *Staphylococcus aureus* isolates in [142, 143, 144], and increase the mortality of observed in the *Caenorhabditis elegans* invertebrate virulence model [146]. Taken together these studies indicate that the PC environment prime contaminating bacteria to be more pathogenic by eliciting the expression of virulence genes. Therefore, the potential risk associated with the transfusion of *C. acnes* contaminated PCs should be given due consideration.

Invertebrates share similar innate responses and regulatory pathways as those found in humans, consequently, they have been used to evaluate the virulence of bacterial pathogens and to assess the efficacy of antimicrobials [200]. The humoral response usually consists of the production of reactive oxygen species (ROS), anti-microbial peptides, and melanization [201]. Additionally, the ROS response triggers the expression of antioxidant mechanisms like superoxide dismutase and catalase to protect against oxidative damage [202]. As a result, these factors are used to measure the innate immune response following bacterial infection [203]. The *Bombyx mori* silkworm invertebrate model has a few advantages over other models that include safer handling due to the

larger size of the larvae and more accurate administration of the test substance to name a few [204]. Notably, Matsumoto et al. were able to successfully demonstrate the utility of the *B. mori* model to assess the virulence of *C. acnes* and have shown that melanization of the hemolymph can be a reliable indicator of the activation of the immune response following *C. acnes* infection [205, 206]. Therefore, in this study, the virulence of PC and culture media derived *C. acnes* isolates were compared in the *B. mori* model. Additionally, the melanization of the hemolymph and SOD activity were measured as indicators of the activation of the innate response of *B. mori* following *C. acnes* infection. Differential expression of genes involved in tissue invasion *(hyl)* and persistence (*roxP* and *PPA2105*) that have been previously assessed in the context of *C. acnes* virulence [207], were compared between PC and media derived bacterial cultures. Additionally, the ability of PC derived bacterial samples to adhere to mammalian epithelial cells was compared with media derived samples, to evaluate the impact of the PC storage environment on the potential for *C. acnes* to cause chronic infection.

4.4 Materials and methods

4.4.1 Bacterial strains

C. acnes PC isolates BPNBT-19195 (phylotype IB) and BPNBT-19329 (phylotype II) representing the two most isolated phylotypes from contaminated PCs were used in this study [208]. A *S. aureus* PC isolate (CBS 2016-05) involved in a septic transfusion reaction [159] was used as a control.

4.4.2 PC production

Buffy coat derived, leukocyte-reduced PC pools were prepared in 100% plasma according to standard procedures at the Canadian Blood Services netCAD Blood4Research Facility (netCAD, Vancouver, Canada). PCs were manufactured and shipped to the Canadian Blood Services Microbiology laboratory in Ottawa, Canada. Ethical approval for this study was granted by the Canadian Blood Services Research Ethical Board.

4.4.3 Silkworm rearing

B. mori eggs were purchased from coastal silk (Jacksonville, Florida) and were hatched at 23-25°C. The larvae were maintained on artificial silkworm chow (coastal silk) containing 300mg of vancomycin/Kg of prepared chow. Fifth in-star larvae were used in all subsequent assays and were fed vancomycin free food for at least 24 hrs prior to inoculation with bacteria.

4.4.4 Determination of the lethal dose 50 (LD_{50}) concentration

C. acnes isolates were grown to stationary phase (~10^8 colony forming units (CFU)/mL) in BACT/ALERT anaerobic culture bottles (BPN) and incubated in the BACT/ALERT 3D system. The cultures were pelleted down and resuspended in insect saline (0.6% NaCl) to a concentration of ~10^{10} CFU/mL. Overnight cultures of the *S. aureus* isolate were prepared in Brain Heart

Infusion (BHI) media at 37°C, pelleted down and resuspended in insect saline. The concentration of bacterial suspensions was verified by plating serial dilutions prepared in BHI media on duplicate blood agar plates and incubated at 37°C (*C. acnes*: anaerobically for 72 hrs, *S. aureus*: aerobically for 24 hrs). Ten-fold dilution series were prepared in insect saline, and 30ul of each dilution was used to inoculate the haemolymph of silkworm larvae (10 larvae/ dilution). The larvae were incubated at 37°C for 72 hrs, during this period larval death was assessed (lack of movement when probed with a sterile pipette). Furthermore, larvae were inoculated with insect saline alone as a control. These assays were performed in biological triplicates. The LD_{50} values were calculated using the online tool AAT Bioquest LD_{50} calculator.

4.4.5 PC and media derived bacterial samples

C. acnes bacterial pellets were obtained as described above and resuspended in 5 mL of BHI media or PCs to a final concentration of 10^{10} CFU/mL. These high concentrations were utilized to enable LD_{50} concentrations to be obtained since *C. acnes* does not proliferate in the PC storage environment. Five milliliter fractions of media and PCs were inoculated with *S. aureus* to a final concentration of 25 CFU/mL. The media and PC samples were incubated for 5 days at 20-24°C with constant agitation in T25 vented cell culture flasks (Thermofisher) in a standard platelet incubator. On day 5, the samples were aliquoted into tubes (1 mL/tube) and frozen at -80C until further use. The bacterial titer of the samples was assessed by serially diluting the suspensions in BHI media and plating the dilutions in duplicates on blood agar plates. Samples were derived from three independent experiments.

4.4.6 Comparison of virulence of media and PC derived bacteria in the *B. mori* model

Bacterial samples derived from media and PCs were thawed on ice. The media derived samples were centrifuged at 10000rpm for 5min at 4°C, after which the supernatant was pipetted out and

the pellet was resuspended with the same volume of insect saline as the supernatant that was removed. When required, the media and PC derived samples were diluted in either saline or un-spiked PCs respectively to obtain bacterial loads equal to the LD_{50} value/ spiking volume as determined earlier. Fifth in-star larvae that had been fed antibiotic free chow for at least 24 hrs were inoculated with spiking suspensions, and with un-spiked PCs and saline that served as background control (10 larvae/ sample tested) and incubated at 37°C. The larvae were examined every 24 hrs, and mortality (no movement when prodded with a pipette tip) was recorded over 72 hrs. These experiments were performed at least three independent times.

4.4.7 Collection of silkworm haemolymph

Virulence assays were performed as described above for the two *C. acnes* isolates. Hemolymph samples were extracted from silkworm larvae 1hr and 24 hrs post inoculation. Briefly, larval skin was disinfected with isopropyl alcohol swabs, once dry the first proleg of the larvae was amputated using sterile scissors and the hemolymph was collected in sterile chilled microcentrifuge tubes containing phenyl thiourea to inhibit further melanization. The samples were centrifuged at 10000 rpm for 10 min at 4°C and the supernatant was used immediately in subsequent assays.

4.4.8 Haemolymph melanization

Melanization of the haemolymph was measured as previously described [206]. Briefly, the haemolymph was diluted in (PBS, pH7.4) to a 1: 10 dilution (v/v), after which the sample was assessed using a spectrophotometer at 490nm.

4.4.9 Haemolymph SOD activity

SOD activity was assessed via a pyrogallol autooxidation inhibition assay, where one unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of pyrogallol autooxidation

in the presence of EDTA, pH 8.2 [209, 210]. Briefly, hemolymph samples were tested in technical duplicates by diluting the sample (1:4, v/v) in TRIS-EDTA (pH 8.2) buffer. The assay reaction mixture consisted of 1 mL of TRIS-EDTA buffer and 50ul of the hemolymph sample (test) or 50ul of sterile water (control). A 1 mL pyrogallol (0.2mM) aliquot was added to the reaction, briefly vortexed and immediately read (T0) using a spectrophotometer at 420nm. Following exactly 1min of incubation at room temperature, the samples were read again (T1) at 420nm. SOD activity (U/mL) was calculated using the following equation:

$$\frac{((\text{Control } A_{420}\,(T1) - (\text{Control } A_{420}\,(T0)) - ((\text{Test } A_{420}\,(T1) - (\text{Test } A_{420}\,(T0)) * 100}{(\text{Control } A_{420}\,(T1) - (\text{Control } A_{420}\,(T0)) * 0.5}$$

Where:
"Control" refers to tubes containing sterile water
"Test" refers to tubes containing hemolymph samples

4.4.10 Bacterial adherence to mammalian cells

C. acnes and *S. aureus* (positive control) bacterial samples (control) derived from media and PCs as described above were used in adherence assays. *C. acnes* has been shown to internalize into human embryonic kidney (HEK) cells [211]. As a result, in this study HEK 293T cells (ATCC) were used to assess adherence and were cultured in DMEM high glucose media containing 10% heat inactivated fetal bovine serum and were used to seed the wells of 6-well cell culture plates (37°C, 5% CO2). Once the cells were 90% confluent (~3.4 x 10^6 cells/ well), the media was refreshed, and ~1.2x 10^5 bacterial cells were added to duplicate wells. Plates were incubated either aerobically at 37°C, 5% CO2 for 3hrs, or anaerobically at 37°C for 3hrs. Following the 3hr incubation period, the media was removed, and the adhered cells were washed twice with 3 mL of PBS by gently rocking the plates and aspirating the supernatant. Ice cold sterile water was added to the wells of the plate (1 mL/ well) and pipetted up and down vigorously to detach and lyse the

mammalian cells. The samples were serially diluted in BHI media and plated on duplicate blood agar plates. These assays were performed with bacteria obtained from three independent PC and media samples in technical duplicates.

4.4.11 RNA extraction

A previously described protocol was modified to extract bacterial RNA from PC derived *C. acnes* samples [212]. Briefly, 2 mL of the PC derived sample was centrifuged at 10000 rpm for 10min at 4°C, the supernatant was removed, and the pellet containing bacteria and platelets was vigorously resuspended in cold 0.02% SDS in phosphate buffered solution (PBS) for 30sec and centrifuged at 4000 x g for 10min. The supernatant was removed, and the pellet was washed twice with 1.5 mL of cold PBS and pelleted down (4000 x g for 10min), and the SDS and PBS washes were repeated. Media derived samples were spun down at 10000rpm for 10min at 4°C, and the supernatant removed. Media and PC derived pellets were resuspended in 1.5 mL Qiagen RNAprotect bacteria reagent for 10 min at room temperature. The suspension was centrifuged at 10000 rpm for 10 min at 4°C, and the excess RNAprotect solution was removed. The RNA was extracted using the FastRNA pro Blue kit (MP Biomedicals), using program 6 on the FastPrep-24 machine (MP Biomedicals) consisting of a 20sec cycle followed by 2min rest on ice, this was repeated 3 times. Chloroform extraction was performed followed by ethanol precipitation.

4.4.12 Differential gene expression

The extracted RNA was DNase treated using the Turbo DNA free kit (Invitrogen), and cDNA was generated using the QuantiTect RT kit (Qiagen). qPCR was performed as previously described [207] using four genes coding for hyaluronate lyase, triacylglycerol lipase, radical oxygenase and 16s ribosomal RNA (housekeeping). The primer sequences have been described in Table 7. The

QuantiNova SYBR green PCR kit (Qiagen) was used according to manufacturer recommendations. Each biological triplicate was tested in technical duplicates.

Table 7. List of primers and their targets used in the evaluation of differential gene expression.

Gene	Description	Primer
hyl	Hyaluronate lyase	Forward: 5'-CAA CAT CGC CGT GTT TAT TG-3' Reverse: 5'-CCC ATG ACG ACG TAG AGG AT-3'
roxP	Reactive oxygenase	Forward: 5'-GCA TCT AGC CCT CTC ACC AT-3' Reverse: 5'-CTG AGA GTC CGG TAG GTG GT-3'
PPA2105	Triacylglycerol lipase	Forward: 5'-GAT TTC CTT AGC ACG TGG AG-3' Reverse: 5'-GAT GAC GGT GTA GGC GAT AC-3'
16s rRNA	16S ribosomal RNA	Forward: 5'- GGG GCT TAA CCC TGA GCG TGC-3' Reverse: 5'- TTC GCT CCC CAC GCT TTC GC-3'

4.4.13 Statistical analyses

Graphpad v10 was used for all analyses. Normally distributed data were analyzed using the student t- test (with Welch's correction for data with unequal variance). If the data were not normally distributed, comparisons were performed using the Mann Whitney test. P values < 0.05 were considered significant.

4.5 Results

4.5.1 *C. acnes* PC isolates are less virulent than the transfusion relevant *S. aureus* in the *B. mori* model

The LD_{50} values for the *C. acnes* isolates BPNBT-19195 and BPNBT-19329 were calculated to be 2.63×10^8 and 1.11×10^8 CFU/ larvae respectively. On the other hand, the LD_{50} titer for *S. aureus* was approximately 100-fold lower at 4.26×10^6 CFU/ larvae.

4.5.2 PC derived *C. acnes* is less virulent than their media derived counterparts

Silkworm larvae injected with PC derived BPNBT-19195 displayed significantly ($p \leq 0.05$) higher survival rates (88%) compared to larvae injected with its media derived counterparts (50%). Similarly, significantly higher ($p \leq 0.05$) survival rates (93.3%) were observed in larvae injected with PC derived than media derived BPNBT-19329 (53.3%) (Figure 17). On the other hand, the inoculation of PC derived *S. aureus* resulted in significantly ($p \leq 0.05$) reduced larval survival (18%) than when media derived counterparts (52%) were used (Figure 17).

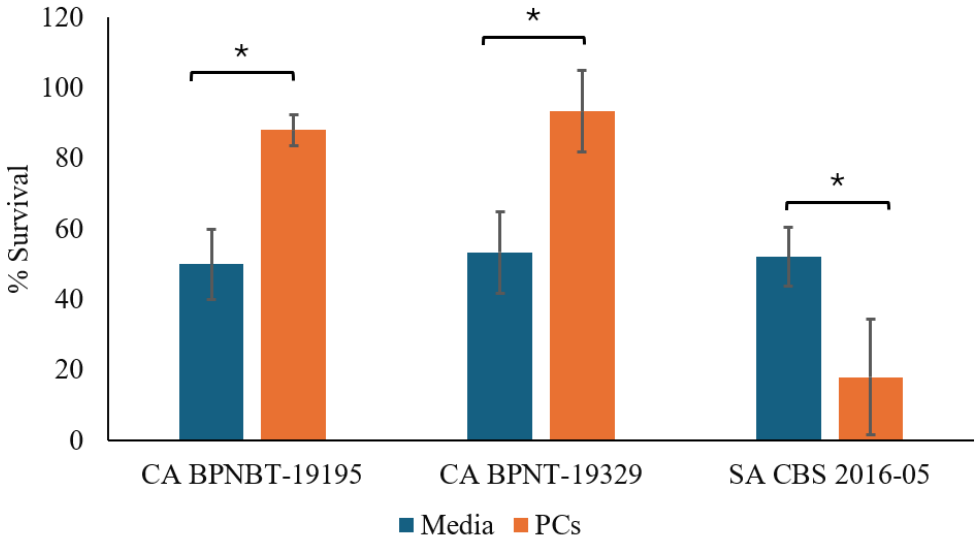


Figure 17. Comparison of virulence of PC and media derived bacteria in the silkworm model. Silkworm survival following the inoculation of larvae with media derived (blue bars) and PC derived (orange) *C. acnes* isolates BPNBT-19195, and BPNBT-19329 and *S. aureus* CBS 2016-05 isolate, following 72 hrs incubation at 37°C. N=3. Student t-test, * -p≤0.05.

4.5.3 PC derived *C. acnes* triggers the silkworm innate immune response to a lesser extent than media derived counterparts

The level of haemolymph melanization (OD490nm) observed on days 0 and 1 were significantly higher in larvae injected with media derived *C. acnes* isolates compared to the control larvae injected with saline (Figure 18A). Though the melanization observed in the hemolymph of larvae inoculated with PC derived BPNBT-19195 significantly increased compared to the PC control on Day 1, the melanization observed in larvae inoculated with media derived *C. acnes* (BPNBT-19195 and BPNBT-19329) was significantly higher than the melanization observed in larvae injected with PC derived sample on both days tested (Figure 18A). However, the observed melanization in larvae injected with media derived BPNBT-19195 decreased on day 1 compared to Day 0, but no significant change was observed in larvae injected with media derived BPNBT-19329 (Figure 18A). Similarly, the hemolymph SOD activity observed in larvae injected with media derived *C.*

acnes was significantly higher than PC derived counterparts (Figure 18B). Significant increases ($p \leq 0.05$) in SOD activity of media and PC derived samples were observed on day 1 compared to day 0 for both bacterial isolates tested. Interestingly, a significant reduction in SOD activity was observed in larvae injected with the PC control on day 1 compared to larvae injected with the saline control (Figure 18B).

4.5.4 PC derived bacteria are more adherent to mammalian HEK 293T cells

Under aerobic conditions, all bacterial isolates tested were more adherent when derived from PC compared to media derived counterparts, and this increase was significant ($p \leq 0.05$) for isolates BPNBT-19329 and CBS 2016-05 (Figure 19). The aerobic adherence profiles of the two *C. acnes* isolates derived from media and PCs were comparable. Under anaerobic conditions, all bacterial isolates derived from PCs were significantly more adherent to HEK cells when compared to media derived samples ($p \leq 0.05$). Significantly reduced adherence was observed in PC and media derived BPNBT-19329 compared to the corresponding *S. aureus* samples under both aerobic and anaerobic conditions ($p \leq 0.05$) (Figure 19). The two *C. acnes* isolates displayed comparable adherence profiles under aerobic and anaerobic conditions, however PC derived *S. aureus* displayed significantly higher ($p \leq 0.05$) adherence under aerobic conditions, compared to anerobic incubation (Figure 19).

4.5.5 *C. acnes* genes involved in tissue invasion and persistence are upregulated in PCs

Genes encoding for hyaluronate lyase (*hyl*), an enzyme generally associated with bacterial tissue invasion, radical oxygenase *(roxP),* and a triacylglycerol lipase (*PPA2105*), which is associated with persistence (207), were assessed in this study and were found to be upregulated in PC derived BPNBT-19195 compared to media derived counterparts, however the increase in gene expression was only significantly ($p \leq 0.05$) higher for *roxP* (Figure 20). In PC derived BPNBT-19329, there

was upregulation in the expression of *hyl* and *roxP* but not in the lipase gene evaluated (Figure 20). None of the changes in the gene expression profile were significantly different in this isolate when grown in PCs versus media.

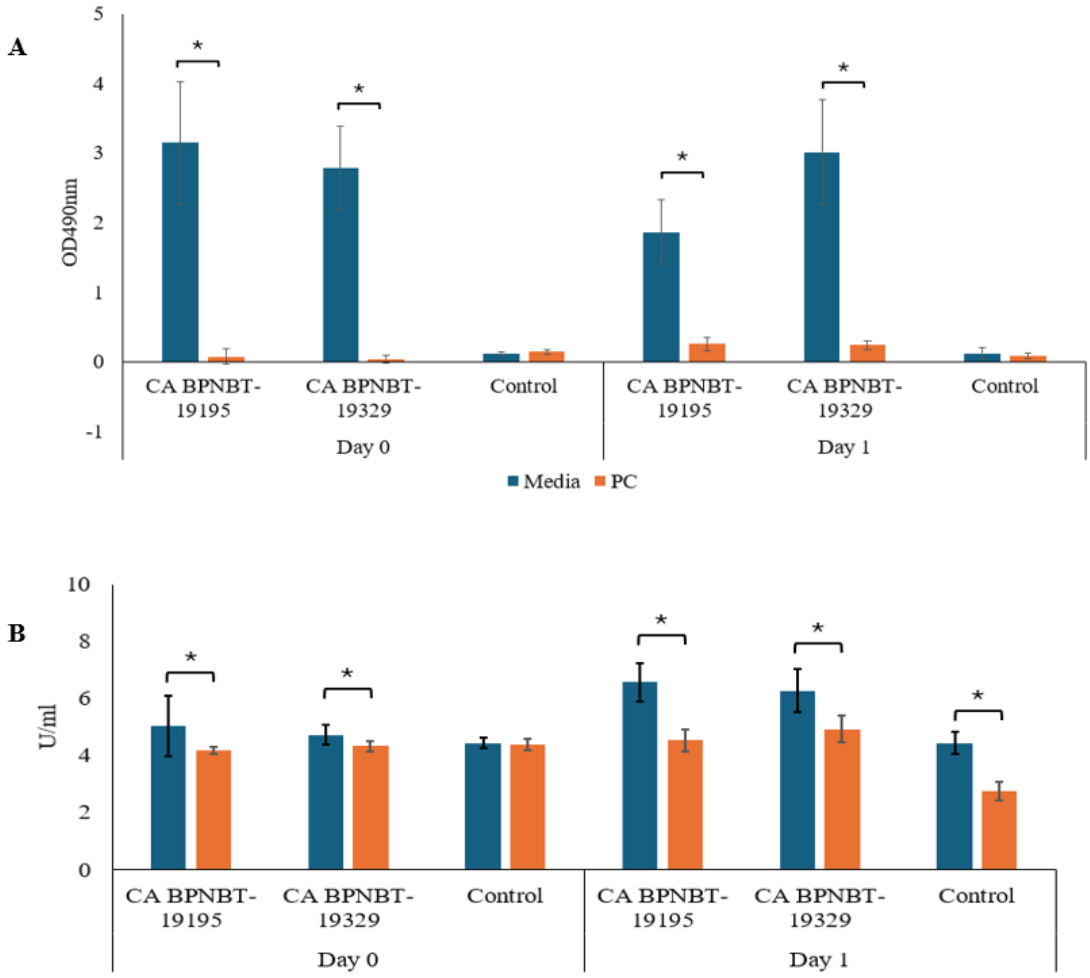


Figure 18. Silkworm innate immune response following *C. acnes* inoculation.

Innate response of silkworms inoculated with media (blue) and PC derived samples (orange) containing *C. acnes* isolates BPNBT-19195 and BPNBT-19329. Controls correspond to analysis performed on haemolymph from larvae inoculated with unspiked insect saline and PCs. A) Melanization of haemolymph measured at OD490nm. B) Superoxide dismutase activity presented as U/mL of haemolymph. N=3, (3 larvae/ repetition), Mann-Whitney test used for melanization assays, student t-test used for SOD activity (Welch's correction for BPNBT-19195 Day 0, comparisons), *- $p \leq 0.05$

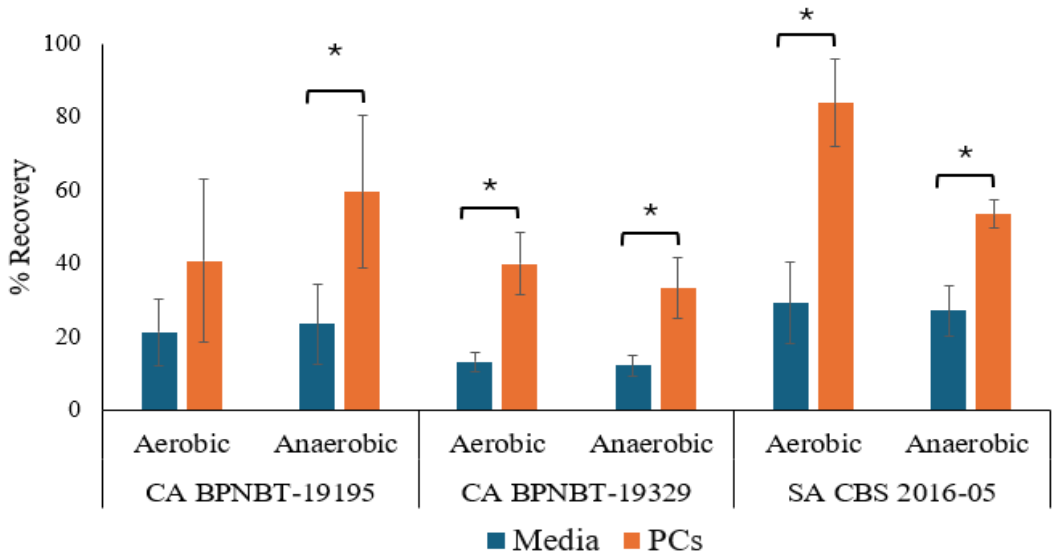


Figure 19. Bacterial adherence to HEK 293T cells.

Comparison of the adherence of media (blue) and PC (orange) derived *C. acnes* isolates BPNBT-19195 and BPNBT-19329, and *S. aureus* isolate CBS 2016-05 to HEK 293T cells following incubation under aerobic and anaerobic conditions for 3 hrs. N=3, Student t-test (BPNBT-19195, SA CBS 2016-05), t-test (Welch's correction, BPNBT-19329 aerobic), and Mann-Whitney (BPNBT-19329, anaerobic), $p \leq 0.05$.

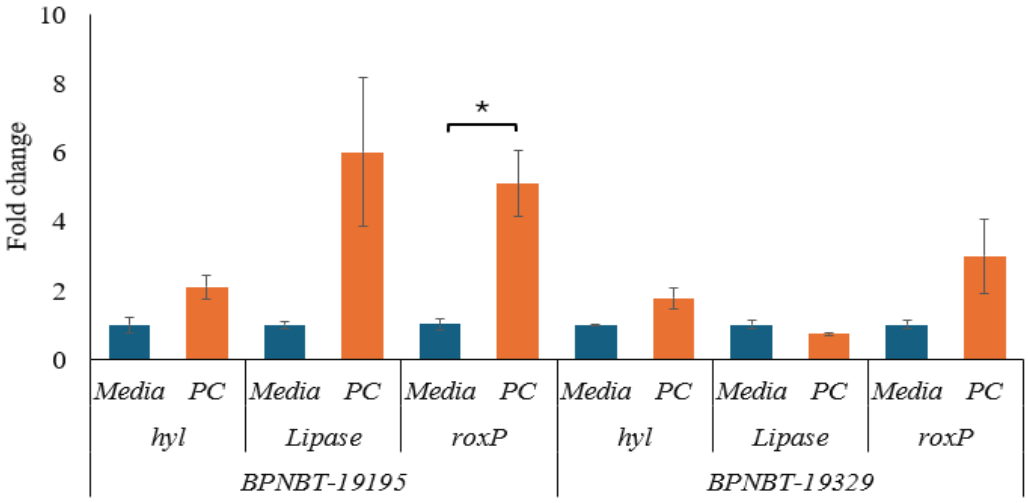


Figure 20. Fold change of virulence gene expression in *C. acnes*.

The fold change (2^-ΔΔCt) of the expression of *C. acnes* virulence genes that code for hyaluronate lyase (hyl), lipase, and radical oxygenase (roxP) relative to the control (media derived samples), using 16S rRNA as a housekeeping gene. Fold change in media derived samples are represented by the blue bars, while PC derived samples are represented by orange bars. N=3, student t-test, *-$p \leq 0.05$.

4.6 Discussion

C. acnes contaminated PCs are often transfused before they can be retrieved due to its slow growing nature in culture media [3, 58, 52]. These transfusion events are frequently disregarded as a cause for concern as they have only been linked to a few mild adverse reactions, however, very little is known about the risk to patients involving delayed or chronic infections. Notably, *C. acnes* can cause slow developing chronic infections in clinical settings and possesses an array of virulence genes that promote biofilm formation, bacterial persistence, and survival. Furthermore, the PC milieu has been shown to have a modulatory effect on the expression of virulence genes in transfusion relevant bacteria [142, 143, 144]. To our knowledge, this is the first report that aims to assess the impact of the PC environment on the virulence of *C. acnes* and provide insight into the potential risks associated with these transfusion events.

Our data indicate that PC derived *C. acnes* samples were less virulent than their media derived counterparts, and these findings were accompanied by a significant reduction in the melanization and SOD responses in silkworm haemolymph. Matsumoto et. al demonstrated that the peptidoglycan fraction of the *C. acnes* cell wall mediated the activation of the innate response in silkworm larvae [206]. The significant reduction in virulence and activation markers suggest that the *C. acnes* peptidoglycan layer may be shielded from the silkworm immune system by PC components. Of note, platelets are known to activate and aggregate around *C. acnes* found at high titers [213, 214], therefore it is plausible, that these platelet-bacterial aggregates may prevent the recognition of bacterial markers like peptidoglycan thereby minimizing the immune response. Given our findings, the mild transfusion events caused by the infusion of *C. acnes* contaminated PCs [52, 58], could potentially be explained by a combination of low bacterial concentration and

the bacterial peptidoglycan shielding effect by PC components that would otherwise trigger an immune response in humans (ex. production of pro-inflammatory cytokines).

Furthermore, our work aimed to evaluate whether the PC environment could elicit the expression of *C. acnes* virulence genes as has been demonstrated in other transfusion relevant bacterial species such as *S. epidermidis* and *S. aureus* [142, 143, 144]. Our data indicate that the PC storage environment enhances the expression of virulence genes that code for hyaluronate lyase, triacylglycerol lipase, and radical oxygenase. It should be noted that most increases observed were not significant due to differences in responses in biological replicates. Differences in gene expression profiles could be explained by donor variability and is highlighted by the relatively low variance observed in gene expression in media derived samples. Due to the difficulty associated with genetically manipulating this bacterial species, only a few studies have assessed the role that *C. acnes* virulence gene products play in the pathogenesis of disease [215]. Interestingly however, *C. acnes* phylotypes IB and II that are normally associated with deep tissue infections [216, 127, 126], have been shown to produce hyaluronate lyase that are enzymatically more potent in vitro than the lyase produced by phylotype IA normally associated with acne [198]. Of note, two isolates belonging to phylotype IB and II were used in this study as it was previously demonstrated to be the most represented phylotypes of *C. acnes* PC isolates [208]. Taken together, these data suggest that the increased expression of *C. acnes* hyaluronate lyase elicited by the PC environment could enhance the ability to degrade hyaluronic acid of the extracellular matrix more effectively and could therefore promote bacterial invasion and spread. Similarly, radical oxygenase was recently described as a secreted enzyme produced by *C. acnes* with antioxidant properties [199]. In the absence of this gene, *C. acnes* was incapable of remaining viable in aerobic conditions for more than 5 days and is thought to play an important role in *C. acnes* persistence and colonization of the

skin [199]. In this study, *C. acnes* was grown in PCs under aerobic conditions, and therefore the observed enhanced expression of *roxP* is consistent with the ability of *C. acnes* to remain viable during PC storage in the PC milieu. We also observed the enhanced expression of triacylglycerol lipase in the PC environment, *C. acnes* lipases are involved in the hydrolysis of sebum components freeing glycerol and other fatty acid that can be used as a nutrient source [217]. Furthermore, in bacteria like *S. aureus*, lipase plays a role in biofilm formation [218], the evasion of the immune response [219, 220], and lipase producing isolates were found more frequently in deep seated infections [221]. Though the role that *C. acnes* lipases play in deep tissue infection is unclear, it has been shown to be involved in the pathogenesis of acne [222]. Interestingly, our data also indicate that the PC environment augments the adherence of *C. acnes* to mammalian epithelia. *C. acnes* has been shown to bind directly and internalize into epithelial cells [211] and is able bind to fibrinogen and promote platelet aggregation [214]. Though the experimental approach does not differentiate between direct bacterial interactions with epithelial cells and indirect interactions via platelets or plasma components like fibrinogen, these findings are noteworthy since the adhesion of bacteria to underlying tissue is an important step to initiate infection. The adherence data obtained, taken together with our findings on differential gene expression, indicate that PC derived *C. acnes* may be primed to be more adherent, persistent, and invasive, and warrants further investigation.

Of the chronic infections caused by *C. acnes*, infectious endocarditis is an example where the current findings may be pertinent. This infection generally begins with the aggregation of activated platelets at the site of injury (cardiac valve), which is secondarily infected by circulating bacteria [223]. This is followed by the recruitment of more platelets and immune cells, while adhered bacteria protected by the fibrin clot proliferates resulting in damage to underlying tissue caused by

an increase in proinflammatory factors [224]. Furthermore, some bacteria like *S. aureus* can bind to healthy cardiac endothelia and internalize, resulting in persistent infection and inflammation causing damage to underlying tissue [225]. To our knowledge, there have been no prospective or retrospective studies that have assessed the risk of chronic infections following the transfusion of *C. acnes* contaminated PCs. Notably, *C. acnes* can internalize into epithelial cells and macrophages [226, 211], therefore, the transfusion of PCs contaminated with *C. acnes* displaying a heightened propensity to adhere and invade mammalian cells could potentially enhance the risk of slow developing chronic diseases like infectious endocarditis. Our results therefore indicate that the risk associated with the transfusion of *C. acnes* contaminated PCs cannot be dismissed, especially in the context of chronic infections.

4.7 Acknowledgements

The authors thank volunteer blood donors and staff at the netCAD Blood4Research Facility in Vancouver (British Columbia, Canada) for PC production. Heartfelt gratitude to Dr. Matsumoto (Meiji Pharmaceutical University) for sharing expertise on sericulture and establishing virulence models. The authors would also like to extend our gratitude to Eika Felipe (sericulture educator) for their time and expertise. Special thanks to Jasminka Bozic (Director of Upstream Manufacturing Development, VBI vaccines) and Rohan Lalani (Research technician, VBI vaccines) for technical support with cell culture. This work was supported by Canadian Blood Services and Health Canada.

Please refer to Appendix A3 for supplementary information.

Chapter 5 *Cutibacterium acnes* Contamination Does Not Enhance the Proinflammatory Profile of Platelet Concentrates

5.1 Statement of contribution and status of manuscript

The manuscript titled "*Cutibacterium acnes* contamination does not enhance the proinflammatory profile of platelet concentrates" has been published online (ahead of print) in Transfusion. 2024 June; doi: https://doi.org/10.1111/trf.17931. Please find the license to reprint in Appendix B. The numbering of sections, tables and figures have been modified (minor) as per Faculty of Medicine requirements for an article-based thesis.

The conceptualization of the study, the development, optimization, and execution of the methodology, as well as data curation and statistical analyses were performed by Dilini Kumaran. The project was supervised by Dr. Ramirez-Arcos who provided guidance with study design, trouble shooting, and resources to execute the study. The original and final draft of this manuscript was written by Dilini Kumaran and was reviewed and edited by Dr. Ramirez-Arcos.

5.2 Abstract

Background: *Cutibacterium acnes*, a common anaerobic platelet concentrate (PC) contaminant, has been associated with rare mild adverse transfusion reactions and is often considered a harmless commensal. Notably, *C. acnes* can cause chronic infections and has been shown to induce the release of proinflammatory cytokines by immune cells. Since elevated concentrations of proinflammatory factors in PCs have been linked to non-infectious adverse reactions, this study aimed to assess whether *C. acnes* could elicit the release and accumulation of proinflammatory factors during PC storage, thereby enhancing the risk of such reactions.

Study Design/Methods: Four ABO matched buffy coat PCs were pooled and split into 6 units, each were inoculated with either saline (negative control), a *Staphylococcus aureus* isolate (positive control, 30 colony forming units (CFU)/unit), or four *C. acnes* PC isolates (10 CFU/mL) and stored at 20-24°C with agitation. Bacterial concentration, platelet activation, and concentration of proinflammatory factors were assessed on days 0, 3, and 5. N=3

Results: *C. acnes* concentrations remained stable, while *S. aureus* proliferated to a concentration of 10^8 CFU/mL by the end of PC storage. No significant differences in the activation or proinflammatory profiles of *C. acnes* contaminated PC were observed compared to the control ($p>0.05$), while there was a significant increase ($p\leq0.05$) in sCD40L concentration (day3), and activation and IL-8 concentration (day5) in *S. aureus* contaminated units.

Discussion: *C. acnes* contamination does not promote the accumulation of proinflammatory factors in the absence of proliferation during storage and may not enhance the risk of inflammatory reactions when transfused to patients.

KEYWORDS: Platelet contamination, *Cutibacterium acnes*, proinflammatory, cytokines, adverse transfusion reactions

5.3 Introduction

Platelet concentrates (PCs) are therapeutic blood products and consist of a concentrated fraction of platelets suspended in 100% plasma or a combination of plasma and a buffer known as platelet additive solution (PAS) [181]. Platelets harbor and release upon activation a host of molecules that aid in platelet adhesion and aggregation, and pro-inflammatory factors such as cytokines and biological response modifiers like soluble CD40 ligand (sCD40L) to name a few [5, 227, 228] These factors play an essential role in maintaining hemostasis and the immune response. Consequently, PCs are used to treat individuals with platelet deficiencies or to control blood loss in acute trauma [229].

Though remarkable strides have been made to ensure the safety of blood transfusions, adverse reactions do occur and can range from mild reactions to life threatening [26]. These reactions can be broadly classified as either being infectious or non-infectious [230]. Febrile non hemolytic transfusion reactions (FNHTR) are the most encountered non-infectious transfusion reactions, with a higher prevalence observed with PC transfusions [231]. These reactions have been described to be mediated by either anti human leukocyte antibodies (HLA) produced by the recipient (immune pathway) or by platelet and residual leukocytes derived pro-inflammatory cytokines (non-immune) [30]. The occurrence of these reactions has been significantly reduced but not entirely eliminated with the implementation of leukoreduction prior to storage [34]. Similarly, the accumulation of high concentrations of sCD40L has been associated with FNHTR [33] and with transfusion related acute lung injury (TRALI) [41]. Infectious reactions on the other hand are caused by the inadvertent transfusion of microbially contaminated blood products like HIV, Hepatitis C, and bacteria [42]. Following the implementation of rigorous virus screening and leukoreduction protocols [43, 44, 232], most reported infectious reactions are those caused by the

transfusion of bacterially contaminated PCs, which can pose a significant risk of sepsis and death [159, 233]. Though rare, these infectious transfusion reactions are driven by the proliferation of contaminating bacteria in PCs, which is promoted by the storage conditions (20-24°C, agitation, neutral pH, in gas permeable containers) used to maintain platelet quality and functionality [26, 2]. At Canadian Blood Services and other blood centers, several strategies have been implemented to mitigate this risk [234] however on occasion, bacterially contaminated PCs are transfused into vulnerable patients leading to severe reactions [159].

Cutibacterium acnes is an anaerobic, aerotolerant member of the skin flora and is the most isolated bacterial contaminant of PCs (~70%) [3]. *C. acnes* has long been considered a harmless commensal; however, recent reports have highlighted this bacterium's role in chronic infections facilitated by its ability to form biofilms and promote inflammation [235, 127]. Hemovigilance data collected at the Canadian Blood Services between 2017 and 2023 indicate that approximately 58% of contaminated PCs are intercepted prior to transfusion due to successful bacterial screening, while around 42% are found to be culture-positive after the units have been issued to hospitals and transfused. Of these PC units, approximately 98% are contaminated with *C. acnes*. This occurs due to the slow growing nature of *C. acnes* in culture bottles used for PC screening, resulting in late detection which prevents timely retrieval of contaminated PCs [3]. Since this anerobic bacterium is incapable of proliferating to clinically significant levels [185, 48] in the aerobic PC environment, these transfusion events have not resulted in severe acute septic reactions [52, 58]. As a result, it is often dismissed as a cause for concern to transfusion patients. It should be noted however, that there have been several reports of retrospective studies that attribute mild transfusion reactions to *C. acnes* contamination, and this number is believed to be an underestimate as reactions are often mild and difficult to differentiate from the recipient's underlying conditions

[52, 58]. It is also important to consider that not all blood centers screen for anaerobic bacterial contaminants, and as a result there is a high probability that *C. acnes* contaminated PCs are transfused at an even higher rate in those instances.

Bacterial-platelet interactions have been demonstrated to result in platelet activation and degranulation [236]. In the context of *C. acnes* contaminated PCs, very little is known about how *C. acnes* interaction with platelets during PC storage impacts the pro-inflammatory composition of the PC milieu. Since many vulnerable PC recipients are transfused with *C. acnes* contaminated product [3, 52] it is important to understand the inherent risk associated with such transfusion events. To that end, this study aimed to determine whether *C. acnes* contamination of PCs could result in the in the release and accumulation of pro-inflammatory factors during storage thereby enhancing the risk of non-infectious adverse reactions like FNHTRs and TRALI.

5.4 Study design and methods

5.4.1 PC production

Four ABO matched leukocyte reduced buffy coat PCs suspended in 100% plasma were prepared as per standard procedures at Canadian Blood Services netCAD Blood4Research Facility (netCAD, Vancouver, Canada). The PC units were pooled, sterility tested, and split into six units of approximately equal volume. It should be noted that the volume and platelet count in each unit met the standards required to maintain the quality and functionality of PCs. Ethical approval for the study was obtained from the Canadian' Blood Services Research Ethical Board.

5.4.2 Bacterial strains

Four *C. acnes* strains isolated from positive culture bottles during routine bacterial screening of PCs at Canadian Blood Services were used in this study. Four *C. acnes* phylotypes were represented in the chosen strains, namely: BPNTBT-19269 (phylotype IA), BPNBT-19195 (phylotype IB), BPNBT-19329 (phylotype II), and BPNBT-19227 (phylotype III) [208]. A *Staphylococcus aureus* isolate (CBS 2016-05) associated with an adverse transfusion reaction [159] was also included in the study and was primarily used as a positive control for the activation of PCs through bacterial interaction. Bacterial suspensions were prepared in Brain Heart Infusion broth supplemented with 15% glycerol to a Densimat reading of 0.6 (~ $1x\ 10^8$ colony forming unit (CFU)/mL) and frozen at -80°C until use. Ten-fold serial dilutions of the enumerated stocks were prepared and plated on Tryptic Soy Broth agar supplemented with 5% sheep blood (BA) (Oxoid) to verify concentrations following incubation at 37°C (*C. acnes* anaerobically for 72h, and *S. aureus*: aerobically for 24h).

5.4.3 Bacterial inoculation of PCs

The experimental procedure followed to inoculate PC units with bacteria has been described in Figure 21. Briefly, of the six PC units obtained following the pool and split, four of the units were inoculated with *C. acnes*, one unit was inoculated with *S. aureus* and the last unit was inoculated with sterile saline served as the un-spiked control. All bacterial spiking suspensions were prepared by serially diluting the enumerated stocks in sterile saline. PC units were inoculated with 1 mL of the spiking suspension to obtain a final concentration of ~10 CFU/mL in units assigned to be spiked with *C. acnes*, while the PC unit inoculated with *S. aureus* had a final concentration of ~30 CFU/ PC. The PC units were stored under standard conditions for 5 days. Samples were obtained on Days 0 (20 minutes post spike), 3 and 5. Approximately 3 mL of the PC samples were centrifuged at 10000 rpm for 10 min at 4°C, and the plasma was transferred to sterile tubes and immediately frozen at -80°C for further testing. Bacterial growth and platelet activation were assessed using PC samples taken on each testing day. These experiments were performed in three biological replicates.

5.4.4 Assessment of bacterial growth

The bacterial growth of *C. acnes* was assessed by plating 1 mL of the PC sample on duplicate BA plates. *S. aureus* growth was assessed by serially diluting (10-fold) the PC sample in sterile saline and plating the dilutions on duplicate BA plates. Bacterial concentrations were determined following the appropriate incubation period at 37°C (*C. acnes* anaerobically for 72h, and *S. aureus*: aerobically for 24h).

5.4.5 Activation of platelets in contaminated PC during storage

Fresh PC samples were analyzed using flow cytometry to assess the activation status of platelets in both bacterially spiked and un-spiked PC units during storage. CD41a was used as a platelet specific marker, while annexin V and CD62P were used as platelet activation markers. Two panels were utilized to assess the PC samples, briefly the first panel consisted of samples diluted in PBS to a concentration of $2x10^6$ platelets/ 100µl of sample and stained with an APC conjugated antibody against CD41a (BD Biosciences) and a PE conjugated antibody against CD62P (BD Biosciences). The second panel consisted of PC samples diluted in annexin V buffer (1X, BD Biosciences) to a concentration of $2x10^6$ platelets/ 100µl of sample and stained with an APC conjugated antibody against CD41a (BD Biosciences) and an Alexa Fluor® 488 conjugated antibody against annexin V (Invitrogen). Samples were analyzed in biological triplicates.

5.4.6 Evaluation of the accumulation of pro-inflammatory cytokines and sCD40L in contaminated PC units during storage

Plasma samples were thawed on ice and since no major differences in activation profiles during storage were observed in the activation assay, plasma samples obtained on the same sampling day from the three repetitions spiked with the same bacteria were pooled. Samples obtained from the un-spiked PC units were also pooled as described above. The cytokine content of pooled samples was assessed using the BD Human Inflammatory Cytokine Cytometric Bead Array kit (BD Biosciences), while sCD40L content was assessed using a commercial human CD40L (soluble) ELISA kit (Invitrogen). In both assays, each pooled sample was tested in duplicate.

5.4.7 Statistical analyses

Statistical difference in activation and pro-inflammatory factors were determined using the Student T-test. P values < 0.05 were considered significant. The analyses were run on GraphPad Prism v10.

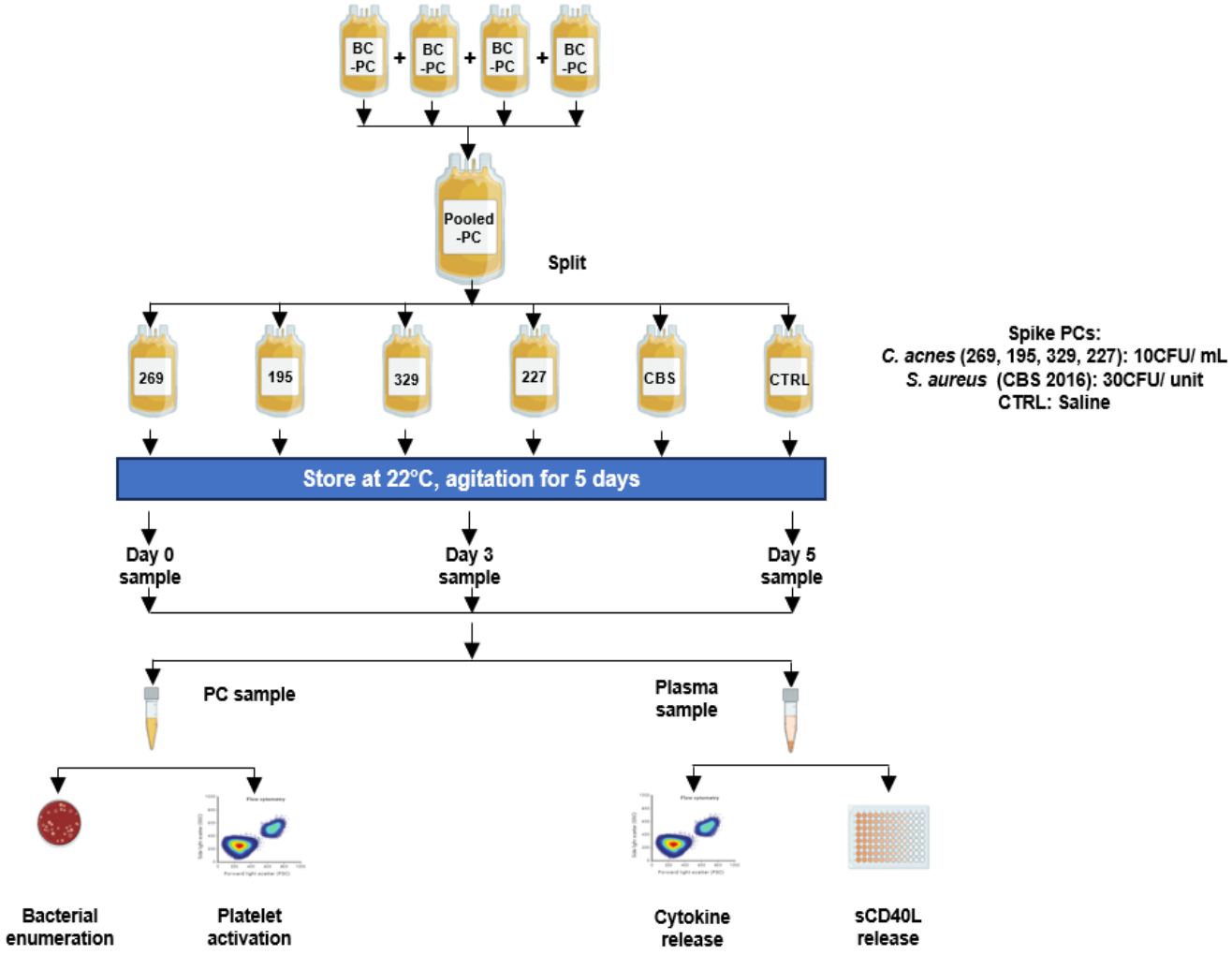


Figure 231. Experimental study design.
Six PC units derived from pooling and splitting four buffy coat PC units were each inoculated with one of four *C. acnes* PC isolates, or a transfusion relevant *S. aureus* isolate. One unit per set served as a negative control (CTRL) and was spiked with sterile saline. PC units were stored under standard PC storage conditions for 5 days, and samples were extracted on days 0, 3, and 5 to assess bacterial growth, platelet activation, and the release of proinflammatory factors. Images were created using Biorender.

5.5 Results

5.5.1 *C. acnes* concentrations remain stable during PC storage

All four *C. acnes* isolates remained viable during PC storage maintaining a concentration of ~10 CFU/mL while *S. aureus* proliferated to a concentration of ~10^8 CFU/mL by day 5 (Figure 22). Platelet aggregates in *S. aureus* inoculated units were observed on day 3 that could not be disassociated with gentle agitation prior to sampling, these aggregates were absent on day 5 of storage.

5.5.2 *C. acnes* contamination does not enhance the activation of platelets during storage

The platelet activation markers tested in *C. acnes* contaminated PC units did not significantly differ from the negative control (Figure 22). The percentage of the CD62P positive population significantly ($p \leq 0.05$) increased from 4 ± 1.3% on day 0 to 12 ± 1.8% by day 5 in *C. acnes* contaminated and control units (of the gated CD41a positive population) (Figure 22). Similarly, a significant ($p \leq 0.05$) increase in the percent of annexin V positive population was observed, increasing from 2.7 ± 1.1% at day 0 to 5.5 ± 0.72% by day 5 for control and contaminated units (Figure 22). On the other hand, in *S. aureus* inoculated PC units, the CD62P and annexin V positive populations were significantly higher ($p \leq 0.05$) on day 5 when compared to the negative control reaching 52 ± 6% and 26 ± 6% respectively. Interestingly the percentage of CD41a events captured on day 5 for *S. aureus* spiked PCs was significantly reduced to 65.5 ± 16.8% ($p \leq 0.05$) compared to the control (97.7 ±0.8%) (Table 7).

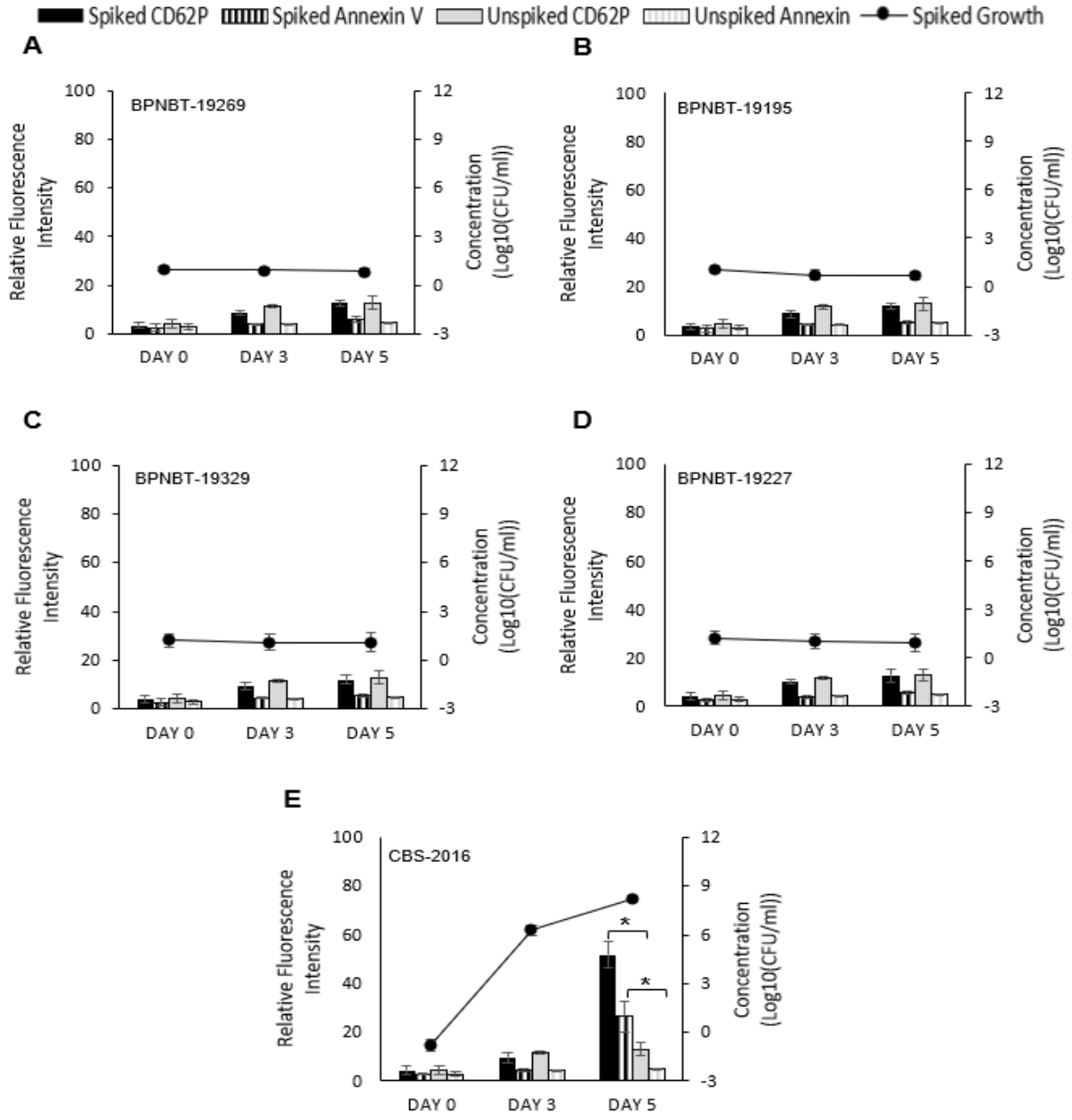


Figure 22. Bacterial growth and platelet activation during PC storage.
Expression of platelet activation markers CD62P (solid bars) and Annexin V (striped bars) on days 0, 3, and 5 of PC storage in PC units contaminated with *C. acnes* PC isolates A: BPNBT-19269, B: BPNBT-19195, C: BPNBT-19329, D: BPNBT-19227, and E: the transfusion relevant *S. aureus* isolate CBS-2016 compared with un-spiked PC controls. Growth curves of each bacterial isolate are represented on the secondary y axis. N = 3, Student t-test, *- $p < .05$

Table 8. Proportion of CD41-positive populations in PC samples obtained on days 0, 3, and 5 of storage.

Unit ID	Sampling time points		
	Day 0	Day 3	Day 5
CONTROL	99.13 ± 0.29	98.5 ± 0.64	97.7 ± 0.87
p value	-	-	-
C. acnes BPNBT-19269	98.33 ± 1.14	98.22 ± 0.79	96.85 ± 1.61
p value	0.15	0.51	0.29
C. acnes BPNBT-19195	98.95 ± 0.55	97.98 ± 1.09	97.43 ± 1.08
p value	0.49	0.34	0.65
C. acnes BPNBT-19329	99.13 ± 0.32	97.8 ± 1.09	97.27 ± 0.77
p value	1.00	0.21	0.38
C. acnes BPNBT-19227	98.87 ± 0.74	98.45 ± 0.66	97.77 ± 0.87
p value	0.44	0.90	0.90
S. aureus CBS 2016-05	99.13 ± 0.29	97.3 ± 1.53	65.5 ± 16.78
p value	1.00	0.12	0.01

The mean RFI values obtained for each unit over three biological replicates have been represented with the standard deviation. Statistical differences in CD41 positive populations were assessed compared to the control using the Student T-test, and p values have been listed.

5.5.3 *C. acnes* PC contamination does not enhance the release of pro-inflammatory cytokines

Over the 5 days of testing, there were no significant differences in the concentration of proinflammatory cytokines on days 3 and 5 compared to baseline values (day 0) obtained in units inoculated with *C. acnes* (Table 8). The evaluated cytokines reached average concentrations of 5.6 ± 0.1 pg/mL (IL-1β), 11.5 ± 0.4 pg/mL (IL-6), 20.5 ± 0.5 pg/mL (IL-8), 8.4 ± 0.4 pg/mL (IL-10), 12.5 ± 0.6 pg/mL (IL-12), and 13 ± 0.4 pg/mL (TNFα) in *C. acnes* inoculated units over the 5 days of testing. A comparison of the cytokine profile of the negative control and the *C. acnes* inoculated units during storage indicate that there was no significant difference in cytokine concentrations

except for IL-1β and IL-6 concentrations in units inoculated with BPNBT-19195 and BPNBT-19227. Specifically, the baseline concentrations of IL-1β and IL-6 were higher ($p \leq 0.05$) in units inoculated with BPNBT-19227 (Table 8), however concentrations on day 3 and 5 of testing were comparable to those obtained for the negative control. Similarly, the average IL-1β concentrations obtained on day 0 and day 3 were higher in units inoculated with BPNBT-19195 compared to those of the control (Table 8), with comparable concentrations in control and *C. acnes* inoculated units on day 5. In units inoculated with *S. aureus*, a significant increase in IL-8 concentrations was observed compared to the control on day 5 of testing, while the cytokine profile of these units on day 0 and 3 were comparable to the control.

5.5.4 *C. acnes* PC contamination does not elicit the enhanced release of sCD40L during storage

There was a gradual increase in the concentration of sCD40L in both the control and *C. acnes* inoculated units during storage, reaching an average concentration of 16.0 ± 1.7 ng/mL (Table 8). On the other hand, there was a significant increase ($p \leq 0.05$) in sCD40L (17.8 ± 0.3 ng/mL) in *S. aureus* contaminated units on day 3, followed by a precipitous drop (6.73 ± 0.2 ng/mL) on day 5 compared to those of the control (Table 8).

Table 9. Concentration of proinflammatory factors in plasma samples derived from PC units contaminated with *C. acnes* and *S. aureus* compared with the control on days 0, 3, and 5 of storage.

Pro-inflammatory factor	Control			*C. acnes* BPNBT-19269			*C. acnes* BPNBT-19195			*C. acnes* BPNBT-19329			*C. acnes* BPNBT-19227			*S. aureus* CBS 2016-05		
	DAY 0	DAY 3	DAY 5	DAY 0	DAY 3	DAY 5	DAY 0	DAY 3	DAY 5	DAY 0	DAY 3	DAY 5	DAY 0	DAY 3	DAY 5	DAY 0	DAY 3	DAY 5
IL-1β (pg/ ml)	5.15 ±	4.77 ±	5.81 ±	5.28 ±	5.55 ±	6.08 ±	5.64 ±	6.03 ±	5.33 ±	5.99 ±	4.85 ±	5.69 ±	5.76 ±	5.71 ±	5.45 ±	4.87 ±	5.48 ±	4.87 ±
	0.04	0.35	0.84	0.72	0.47	0.00	0.02	0.11	0.38	1.09	0.20	0.50	0.00	0.75	0.07	0.40	0.38	0.13
p value	-	-	-	0.822	0.205	0.694	0.005	0.041	0.537	0.393	0.812	0.883	0.003	0.250	0.611	0.425	0.194	0.258
IL-6 (pg/ ml)	11.58 ±	10.75 ±	11.46 ±	12.00 ±	11.49 ±	11.47 ±	12.05 ±	13.03 ±	11.12 ±	11.30 ±	10.93 ±	11.49 ±	9.90 ±	12.13 ±	11.3 ±	10.63 ±	10.02 ±	10.97 ±
	0.13	0.96	1.01	1.33	0.62	1.27	0.27	0.76	0.35	0.09	0.35	0.35	0.51	2.22	0.26	0.87	0.34	0.92
p value	-	-	-	0.705	0.455	0.999	0.155	0.119	0.694	0.130	0.824	0.974	0.046	0.504	0.850	0.264	0.413	0.658
IL-8 (pg/ ml)	16.84 ±	20.05 ±	23.59 ±	17.27 ±	19.63 ±	23.69 ±	18.89 ±	20.92 ±	23.66 ±	19.18 ±	19.85 ±	21.30 ±	19.71 ±	21.56 ±	20.96 ±	19.39 ±	19.22 ±	32.88 ±
	1.13	0.6	1.28	0.28	0.76	2.74	1.45	0.24	3.53	0.58	2.46	0.77	0.41	2.08	0.53	0.76	0.75	0.46
p value	-	-	-	0.649	0.617	0.967	0.240	0.214	0.982	0.121	0.925	0.161	0.077	0.430	0.115	0.117	0.361	0.010
IL=10 (pg/ ml)	7.73 ±	7.85 ±	8.45 ±	8.03 ±	8.03 ±	9.20 ±	9.18 ±	8.80 ±	8.50 ±	7.80 ±	7.53 ±	8.00 ±	8.35 ±	8.75 ±	8.65 ±	7.31 ±	7.16 ±	7.78 ±
	0.94	0.28	0.64	0.53	1.30	0.43	0.04	0.71	0.49	0.63	0.10	0.63	0.63	1.21	0.50	0.97	0.28	0.73
p value	-	-	-	0.737	0.866	0.300	0.163	0.221	0.939	0.937	0.268	0.553	0.522	0.411	0.760	0.705	0.132	0.432
IL-12 (pg/ ml)	11.66 ±	12.16 ±	13.66 ±	12.62 ±	12.20 ±	12.83 ±	14.15 ±	12.68 ±	13.22 ±	12.47 ±	11.67 ±	11.78 ±	12.00 ±	12.43 ±	11.83 ±	11.37 ±	11.46 ±	10.24 ±
	0.88	0.88	2.30	0.59	0.65	0.30	0.49	1.84	3.42	2.01	1.34	0.23	1.00	2.19	1.11	0.58	1.40	0.11
p value	-	-	-	0.330	0.926	0.663	0.072	0.756	0.894	0.656	0.707	0.370	0.757	0.887	0.418	0.734	0.611	0.170
TNFα (pg/ ml)	11.63 ±	12.26 ±	13.3 ±	12.22 ±	13.10 ±	13.77 ±	13.53 ±	13.61 ±	13.53 ±	13.57 ±	11.95 ±	12.97 ±	11.91 ±	14.59 ±	11.83 ±	12.15 ±	11.25 ±	10.7 ±
	0.06	0.61	1.74	0.89	2.02	0.62	1.07	0.73	0.17	4.11	0.28	0.84	0.55	3.02	0.55	1.11	0.05	0.49
p value	-	-	-	0.448	0.630	0.753	0.130	0.184	0.869	0.574	0.575	0.835	0.558	0.397	0.374	0.581	0.144	0.180
sCD40L (ng/ ml)	12.22 ±	14.46 ±	14.64 ±	12.29 ±	15.44 ±	15.05 ±	13.71 ±	15.27 ±	16.92 ±	12.64 ±	17.13 ±	18.63 ±	11.37 ±	14.78 ±	14.93 ±	11.05 ±	17.8 ±	6.73 ±
	0.35	0.24	0.30	0.26	1.25	0.13	2.52	0.16	0.86	0.34	0.55	2.68	0.36	0.30	0.14	0.08	0.34	0.20
p value	-	-	-	0.844	0.465	0.272	0.555	0.071	0.136	0.342	0.058	0.279	0.140	0.364	0.388	0.117	0.011	0.002

The concentration of pro-inflammatory factors as a mean of duplicate values obtained from plasma samples pooled from three biological replicates have been represented with the standard deviation. Statistical differences were assessed compared to the control using the Student T-test, and p values have been listed.

5.6 Discussion

C. acnes is able to evade mitigation strategies adopted by blood centers like donor skin disinfection and bacterial screening leading to the transfusion of contaminated PCs [3]. Due to the strong immunostimulatory properties displayed by *C. acnes* in humans and other animal models [236, 237], it has been investigated as a vaccine adjuvant to treat certain cancers and infections [238, 239, 240]. Of note these toll-like receptor mediated pro-inflammatory responses are elicited by *C. acnes* cell wall components even when the bacterium is nonviable [241, 242]. PCs contain platelets and residual leukocytes (max 1.2 x 10^5/ pooled buffy coat PC unit) that can release immunomodulatory factors following pathogen interaction. Therefore, this study aimed to determine whether the interaction of *C. acnes* with platelets in contaminated PCs could result in platelet activation and result in the release of pro-inflammatory factors during storage.

Previous work has demonstrated that *C. acnes* can elicit platelet activation and aggregation in a phylotype and donor dependent manner, mediated through the binding of plasma proteins like fibrinogen [214]. However, the ability of *C. acnes* to activate platelets during prolonged exposure at low concentrations as observed in the transfusion setting has not been previously assessed. The data obtained in our study show that *C. acnes* contamination does not enhance the activation of platelets in PCs at relatively low concentrations (10 CFU/mL) compared to an uncontaminated control, and this was independent of the phylotype of *C. acnes* being assessed. Since *C. acnes* cannot proliferate in the aerobic PC environment, it is unlikely that high concentrations of *C. acnes* will be achieved in contaminated PCs during storage as those that elicited activation in previous work [214]. In addition to the lack of platelet activation, there was no significant increase in the concentration of factors like pro-inflammatory cytokines and soluble CD40L even with prolonged exposure (5days) of PC components to the bacterium. Conversely, PCs inoculated with *S. aureus*

displayed visible signs of activation by day 3 (platelet aggregate clot), though this was not captured when activation markers were assessed. This could have been caused by the fact that activated platelets were associated with the large aggregate observed, while un-activated platelets remained in suspension which was sampled. Furthermore, the significant reduction in CD41 positive populations on day 5 is consistent with platelet aggregation and studies that have shown that exotoxins induce platelet activation and apoptosis [243]. This was accompanied by an increase in the expression of activation markers and proinflammatory cytokine IL-8. Consistent with the enhanced activation and aggregation observed on day 3, concentrations of sCD40L peaked, reducing significantly on day 5 suggesting that sCD40Lmay have bound to CD40 expressed on platelets [244] resulting in lower concentrations in the plasma tested on day 5. These data suggest that there may be no enhanced risk of non-infectious reactions driven by pro-inflammatory factors caused by *C. acnes* contamination of PCs. Importantly, since *C. acnes* has been shown to exert a proinflammatory response driven by cell wall components even when nonviable, our findings indicating that low concentrations of *C. acnes* cannot elicit similar responses in PCs can therefore be extended to *C. acnes* contaminated PCs that have undergone pathogen reduction which renders the bacterium nonviable [245] but does not alter its immunogenic surface properties.

Though the results of our study are heartening, it should be noted that one of the hallmarks of *C. acnes* infections is that they progress slowly and may take up to a decade to manifest symptoms [246]. These infections result in severe morbidity caused by extensive localized damage, as is observed in infective endocarditis [223]. Infective endocarditis is an infection of the endocardium and effects both native and prosthetic valves. The onset of infection is characterized by the attachment of a platelet clot to a cardiac valve, followed by attachment of circulating bacteria. This results in the recruitment of more activated platelets and other immune cells, which inadvertently

aids in immune evasion, allowing bacterial proliferation [223] and the establishment of bacterial aggregates called biofilms causing damage to underlying tissue [224]. We have previously demonstrated that the PC milieu elicits the expression of virulence genes including those involved in biofilm formation in transfusion relevant bacteria like *Staphylococcus epidermidis* [143], furthermore, we have also shown that *C. acnes* is able to adhere strongly to activated platelets [213]. In view of these findings, it is plausible that *C. acnes* from contaminated PCs are associated with activated platelets and display a heightened ability to form biofilms thereby enhancing the risk of infections like infective endocarditis. Future work aimed at elucidating the impact of the PC milieu on the virulence gene expression profile of *C. acnes* may provide insight into the risk associated with developing such infections.

5.7 Acknowledgements

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Chapter 6 Conclusion

6.1 General discussion

The Canadian Blood Services mandate to be Canada's biological lifeline begins and ends with ensuring the safety of the blood supply. Since the bacterial contamination of PCs poses one of the most significant safety risks to transfusion patients [2], the work described in this thesis focuses on the different aspects of the PC production process and its impact on *C. acnes* contamination and blood product safety.

C. acnes is one of the most abundant bacterial species in the skin microbiome and this commensal has an important role in preventing the colonization of the skin by more pathogenic bacteria like *S. aureus* [104, 107, 108, 174]. However, over the years, the pathogenic potential of *C. acnes* has been reviewed by several groups that have highlighted its ability to form biofilms and the presence of putative virulence genes that encode for adhesins, lipases, glycosidases, neuraminidases, toxins, and heat shock proteins to name a few [247, 248]. Though the role of these factors is yet to be clearly determined in the context of *C. acnes* pathogenicity, it is known through transcriptomic and proteomic studies that adhesins, lipases and toxins like the CAMP factor are highly expressed in *C. acnes* [249, 250]. In vitro studies indicate that lipases expressed by *C. acnes* may contribute to its ability to colonize the pilosebaceous unit and elicit inflammation [197]. Glycosidases have been shown to aid in nutrient acquisition and contribute to biofilm formation, persistence and immune evasion in pathogenic bacteria like *Streptococcus* spp [251], and purified glycosidases from *C. acnes* have been demonstrated to show similar activity in vitro to purified streptococcal glycosidases [252]. The disruption of the extracellular matrix by hyaluronate lyase has been shown to be an important step in nutrient acquisition and the spread and pathogenesis of *S. aureus* and *Streptococcus pneumoniae* [253, 254]. Interestingly *C. acnes* has been shown to express two

different variants of hyaluronate lyase, with the lyase produced by phylotype IB and III displaying more potent hydrolyzing activity against hyaluronic acid substrates than the lyase produced by phylotype IA [198]. Taken together, these studies indicate that *C. acnes* has an arsenal of virulence factors that can be mobilized to cause infection, and this has been substantiated by numerous clinical reports of slow developing infections caused by *C. acnes* like infectious endocarditis, prosthetic joint infections, spinal disc infections, brain abscesses, and sarcoidosis and has also implicated in the progression of prostate cancer [124-132, 211]. Since *C. acnes* contaminated PCs are often transfused, it is important to understand the potential risk associated with these transfusion events and to improve current processes and identify opportunities to prevent the contamination of blood products and its subsequent transfusion.

One of the first steps in the PC production process is the collection of blood products from donors which is preceded by the disinfection of the donor's skin with the standard donor disinfectant (2% chlorhexidine, 70% isopropyl alcohol). The frequent isolation of *C. acnes* from PCs indicates that the current disinfection protocol may not be effective at completely eradicating *C. acnes* at the venipuncture site. The skin is rich in microbial diversity and the composition of these populations are impacted by environmental factors [149], diet [148], and gender [147]. Balanced interactions within these populations are essential and can impact biofilm formation and sensitivity to antimicrobials thereby modulating skin health [255, 256, 174].

I optimized an in vitro biofilm assay that would facilitate the assessment of biofilm formation and disinfectant efficacy in a sebaceous environment. Only *C. acnes* isolates obtained from contaminated PCs were assessed in this model as a previous study with *S. epidermidis* showed that biofilm forming capabilities of skin derived isolates were similar to those isolated from PC units [257]. The work described herein, demonstrates that sebum components that feature prominently

in the niches that *C. acnes* occupies on the skin, significantly reduces the efficacy of the primary blood donor disinfectant used at Canadian Blood Services. Furthermore, I have demonstrated, using a dual species biofilm model, that the sensitivity of *C. acnes* to disinfectants may be modulated by the presence of other bacterial species like *S. aureus*. My data indicated that the combination of ineffective bacterial eradication and poor penetration into the skin by the standard disinfectant [164] could contribute to the dominance of *C. acnes* as a PC contaminant. My work has also highlighted the need for the evaluation of skin disinfectants that are not neutralized by sebum components with stronger penetration properties.

The next mitigation step in the PC production process is the active screening of platelet concentrates using the automated BACT/ALERT culture system. Though this strategy has prevented the transfusion of pathogenic bacteria, slow growing bacteria like *C. acnes* are often only detected after the PC units have been transfused [3]. I have demonstrated that supplementation of BACT/ALERT BPN culture media with the commercially available nutrient supplement BD Vx can reduce the time to detection of *C. acnes* by up to 24 hrs. The use of this supplement could allow for the early retrieval of *C. acnes* contaminated PCs prior to transfusion thereby enhancing the safety of the blood supply. Furthermore, I have shown that Tween80; a readily available reagent can significantly reduce the detection of very slow growing isolates of *C. acnes* from culture media. Therefore, I have provided evidence for the potential use of tween 80 to enhance the growth of *C. acnes* from non-blood derived samples like those used in pharmaceutical sterility testing [258], and in the clinical setting for samples like sonication or debridement fluid [259].

As mentioned above, the late detection of *C. acnes* in the BACT/ALERT system results in the transfusion of contaminated PCs. Unfortunately, very little is known about how these contamination events impact the properties of PCs or whether these slow growers are primed by

the PC environment to cause delayed infections in those transfused. In my work, I evaluated the risk associated with these contamination events, given that platelet-bacteria interactions can elicit changes in bacterial gene expression [142, 143, 144] and platelet activation profiles [236]. I have demonstrated that *C. acnes* contamination does not elicit the accumulation of proinflammatory factors during PC storage, and therefore it does not enhance the risk of acute adverse reactions driven by these factors (ex. febrile non hemolytic transfusion reactions). Furthermore, using a silkworm virulence model, I have been able to demonstrate that peptidoglycan shielding by PC components may prevent the activation of acute innate responses in the silkworm and may explain the mild reactions observed in the transfusion setting despite the frequent transfusion of *C. acnes* contaminated PCs. On the other hand, I have shown that the PC environment enhances the potential of *C. acnes* to adhere to mammalian cells, and results in the increased expression of genes involved in tissue invasion and persistence, which could prime contaminating *C. acnes* to cause delayed chronic infections as those seen in the clinical setting [235, 127]. An example of a potential risk associated with the transfusion of *C. acnes* contaminated PCs is the establishment of infective endocarditis (Figure 23). My work has shown that *C. acnes* in contaminated PCs may be primed to adhere, persist and invade underlying tissue, and in the context of infectious endocarditis this would enable transfused *C. acnes* to establish infection and form biofilms in compromised cardiac valves resulting in persistent infection, inflammation and ultimately tissue damage. The validity of this hypothesis would have to be studied either using animal models or by conducting prospective studies undertaken in collaboration with clinicians. These investigations would ultimately aid in elucidating the risk following the transfusion of *C. acnes* contaminated PCs.

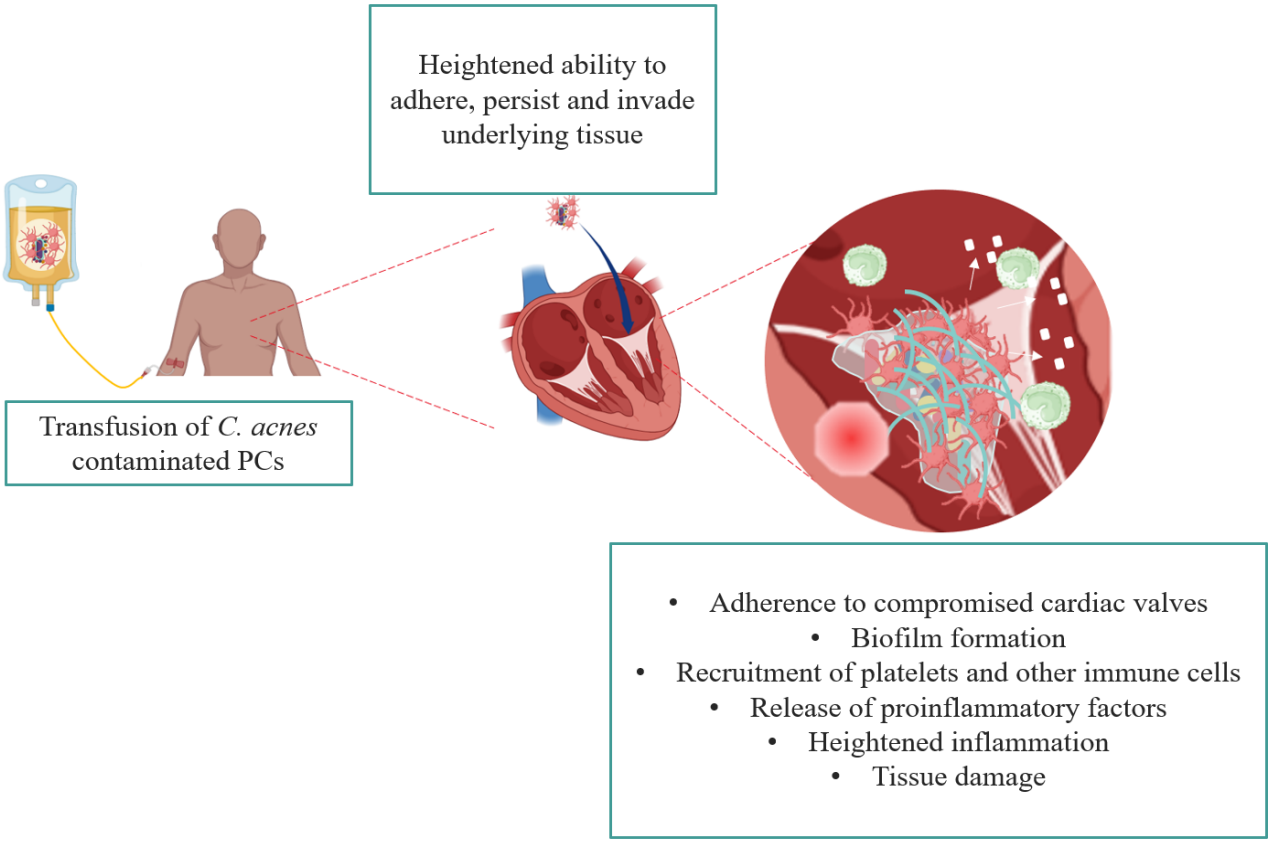

Figure 253. Potential mechanism for transfused *C. acnes* to cause infective endocarditis.

(From left to right) In the PC storage environment *C. acnes* is primed to adhere more strongly to host tissue, and expresses genes involved in persistence and tissue invasion and may be surrounded by activated platelets. This platelet-bacterial aggregate is then transfused into a vulnerable patient and can therefore adhere to the compromised cardiac valve (native or prosthetic). The enhanced ability to persist and invade tissue allows *C. acnes* to initiate infection and establish biofilms. The presence of *C. acnes* and activated platelets leads to the recruitment and activation of more platelets at the site which results in the release of proinflammatory factors including cytokines and chemokines. This results in the recruitment of other immune cells including monocytes, and neutrophils. Since *C. acnes* is protected within the biofilm and the platelet aggregate, it is not cleared by the immune response, resulting in persistent inflammation and tissue damage. Image created on Biorender

The driving force behind the dismissal of *C. acnes* as a cause for concern in the transfusion setting is the fact that it does not cause severe acute adverse reactions, furthermore, historically, it has been considered a harmless contaminant in clinical settings. As a result, not a lot of literature is available that discusses measures to improve the detection of *C. acnes* during bacterial screening or the impact that *C. acnes* contamination had on blood product safety. The work presented in this thesis aims to fill this void in our understanding and highlights areas of improvement of PC production practices and processes implemented by blood suppliers.

It should be acknowledged that the adoption of the pathogen inactivation technology INTERCEPT at Canadian Blood Services countrywide (excluding Quebec) in 2024 to treat PCs overcomes some of the challenges of bacterial contamination associated with this product experienced by blood suppliers. More importantly in context of the work described herein, this technology has been shown to be effective at inactivating *C. acnes* in PCs [87]. However, it should be noted that not all blood suppliers have implemented or plan to implement technologies like INTERCEPT [260], and therefore the data generated in this thesis would still be relevant to those suppliers. Furthermore, one of the advantages of the implementation of the large volume delayed sampling algorithm at Canadian Blood Services, was the ability to prevent the transfusion of bacterially contaminated red blood cell concentrates (RCCs) by screening PCs [3]. Notably, with the implementation of INTERCEPT, all PCs are released following treatment, and there are no measures in place to test companion products like RCCs. Hemovigilance data indicate that *C. acnes* accounts for 72% of bacterially contaminated buffy coat derived PCs, which suggests that one or more of the companion RCCs could be contaminated, and this was confirmed for ~30% of the 72% of confirmed cases through confirmatory testing with RCCs [3]. In the absence of pathogen inactivation technologies that are effective in RCCs, these contaminated products will be

transfused to vulnerable patients. The work presented in this thesis therefore stands as a foundation on which future studies involving RCCs can be built on. Overall, I have advanced knowledge and provided insight into enhancing the safety of transfusion patients in Canada and worldwide.

6.2 Limitations

Though each of the studies undertaken in this thesis aid in expanding our understanding of the impact *C. acnes* has in the different PC production processes and product safety, there are some limitations that should be considered. One of the weaknesses is the use of only one bacterial isolate per phylotype tested. This approach does not capture differences in behavior of isolates belonging to the same phylotype, and it is important to not infer generalizations about the behavior of certain *C. acnes* phylotypes in the PC storage environment.

In the study that assessed donor skin disinfectant efficacy, the established in vitro model aimed to reflect the sebaceous niche of the skin that *C. acnes* occupies, however this model did not include sapienic acid a fatty acid unique to humans known to have antibacterial activity and it does not take into account the interaction of *C. acnes* with the host which could shape biofilm formation and its properties. As a result, there might be an overestimation of biofilm formation and resistance to disinfectants, which cannot be verified, unless at the very least, a skin model with functional sebocytes can be established in vitro.

An important question that this thesis aimed to answer was whether the transfusion of *C. acnes* contaminated PCs could enhance the risk of adverse reactions in those transfused. To that end, I utilized a silkworm virulence model to assess the potential to cause acute reactions. Though this is an elegant model to assess the virulence of bacteria, it should be noted that due to the formation of pupa, the time frame within which the virulence assay is performed is quite short (3-5 days).

Consequently, it is difficult to accurately assess the impact of the proliferation of slow growing bacteria like *C. acnes* on the survival of silkworm larvae. Therefore, it may be prudent to use other in vivo models that allow for longer observation periods post inoculation. Furthermore, in order to gain insight into the ability of PC derived *C. acnes* to adhere to mammalian cells, I used HEK 293T cells as previously described [211]. However, following transfusion, contaminating bacteria like *C. acnes* would encounter endothelial cells, and though some surface markers used by bacteria to adhere can be found on both epithelial and endothelial cells [261], further studies using endothelial cells will have to be performed to ensure that the adherence data is reproducible in endothelial cells.

6.3 Future direction

An interesting avenue of investigation to improve skin disinfection protocols would be to assess disinfectants that are not neutralized by sebum components like oleic acid. Currently available bis-phenol or aldehyde-based disinfectants may be poorly neutralized by oleic acid [161], however these disinfectants are generally harsh on the skin so it may be difficult to implement routinely [219]. Therefore, screening a panel of chemically synthesized and/or naturally derived compounds with antimicrobial properties, that can be used individually or in combination with the standard blood donor skin disinfectant could help identify alternatives or supplements to the current process to improve disinfectant efficacy against *C. acnes*. The model described in this thesis could also be improved by including sapienic acid to the mix of fatty acids to obtain a sebum like mixture that better reflects human sebum. Furthermore, it would be beneficial to test these models with a panel of bacterial isolates representing each *C. acnes* phylotype, individually and in multispecies models with other more commonly isolated skin flora so that patterns of disinfectant efficacy can be uncovered that corresponds more closely to what occurs on the skin. The composition of the

extracellular matrix of the biofilm can impact disinfectant efficacy, in the current model. it is impossible to assess the matrix without contamination by the sebum components. Therefore, optimizing a new biofilm model that allows for the free flow of nutrients (membrane or glass filters) from the sebum like suspension to the bacteria whilst still occupying physically distinct spaces would allow for the evaluation of the biofilm matrix and might provide further insight into the mechanisms involved in the reduced efficacy of disinfectants against *C. acnes*.

The expression of specific virulence genes was assessed in PC derived *C. acnes* samples, however, by using this approach it is not possible to assess global expression of virulence genes. Comparison of the transcriptome of PC and media derived cultures will provide a more comprehensive understanding of how the PC environment impacts *C. acnes* gene expression, and how that in turn affects the residual risk to transfusion patients. Furthermore, it would be beneficial to develop an in vitro model that assesses bacterial invasiveness. A potential model could consist of well inserts containing mammalian cells followed by the evaluation of the ability of *C. acnes* to infiltrate the extracellular matrix and travel into the lower chamber. This could prove to be an important tool to evaluate whether enhanced expression of the *hyl* gene translates to increased invasiveness.

Following the implementation of INTERCEPT, all PCs will be solely prepared in a mix of plasma and platelet additive solution. Since the studies performed in this thesis utilized PCs suspended in 100% plasma, it would be interesting to evaluate how the reduction of plasma in INTERCEPT treated PCs impacts the observations made in this work. Furthermore, since there are no technologies currently available to treat RCCs, it would be informative to evaluate the impact of the RCC environment on the virulence expression profile of *C. acnes* and patient safety.

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Appendix A1: Supplementary information for Chapter 2

Assessment of the neutralizer used in this study as per the ASTM E1054-08 Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents.

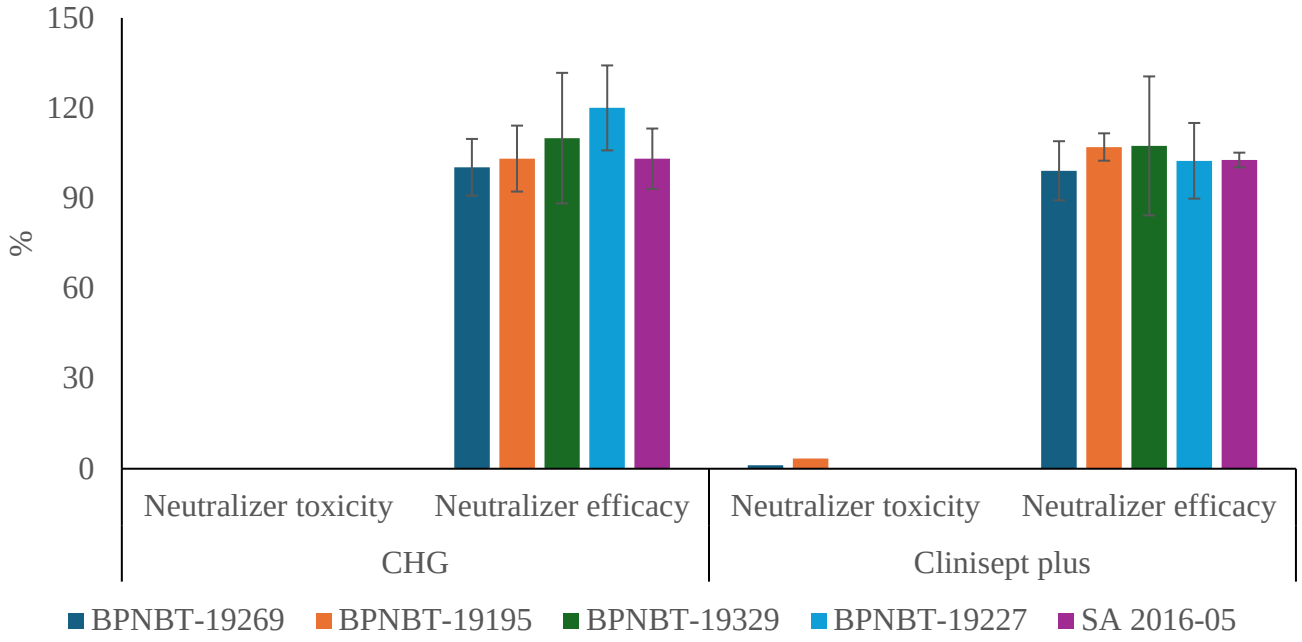

Figure A1.1: Assessment of neutralizer against the standard donor skin disinfectant and Clinisept plus. The toxicity of the neutralizer (10% T80. 3% lecithin, 0.3% sodium thiosulfate) on the four *C. acnes* isolates (BPNTBT-19269, BPNBT-19195, BPNBT-19329, and BPNBT-19227) and *S. aureus* 2016-05. The efficacy of the neutralizer to inactivate the standard donor skin disinfectant and an alternative Clinisept plus. The starting concentration of bacteria in each tube was ~3000 CFU/mL. Toxicity was determined by diluting bacterial suspensions in the neutralizer (1:10, v/v) and the neutralizer efficacy was assessed by diluting the disinfectants in neutralizer (1:100, v/v). the surviving bacteria were enumerated following 5min of contact time. N=3.

Determination of the minimal antimicrobial concentration (MIC) of chlorhexidine for *C. acnes* isolates as per Clinical and Laboratory Standard Institute (CLSI, Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria M11, 9th edition)

Table A1.1: The MIC values of chlorhexidine (CHG) against bacterial isolates used in the biofilm study. The four *C. acnes* PC isolates, the *S. aureus* CBS 2016-05 isolate, and the control ATCC 6919 *C. acnes* isolate were tested using the agar dilution method to assess the MIC endpoints of CHG. Bacterial samples were spotted (10^4 CFU/10µL) in duplicate on cooled brain heart infusion agar plates that were freshly prepared by serially diluting CHG in molten agar (0.125 µg/mL to 16 µg/mL). Bacteria spotted on plates containing no CHG served as a viability control. The plates were incubated at 37°C (*C. acnes* anaerobically for 96 hrs, and *S. aureus* anaerobically for 24 hrs) and assessed for bacterial growth at every concentration tested. Experiments were performed three independent times.

Strain	MIC REP -1 (ug/mL)	MIC REP -2 (ug/mL)	MIC REP -3 (ug/mL)
BPNBT-19269	1	1	1
BPNBT-19195	0.25	0.25	0.25
BPNBT-19329	0.5	0.25	0.25
BPNBT-19227	0.5	0.5	0.25
ATCC 6919 (*C. acnes*)	0.5	0.5	0.5
S. aureus (CBS 2016-05)	2	2	2

Appendix A2: Supplementary information for Chapter 3

When oleic acid was used to supplement BPN bottles, the bottles flagged false positive in ~0.5 days. Two different sources of oleic acid were used to supplement BPN culture bottles namely olive oil and sodium oleate and were used to detect *C. acnes* in media, PCs and RCCs.

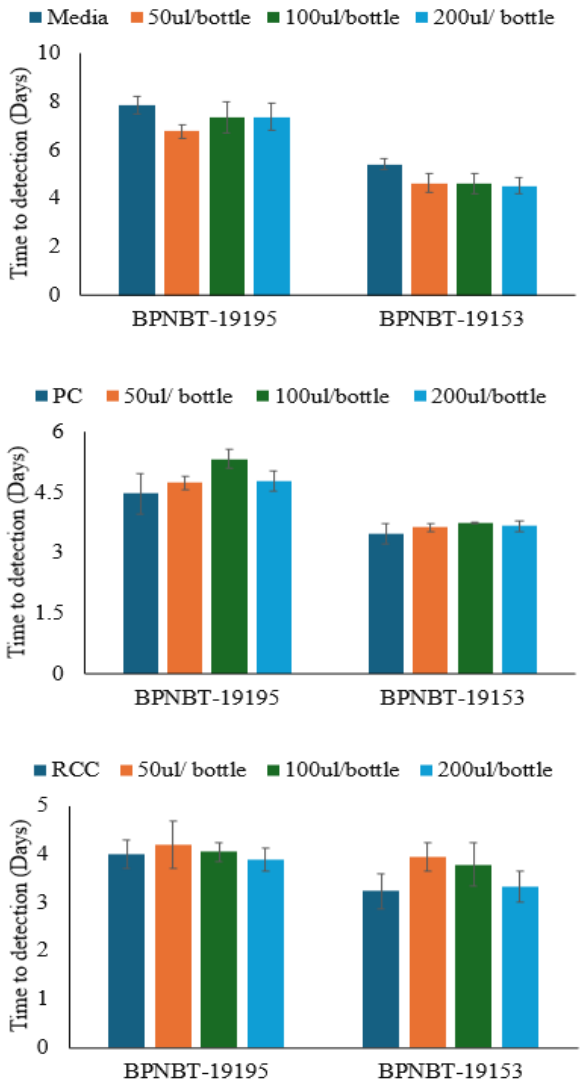

Figure A2.1: Time to detection of *C. acnes* PC isolates in olive oil supplemented BPN culture bottles. Time to detect *C. acnes* (10CFU/ bottle) from A- media, B- PCs, and C- RCCs. Time to detection compared in BPN bottles supplemented with 50 uL (orange), 100 uL (green), and 200 uL (light blue) of olive oil to unsupplemented bottles (dark blue). N=3

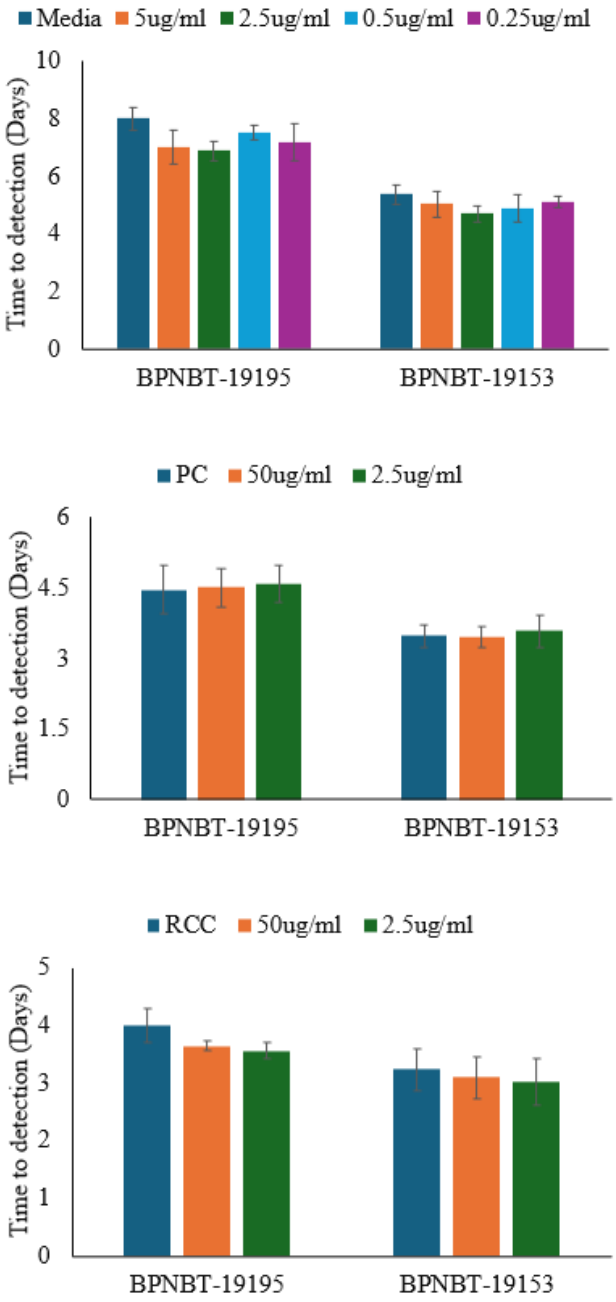

Figure A2.2: Time to detection of *C. acnes* PC isolates in sodium oleate supplemented BPN culture bottles. Time to detect *C. acnes* (10 CFU/ bottle) from A- media, B- PCs, and C- RCCs. Time to detection compared in BPN bottles supplemented to a final concentration of 5 ug/mL (orange), 2.5 ug/mL (green), 0.5 ug/mL (light blue), and 0.25 ug/mL of sodium oleate to unsupplemented bottles (dark blue). N=3.

Two commercial supplements were tested (according to manufacturer recommendations) in BPN culture bottles to enhance the growth of *C. acnes*. BDVx supplement and Oxoid supplement. The Oxoid HTM supplement did not change time to detection and was not used in further studies.

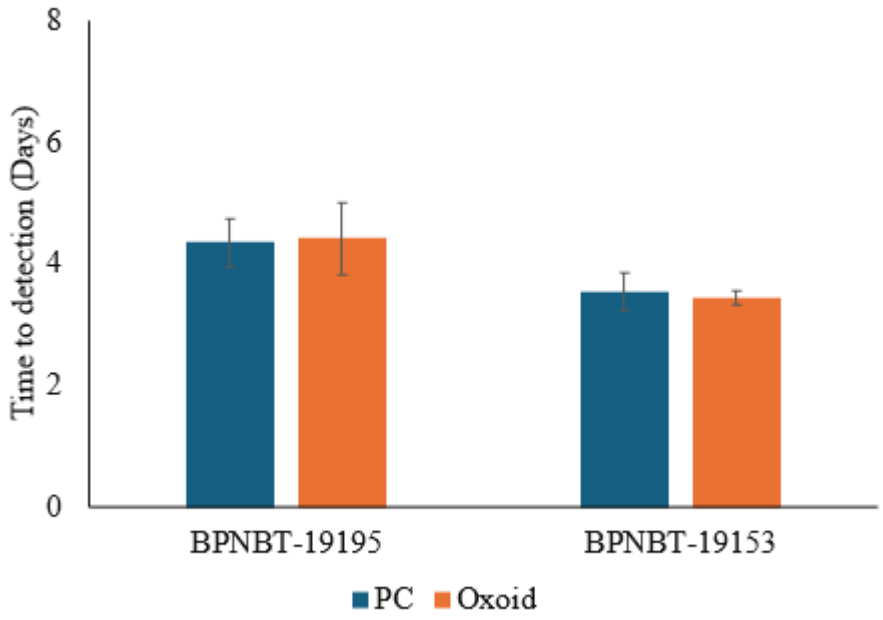

Figure A2.3: Time to detection of *C. acnes* PC isolates Oxoid supplemented BPN culture bottles. Time to detect *C. acnes* (10CFU/bottle) in bottles supplemented with Oxoid supplement (orange bars) was compared to unsupplemented bottles (blue). N=3

Appendix A3: Supplementary information for Chapter 4

The LD_{50} sigmoidal graphs generated by AAT Bioquest

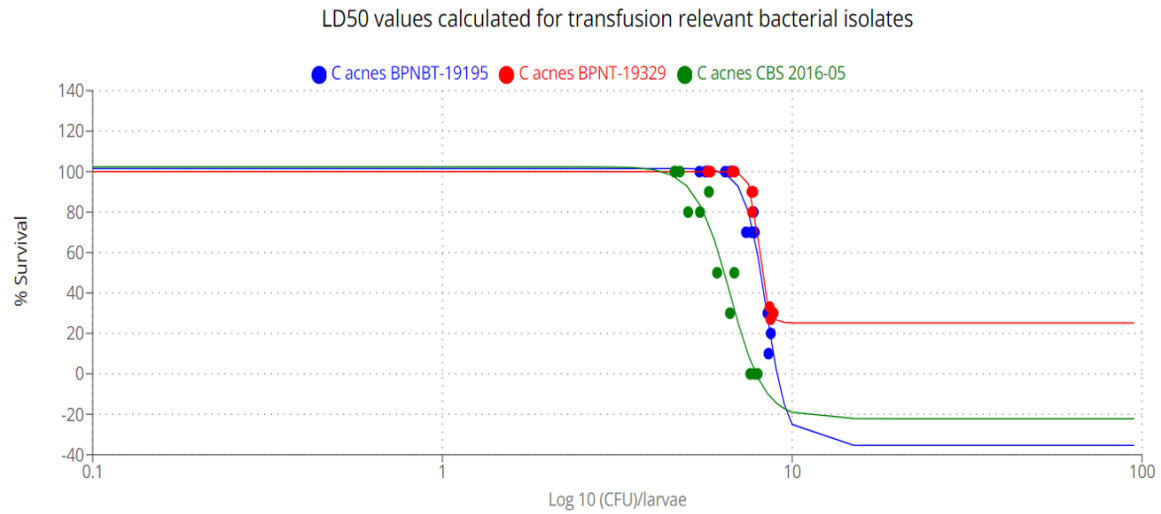

Figure A3.1: LD50 values of *C. acnes* and *S. aureus* PC isolates. Survival of silkworm larvae following inoculation with varying concentrations (1/10 dilution series) of two *C. acnes* isolates (BPNBT-19195 and BPNBT-19329) and a *S. aureus* isolate (CBS 2016-05) following three days of incubation at 37°C. The graph was created using the data from three biological replicates for each dilution tested. The LD_{50} values calculated by the online AAT Bioquest tool were 2.63 x 10^8/ larvae (BPNBT-19195), 1.11 x 10^8 CFU/ larvae (BPNBT-19329) and 4.26 x 10^6 CFU/ larvae (CBS 2016-05)

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