# PREVENTION OF BACTERIAL GROWTH IN PLATELET PRODUCTS

# VIA INCLUSION OF IRON CHELATORS

by

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## ABSTRACT

Bacterial infection is a leading cause of morbidity and mortality arising from platelet transfusions (1, 2). Storage of platelet products at room temperature (20 to 24°C) provides ideal conditions for bacterial proliferation (1, 3-6). Furthermore, platelets are stored in plasma containing bioavailable iron that bacteria require to survive (7). Thus we hypothesize that the inclusion of iron chelators will bind and remove iron, thereby inhibiting bacterial growth in both culture medium and platelet concentrates. Additionally, we hypothesize that residual red blood cells (RBCs) in platelet units may contribute bioavailable iron that promotes bacterial growth.

To test these hypotheses, we first assessed growth of *Staphylococcus epidermidis* in culture medium after treatment with the iron chelators deferoxamine (DFO) or phytic acid. DFO significantly inhibited bacterial growth in a dose dependent manner (p < 0.009). Conversely, phytate only inhibited bacterial growth at concentrations  $\geq 100$  mM (p < 0.001); at  $\leq 5$  mM, phytate supplied *S. epidermidis* with additional nutrients and significantly promoted growth (p < 0.001). Subsequently, we monitored the change in RBCs over time. Hemolysis, methemoglobin, and iron levels all significantly increased over the 7-day storage period (p < 0.001) releasing bioavailable iron. Indeed, we found that *S. epidermidis* growth in iron-poor medium drastically increased with the addition of RBCs, thus supporting our second hypothesis. Surprisingly, the inclusion of DFO in minimal medium did not demonstrate a bacteriostatic effect in the presence of RBCs. The inhibitory effect of DFO was likely overcome by iron released from the elevated methemoglobin levels arising from the direct interaction of DFO with hemoglobin. Previous studies demonstrate that methemoglobin releases iron more quickly than normal hemoglobin (8).

Lastly, we evaluated the effect of DFO on microbial growth in platelet concentrates using the BacT/ALERT system. The presence of DFO significantly inhibited *S. epidermidis* growth in buffy coat platelets in a dose dependent manner (p < 0.001). With these findings, the inclusion of iron chelators is a promising approach to preventing transfusion-transmitted bacterial infection and providing patients with a safer platelet product.

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# LIST OF SYMBOLS

g	Relative centrifugal force	
g%	Weight of hemoglobin per 100 mL volume	
$H_1$	Initial hematocrit	
H <sub>2</sub>	Final hematocrit	
$\mathbf{V}_1$	Initial volume	
$V_2$	Final volume	
Х	cfu/mL	
у	$OD_{600}$	

# LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
Ca <sup>2+</sup>	Calcium
CBS	Canadian Blood Services
cfu	Colony forming units
$CO_2$	Carbon dioxide
DFO	Deferoxamine
DIC	Disseminated intravascular coagulation
Fe <sup>2+</sup>	Ferrous iron
Fe <sup>3+</sup>	Ferric iron
FDA	Food and Drug Administration
HBSS	Hank's Balanced Salt Solution
HUS	Hemolytic uremic syndrome
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HPD	Human peritoneal dialysate
ICL670	Deferasirox
ITP	Idiopathic thrombocytopenic purpura
K <sub>d</sub>	Dissociation constant
kDa	kilodalton
L1	Deferiprone
mM	Milimolar
М	Molar

NTBI	Non-transferrin bound iron
NB	Nutrient broth
OD	Optical density
O <sub>2</sub>	Oxygen
Pall eBDS	Pall enhanced bacterial detection system
PRP	Platelet-rich plasma
RBC	Red blood cells
sp.	Species
TTP	Thrombotic thrombocytopenic purpura
TXA <sub>2</sub>	Thromboxane A <sub>2</sub>
Tpn	Transferrin binding protein
UV	Ultraviolet
vWF	Von Willebrand's Factor

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### I. INTRODUCTION

#### **BACTERIAL CONTAMINATION OF PLATELET PRODUCTS**

Bacterial infection is a leading cause of morbidity and mortality arising from platelet transfusions in the developed world (1, 2). Studies report that approximately 1 in 3,000 platelet units are bacterially contaminated (3, 9), and 1 in 25,000 platelet transfusions result in sepsis (2, 3, 10-13). The most common source of contamination is the skin, due to inadequate sterilization of the donor's arm prior to the phlebotomy process (3, 6, 13-15). Further, storage of platelet products at room temperature (20 to 24°C) provides ideal conditions for bacterial proliferation (1, 3-6). Consequently, with approximately 9 million platelet units transfused annually in the United States, and over 400,000 units in Canada (16), a cost-effective means to increase the safety of this essential blood product is needed (17).

#### **PLATELETS**

Platelets are anuclear particles (2 to 4  $\mu$ m in diameter) derived from the highly granular cytoplasm of bone marrow megakaryocytes (18, 19). Approximately 70% of platelets produced are found in the bloodstream, while 30% reside in the spleen as functional reserves (18). The normal platelet count in the blood is 150 to 400 x 10<sup>9</sup>/L (18, 19). Circulating platelets survive for 7 to 10 days, and play a prominent role in hemostasis and blood clotting (18).

Although platelets lack a nucleus, they have abundant cytoplasmic granules (18). In particular, alpha and dense granules contain a variety of substances, such as fibrinogen, von Willebrand's Factor (vWF), serotonin, and adenosine diphosphate (ADP), which are secreted in the event of vascular injury (18, 19). This process is known as the release reaction, and is essential to the clotting function and hemostatic role of platelets (18, 20, 21).

## **Platelet Function in Hemostasis**

Primary hemostasis is the major function of platelets and involves three mechanisms: adhesion, degranulation, and aggregation (see Figure 1) (18, 22). At the site of vascular injury, vessels constrict to decrease blood loss, and to facilitate platelet contact with the damaged endothelium (18, 23). Importantly, vWF, a plasma protein that is also secreted from the alpha granules of platelets, binds subendothelial collagen exposed to the blood (18, 23, 24). Consequently, vWF binds its platelet receptor, glycoprotein Ib/IX (GPIb/IX), thus allowing platelets to adhere to the injured site (see Figure 1A) (18, 24, 25).

Following adhesion, platelets release calcium (Ca<sup>2+</sup>), which initiates thromboxane A<sub>2</sub> (TXA<sub>2</sub>) formation, and granule release of serotonin and ADP (18, 24). TXA<sub>2</sub> acts as a vasoconstrictor, and promotes platelet degranulation (see Figure 1B) and aggregation (18, 24). Serotonin also supports vasoconstriction, while ADP attracts more platelets to the site for aggregation (18, 24). Specifically, Ca<sup>2+</sup> promotes binding of plasma fibrinogen to the GPIIb/IIIa platelet receptor causing platelets to aggregate and create a plug (see Figure 1C) (18, 26-31). Formation of the platelet plug occurs within seconds after vascular injury and temporarily prevents bleeding until a permanent fibrin clot is produced (18).



**Figure 1. Platelet (A) adhesion, (B) degranulation, and (C) aggregation promote blood clotting following vascular injury.** (A) At the site of vascular damage, vWF binds subendothelial collagen exposed to the blood. Platelets possess a GPIb/IX receptor that binds to vWF, thus allowing platelets to adhere to the injured site. (B) At the platelet level, adhesion promotes platelet degranulation, and release of Ca<sup>2+</sup> and ADP. (C) ADP attracts more platelets to the site, while the platelet GPIIb/IIIa receptor binds Ca<sup>2+</sup> and fibrinogen to form a platelet plug that will prevent further bleeding.

## **Platelet Disorders**

Treatment and prevention of bleeding requires adequate platelet quantity and quality (18). Unfortunately, there are various disorders where platelet number and/or function are abnormal, and hemostasis is impaired.

As previously stated, the normal platelet count in the blood is 150 to 400 x 10<sup>9</sup>/L (18, 19). Thrombocytopenia is an abnormal decrease in the number of circulating platelets (18). Common causes of thrombocytopenia include increased platelet loss (hemorrhage), increased platelet destruction via autoantibodies, and decreased platelet production due to bone marrow failure (32). Idiopathic thrombocytopenic purpura (ITP) is one of the most common quantitative platelet disorders with prevalence in children under ten years of age (32-34). Characterized by easy bruising and severe thrombocytopenia, ITP is a disorder of increased platelet destruction in the blood due to autoantibodies against platelet membrane antigens, GPIIb/IIIa or GPIb/IX (32, 33, 35).

Defective platelet production can arise from inherited aplastic anemia or acquired bone marrow failure from chemotherapy or medications (36). For example, in leukemia, immature cells accumulate in the bone marrow leaving no room for normal cells (*i.e.*, megakaryocytes) to exist and proliferate (32, 36). Other quantitative platelet disorders include disseminated intravascular coagulation (DIC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (32, 37-39).

Qualitative platelet disorders are based on abnormal platelet function or response, due to an inherited defect or as a symptom of an acquired disease (32). Bernard-Soulier Syndrome and Glanzmann's Thrombasthenia, for instance, are rare, autosomal recessive disorders characterized by a platelet membrane protein defect (25, 32, 40-43). Individuals with Bernard-Soulier do not express enough or have non-functional platelet GPIb/IX receptors, thus reducing platelet adhesion following endothelial damage (25, 32, 40, 42, 43). In contrast, patients with

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Glanzmann's Thrombasthenia have a dysfunctional or absent GPIIb/IIIa complex, resulting in a loss of platelet aggregation (31, 32, 41, 42). Other functional platelet disorders include storage pool disease, where granules are deficient or the granule release mechanism is defective, (32, 42) and aspirin-induced TXA<sub>2</sub> inhibition, which prevents the vasoconstriction, granule release, and platelet aggregation normally induced by TXA<sub>2</sub> (32, 40-48).

## PLATELET TRANSFUSIONS

Individuals who are bleeding due to abnormal platelet number and/or function may require platelet transfusions to re-establish hemostasis (49, 50). Specific indications for platelet transfusions include cancer patients undergoing chemo- or radiation therapy resulting in decreased platelet production, preoperative patients with low platelet counts, patients recovering from bone marrow or organ transplant surgery, and bleeding patients with an inherited or acquired platelet dysfunction (49-51).

Platelets for transfusion are obtained in three ways: apheresis, platelet-rich plasma, and buffy coat. Single donor platelet concentrates are produced by apheresis, whereby platelets are removed from a donor's whole blood, and the remaining blood components are immediately returned to the donor (51). Alternatively, random donor platelets or platelet-rich plasma (PRP) is prepared by centrifuging whole blood at certain speeds to separate red blood cells (RBCs) and plasma from platelets (51-53). The resulting platelet concentrates from 3 to 5 donors are then pooled into a single unit, while the remaining RBCs and plasma are stored at 4°C and -18°C, respectively, until required for transfusion (51, 53). This PRP method is extensively used in the United States. In Europe (54) and recently in Canada (55), whole blood derived platelets are prepared via the buffy coat method, which involves: (1) a hard spin, to separate the buffy coat layer from the RBCs and platelet-poor plasma; (2) pooling of buffy coats from 4 to 6 donors; (3) a soft spin, to isolate the platelets; and (4) a final filtration step, to leukoreduce the platelet concentrate (54-57).

All three procedures produce an adequate number of quality platelets to prevent and treat bleeding (56). Apheresis platelets are preferred for individuals with compromised immune systems (*e.g.*, leukemia and bone marrow transplant patients) as they limit the recipient's exposure to multiple donors (10, 51, 56, 58). Apheresis technology, however, involves potential donor risks such as toxicity from the citrate anticoagulant used to prevent blood clotting during separation, significant bruising or hematoma, and localized or systemic infection (54, 56, 58-62). This process is also expensive and does not utilize the whole donor unit as in PRP and buffy coat procedures (54, 56).

Because whole blood derived platelets are pooled from multiple donors, the risk of transfusion-transmitted infection increases. Figure 2 illustrates this problem and the fact that only one contaminated donor unit is needed to spoil a complete PRP or buffy coat product. Importantly, if a whole blood unit contains a very low number of bacteria, pooling it with multiple units will dilute the initial inoculum, making it difficult to detect by Gram stain or culture during the short interval between pooling and transfusion.



Figure 2. Bacteria present in one donor unit of whole blood will contaminate the final pooled platelet product. Platelets removed from WB units of multiple donors are processed and pooled to make a single PRP or buffy coat platelet product. If donor unit 4 is bacterially contaminated, the final pooled platelet product will also be contaminated. Furthermore, if the initial bacterial inoculum is very low (*e.g.*, 10 bacteria or 0.02 cfu/mL), the final concentration in the pooled platelet unit could be as low as  $7.4 \times 10^{-5}$  cfu/mL.

\* Denotes bacterial contaminated units.

#### **PROBLEMS WITH PLATELET PRODUCTS**

#### Storage

In 1969, Murphy and Gardner found that platelets stored at room temperature are most optimal in viability and function for transfusion (63). In contrast, the lifespan of platelets decreased by  $\sim$ 30% after storage at 4°C (63). Further studies have shown that unlike RBCs, platelets become activated at 4°C and undergo a morphological change from discoid to spherocytic (64-68). Subsequently, activated platelets progressively deteriorate in function and lifespan during storage and in circulation after transfusion (66). These findings led to the current standard of storing platelets at 20 to 24°C (1, 51, 67).

This practice, however, created two problems. First, platelets suspended in nutrient-rich plasma and stored at room temperature with gentle agitation make an excellent growth environment for a wide spectrum of bacteria (see Table 1) (1, 3-5). Second, to reduce the risk of bacterial proliferation, platelet storage is limited to 5 days (14, 67). Unfortunately, this short shelf life increases the number of uncontaminated units discarded, resulting in platelet shortages and increased demand for platelet donors.

Organism	Number of times isolated (number of fatalities)	Percentage of total contaminated products (%)
Staphylococcus epidermidis	98 (3)	38.6 (1.2)
Diphtheroids	95	37.4
Staphylococcus aureus	17 (1)	6.7 (0.4)
Enterobacter species	13 (2)	5.1 (0.8)
Bacillus species	14 (2)	5.5 (0.8)
<i>Escherichia</i> coli	8 (2)	3.1 (0.8)
Pseudomonas species	6	2.4
Flavobacterium species	3	1.2
Total	257 (10)	

 Table 1. Bacterial contaminants isolated from transfused platelet products.\*

\*Data summarized from 1971 to 2005 (1, 3, 6, 11, 12, 14, 69-75).

## Contamination

The risk of acquiring an infectious disease from a blood product is a longstanding problem (1-3, 9-12, 76). With improvements to donor screening of blood-borne viruses, the rates of transfusion-transmitted viral infections (*e.g.*, hepatitis B virus (HBV), human immunodeficiency virus (HIV)) have dramatically decreased (3, 77-80). Thus the most prevalent infectious risk associated with transfusion is bacterial contamination, particularly, of platelet products (1-3, 9, 81, 82).

As noted, studies report that approximately 1 in 3,000 platelet units are bacterially contaminated (3, 9). Contaminants predominantly originate from a donor's skin flora, and are introduced into the platelet product at low concentrations during phlebotomy (3, 6, 9, 13-15, 76). Subsequent storage at room temperature in a nutrient-rich platelet bag enables bacteria to rapidly proliferate from  $\leq 10$  to  $\geq 10^6$  colony forming units/mL within the allowed storage time of platelets (1, 3, 76). Other possible although less frequent, mechanisms for bacterial contamination are asymptomatic donor bacteremia and contamination during processing of the platelet product. Approximately 1 in 25,000 platelet transfusions have been estimated to result in sepsis and 1 in 60,000 result in death (2, 3, 9-12, 76).

#### **Bacterial Contaminants of Platelet Products**

Many of the organisms recovered from contaminated platelet units (Table 1) are a part of the normal skin flora, such as *Staphylococcus* sp., *Bacillus* sp., and diptheroids (1, 6, 9, 14, 71, 72, 74). Other contaminating microbes originate from either the environment (*Pseudomonas* and *Flavobacterium* sp.) or from a blood donor with asymptomatic bacteremia (*Enterobacter* sp., *Staphylococcus aureus*, *Escherichia coli*) (1, 70, 71, 74).

Among these contaminants, *Staphylococcus epidermidis* is the most frequently isolated and implicated in transfusion-associated sepsis (74). Wagner and colleagues compiled the results

of eight separate studies and revealed that *S. epidermidis* was the infectious agent in 25% of the septic events from transfused platelets (74). Likewise, te Boekhorst *et al.* performed a two year bacterial screening study and found that 25.2% of platelet product contamination was caused by *S. epidermidis* (73). Fatalities consequent to transfusion of platelets contaminated with *S. epidermidis* have also been reported (69, 70, 75), revealing the pathogenic potential of this skin bacterium.

# PREVENTION OF BACTERIAL CONTAMINATION

## **Bacterial Avoidance**

Skin disinfection is the first line of defense in reducing transfusion-transmitted infection (83). Several studies have shown that disinfection, specifically using isopropyl alcohol and iodine, drastically reduces the number of bacteria present at the phlebotomy site (83-86). However, certain skin flora such as diptheroids and *Staphylococcus* sp., reside in the deeper skin layers, and may avoid surface decontamination (6, 71, 74, 84).

Investigators have suggested that these bacteria are introduced into the donated blood via a skin plug formed from the needle puncture during phlebotomy (87). Since the majority of the contaminating bacteria can be detected in the first few milliliters of each blood unit (2), diversion pouches have been designed to exclude the initial 15 to 30 mL of whole blood from the main collection bag (3, 84, 88-90). Several studies have found that diversion alone decreases the prevalence of skin flora in whole blood units by approximately 50% and with improved skin disinfection the prevalence decreases by 70% (84, 89, 91). Consequently, a residual contamination risk of at least 30% remains (91) and is evident from the data summarized in Table 1. Furthermore, neither diversion nor improved skin disinfection techniques prevent transmission from rare asymptomatic bacteremic donors (15, 91-93). Therefore, to assure safety,

additional testing of platelet concentrates is necessary to detect bacteria prior to transfusion (15, 92).

# **Bacterial Detection**

Blood suppliers in both Europe and North America have implemented the BacT/ALERT system (bioMérieux, St. Laurent, QB, Canada) for routine bacterial testing of apheresis platelets (94-97). The BacT/ALERT is a standard automated culture system that utilizes a colorimetric sensor of culture bottles to detect bacterial growth (1, 98, 99). Samples of platelet concentrates are transferred to a sampling pouch via a sterile connection device, which maintains a closed system (91, 96). After the pouch is detached, the contents are inoculated into culture bottles containing tryptic soy broth. The bottles are then placed in the BacT/ALERT instrument and incubated at 37°C. If bacteria are present in the sample, they proliferate and generate carbon dioxide. The presence of carbon dioxide causes a sensor in the bottle to change color (1, 98, 99). This color change is detected by the BacT/ALERT system, which produces audible and visual alarms to notify lab personnel of a positive sample (98). The BacT/ALERT detects most aerobic and anaerobic bacteria within 24 hours of bottle inoculation (93, 94, 96, 100).

Another automated detection system approved by the Food and Drug Administration (FDA) for use in the United States is the Pall enhanced bacterial detection system (Pall eBDS) (1). Unlike the BacT/ALERT, the Pall eBDS is limited to detecting aerobic bacterial growth as the presence of bacteria is determined by measuring oxygen consumption in the sample (1, 92, 96, 99). Furthermore, the Pall eBDS requires a minimum 24 hour incubation before the oxygen level can be measured (1, 92). However, an advantage of the Pall eBDS is that samples of platelets to be tested are transferred directly into a culture pouch via a sterile connecting device (1, 92, 99). This provides a closed system, which minimizes the risk of false-positive results from environmental contaminants (92, 99).

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Numerous studies have shown that the BacT/ALERT and Pall eBDS are capable of detecting a wide array of potentially contaminating bacteria and that their sensitivity levels are comparable (92, 94, 101-103). Implementation of these systems, however, has not eliminated the risk of septic reactions (73, 76, 82, 104). False-negative screens have led to life threatening and fatal cases of sepsis following platelet transfusions (73, 104). Further investigations, therefore, are needed to improve the sensitivity of bacterial detection systems.

## **Bacterial Elimination**

In contrast to the above, a variety of pathogen reduction strategies are being tested against bacteria (99, 105, 106). The best studied technology is the Intercept Platelet System, which uses a psoralen compound, amotosalen that can cross the membrane or cell wall of pathogens and intercalate into their DNA (99, 105, 107). Subsequently, amotosalen is activated in the presence of ultraviolet (UV) light and forms permanent crosslinks in the DNA that prevents bacterial replication (99, 105, 107). Janetzko *et al.* demonstrated that the Intercept system had no influence on *in vitro* platelet function (105). However, clinical trials have shown a reduction in post-transfusion platelet recovery (~11-26%) and survival (1.5 days) following 5-day storage of amotosalen/UV treated platelets (108, 109). Furthermore, the impact of this treatment and evaluation of the potential toxicity of such chemicals on the platelets and/or the transfusion recipient remains to be established (1, 3).

## STAPHYLOCOCCUS EPIDERMIDIS AND IRON IN PLATELET PRODUCTS

## Staphylococcus epidermidis

*S. epidermidis* is ubiquitous in nature, and a major constituent of the normal flora of human skin and mucus membranes (99, 110-115). These bacteria are regularly shed from the skin, and adapt easily to dryness and temperature changes in the environment (112). Originally, *S. epidermidis* was considered to be a non-pathogenic contaminant of clinical specimens (110-112, 114). The organism, however, has emerged as the most common *Staphylococcus* sp. isolated and implicated as the causal agent in human diseases (110, 112, 114, 115).

#### Classification

*S. epidermidis* is classified in the bacterial family *Micrococcaceae* under the genus *Staphylococcus* (110, 112, 116). Staphylococci are spherical gram positive cocci occurring in singles, pairs, short chains, or irregular clusters (110, 112, 115). Colonies of most Staphylococci grow on agar plates within 24 hours, and are typically small (1 to 2 mm in diameter), circular, entire, and white or beige pigmented (110, 115). Differentiation of *Staphylococcus* sp. is important in the prognosis and treatment of bacterial infections. The first test usually performed is the coagulase test. This determines whether the isolated bacteria can convert plasma fibrinogen into a fibrin clot, and differentiates the coagulase-negative, *S. epidermidis* from the more predominant coagulase-positive pathogen, *S. aureus* (110, 112).

## Infections

*S. epidermidis* is predominantly an opportunistic nosocomial pathogen that affects immunocompromised patients (99, 117). For instance, breaks in the host's natural barriers (*i.e.*, skin) via surgery or insertion of indwelling devices allows *S. epidermidis* at the site of the break to colonize and cause infection (111-113, 118). Frequent infective complications include

endocarditis after cardiac surgery and prosthetic heart valve implantation, wound infections following orthopedic joint replacement, and central nervous system infections with cerebrospinal fluid shunts (111, 113, 118-123). Neonates and bone marrow recipients often require central venous lines to receive fluids, medications, and blood products, and are often immunosuppressed, making them especially vulnerable to opportunistic *S. epidermidis* infection (110, 112, 113, 117, 124-126).

#### Iron and Staphylococcus epidermidis

Progression to infection is largely dependent on the bacteria's ability to acquire iron from their host (7, 127-132). Virtually all bacteria require iron to survive and proliferate (7, 127, 128, 130, 133-135). Several essential biological processes require iron including DNA synthesis, dinitrogen reduction, and aerobic and anaerobic respiration (136-142). Iron also serves as a cofactor of enzymes that function to protect bacteria from host defense mechanisms. For instance, microbial catalase combats toxic oxygen species, such as superoxide and hydrogen peroxide, released from human phagocytic cells (143). Other bacteria produce beta-lactamase, which inactivates certain widely prescribed antibiotics including penicillin, ampicillin, and cefazolin (144-146).

The importance of iron is further illustrated by its limited availability *in vivo* (128, 132, 136). Iron exists primarily in its insoluble and highly toxic ferric (Fe<sup>3+</sup>) iron form, and thus must be bound intracellularly or to proteins (131, 133, 137). In humans, plasma iron circulates bound to transferrin, an 80 kilodalton (kDa) glycoprotein with two N- and C-terminal iron-binding domains (128, 131, 135, 147-151). Transferrin has a strong affinity for Fe<sup>3+</sup> as indicated by its low dissociation constant ( $K_d$ ) of ~10<sup>-22</sup> M (150-152). Normally, only one third of transferrin is saturated allowing it to uptake any free iron and transport it around the body (128, 131, 134, 149). Alternatively, iron is incorporated into the heme molecule of the oxygen carrier proteins

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hemoglobin and myoglobin, or is bound to the main intracellular iron-storage protein ferritin (128, 132, 140, 149). Because iron is predominantly bound, free ionic iron levels are too low to support bacterial growth (127, 128, 133, 135, 148). Thus bacteria have developed efficient mechanisms to acquire this essential metal (127, 128, 134-136, 149, 153).

One major iron acquisition system is mediated by siderophores (127, 128, 130, 131, 133, 134, 136, 148, 149, 153, 154). By virtue of their high binding affinity, many siderophores can remove iron from ferritin or transferrin, and transport it into the microbe where it is utilized to maintain bacterial function or deposited within iron storage proteins (*e.g.*, bacterioferritin) for future reserve (127, 128, 136, 153). These  $Fe^{3+}$ -specific chelating compounds are synthesized by certain microbes in response to iron deficiency (127, 128, 133). In 1994, Lindsay and Riley showed that *S. epidermidis* grows poorly in iron-depleted media, owing to their inability to secrete siderophores (133). Earlier work by Schade demonstrated that *S. epidermidis* only proliferates in human serum containing transferrin when excess iron is added (129). These findings suggest that *S. epidermidis* must possess a mechanism other than siderophores for obtaining iron.

Information on the iron acquisition mechanism used by *S. epidermidis* is limited. By 1992, several studies showed that the growth of *S. epidermidis* in human peritoneal dialysate (HPD), an iron-restricted medium containing transferrin, resulted in the expression of a 42-kDa surface protein (154, 155). Upon further investigation, Modun and coworkers demonstrated that growth in HPD promoted binding of human transferrin to the *S. epidermidis* cell wall and specifically to this 42-kDa protein (see Figure 3) (148). The above findings confirmed that *S. epidermidis* acquires iron via a siderophore-independent process, involving a transferrin-specific binding protein (Tpn) located within the bacterium's cell wall (127, 148, 156). Although the bacterial mechanism by which iron is released from Tpn-bound transferrin remains unknown, evidence of Tpn expression *in vivo* during infection suggests that iron must be internalized to be

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used, and that this receptor, therefore, may contribute to the virulence of *S. epidermidis* (127, 135, 157).



Figure 3. S. epidermidis acquire transferrin-bound iron via a 42-kDa transferrin specific membrane protein (Tpn). (A) Plasma iron (Fe<sup>3+</sup>) circulates in the body bound to transferrin. To acquire this Fe<sup>3+</sup>, S. epidermidis possess a cell wall receptor, Tpn, which recognizes and specifically binds transferrin. (B) Once removed from transferrin, Fe<sup>3+</sup> is internalized and used by the bacterium. (C) The unsaturated transferrin is then free to uptake any free plasma Fe<sup>3+</sup>.

\* Modified from reference (136).

## **Iron in Platelet Products**

Plasma, the medium in which platelets are stored, has proven to be favorable for S.

epidermidis growth. Thus, given the frequency of S. epidermidis as a platelet contaminant (~25%

of contaminated units), plasma must contain available sources of iron to permit bacterial survival

and propagation (see Figure 4).

Bioavailable or free iron (Fe<sup>3+</sup>) exists at very low levels (10<sup>-18</sup> M) in the environment (128, 148). However, during various pathologic conditions such as acute leukemia, thalassemia, myelodysplastic syndromes, and hemochromatosis, these levels are elevated due to the iron-saturation of transferrin (158-163). Incidentally, patients with these conditions have an increased incidence of infection, indicating that the free iron exists in a form available for bacterial uptake (162).

Hershko and colleagues first identified the free iron as non-transferrin bound iron (NTBI) (162). Matinaho *et al.* later showed that NTBI exists in platelet concentrates (7). More importantly, the authors showed that *S. epidermidis* growth in platelet concentrates was dependent on the presence of NTBI (7). This supports previous studies and indicates that NTBI is critical for the proliferation of *S. epidermidis* (7, 129, 134, 164-166).

Another possible source of iron in plasma is RBCs (167, 168). Platelet concentrates are derived from whole blood and processed to separate the blood components (51-53). Apheresis or centrifugation of the whole blood is designed to remove donor RBCs; however, a small number of red cells remains. Over time, the RBCs age and lyse, releasing their hemoglobin, which degrades and releases iron (149, 167, 169). The findings that NTBI levels increased during the storage of plasma-depleted RBC units and in platelet concentrates prepared from whole blood stored overnight, supports the latter (167, 168). Consequently, as hypothesized in this thesis, the risk of bacterial growth and transmission likely increases with storage time, in part due to the saturation of plasma transferrin, and the subsequent presence of bioavailable iron arising from the breakdown of residual RBCs (see Figure 4) (168).



Figure 4. Bacterial growth in platelet products is dependent on bioavailable iron.

(A) Platelet concentrates are isolated from whole blood and stored in plasma at room temperature for a maximum of 5 days. Initially, the plasma contains bioavailable iron (Fe<sup>3+</sup>) and partially saturated transferrin. The latter is a plasma glycoprotein that can mop up the Fe<sup>3+</sup> to an extent. (B) Once transferrin is saturated, excess Fe<sup>3+</sup> exists as NTBI (arrow), a chelatable form available for bacterial uptake. Plasma also contains residual RBCs. Apheresis or centrifugation of whole blood is designed to remove donor RBCs; in reality, a small number remains in platelet concentrates. Over time, residual RBCs age and lyse, their hemoglobin breaks down, and Fe<sup>3+</sup> is released (dashed circle). (C) Thus the risk of bacterial growth and transmission may increases with storage time, in part due to the saturation of transferrin and the subsequent presence of Fe<sup>3+</sup> arising from residual RBCs.

#### **IRON CHELATORS**

## **History of Iron Chelation Therapy**

Most free plasma iron originates from old or damaged RBCs that have been degraded by liver and spleen macrophages (170-172). Ordinarily, transferrin in healthy individuals is partially saturated and can readily uptake this free iron (128, 131, 134, 159). Certain diseases, however, particularly thalassemia, require regular RBC transfusions, which accelerate iron release and saturation of transferrin (159, 161, 162, 170, 172, 173). As the body has no physiological mechanism for excreting iron, NTBI circulates in the plasma eventually accumulating in and damaging the liver, spleen, bone marrow, pancreas, and heart (152, 159, 161, 170, 172-175). Toxicity to the heart results in cardiac failure, the most common cause of morbidity and mortality in thalassemia patients, followed by bacterial infection (172, 173, 176-178).

Chronically transfused patients, therefore, require concomitant chelation therapy to avoid the effects of iron overload (163, 173, 176, 179). Chelators are chemical compounds that bind and remove free metal ions from the body. In the early 1970s, Richard Propper and his colleagues demonstrated that continuous subcutaneous infusion of the iron chelator DFO to iron overload patients promoted urinary iron excretion (180, 181). Subsequent studies showed that DFO drastically reduced the incidence of cardiac failure and organ toxicity, and improved the longevity and quality of life of thalassemia patients (170, 172-175, 177, 179, 182-187).

Since Propper *et al.*'s demonstration, adverse side effects have been associated with DFO therapy and driven researchers to develop an alternate, but equally effective pharmaceutical chelator. The efficacy of a chelator depends on many factors including administration route, metal affinity, bioavailability, and toxicity (188, 189). Ideally, a chelator should be administered orally and absorbed by the gastrointestinal tract, should preferentially bind and remove  $Fe^{3+}$  (including NTBI), be available in the plasma to continuously bind NTBI between doses, and cause minimal side effects to the iron-overload patient (173, 188-190). Moreover, an optimal

iron chelating agent must be affordable to accommodate chronically transfused patients worldwide (173).

To date, two effective, oral chelators are approved for use in certain countries. Deferiprone (L1) was first licensed in India in 1995, followed by Europe in 1999 where it is prescribed for patients who do not respond to DFO therapy (188). Deferiprone is a bidentate chelator and binds  $Fe^{3+}$  at a 3:1 ratio ( $K_d \sim 10^{-35}$  M) (179, 191, 192). Consequently, the deferiprone-iron complex has no net charge enabling it to cross membranes and remove excess intracellular iron (179). Iron excretion by deferiprone occurs primarily via the urine, and is comparable to that achieved with DFO (191, 193). Reported side effects of deferiprone are usually abdominal pain, nausea, and vomiting; joint problems and mild neutropenia are less frequent (179, 188, 194). Agranulocytosis, characterized by severe reduction of circulating granulocytes, is the most serious complication of deferiprone, but occurs rarely ( $\leq 0.5\%$  of patients) (179, 188, 191, 194).

The more recently approved (2005) and first oral iron chelator to be used in North America is deferasirox (195). Commonly known as ICL670, this tridentate chelator is highly selective for iron and binds the metal at a 2:1 ratio (179, 196, 197). The resultant ICL670-iron complex is stable, uncharged, and excreted in the stool (179, 197). Remarkably, ICL670 has a long plasma half-life (8 to 16 hours) compared to DFO (minutes) and deferiprone (< 2 hours), allowing it to scavenge and excrete iron between daily doses (179, 197). ICL670 was also initially viewed as being well-tolerated with minor, infrequent adverse events (*e.g.*, headaches, mild diarrhea, and abdominal discomfort) upon initiation of therapy (179, 196-198)

Although once-daily oral administration of ICL670 would increase patient compliance, its hefty cost is a burden to patients worldwide (170, 179). Furthermore, the United States FDA and Health Canada recently issued an advisory warning following reports of fatal acute renal failure and cytopenias in patients receiving ICL670 (199). Deferiprone is the least expensive

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chelator. However, its ability to penetrate the blood brain barrier may result in removal or redistribution of iron in the brain, leading to neurotoxic effects (179, 189, 200). These limitations and evidence that DFO reverses cardiac damage have made DFO the gold standard for treating iron overload (172, 177, 179).

## **Deferoxamine (DFO)**

DFO (see Figure 5) is a natural bacterial siderophore isolated from *Streptomyces pilosus* (173, 175, 201, 202). It has a high, specific affinity for Fe<sup>3+</sup> ( $K_d \sim 10^{-31}$  M) and binds the metal at a 1:1 ratio (170, 172, 173, 179, 188, 192, 201, 203-205). As a hydrophilic molecule with a molecular weight of 561 g/mol, DFO cannot easily cross cell membranes. Hence, DFO has two pathways for removing excess iron from the body (172, 201, 206, 207). First, DFO readily chelates plasma iron (*i.e.*, derived from hemoglobin) and excretes it in the urine (201, 206, 208). Alternatively, DFO enters hepatocytes via facilitated uptake, where it binds cytosolic iron for excretion in the stool (201, 206-208). Both pathways generate a stable complex that renders iron inactive, and prevents it from producing toxic free radicals that destroy cellular proteins, membranes, and DNA (170, 172-174, 188, 202, 209-213).



Figure 5. Chemical structure of deferoxamine.

Use of DFO, therefore, to treat transfusion induced iron overload has greatly reduced morbidity and mortality of thalassemia patients for over 30 years (170, 176, 179, 214). Additionally, DFO is used to treat acute iron intoxication of children who have ingested concentrated iron supplements, and has been beneficial in other situations of progressive iron overload, such as hereditary hemochromatosis, sickle cell anemia, myelodysplastic disorders, and acute leukemia (158-161, 163, 201, 208, 215, 216). Despite success with DFO, a number of factors illustrate the need to develop an alternate chelator.

For instance, DFO is not orally absorbed and has a short plasma half-life of only 5 to 10 minutes (173, 179, 202, 217). To address this issue, DFO is administered as a bolus subcutaneous injection, an implantable pump, or through a central venous line, all of which allow for continuous high-dose intravenous therapy (173, 180, 181, 217). These procedures are invasive and inconvenient, and result in poor patient compliance (173, 179, 182, 202, 217). The high costs of DFO and infusion delivery systems make chelation therapy unaffordable for patients in both developing and developed countries (172, 173, 186, 209, 217). Lastly, DFO therapy is associated with various side effects including local skin and allergic reactions, vision and hearing impairment, bone and growth abnormalities, and pulmonary and renal toxicity (172, 192, 214, 215, 218). Importantly, the majority of these events occur in patients with a low iron burden who have received high doses of DFO (177, 218, 219). Thus an appropriate dosage of DFO must chelate excess iron while permitting sufficient levels of iron for biological processes and consequently avoiding toxic complications (177).

# Phytic Acid (phytate)

A potential, non-toxic alternative to the above chelators is phytic acid ( $K_d \sim 10^{-25}$  M) (see Figure 6), a plant constituent abundant in legumes, cereals, seeds, and nuts (220-224). These foods are major staples in developing nations and are important sources of protein in Western

vegetarian diets (223, 225-229). Plant foods are also a rich source of essential trace elements including zinc, magnesium, calcium, and iron (223, 227). Interestingly, the bioavailability of these elements is low in plants characterized by high levels of phytate (220-223, 225-228).



Figure 6. Chemical structure of phytic acid.

Phytate has been shown to possess iron chelating activity (221-223). Specifically, phytate binds iron in the gut, impairing its absorption and promoting its excretion from the body (225, 230, 231). This phenomenon may contribute to iron deficiency commonly seen in populations that rely on plant-based foods; conversely, it may play a beneficial health role in preventing iron overload and iron-driven oxidative damage (221, 222, 231). Investigators have shown that phytate inhibits iron-mediated hydroxyl radical formation and reduces membrane lipid peroxidation in a manner comparable to DFO (221, 222, 232, 233). These antioxidant properties combined with its low toxicity make phytic acid a desirable iron chelator for therapeutic application in iron overload states (222). However, at present, phytate has not yet been developed as a pharmaceutical chelator for treating iron overload.

# **HYPOTHESES**

As previously described, platelet transfusions are critical to treat and prevent bleeding. Platelet concentrates, however, are still frequently associated with transfusion-transmitted infection and most current strategies (see Table 2) only reduce, not eliminate, bacterial contamination. While new methods which do eliminate bacteria by targeting DNA are now available, they are very expensive, and associated with poor platelet survival and some potential risk to the recipient. Importantly, plasma in which platelets are stored provides bacteria with the bioavailable iron they need to survive and proliferate (see Figure 3) (7). Withholding iron from bacteria, therefore, may be an effective approach to preventing microbial growth during platelet storage (7, 234).

Strategy	Example(s)	Benefit(s)*	Limitation(s)*
Bacterial avoidance	<ol> <li>Skin antiseptic (isopropyl alcohol, iodine)</li> <li>Diversion pouch (remove first 15-30 mL of whole blood)</li> </ol>	Risk of bacterial contamination reduced by approximately 70%.	Bacteria residing in deeper skin layers may avoid surface decontamination; cannot prevent transmission from donors with asymptomatic bacterial infections.
Bacterial detection	<ul> <li>(1) BacT/ALERT system (CO<sub>2</sub> production)</li> <li>(2) Pall eBDS (O<sub>2</sub> consumption)</li> </ul>	Wide array of aerobic/anaerobic bacteria detected in $\leq$ 24 hours.	Cannot always detect slow growing organisms; false negative screens have led to recipient sepsis and/or death.
Bacterial elimination	(1) Intercept Platelet System (prevents bacterial replication using amotosalen/UV light)	<i>In vitro</i> platelet functions are not affected by chemical treatment.	Associated with reduced <i>in</i> <i>vivo</i> platelet recovery and survival; toxicity to platelets and transfusion recipients unknown.

Table 2. Current strategies to prevent transmission of bacteria from platelet products.

\*Data from references (1, 6, 15, 71, 73, 74, 83-86, 89, 91-93, 99, 101-105, 108, 235)

In this thesis, we hypothesized that the inclusion of iron chelators in platelet concentrates will have a bacteriostatic effect on bacterial growth (see Figures 4 and 7). Our second hypothesis is that residual RBCs in platelet concentrates contribute bioavailable iron during storage that promotes bacterial survival and growth (see Figure 4B).



**Figure 7. Schematic of hypotheses.** (A) Inclusion of iron chelators in a bacterially contaminated platelet product will (B) bind any plasma  $Fe^{3+}$  or NTBI (arrow), as well as iron released from residual RBCs (dashed circle). (C) Consequently, bacterial growth will cease under these iron-poor conditions, thus decreasing the risk of bacterial transmission and infection in the recipient.

To test our hypotheses, *S. epidermidis*, the normal skin bacterium most frequently isolated from platelets and implicated in transfusion-associated sepsis, was chosen. First, *S. epidermidis* was cultured in nutrient broth (NB) to establish a standard growth curve. After 24 hours, a series of dilutions of the bacterial culture were made, and the optical density measured at

a wavelength of 600 nm ( $OD_{600}$ ). The dilutions of the 24-hour culture were immediately plated on nutrient agar and incubated overnight to count the number of viable bacteria. The relationship between the  $OD_{600}$  readings and the viable counts were then graphed to derive a standard calibration curve from which all further  $OD_{600}$  readings were converted to bacterial concentration.

Second, experiments were conducted to determine the effect of iron chelators on bacterial growth in culture medium. *S. epidermidis* was grown in NB treated with various concentrations of DFO or phytate, and the  $OD_{600}$  was followed over 72 hours. DFO is the gold standard drug for treating iron overload. In contrast, phytic acid was chosen as a non-toxic alternative chelator. Importantly, performing these experiments with culture medium provided a controlled environment (*i.e.*, defined nutrient concentrations) to validate our system for determining the effect of iron chelators on bacterial growth and to verify previous findings by Matinaho *et al* (7).

Regarding our second hypothesis, *S. epidermidis* was cultured in 1% hematocrit suspensions prepared in Hank's Balanced Salt Solution (HBSS), with or without DFO, for 7 days. Preliminary experiments in our lab had shown immediate lysis of RBCs when added to the NB. To avoid hemolysis and to limit bioavailable iron, we chose HBSS, a solution designed to maintain the physiological pH range and to provide cells with minimal nutrients for survival. On days 0, 1, 3, 5, and 7, bacterial growth was determined by the standard plate count method, and RBC lysis, methemoglobin, and iron levels were measured spectrophotometrically to determine the effect of storage time on RBCs.

Last, the automated BacT/ALERT system was used to determine the effect of DFO on bacterial growth in platelet concentrates. Aerobic culture bottles containing buffy coat platelets were spiked with DFO and/or *S. epidermidis* and the presence or absence of growth was detected by the BacT/ALERT instrument over 6 days. Samples positive for bacterial growth were plated to determine the bacterial concentration and to verify the instrument results.

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The issue of bacterial contamination in platelet units is of serious concern. Using chelators to remove iron from platelet concentrates may provide a new approach to inhibiting microbial growth and eliminating transmission of bacteria during transfusion. Furthermore, successful implementation of iron chelators will provide patients with a safer platelet product, and may improve the storage conditions and increase the inventory of platelet concentrates.

## II. MATERIALS AND METHODS

#### **BACTERIAL STRAIN AND PREPARATION**

*S. epidermidis*, strain 12228 (American Type Culture Collection (ATCC), Manassas, VA, USA) was cultured on nutrient agar (Becton Dickinson, Franklin Lakes, NJ, USA) in 100 x 20 mm petri plates (Sarstedt Inc., Montreal, QB, Canada) for 24 hours, in a 37°C incubator. Plated colonies were resuspended in nutrient broth (NB, Becton Dickinson) containing a 15% glycerol cryoprotectant (Fisher Scientific, Ottawa, ON, Canada) and aliquoted into 2 mL cryotubes (Nalgene Nunc International, Rochester, NY, USA). Stocks of *S. epidermidis* aliquots were then frozen and stored at -80°C. For subsequent experiments, one stock was thawed at room temperature, sub-cultured on a nutrient agar plate, and incubated at 37°C overnight. Sub-culture plates were wrapped with Parafilm and stored at 4°C for up to 1 month.

Twenty-four hours before performing a bacterial spiking experiment, a single, isolated colony from a *S. epidermidis* sub-culture plate was inoculated into a 16 x 150 mm flint glass culture tube (VWR International, Mississauga, ON, Canada) containing 10 mL of NB. The tube was vortexed and incubated overnight in a 37°C water bath, with constant agitation (140 rpm).

#### **BACTERIAL STANDARD CALIBRATION CURVE**

Following overnight incubation, a series of dilutions of the bacterial culture were prepared (see Figure 8), and the OD<sub>600</sub> was measured using a Helios Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The dilutions of the 24-hour culture were immediately plated on nutrient agar and incubated overnight at 37°C. Isolated colonies were subsequently counted using an Electronic Colony Counter (Carolina Biological Supply Company, Burlington, NC, USA), and expressed as colony forming units per milliliter (cfu/mL).

## Figure 8. Serial dilutions and standard plates for S. epidermidis calibration curve.



Culture of *Staphylococcus epidermidis* on nutrient agar

↓ ↓

Inoculate one colony into 10 mL nutrient broth (NB); incubate in 37°C water bath, with agitation (140 rpm) for 24 hours



The relationship between the  $OD_{600}$  readings and the viable counts were then graphed, and the equation of the best-fit line derived as,

$$y = 0.026158 - 0.26086x \tag{1}$$

or

$$1 \text{ OD}_{600} = 3.73 \text{ x } 10^8 \text{ cfu/mL}.$$
 (2)

The latter was used to convert all further  $OD_{600}$  readings to bacterial concentration.

#### **BACTERIAL GROWTH IN NUTRIENT BROTH (NB)**

Prior to bacterial spiking in NB, stock solutions of DFO or phytate were prepared (as described below) and mixed with fresh NB dispensed in sterile 16 x 150 mm flint glass culture tubes. Each tube was spiked with 0.1 mL of a 24 hour *S. epidermidis* culture and vortexed. Tubes containing NB only, and NB with DFO or phytate only were also prepared as negative controls.

For estimation of the initial bacterial concentration, 0.8 mL of each control and spiked samples were immediately aliquot into disposable cuvettes (Sarstedt). The NB only control was used as a blank to adjust the spectrophotometer to zero. Readings of the remaining samples were measured by  $OD_{600}$  and converted into bacterial concentration using equation (2). The initial concentration of *S. epidermidis* was standardized to ~5 x 10<sup>6</sup> cfu/mL. All samples were subsequently incubated in a 37°C water bath with constant agitation (140 rpm), and  $OD_{600}$  readings were taken over 72 hours to establish a growth curve.

#### **IRON CHELATORS**

Deferoxamine (DFO) mesylate salt and phytic acid (phytate) sodium salt were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). For bacterial spiking experiments in NB, a stock solution of DFO (1 mM) or phytate (1 M) was prepared in a 50 mL conical tube (Sarstedt) using sterile saline. DFO and phytate were weighed out using a Denver Instrument Company XT Series Model 400D balance (Denver, CO, USA). Samples of each stock solution were subsequently aliquot into 12 x 75 mm borosilicate glass culture tubes (VWR International), and diluted with saline to obtain 35, 50, 100, and 500  $\mu$ M, or 5, 10, and 50 mM solutions of DFO or phytate, respectively. All DFO solutions were immediately wrapped in aluminum foil, as DFO is light sensitive. Additional experiments were conducted to test high phytate concentrations of 100 and 1000 mM. As phytate is difficult to dissolve at higher concentrations, for these experiments, the appropriate amount of phytate was weighed directly into the culture tube and suspended to yield the desired final concentration.

#### **RED BLOOD CELL AND HEMATOCRIT PREPARATION**

On the designated days for bacterial spiking in 1% hematocrit suspensions, whole blood was collected from normal, healthy, human donors in ethylenediaminetetraacetic acid (EDTA) additive tubes. To remove the plasma and buffy coat layer, the tubes were centrifuged at 1,000 x g for 5 minutes at room temperature using a Clay Adams<sup>TM</sup> Sero-fuge Model 2002 (VWR). The remaining packed RBCs (pRBCs) were washed three times with sterile saline and the hematocrit determined using a Clay Adams<sup>TM</sup> Autocrit Ultra 3 Centrifuge Model 420575 (VWR). Based on the hematocrit ( $H_1$ ), the volume ( $V_1$ ) of pRBCs needed to prepare a 1% hematocrit ( $H_2$ ) suspension with HBSS was calculated using the following equation:

$$H_1 V_1 = H_2 V_2. (3)$$

where  $V_2$  is the final volume. The hematocrit solutions were prepared in 16 x 150 mm flint glass culture tubes, covered with Parafilm, and gently inverted to evenly distribute the pRBCs.

#### SPIKING PROTOCOL FOR HEMATOCRIT SUSPENSIONS

One percent hematocrit suspensions were spiked with 0.1 mL of a 24 hour *S. epidermidis* culture. The initial bacterial concentration was  $\sim 5 \times 10^6$  cfu/mL as determined from the OD<sub>600</sub>

and equation (2) (as described above). Working DFO solutions prepared from a 15 mM stock were added to the bacterial spiked hematocrit suspensions to obtain final DFO concentrations of 25, 50, 500, and 1000  $\mu$ M. HBSS solutions with pRBCs and/or DFO were also prepared as negative controls, and to compare RBC changes in the presence and absence of an iron chelator. All samples were mixed several times by inversion and kept agitated in a 37°C water bath for 7 days. On days 0, 1, 3, 5, and 7, spiked suspensions were sampled for standard plate counts and RBC experiments (described below).

## STANDARD PLATE COUNT PROTOCOL

To assess bacterial growth in the presence of RBCs and/or DFO, 0.1 mL aliquots were removed from each spiked suspension, and serially diluted  $(10^{-1} \text{ up to } 10^{-8})$  in sterile saline. Final dilutions of  $10^{-2}$  to  $10^{-4}$  were made for samples without RBCs, while higher dilutions  $(10^{-6} \text{ to } 10^{-8})$ were chosen for spiked hematocrit suspensions. For each sample, a 0.1 mL volume of the desired final dilution was dispensed onto the surface of a nutrient agar plate. Drops were spread around the agar surface using sterile disposable spreaders (30 mm blade width) (VWR). Once dry, the plates were inverted and stored in a 37°C incubator.

After 18-24 hours, the plates were removed from the incubator and the number of cfu/mL counted using an Electronic Colony Counter. The number of viable bacteria was subsequently determined from the following equation:

<u>Number of colonies (cfu)</u> = Number of viable bacteria/mL. (4) (Dilution)(Volume plated)

## **RED BLOOD CELL EXPERIMENTS**

The effect of storage time (*i.e.*, 7 days) on RBCs was assessed based on RBC lysis, hemoglobin oxidation status, and levels of  $Fe^{3+}$ . These results were then compared to the counts

of viable bacteria counts to determine whether RBCs contribute to the growth of *S. epidermidis* in minimal medium (HBSS).

## Hemoglobin Concentration and Red Cell Lysis

Total hemoglobin and percent RBC lysis were determined using the Drabkin's Assay (236). Briefly, Drabkin's Reagent converts hemoglobin to cyanomethemoglobin, which absorbs light at 540 nm. Aliquots of the spiked hematocrit solutions were removed and immediately mixed with Drabkin's to measure total hemoglobin. A second aliquot of each sample was centrifuged, and the supernatant removed and mixed with Drabkin's. Both total and supernatant hemoglobin concentrations were calculated from the OD at 540 nm as grams of hemoglobin per 100 mL of solution (g%):

$$[g\% hemoglobin] = \underline{OD_{540} x (Final volume/Sample volume)}_{6.8}.$$
(5)

Subsequently, the supernatant hemoglobin was calculated as a percent of total hemoglobin, to give percent RBC lysis:

Percent RBC lysis = (Supernatant hemoglobin/Total hemoglobin) x 100%. (6)

## **Hemoglobin Oxidation**

For hemoglobin oxidation status, RBC hemolysates were prepared by mixing 80  $\mu$ L of each spiked hematocrit suspension with 1 mL of double distilled water. Hemolysates were then scanned from 500 to 701 nm. Absorbances at 560, 577, 630, and 700 nm were recorded to calculate the oxyhemoglobin, methemoglobin, and hemichrome levels (mM) as described previously (237):

Oxyhemoglobin (mM) = 29.8 (
$$OD_{577}$$
- $OD_{700}$ ) - 9.8 ( $OD_{630}$ - $OD_{700}$ ) - 22.2 ( $OD_{560}$ - $OD_{700}$ ) (7)

$$Methemoglobin (mM) = 7 (OD_{577}-OD_{700}) - 76.8 (OD_{630}-OD_{700}) - 13.8 (OD_{560}-OD_{700})$$
(8)

Hemichrome (mM) = 
$$33.2 (OD_{577} - OD_{700}) - 36 (OD_{630} - OD_{700}) - 58.2 (OD_{560} - OD_{700})$$
 (9)

#### **Determination of Bioavailable Iron**

Bioavailable or Fe<sup>3+</sup> iron was measured by spectrophotometry using the ferene iron assay (238). Sample aliquots were mixed with 40% trichloroacetic acid (TCA) and centrifuged at 1,310 x *g* to precipitate out any protein. The supernatants were then removed and mixed with thiourea/ascorbate and Ferene S solutions. The thiourea/ascorbate solution was added to convert Fe<sup>3+</sup> to the ferrous (Fe<sup>2+</sup>) state; subsequently, ferene S (3-(2-pyridyl)-5,6-bis(2,5-furylsulfonic acid)-1,2,4-triazine) chelated Fe<sup>2+</sup> to form a bright blue colored product, with an absorbance at 594 nm. The OD<sub>594</sub> readings were converted to iron concentrations ( $\mu$ M) using the equation below:

$$[Fe^{3^+}] = (OD_{594} - Blank) \times 3.$$
(5)  
0.0355

#### SPIKING PROTOCOL FOR OUTDATED BUFFY COAT PLATELETS

The following procedures were done in a biological safety cabinet. All necessary materials were labeled and disinfected with 70% ethanol prior to entering the sterile environment. Aseptic technique was maintained throughout each experiment to avoid exogenous contamination of the platelets.

## Preparation of Staphylococcus epidermidis and DFO

A 24 hour *S. epidermidis* culture (prepared as described above) was serially diluted in sterile saline to obtain a  $10^3$  and a  $10^2$  cfu/mL suspension. The amount of dry DFO needed to

produce 25, 50, and 500 mM stock solutions was calculated and weighed out in 15 mL conical tubes. DFO aliquots were wrapped in aluminum foil, and dissolved in sterile saline in the biological safety cabinet immediately before inoculating BPA bottles.

#### **Sampling of Unspiked Platelet Unit**

Outdated, leukoreduced buffy coat platelets were generously donated from normal, healthy volunteers, and prepared by Canadian Blood Services (CBS) in Vancouver, British Columbia. Platelet units were kept on a platelet agitator at 22°C until ready to use and, after experiments, discarded according to the CBS biohazard protocol.

Strict aseptic technique was used when sampling from platelet units. First, the plastic closure of the platelet bag was disinfected with an alcohol swab for 30 seconds, and removed and discarded into a biohazard container. A sterile sampling site coupler (Fenwal Inc., Round Lake, IL, USA) was then inserted in place of the plastic closure and swabbed with alcohol for 30 seconds. A 21G-1 inch bevel needle (Becton Dickinson) was threaded into a vacutainer holder (Becton Dickinson) and carefully inserted into the sterile sampling site coupler. To confirm that the platelet unit was not previously contaminated, a 3 mL sample was drawn into a no additive Vacutainer tube (Becton Dickinson), and later Gram stained and plated on nutrient agar. Nine mL of buffy coat platelets were subsequently inoculated into each labeled, BacT/ALERT aerobic culture bottle (BPA bottle) (bioMérieux, St. Laurent, QB, Canada), as illustrated in Figure 9. BPA bottles were disinfected with an alcohol swab before and after adding platelets.

#### **Spiking BPA Bottles with DFO**

A 1 mL syringe (Becton Dickinson) attached to a 20G-1 inch bevel needle (Becton Dickinson) was used for each stock DFO solution. After disinfecting the BPA bottles containing platelets, 1 mL of the 25 mM DFO solution was injected into each of its corresponding BPA

bottles, giving a final concentration of 0.5 mM DFO. Spiking continued with the 50 and 500 mM stock DFO solutions to give final concentrations of 1 and 10 mM DFO, respectively.

## Spiking BPA Bottles with Staphylococcus epidermidis

*S. epidermidis* suspensions (10<sup>3</sup> and 10<sup>2</sup> cfu/mL) were placed on a vortex and inoculated into their designated BPA bottles as explained above for DFO. The initial bacterial concentrations relative to the volume of platelets (9 mL) added were 10<sup>2</sup> and 10<sup>1</sup> cfu/mL, respectively. To validate these concentrations, 0.1 mL from each *S. epidermidis* suspension was spread around a nutrient agar plate and incubated at 37°C for 18-24 hours.

## **BacT/ALERT Automated Microbial Detection System**

Within 1 hour of inoculation, spiked BPA bottles were taken to the BacT/ALERT 3D Combination Module System (bioMérieux) located at the Canadian Blood Services' NetCAD Laboratory (University of British Columbia, Vancouver, BC, Canada). Samples were identified to the instrument by scanning the barcode on the bottles and then typing in an identification name. Bottles were placed in the BacT/ALERT system until recognized as positive, or, if negative, for a maximum of 6 days. Figure 9. Schematic for spiking BPA bottles with platelets, DFO, and S. epidermidis.



## VALIDATION OF BACT/ALERT SYSTEM

All samples reported as positive by the BacT/ALERT System were subjected to manual tests (described below) to validate the presence of bacteria.

## **Gram Staining**

Ten μL samples from each positive BPA bottle was removed and spread evenly across a 75 x 25 x 1 mm frosted microscope slide (Corning, Corning NY, USA). Once dry, slides were heat fixed over a Bunsen burner, and stained using a Gram's Stain Kit (Becton Dickinson). Slides were looked at under a Zeiss light microscope (Carl Zeiss Canada Ltd., Toronto, ON), using a 100 X oil immersion lens. Digital photographs of representative fields were captured as TIF files using a digital camera (Sony DXC-950P) mounted on the microscope. Images were saved as tiff files.

#### **Viable Counts**

Additionally, positive samples were serially diluted  $10^{-1}$  to  $10^{-8}$  (as described above), and plated on nutrient agar to further verify the presence of bacteria.

#### STATISTICAL ANALYSIS

In order to investigate the influence of deferoxamine or phytic acid on *S. epidermidis* growth, for each concentration of each chelator the mean and standard deviation (SD) were calculated. Using the mean and SD values, growth curves were plotted for each chelator concentration over 72 hours, and the curves were compared using ANOVA. The statistical software used was SigmaStat Version 3.11 (Systat Software Inc., San Jose, CA, USA). When significant differences were found by ANOVA, concentrations were compared using Holm-Sidak paired *t*-tests.

For bacterial growth in the presence of RBCs and/or DFO, the number of cfu/mL for each condition was normalized to the positive *S. epidermidis* control. The normalized data was then log transformed to better approximate the distribution. Duplicate values for each condition were averaged and compared using ANOVA. When ANOVA revealed significant differences, Holm-Sidak *t*-tests were performed to compare the mean values between conditions.

For assessing RBC degradation, the percentage of RBC lysis and methemoglobin, and the levels of  $Fe^{3+}$  were determined on day 0, 1, 3, 5, and 7. At each time point, the mean and SD for each test was calculated, and the mean values subsequently compared using ANOVA. A Holm-Sidak *t*-test was then performed when ANOVA identified significant differences.

Lastly, for bacterial growth in buffy coat platelets, at each condition the mean and SD were calculated and compared by ANOVA. Pair wise comparisons were performed using the Holm-Sidak *t*-test when significant differences were found by ANOVA.

## III. RESULTS

#### STANDARD GROWTH CURVE FOR STAPHYLOCOCCUS EPIDERMIDIS

Bacterial cultures were prepared in NB and incubated at 37°C for 24 hours. To establish a growth curve for *S. epidermidis*, samples were prepared as described in the Materials and Methods section, and the OD<sub>600</sub> of each was measured (n=4). Figure 10 shows the average OD readings versus time in hours, from which we established the lag, log, stationary, and death phases of bacterial growth. To determine the relationship between OD<sub>600</sub> and the bacterial concentration, tenfold serial dilutions (Figure 8) of 24 hour cultures were plated and bacterial colonies were counted following incubation at 37°C for 24 hours (n=3). A graphic presentation of the positive linear correlation between the mean OD<sub>600</sub> (y) readings and the number of bacteria counted (x) is shown in Figure 11. The equation of the best-fit line was derived as y = 0.026158 - 0.26086x (r = 0.996), or alternatively, 1 OD<sub>600</sub> = 3.73 x 10<sup>8</sup> cfu/mL. Using the latter equation, we standardized the initial inoculum concentration of bacteria for all subsequent experiments.



Time (hours)

**Figure 10.** Growth curve of *S. epidermidis* cultured in nutrient broth and incubated for 72 hours at 37°C (n=4). The graph is of OD at 600 nm versus time in hours, and highlights the (A) lag, (B) log, (C) stationary, and (D) death phases of *S. epidermidis* growth. (A) During the lag phase, growth marginally increased as the bacteria adapted to their environment. (B) After 4 hours, *S. epidermidis* entered their log phase of growth, as illustrated by the rapid increase in growth. (C) Subsequently, *S. epidermidis* growth reached a plateau or stationary phase, indicating a decrease in nutrients and the number of living bacteria. (D) As nutrient levels further decreased, the number of living bacteria further declined over time in the final death phase of growth.



Figure 11. Optical density readings at 600 nm have a linear relationship with bacterial concentration of a 24 hour *S. epidermidis* culture (n=3).

# EFFECT OF IRON CHELATORS ON BACTERIAL GROWTH IN CULTURE MEDIUM

## Deferoxamine

To assess the effect of DFO on bacterial growth in culture medium, *S. epidermidis* was cultured in NB and treated with various concentrations of DFO (0, 3.5, 5, 10, 50, 100  $\mu$ M) (n=3). Samples were measured by OD<sub>600</sub>, and the mean of the readings were plotted versus time in hours in Figure 12. All conditions were significantly greater than the negative control at all time points (p < 0.002). From 0 to 6 hours, there was no significant difference between the conditions; however, at 8 hours, the positive control (0  $\mu$ M DFO) was significantly greater than the 5, 10, 50, and 100  $\mu$ M DFO groups (p < 0.002), but not significantly different from the 3.5  $\mu$ M DFO group. These results suggest that the bacteria initially used pre-existing intracellular iron stores arising from growth in iron-rich NB medium to support their growth (136, 153). After 8 hours, the OD<sub>600</sub> readings significantly decreased with increasing concentrations of DFO up to 48 hours (p < 0.009). Thus bacterial growth was inhibited by DFO in a dose dependent manner.



Figure 12. DFO inhibits *S. epidermidis* growth in NB in a dose dependent manner (n=3). Importantly, after 6 hours bacterial growth significantly decreased in the presence of increasing concentrations of DFO (p < 0.009).

#### Phytic Acid

Phytate, a natural iron chelator with a high affinity for Fe<sup>3+</sup>, was expected to behave similarly to DFO. Surprisingly, in the presence of low phytate concentrations (0.5, 1, 5 mM), bacterial growth significantly increased in a dose dependent manner in comparison to that of the positive control (p < 0.001) (Figure 13A), suggesting that the phytate-iron complex was readily used by the bacteria. After 24 hours, no difference in growth was found between these phytate concentrations and the positive control.

Subsequently, we investigated whether this growth enhancement could be overcome by increasing the concentration of phytate. In the presence of 100 and 1000 mM phytate, bacterial growth was significantly less than *S. epidermidis* grown without phytate up to 48 hours (p < 0.001) (Figure 13B). As shown in the graph, bacterial growth in presence of 100 mM phytate was significantly decreased, but initially followed the same curve as the positive control. After 12 hours, however, the growth curve began to increase, eventually becoming greater than all other conditions at 72 hours (p < 0.001). In contrast, the OD<sub>600</sub> of the 1000 mM condition was significantly greater than all other conditions from 0 to 4 hours (p < 0.008), but decreased over time. By 6 hours, the 1000 mM phytate sample was significantly lower than the positive control and 100 mM phytate, and similar to that observed in the negative control, suggesting that very high phytate levels exhibited bactericidal activity.



Figure 11. Phytic acid (A) promoted and (B) inhibited *S. epidermidis* growth in NB at different concentrations. (A) At  $\leq$  5 mM, phytate significantly enhanced bacterial proliferation (p < 0.001) (n=2). (B) Conversely, phytate decreased bacterial growth at 100 mM, and dramatically inhibited growth at 1000 mM (p < 0.001) (n=3).

#### **RED BLOOD CELLS DEGRADE DURING 7-DAY STORAGE**

To determine the effect of storage time on red blood cells, RBCs were suspended in HBSS to a final hematocrit of 1 and 5%, and incubated at 37°C for 7 days. On days 0, 1, 3, 5, and 7, the percent lysis, percent methemoglobin, and iron levels were measured spectrophotometrically to assess red cell changes. For the 1% hematocrit suspension, the percent RBC lysis steadily increased between days 0 to 5, and significantly increased by day 7 (p < 0.001) (Figure 14A). Similarly, the methemoglobin levels in the 1% hematocrit remained constant up to day 5, and then significantly increased by day 7 (p < 0.001) (Figure 14B). With the 5% hematocrit, change in percent hemolysis and methemoglobin (Figure 14A and B, respectively) was gradual between days 0 to 3, but significantly increased by day 5 and furthermore by day 7 (p < 0.001).

These results demonstrate that iron is clearly released by residual RBCs over the storage time of platelet concentrations. This iron would potentially be bioavailable for bacterial replication. The accelerated lysis, methemoglobin generation, and iron release are likely accelerated by glucose depletion and the subsequent metabolic failure of the residual RBCs. The presence of platelets or bacteria would further exacerbate the rate of glucose utilization.



Figure 14. RBCs break down as storage time increases. During 7-day storage at 37°C, both 1% ( $\triangle$ ) and 5% hematocrit  $(\Box)$  suspensions showed an increase in (A) percent RBC lysis, (B) percent methemoglobin, and (C) iron levels, verifying that break down of RBCs over time (n=3). HBSS, in which RBC suspensions were prepared, served as a negative control  $(\bullet)$ .

## BACTERIAL GROWTH INCREASES IN THE PRESENCE OF RBCS AND/OR DFO

Based on the above finding, 1% hematocrit solutions treated with various concentrations of DFO (25-1000  $\mu$ M) were prepared to determine whether: (a) iron released from aged RBCs contributes to bacterial growth, and (b) DFO can chelate this iron and inhibit bacterial proliferation. Samples were prepared in HBSS, which provided minimal nutrients and an appropriate pH for cell survival, but did not support bacterial growth as compared to growth in NB (Figure 15). As shown by the positive control, in the absence of an iron source, *S. epidermidis* actually died out over 24 hours. However, proliferation in the presence of RBCs was drastically increased over 24 hours. Interestingly, growth also increased in the presence of RBCs and different DFO concentrations, and no significant difference was found between growth with 1% hematocrit and with RBCs and DFO (p > 0.05). This may have resulted from a known adverse interaction between DFO and RBCs (239).

To test this, we compared RBC break down over time in the presence and absence of various concentrations of DFO. First, we observed a similar trend in percent lysis of RBCs, with and without DFO (Figure 16), as compared to our earlier results (Figure 14A). Specifically from day 3 to day 7, the positive control and RBCs mixed with 500 and 1000  $\mu$ M DFO showed a significant increase in hemolysis (p < 0.007). On day 3 and day 5, the positive control and the 500  $\mu$ M DFO condition, respectively, showed the greatest increase in percent lysis (p < 0.002 and < 0.001, respectively) in comparison to all other conditions. By day 7, all samples showed a significant increase in hemolysis from that on day 0 (p < 0.007), but all conditions were not significantly different from each other (p > 0.02). Second, in contrast to the gradual increase in percent methemoglobin seen in Figure 14B, Figure 17 shows that methemoglobin levels increased more rapidly over time in the presence of increasing concentrations of DFO (p < 0.005). Similarly, the change in iron levels during the 7-day storage period was dependent on DFO concentration (Figure 18). Over time, iron levels significantly increased with decreasing

concentrations of DFO (p < 0.005). Importantly, iron levels were below the detectable limits of the spectrophotometer for the 500 and 1000  $\mu$ M DFO conditions. Thus DFO clearly increased methemoglobin formation, which may have accelerated iron release within the RBC, but did not increase hemolysis. While DFO did chelate iron, it would be in competition with any bacteria to capture this iron.



Figure 15. RBCs promoted bacterial proliferation from 0 ( $\blacksquare$ ) to 24 hours ( $\Box$ ) in minimal medium in both the absence and presence of DFO (n=3). Conversely, neither HBSS nor HBSS + 1000  $\mu$ M DFO supported bacterial growth, as illustrated by the decline of the log scale number of cfu/mL from 0 to 24 hours.



Figure 16. Presence of DFO did not have an impact on RBC lysis during 7-day storage (n=3).



Figure 17. Production of methemoglobin increased over time in the presence of increasing concentrations of DFO (p < 0.005) (n=3).



Figure 18. Iron released from RBCs was effectively chelated by DFO in a dose dependent manner (n=3).

## **DEFEROXAMINE INHIBITS BACTERIAL GROWTH IN BUFFY COAT PLATELETS**

To more closely approximate actual platelet storage conditions, the effect of DFO on microbial growth in platelet concentrates was assessed using the BacT/ALERT system. In contrast to our experiments in NB, buffy coat platelet samples were treated with various concentrations of DFO (0, 0.5, 1, 10 mM) and subsequently spiked with *S. epidermidis* to a final concentration of  $10^1$  or  $10^2$  cfu/mL. Bacterial concentration of the inoculums was determined by  $OD_{600}$  using equation (2). Figure 19A shows the time needed for the BacT/ALERT to detect the presence of bacteria in buffy coat platelets with the inclusion of DFO.

The average detection time for the positive *S. epidermidis* control at an initial inoculum of  $10^{1}$  cfu/mL was ~19 hours (SD, 0.24 hours) and was ~17 hours (SD, 0.13 hours) at the  $10^{2}$  cfu/mL level (Figure 19A). Both average detection times correspond with the literature and reflect the mean time it took *S. epidermidis* of the same inoculum levels to reach the log phase of growth in NB (Figure 19B) (93, 240). Therefore, the time to detect *S. epidermidis* growth is dependent on the initial bacterial concentration, and is comparable between the BacT/ALERT system and by OD<sub>600</sub>.

More importantly, the addition of DFO dramatically delayed the time needed to detect *S. epidermidis* growth by the BacT/ALERT system (p < 0.001) (Figure 19A). For example, in samples with a 10<sup>2</sup> cfu/mL inoculum, the presence of 1 mM DFO prolonged the detection time to ~40 hours, while 10 mM DFO was not positive until ~52 hours. These DFO concentrations further delayed growth in platelets with 10<sup>1</sup> cfu/mL to ~49 and ~58 hours, respectively. Evidently, the inclusion of DFO had a significant bacteriostatic effect on *S. epidermidis* in platelet concentrates, which supports our hypothesis (see Figure 7). Moreover, our data demonstrate that iron chelators have potential for creating a safer platelet product and eliminating the transmission of bacteria during transfusion.



Figure 19. (A) DFO inhibits bacterial growth in buffy coat platelets in a dose dependent manner (p < 0.001), as determined by the BacT/ALERT system (n=3). The time to detect *S. epidermidis* growth is dependent on the initial bacterial concentration, and is comparable between (A) bacteria cultured in platelets and measured by the BacT/ALERT system, and (B) bacteria cultured in NB and measured by OD<sub>600</sub>.

#### V. DISCUSSION

Platelets are responsible for the majority of transfusion-transmitted bacterial infections (1, 2, 93). Table 2 outlines the strategies that have been developed and implemented to avoid and detect bacterial contamination of platelet products. As previously noted, both topical antiseptic and/or diversion pouches do not fully eliminate the risk of contamination from the skin plug or from donors with asymptomatic bacterial infections (15, 92). Likewise, bacterial detection systems can miss the presence of slow growing microbes, resulting in recipient sepsis and even death. When bacteria are detected, contaminated units must be discarded. This is especially problematic when blood banks experience platelet shortages or when uncontaminated units are available but are not the appropriate blood-type for recipients that urgently require a platelet transfusion. Discarding units is also wasteful and costly, in terms of materials, processing the units, and training lab personnel, as well as a loss to recipients who need platelets.

A more cost-effective approach would be to target any bacteria present in the bag by inhibiting their growth. Table 3 is a summary of our hypotheses, and the corresponding results and their implications. We first investigated whether the inclusion of the iron chelator, DFO, in culture medium and in platelet concentrates would inhibit bacterial growth. Upon addition of DFO to nutrient broth, the growth of *S. epidermidis* was significantly inhibited in a dose dependent manner (see Figure 12), thus verifying the results by Matinaho *et al* (7). We observed a similar dose dependent response to DFO in buffy coat platelet samples, as will be discussed later in this section.

In addition to DFO, we evaluated the effect of phytic acid on bacterial growth in nutrient broth. Despite its known ability to bind iron and our hope to use it as a non-toxic chelator, phytate at 0.5, 1, and 5 mM promoted bacterial growth in a dose dependent manner (see Figure 13A). In contrast, bacterial growth decreased with the addition of 100 mM phytate and was prevented at 1000 mM (see Figures 13B). These findings suggest that phytate has a bacteriostatic

Hypothesis	Result (Yes/No)	Figure (page)	Implication(s)
(1) DFO inhibits bacterial growth in nutrient broth.	Yes	12 (p. 44)	DFO effectively removed iron in culture medium, thus limiting <i>S. epidermidis</i> growth.
(2) Phytic acid inhibits bacterial growth in nutrient broth.	No (≤ 5 mM) Yes (≥ 100 mM)	13 (p. 46)	Low concentrations of phytic acid supplied <i>S. epidermidis</i> with nutrients to grow. Higher concentrations inhibited growth, thus demonstrating phytate's bacteriostatic/bactericidal capability.
(3) RBCs degrade over time and release iron.	Yes	14 (p. 48)	Bioavailable iron $(Fe^{3+})$ released from RBCs increased over time and was dependent on hematocrit.
(4) RBCs release iron during storage that promotes bacterial growth in minimal medium.	Yes	15 (p. 51)	As storage time increases, residual RBCs in platelet bags may provide additional nutrients for contaminating bacteria to use to proliferate.
(5) DFO inhibits bacterial growth in the presence of RBCs ( <i>i.e.</i> , iron) in minimal medium.	No; however, DFO increased methemoglobin production in a dose dependent manner.	15 (p. 51); 17 (p. 53)	Interaction between DFO and free hemoglobin may contribute to bacterial growth by facilitating iron release from RBCs. Other iron chelators may be more suitable and should be investigated (see Table 4).
(6) DFO chelates iron released by RBCs during storage in minimal medium.	Yes	18 (p. 54)	DFO effectively removed iron released from RBCs in a dose dependent manner, but becomes saturated due to high iron content of RBCs.
(7) DFO inhibits bacterial growth in platelet concentrates.	Yes	19A (p. 56)	In platelet concentrates, DFO effectively reduced <i>S.</i> <i>epidermidis</i> proliferation, thus increasing the "safe" status of the platelet sample from ~19 hours to ~58 hours.

# Table 3. Summary of our hypotheses, results, and their implications.

effect at higher concentrations. However, because phytate at concentrations  $\leq 5$  mM enhanced bacterial growth, it is not a favorable chelator for preventing microbial growth in platelet products. Additionally, phytate was very difficult to dissolve at 100 mM and 1000 mM, and thus would not be practical to prepare and infuse into platelet concentrates.

Our second hypothesis was to determine whether RBCs released iron that would promote bacterial proliferation in platelet products. First, we assessed RBCs changes over time by measuring hemolysis, methemoglobin, and iron. A significant increase was observed for all three tests during the 7-day storage period (see Figure 14), indicating that RBCs, and consequently hemoglobin, break down over time. This corresponds to the normal hemolysis of old or damaged RBCs in the body, and the release of iron from degraded hemoglobin, as described in the introduction. Second, knowing that  $Fe^{3+}$  is released from RBCs over time, we evaluated bacterial growth in the presence and absence of RBCs (*i.e.*, iron). In an iron-poor medium, S. epidermidis did not proliferate; the addition of RBCs, however, provided the necessary bioavailable iron for bacterial replication (see Figure 15). Surprisingly, no bacteriostatic effect was seen with the addition of DFO. This outcome may be associated with the elevated methemoglobin levels we observed in the presence of DFO (see Figure 17). Our finding is supported by Rice-Evans et al. who showed that free hemoglobin released from lysed RBCs interacts with DFO, resulting in methemoglobin formation (239). Incidentally, methemoglobin is known to lose iron at a faster rate than normal hemoglobin (8). Thus the inclusion of DFO in RBC suspensions may have accelerated hemoglobin oxidation and iron release, which consequently overcame the inhibitory effect of DFO.

Although DFO did not reduce bacterial growth in 1% hematocrit suspensions, it had a dramatic bacteriostatic effect in buffy coat platelet concentrates. As predicted, platelets inoculated with  $10^2$  cfu/mL at a given DFO concentration were detected by the BacT/ALERT earlier than those with  $10^1$  cfu/mL. Subsequently, the time it took to detect a positive reading for

each bacterial concentration significantly increased with increasing concentrations of DFO (p < 0.001). In fact, as illustrated in Figure 19A, DFO sustained a bacteriostatic effect for up to 58 hours — more than three times longer than the time to detect bacterial growth in the absence of DFO. This delay in growth would add a substantial margin of safety during platelet storage. Furthermore, our results show that the inclusion of an iron chelator is a positive step towards preventing transfusion-related sepsis in recipients.

Despite these promising results, a disadvantage of DFO is that it is a microbial siderophore produced as a means to obtain iron from the environment or from host iron-binding proteins (127, 128, 130, 131, 133, 134, 148, 149, 154, 241). Although we showed that DFO does not promote *S. epidermidis* growth in culture medium or in buffy coat platelets, other bacterial platelet contaminants (Table 1) may be able to utilize DFO if it is added to platelet products. Since the purpose of adding chelators to platelet products is to inhibit microbial growth, further studies subjecting an array of bacterial species to DFO would help to determine this chelator's potential as a bacteriostatic agent in platelet concentrates.

Additionally, with phytic acid's ability to act as a nutrient, and with the known clinical toxicities of DFO and its potential to help bacteria sequester iron, other iron chelators must be investigated. Table 4 describes the properties of various iron chelators that have been studied as an alternative to DFO in chelation therapy. One synthetic chelator of particular interest is HBED  $(N,N^{-})$ -bis(o-hydroxybenzyl)ethylenediamine- $N,N^{-}$ -diacetic acid), which, like DFO, binds Fe<sup>3+</sup> in a 1:1 ratio with high affinity and specificity (188, 209, 242, 243). Moreover, numerous *in vitro* and *in vivo* models have demonstrated the lack of toxicity of HBED, as well as its efficiency in excreting iron when administered orally or parenterally to rodents and primates (188, 209, 242, 243). Another attractive chelator is a modified version of DFO called 40SD02. Attaching a starch polymer to DFO creates a high molecular weight compound (260 kDa) with the same specificity and affinity for iron (173, 188, 205, 244). The 40SD02 chelator is also able to remain in the

## Table 4. Properties of chelators of interest for inhibiting bacterial proliferation in platelet products.

Chelator	Natural or Synthetic	Size	Chelator to Iron ratio	Route of administration	Adverse effect(s) & Limitations*
<b>DFO</b> (deferoxamine)	Natural ( <i>S. pilosus</i> )	561 Da	1:1	Intravenous or subcutaneous infusion	Associated with local and systemic reactions; expensive; limited access to intracellular iron pools as DFO cannot penetrate cell membranes; short plasma half-life; low patient compliance.
<b>Phytic acid</b> (phytate)	Natural (plant)	660 Da	ND**	ND	ND
ICL670 (deferasirox)	Synthetic	373 Da	2:1	Oral	Mild nausea, vomiting, headaches; acute renal failure; cytopenias; hypersensitivity reactions; expensive.
LI (deferiprone)	Synthetic	139 Da	3:1	Oral	Agranulocytosis; may penetrate blood brain barrier.
HBED ( <i>N</i> , <i>N</i> '-bis(o- hydroxybenzyl) ethylene- diamine- <i>N</i> , <i>N</i> '- diacetic acid	Synthetic	386 Da	1:1	Oral or subcutaneous infusion	
40SD02 (starch-DFO)	Synthetic	260 kDa	1:1	Intravenous	Pruritis, urticaria

\*Data from references (172, 173, 179, 188, 192, 194, 196, 198, 199, 205, 209, 214, 215, 218, 242-244)

\*\*ND, not defined.

plasma longer, and does not cause the acute adverse effects of DFO. Both these chelators are potential candidates for the treatment of iron overload and, therefore, may be suitable agents for removing iron in platelet products.

In summary, we showed that DFO has a dose dependent bacteriostatic effect on microbial growth in culture medium. In comparison, phytic acid was not a consistent bacteriostatic agent as it both promoted and inhibited *S. epidermidis* growth at different concentrations. We also demonstrated that RBCs contribute bioavailable iron necessary for microbial growth. Thus residual RBCs in platelet concentrates may be an important food source for bacterial contaminants thereby prolonging their survival and increasing their chances of infecting platelet transfusion recipients. Subsequently, we found that DFO did not reduce bacterial growth in a 1% hematocrit solution. The addition of DFO, however, appeared to increase methemoglobin production in a dose dependent manner suggesting a possible interaction between DFO and free hemoglobin. Lastly and most importantly, the inhibitory effect of iron chelation was demonstrated in platelet concentrates spiked with *S. epidermidis*. In a dose dependent manner, DFO dramatically prolonged the time to detect bacterial growth by the BacT/ALERT system.

#### **FUTURE DIRECTIONS**

Prevention of bacterial growth in platelet concentrates using iron chelators seems very promising. In addition to evaluating various chelators in platelet concentrates, iron chelators should be assessed in platelet additive solutions (PAS). PAS are synthetic, glucose-free solutions designed as an alternative medium for storing platelets (245). The use of PAS has been shown to improve platelet preservation and has been well-received by recipients after 4 to 12 day storage (56, 245, 246). PAS, however, does not possess transferrin or ferritin, which are natural bacteriostatic chelating agents found in plasma. Supplementing PAS with iron chelators,
therefore, would not only diminish the risk of transfusion-induced infection, but also improve the storage conditions and quality of platelet concentrates.

Subsequently, it is important to determine how chelators impact platelets in both plasma and PAS. Assessing platelets *in vitro* by morphology, platelet counts, ability to aggregate, hypotonic shock response, and protein and CD marker expression will determine whether iron chelators alter platelet structure and/or function. The latter can be further evaluated *in vivo* via bleeding times and platelet survival studies to help determine whether iron chelators are feasible as a means to inhibit microbial growth in platelet concentrates. Pending these results, inclusion of iron chelators could prevent transfusion-transmitted bacterial infection and provide patients with a safer platelet product.

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