UNIVERSITY OF BIRMINGHAM

ANTIMICROBIAL ACTIVITY OF BLOOD-DERIVED BIOMATERIALS AGAINST DIFFERENT BIOFILM-FORMING BACTERIA STRAINS COMMONLY FOUND IN WOUNDS

by

FEDERICA D'ASTA

A thesis submitted to the University of Birmingham

For Post-graduate Master of Science degree

Supervisor: Prof Janet Lord, Dr Paul Harrison

Institute of Inflammation & Ageing
College of Medical and Dental Sciences
Institute of Biomedical Research
University of Birmingham
Edgbaston, Birmingham

July 2017

Abstract

Platelet-Rich Plasma (PRP) and Leucocytes-PRP (L-PRP) are blood products widely used to promote wound healing that have also shown antimicrobial activity. The aim of the study was to perform a systematic review of the literature on the contribution of leucocytes included in PRP preparations in terms of their antimicrobial properties and to conduct in vitro studies to explore the antimicrobial properties of blood products against free bacteria and bacteria-forming biofilm. Assays included growth kinetics and crystal violet biofilm formation assays.

The systematic review revealed that the limited numbers of studies so far conducted did not allow determination of the specific contribution of leucocytes to the bactericidal effect of PRP preparations. However, overall, PRP preparations do de monstrate bacteriostatic properties against the majority of the bacterial strains tested in the literature. From the in vitro studies carried out here the only preparation showing growth control of *Staphylococcus aureus* was the L-PRP preparation, where the leukocyte and neutrophil content of the preparation both significantly influenced bacterial growth kinetics. PRP and PPP (Platelet Poor Plasma) preparations significantly reduced *Pseudomonas* and *Acinetobacter* growth over a 12-hour period. Against the Gram-negative bacteria, all the biomaterials tested gave a reduced level of biofilm biomass formation.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisors Prof Janet Lord and Dr Paul Harrison for their continuous support of my research, for their patience, motivation, and immense knowledge.

I would like to express the deepest appreciation to Mark Webber (Institute of Microbiology and Infection) for his substantial guidance in regards the microbiological aspect of my research. Also a sincere thank you goes to Hemalata Chahal, Robert Dinsdale, and Fenella Halstead, who have patiently taught me all the laboratory techniques used in this project. Without their guidance and help, this research would not have been possible.

Results described in this thesis would not have been obtained without a close collaboration with Jonathan Bishop, who has considerably contributed to the statistical analysis of the data.

I also would like to thank the volunteers who donated blood for my experiments during my research.

I take this opportunity to sincerely acknowledge the Scar Free Foundation, for providing financial support for my study, and Mr Naiem Moiemen for his guidance, support, and constructive criticism. My sincere thanks also go to Birmingham Children's Hospital, Burns Centre Team, in particularly Miss Yvonne Wilson and Sister Clare Thomas, who provided me with the opportunity to dedicate time to this research. Without their precious moral support and encouragement it would not be possible to conduct this research.

Finally, I would like to acknowledge friends and family who supported me during these two years.

Table of Contents

List of tables and illustrations

Figure 4.24 MSSA_F77 (expressed as absorbance) over time plotted for different concentration of isolated neutrophils

List of abbreviations

AA: Acetic acid

ACD-A: citrate dextrose A

CI: confidence interval

EDTA: ethylene diamine tetra acetic acid

EPS: extracellular polymeric substance

ESBL: extended spectrum beta lactamase

HSSB: Hanks' Balanced Salt Solution

IGF-1: insulin-like growth factor 1

ISTH: international society on thrombosis and haemostasis

LB: lysogeny broth

L-PRP: leucocyte platelet rich plasma

MMPs: matrix metalloproteinases

MH: Mueller Hinton

MRSA: methicillin resistant Staphylococcus aureus

MRSE: methicillin-resistant Staphylococcus epidermidis

MSSA: methicillin-sensitive Staphylococcus aureus

MSSE: methicillin-sensitive Staphylococcus epidermidis

PBS: phosphate buffered saline

PDGF: platelet-derived growth factor

PDGF-BB: platelet-derived growth factor BB

PLT: platelet

PMPs: platelet microbicidal proteins

PPP: platelet poor plasma

PRP: platelet rich plasma

ROS: reactive oxygen species

RANTES: regulated on activation normal T cell expressed and secreted

SCC: scientific and standardisation committee

TGF: transforming growth factor

TNF: tumor necrosis factor

VEGF: vascular endothelial growth factor

WBC: white blood count

CHAPTER 1: INTRODUCTION

Over the last few years, one of the hottest areas in regenerative medicine has been research focusing on enhancing and promoting tissue repair and regeneration [1]. Burns and chronic wounds represent a continuing challenge for physicians in terms of treatment, and for healthcare services in terms of elevated costs [2, 3]. The infection of a wound is one of the major contributors to delays in wound healing and tissue regeneration [4, 5]. As multidrug resistance to antibiotics is becoming a serious threat [6], research in this field has focused on finding new agents and strategies to fight infection [7] and additionally to reduce healing times. Recent literature suggests that Platelet Rich-Plasma (PRP), a blood-derived component used in wound care as a topical biological accelerator of the healing process, can also exert antimicrobial activity. Leucocyte-Platelet Rich-Plasma (L-PRP) is the terminology used when leucocytes are included in PRP products [8].

This research study aims to explore the antimicrobial properties of PRP products against free bacteria and bacteria forming biofilms commonly found in wounds.

1.1 Definition of PRP

The topical use of autologous Platelet Rich-Plasma (PRP) has been safely used as a biological accelerator of the healing process for wounds since the 1990s [9, 10]. PRP is a derivative of whole blood that contains an increased concentration of platelets compared to the circulating blood platelet count. A working definition of PRP has been suggested as a concentration of cells of between 4 to 5-fold higher than the circulating blood platelet count [11]. PRP can be prepared in several different methods and according to the preparation methodology the final product obtained varies widely. Many different procedures to obtain PRP have been proposed and used in clinical settings. During the

extraction and concentration of platelets from whole blood, leucocytes can either be included in the final product at different concentrations or totally excluded from the preparation. By most authors, platelet concentrates are generically termed as PRP. This term is extremely vague and incomplete as the specific biological components are not considered [12, 13]. Such variations of cellular components included in PRP are often poorly defined in published studies making it challenging to interpret and compare the clinical and antimicrobial effects of various PRP preparations between different studies [8, 14]. Recently this issue has been addressed and in an attempt to classify PRP products, two classifications have been suggested by Ehrenfest [15] and Mishra [16]. Ehrenfest suggested a classification based on the cellular components and fibrin content included in the blood product (Table 1.1).

PRP Class	Leucocytes	Fibrin
P-PRP	No	Low
L-PRP	Yes	Low
P-PRF	No	High
L-PRF	Yes	High

Table 1.1 Ehrenfest PRP classification system

In the Mishra classification system PRP products are divided according to their white cell inclusion, method of activation and platelet concentration (Table 1.2).

	Leucocytes	Activation	Platelet Concentration
Type 1	Increased	No activation	A >5x B <5x
Type 2	Increased	Activated	A >5x B <5x
Type 3	Minimal or no WBC	No activation	A >5x B <5x
Type 4	Minimal or no WBC	Activation	A >5x B <5x

Table 1.2. Mishra PRP classification system

1.2 Methods of PRP preparation

It has been shown that several factors contribute to the possible antimicrobial and healing efficacy of PRP products [19]. The presence and concentration of the different components included in PRP preparation, the methodology used to prepare the product and the methodology used to activate the platelets will all contribute to efficacy.

1.2.1 Blood collection and anticoagulant choice

To prevent activation/damage of platelets due to mechanical stress, a large bore needle is recommended to draw blood and usually 10% recovery of PRP is gained from a set blood volume. In order to prevent early platelet activation and clot formation, it is important to include an anticoagulant. Different anticoagulants have been suggested to be valid such as citrate dextrose A (ACD-A) and trisodium citrate solution [17, 18]. The use of Ethylene Diamine Tetra Acetic acid (EDTA) is not recommended as it has been shown to activate/damage a large number of platelets [12].

1.2.2 Centrifugation

At the International Society on Thrombosis and Haemostasis (ISTH), the platelet physiology subcommittee of the Scientific and Standardisation Committee (SSC) recommended to use a set g value for platelet preparation [19]. Using a single step low g centrifugation (170 – 200 g) of anticoagulated blood for 10 minutes at 21°C, a relatively pure preparation and reasonable harvest of PRP can be easily achieved [20]. The temperature recommended is 21°-24°C [12]. Several methods using different centrifugation speeds are available to prepare PRP have been reported but most authors agree a two-step centrifugation technique is best. Following a ‘soft’ spin centrifugation three layers become apparent: the bottom layer comprises red blood cells, the middle layer includes platelets and white blood cells (also referred as “buffy coat”) and the top layer

contains plasma and platelets. The obtained supernatant plasma is transferred into another tube and a second 'high' speed centrifugation is performed and a platelet pellet at the bottom of the tube is obtained. The upper layer consists of platelet-poor-plasma (PPP) and it is used to re-suspend the pellet to a desired final platelets concentration. After multiple attempts, Dhurat *et al* [12] described that a first centrifugation of 900g x 5 min, followed by a second centrifugation of 1000g x 10 min at 16°C, gave over 1 million/ml platelets yield. With this method a large variability of red cell and leukocyte contamination must be addressed. If the collection and inclusion of leucocytes into the PRP preparation is instead the aim of the procedure, 2 methods to obtain L-PRP are possible. The first method is the single step 'high' spin centrifugation, with the discharge of the upper layer plasma and the collection of the buffy coat layer. The second method is the same as previously described [12]: two-steps of centrifugation, including, after the first centrifugation, the collection of the entire buffy coat.

1.2.3 Activation of PRP

PRP routinely has to be activated for the platelets to release their granule constituents for full expression of their potential biological activities *in vivo* [21]. The platelets can either be activated immediately either prior to or during their application to the wound site or even left to be activated *in situ* within the damaged tissue site by naturally expressed platelet agonists e.g. collagen and thrombin [21]. As collagen is naturally present in human tissue, the activation by other means may not always be required when PRP is used clinically [22-24]. When PRP is activated, fibrinogen is converted to fibrin and a fibrin network is formed. The fibrin clot allows the capture of the released proteins, cytokines, growth factors and cells. This fibrin matrix can serve as a natural matrix which has also shown promising results in wound healing, mainly in dental applications [25]. Different

activators have been suggested and studied including thrombin, calcium chloride, collagen or mechanical trauma [12]. The safest activator seems to be calcium chloride. It has been shown that bovine thrombin can cause coagulopathy due to the cross-reactivity of anti-bovine factor V antibodies and human factor V [26, 27]. The activation with calcium chloride has the disadvantage that it takes at least 20 minutes [10], which can be a long time in a clinical setting. Autologous thrombin is another safe and fast activator and lately, autologous thrombin preparation kits are now available [28].

1.3 Why topical use of PRP in skin wound healing?

1.3.1 Wound healing

Skin is the largest organ in the human body, with a total area of about 2.0 square meters and a weight of about 6 to 10 kg in adults. Several essential functions are exerted by the skin including protection against infection, thermoregulation, and homeostasis [29]. Acting as a barrier [30], the skin provides protection from micro-organisms, photo-damage and chemicals, mechanical forces, and changes in temperature. The skin has also relevant immunological and metabolic functions. The skin is for example the major natural source of the vitamin D[31].

Skin has two anatomical layers: epidermis and dermis (figures 1.1 and 1.2).

The epidermis is the external layer of skin which is a nonvascular structure derived from ectoderm and it is composed of several layers of epidermal cells [30]. Different parts of the human body have different thickness of epidermis, being the thinnest on the eyelids (0.05mm) and the thickest on the palms and soles (1.5mm). The stratum basale is the inner part of the epidermis, composed of perpendicularly arranged columnar cells, covered by several layers of flattened cells. Stratum basale, spinosum, granulosum, lucidum and corneum comprise the overall epidermis. The skin’s colour is created by melanocytes

located within the stratum basale. Due to the production of pigment melanin, they are responsible for variations in individual skin colour. The aim of melanogenesis is to protect the lower layer of the skin, the hypodermis from the UV-B sun radiation (DNA photo-damage).

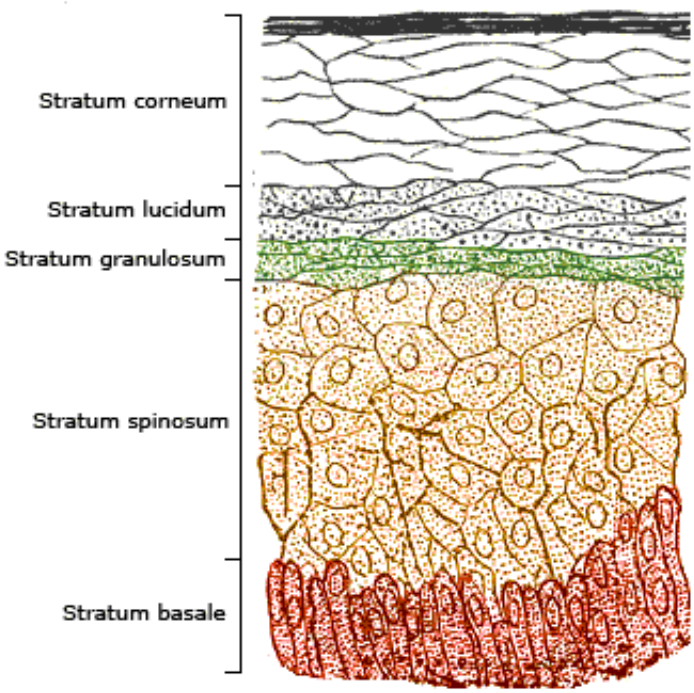


Figure 1.1: layers of epidermis
Source: https://www.boundless.com/physiology/textbooks/boundless-anatomy-and-physiology-textbook

Beneath the epidermis is the dermis which is the mesoderm derived layer. It is vascularised and largely made of collagen containing hair follicles, sweat, and sebaceous glands. The loss of epidermis, due to burns or other injuries, can be replaced by the dermal layer able to reproduce new epithelial cells. Finally, the hypodermis, beneath the dermis, is the layer subcutaneous rich fat and connective tissue. The entire epidermis is completely and spontaneously replaced over a period of about 48 days.

The stratum basale constantly replaces the squamous epithelium by cell division. Cells undergo multiple stages of differentiation, lose their nucleus, change their shape and are finally shed from the surface. Therefore, micro trauma or minor injuries affecting only the epidermis will heal itself restoring the damaged epidermis, whereas when injuries affect the dermis the body responds with a process named “wound healing”.

The aim of wound healing is to obtain a similar if not identical tissue to the one that has been lost or severely damaged. A complete tissue restoration is only seen in fetal healing [32], whilst in children and adults, the wound healing process allows to substitute the lost skin with the formation of the scar tissue, a similar tissue in function and structure, but never identical from its original condition. Several factors influence the outcome of tissue repair, and each step of the healing process is extremely relevant [33]. Any variations disturbing the controlled timely healing processes would contribute with an extension of tissue damage, a prolonged tissue repair and a worsening of cosmetic scar outcome [34]

1.3.4 PRP and wound healing

PRP has been proposed as a wound healing accelerator since the early 1990s [35]. The beneficial effects of PRP on the healing process and its reduction of local inflammation have been extensively reported in dental, orthopaedic, cardiac, gynaecologic, general surgery and plastic surgery fields [9, 13-15].

Platelets promote the wound healing process through their release of a vast array of granule components and biological mediators, including alpha-granular growth factors which promote the chemotaxis of leucocytes, fibroblasts and myofibroblasts, as well the synthesis of extracellular matrix (ECM) and promotion of neoangiogenesis [9, 11, 12]. PRP is thought to restore the complex natural physiological balance required for wound healing, and in doing so, acts to accelerate this process [8].

I*n vitro* studies showed the effect of PRP preparations added to serum-free Dulbecco's Modified Eagle Medium for the culture of human adipose–derived stem cells and human dermal fibroblasts. The effect of PRP seems to be dose dependent with a concentration of 5% of activated PRP stimulating the proliferation of dermal fibroblast and adipose-derived

stem cells, whereas a concentration of 20% did not show promotion of fibroblast proliferation [36]. Clinical studies in regards the beneficial effects of topical PRP in skin wound healing so far conducted report contrasting results [37, 38]. Positive results with significant enhancement of wound closure have been demonstrated with the use of PRP in different type of wounds [37-39]. In contrast, no benefits in terms of enhancement of epithelialisation were reported when PRP was used in donor sites and meshed split skin graft healing in chronic leg ulcers [40]. Although, in the same population, less pain was experienced by patients during the dressing when platelet concentrate was applied.

Even though several systematic reviews of the use of PRP in wound healing suggest that the application of PRP significantly improves wound healing [9, 41, 42], in 2012 a Cochrane review emphasised that there was no strong evidence to recommend the use of PRP in chronic wounds [14]. This is due to the variability of the methods used to prepare PRP and the small sample size of the trials.

In 2014, the effect of PRP specifically in treating burns wounds was summarised in a systematic review [43]. There is still currently a paucity of studies that explore the use of PRP in burns and results reported are dissimilar. When compared to silver sulfadiazine, burns treated with topical PRP showed an acceleration of the healing time [44]. Encouraging responses have been reported by the use of single growth factors (such as platelet-derived growth factor-BB, epidermal growth factor) in the treatment of burn wounds in terms of acceleration of wound healing [45, 46]. It has also been reported that the use of fibroblast growth factor can in the long term contribute to a better colour match when combined with a split skin graft [47]. Enhancement of wound healing and improvement of scar outcome has also been reported when fibroblast growth factor has been included in the treatment of second degree burns compared to placebo [48, 49].

Recently Marck *et al*. [50] conducted a randomised, double blind, intra-patient controlled study using PRP in the treatment of deep dermal burns. The authors have reported that no significant improvement was detected in terms of graft take, epithelisation and scar quality when PRP was used in conjunction to skin graft compared to skin graft only. It is relevant to notice the considerable variation of the burn population included in the study and the great difference in the PRP preparation used (concentration of platelets and leucocytes). Moreover, PRP applied in skin graft was extracted from blood of acutely burned patients. It is well known that in this population platelets are massively activated due to the peculiar nature of the burn trauma [51]. Not surprisingly, in these patients, a great fluctuation in the platelet count is seen. Platelet counts in whole blood significantly decrease by day 3 post-burn and reach a peak at day 15 post-injury [52, 53]. As the skin graft and PRP were applied at different time points between patients, it is sensible to consider that the outcomes may be also affected by this variation.

Additionally, the impact of PRP on long term scarring has not been adequately evaluated. The chemotactic effect of platelets, growth factors, and leucocytes, when included in the preparation, may contribute to a pro-inflammatory environment that may negatively influence tissue regeneration [54]. So far there are no reports of hypertrophic scarring related with PRP application in wounds. Moreover, the inclusion of leucocytes in a PRP preparation could be detrimental. The activation of platelets with the release of growth factors, could induce fibroblasts to proliferate [55] and increase collagen deposition [56] and formation of granulation tissue. TGF-β has regulatory function for the extracellular matrix formation and deposition. An animal study conducted in horses showed that the use of PRP in deep dermal burns accelerates wound repair but also induced fibrosis, which is

not a desirable long-term outcome in terms of scarring, because of the aesthetic consequences [57].

The addition of leucocytes to the wound site can also result with an increase of TGF-β1 and TGF-β2 levels [54, 58] that have both been previously associated with higher risk of hypertrophic scarring and keloid formation [59-64].

Overall, at present, even if strong evidence on the use of PRP in burns and wound healing have not been shown, negative effects of the use of PRP have also not been reported. This should encourage clinicians to continue to consider PRP as a strategy to treat wounds and to stimulate scientists to continue researches in this field.

1.4 PRP and prevention of infection

The infection of a wound is one of the major contributors to the delay in wound healing and tissue regeneration [4, 5]. With the skin significantly disrupted or destroyed following a trauma affecting the skin, patients lose the body's primary defense to infection. Plasma components, platelets, and leucocytes included in a PRP preparation are all potential contributors to the microbicidal effect of PRP documented in *in vitro* and *in vivo* studies [65]. Platelets can contribute to host defense through several mechanisms of action [66-71]. Three different types of granules are found in the platelets: α, δ and λ granules. Whilst δ and λ granules contribute mainly to the vascular tone and to the promotion of tissue regeneration, the contents of α granules support to tissue regeneration with around 30 growth factors, as well as host defense [66, 67]. Alpha granules in fact, contain various groups of proteins with antimicrobial properties including kinocidins and 7 platelet microbicidal proteins (PMPs): fibrinopeptide A and B, thymosin beta 4, platelet basic protein, protein activating the connective tissue 3, RANTES (Regulated on Activation Normal T Cell Expressed and Secreted) and platelet factor 4 [66]. These peptides have

been linked to the antimicrobial action against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Candida albicans, Cryptococcus neoformans[67]*. In tuberculosis and in *Pneumocystis carinii* and *Helicobacter pylori* infection RANTES levels were also noted to be increased [66, 69]. When the antimicrobial properties of proteins and peptides secreted and collected in platelet gel supernatants were studied [72] dissimilar bactericidal effect against different bacteria strains (*Acinetobacter baumannii, Pseudomonas aeruginosa* and *Staphylococcus aureus*) were detected. In fact, platelet gel supernatants showed strong antimicrobial property against *A. baumannii*, moderate activity against *S. aureus* and only a low bactericidal activity was observed in *ex vivo* skin model, but no bactericidal effect was detected *in vitro* against *P. aeruginosa* [73, 74].

Another indirect mechanism of how platelets contribute to the host defense is by inducing expression of antimicrobial protein in other cells. It has been reported that platelet-derived growth factors induce the expression of peptide human β-defensin-2 in keratinocytes [75]. Human β-defensin-2 has been advocate to be one of possible contributors to the inhibitory effect of PRP against Gram + and Gram – bacteria tested in an *in vitro* study [76]. Also, platelets facilitate bacterial clearance through the internalisation of the pathogens [68, 71, 77]. The mechanism of action has not been clearly understood, but it is known that once activated by various stimuli, such as bacterial lipopolysaccharide, platelets can modify their shape from spheroid cells to cells with pseudopods. Changing their shape, they will gain the capability to interact with pathogens and contribute to the production of reactive oxygen species (ROS) [70, 78, 79]. Moreover, platelets are capable to enhance neutrophils locomotion, phagocytosis and ROS production [80-82].

It is reasonable to assume that when leucocytes are included in a PRP, their presence contributes to the antimicrobial effect detected in PRP preparation [65, 83, 84]. In most of the published literature on PRP, the presence of leucocytes has been largely neglected. It is known, though, that in the classical 2 steps PRP preparation procedure and when the commercial gravitational platelet separation system is used, a significant amount of leucocytes are included in the final preparation. When the composition of L-PRP attained by these preparation protocols was studied, granulocytes, T lymphocytes, B lymphocytes, and monocytes were found [85]. Thomsen *et al.* [86] investigated the phagocytic fitness of leucocytes entrapped in a multilayer matrix of fibrin and platelets. The authors observed that the neutrophils included in this preparation were active and capable of chemotaxis, phagocytic activity, and respiratory burst. Interestingly, the authors contradicted the general assumption that neutrophils have a short lifespan of less than 24 hours. Neutrophils, in fact, showed reduced but still present oxidative burst after 7 days, confirming another study's finding revealing a neutrophil lifespan of more than 5 days [87].

Within the family of granulocytes, neutrophils are crucial components of the immune system as a first line defense against bacteria, yeats and fungi. Neutrophils migrate to the infection site and exert their antimicrobial action through phagocytosis and the microbes are incorporated into the phagosome. Granules present in neutrophils contain several antimicrobial peptides, proteins, and enzymes, such as lactoferrin, cathelicidins, azurocidin, bactericidal/permeability-increasing protein, defensins, phospholipases A2 [83]. The content of granules is released once the activated neutrophils have migrated to the infection site, the microbe has been internalised and included in the phagosome. The microbe is therefore exposed to a mixture of antimicrobial proteins and enzymes. Through

the rapid release of ROS, superoxide radical and hydrogen peroxide, (oxidative burst) internalised particles can be degraded. Myeloperoxidase (MPO), one of the enzymes stored in the granules further, contributes greatly to the bactericidal activity. MPO in fact, catalyses the reaction between chloride ion and hydrogen peroxide enable the production of hypochlorite, a strong bactericidal agent [88].

In PRP preparations T and B lymphocytes can be present and are the major contributors to the adaptive immune response, a pathogen-tailored response. B cells are responsible for humoral immunity and their antibodies opsonise the bacteria so that it can be recognised by phagocytes and removed. T cells are instead involved in cell-mediated immunity through the production of cytokines and the release of enzymes included in granules as well as direct killing of virus infected cells [71].

Various published studies have now acknowledged the antimicrobial properties of PRP and L-PRP preparations [65]. Results are promising but also often dissimilar. The diversity of outcomes is likely due to the large variability of experimental conditions, the methodology used to prepare PRP, the inclusion or exclusion of leucocytes within the preparations, and the activation method of the preparation. The bacterial growth inhibition is mainly detected from the first hour of the PRP application. Antimicrobial effects of PRP preparation have been recorded against periodontal disease–associated bacteria (*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleate*) [89] and against oral cavity microorganisms (*Enterococcus faecalis, Candida albicans, Streptococcus agalactiae, Streptococcus oralis* and *Pseudomonas aeruginosa*) [90]. PRP has also been shown to have antimicrobial activity against *Staphylococcus epidermis* [91], *Staphylococcus aureus* [91-93], *Escherichia coli* [93, 94]. Contradictory results have been published against other strains. Microbicidal effects of PRP against *Klebsiella pneumoniae*

and *Pseudomonas aeruginosa* were noted in Mariani *et al.* [94] study but not in the study conducted by Bielicki *et al.* [93] when L-PRP preparation was tested against the same strains.

Which components of PRP preparation contribute to the antimicrobial effect seen is still under debate. A few studies showed that the antimicrobial effect of PRP is independent of platelet concentration [91, 93, 95]. As PRP preparation also incorporates plasma components, complement has been suggested to be one of the major contributors to the antimicrobial effect of PRP preparation. The complement system is in fact, a major supplier of innate defense acting in both antibody-dependent and -independent immunity [96]. Studies suggest that the plasma complement rather than platelets or leucocytes contributes to the antibacterial effect [95, 97].

It is important to point out that studies so far published, have mainly tested the antimicrobial properties of PRP against planktonic cultures of bacteria and have not considered effects on bacteria biofilm formation. Bacteria in biofilms have become an emerging and serious clinical issue as a frequent cause of persistent infection [98, 99]. Biofilm is an aggregate of bacteria where pathogens are able to produce an extracellular polymeric substance (EPS) matrix. Bacteria initially adhere to a surface or to each other and consequentially, through a self-release of quorum-sensing molecules, start producing EPS [100]. Compared to a single free-floating bacteria life-style, living in biofilm seems to be convenient for bacteria for several reasons: exchange of genomic materials, a way to share food, protection to unfavourable environmental conditions, including when bacteria have to fight against antimicrobial, antibiotic and innate human defenses. It has well recognised that biofilms of *Pseudomonas aeruginosa* are one of the major mechanisms responsible for chronic infections such as otitis media[101], cystic fibrosis [102],

periodontal disease [102, 103], skin infection [104], burns [105] and recalcitrant wounds [106, 107].

To the best of our knowledge, only two studies have been conducted testing the effect of PRP against biofilm [86, 108]. Rozalski *et al*. showed bactericidal effect of 'expired' platelet-rich plasma against planktonic and biofilm cultures of *S. aureus*, whilst Thomsen *et al.* showed that leucocytes included in a multi-layered rich platelet-rich fibrin patch (LeucoPatch®) were able to control bacterial growth of an alginate-embedded *P. aeruginosa* model, that mimic the biofilm mode of growth.

1.5 Study aims

I. To perform a systematic review of the literature on the specific contribution of leucocytes included in PRP preparations in terms of their antimicrobial properties.

II. To define methods of preparation of PPP, PRP, L-PRP and to demonstrate ability to produce consistent results.

III. To evaluate *in vitro* antimicrobial effects of different PRP preparations against most frequent, resistant bacteria present in burn wounds (*Staphylococcus Aureus*, *Acinetobacter Baumanni*, *Pseudomonas Aeruginosa* testing the capability of the preparations to reduce bacteria growth and prevent biofilm formation.

CHAPTER 2. SYSTEMATIC REVIEW

2.1 Background

In this review the aim was to review the literature on the contribution of leucocytes included in PRP preparations in terms of their antimicrobial properties. In view of the vast variation and heterogeneity in terms of classification, preparation methodologies, and activation methods, the studies included in this review have been analysed in terms of the methodology used i) for the preparation of L-PRP, and ii) to study the antimicrobial activity. This should help to inform clinical practice and additional research in this promising field.

2.2 Methods

2.2.1 Data Sources

A literature review of publications was performed by two independent reviewers in May 2016 in the Ovid MEDLINE, PubMed (1946-2016) and EMBASE (1974-2016) database. In addition, the reference lists of all identified articles were examined to identify relevant papers that were not captured by electronic searches. MeSH Terms, Headings with Boolean operator for PubMed search were: ["platelet-rich plasma"[MeSH Terms] OR "platelet-rich"[All Fields] AND "plasma"[All Fields]] OR "platelet-rich plasma"[All Fields] OR ["platelet"[All Fields] AND "rich"[All Fields] AND "plasma"[All Fields]] OR "platelet rich plasma"[All Fields]] AND ["anti-infective agents"[Pharmacological Action] OR "anti-infective agents"[MeSH Terms] OR ["anti-infective"[All Fields] AND "agents"[All Fields]] OR "anti-infective agents"[All Fields] OR "antimicrobial"[All Fields]].

In addition, the terms “exp Platelet-Rich Plasma/ OR “exp Platelet-Derived-Growth Factor/” OR the following keywords: “L-PRF” OR “L-PRP” OR “Platelet Rich Fibrin” OR [“PLG” AND “Platelet-Rich Plasma"] OR [“PRF” AND “Platelet-Rich Plasma"] and “exp antimicrobial/” were used in MEDLINE and EMBASE to search all articles including PRP and antimicrobial effect exploded subheadings.

2.2.2 Study selection criteria

Study eligibility was defined using the population, intervention, comparator, outcome, and study design approach (PICOS) [109]. The inclusion and exclusion criteria are summarized in Table 2.1. Study selection was performed through two levels of screening. In the first level, abstracts were reviewed for the inclusion and exclusion criteria. In the second level screening, all articles filtered through the first level were fully read and the selected inclusion and exclusion criteria applied. The eligibility of the studies was assessed independently by two authors. Discrepancies were resolved by discussion. The ultimate list of included articles was designated with the agreement of all the authors. To be included, studies needed to clearly address the leucocyte inclusion in the composition of PRP and haematology value reported. Comment, case reports and review papers were excluded. Studies with duplicate publication were only included once, using the most recent publication. Only in vitro and human studies were included. Animal studies were excluded in this review, since i) none of the animal studies extracted from the literature search compared PRP preparations including leucocytes to preparations only including PRP, ii) the environment in an animal model is different to a human model and does not translate to the full potential of PRP-like products in terms of bactericidal capabilities.

Table 2.1. Inclusion and exclusion criteria for literature review

	INCLUSION CRITERIA	EXCLUSION CRITERIA
POPULATION	Human In vitro	Animal
INTERVENTION	Clear inclusion of leucocytes in PRP/L-PRP preparation	Cellular components into PRP/L-PRP preparation not stated
COMPARATOR	Any preparation with negligible concentration of Leucocytes	No comparators
OUTCOME	Antimicrobial effect of L-PRP	-
STUDY DESIGN	Experimental studies Observational studies	Review Comments Studies with duplicate publication Case reports

2.3 Results

2.3.1 Articles included

The literature search yielded a total of 686 articles. From a first screening of the titles and abstract and removal of duplicates, 643 citations were found to be not relevant to the topic or removed as they were duplicates. Therefore 43 articles progressed to the second level of screening. After retrieval of full text, review of each article, and application of inclusion and exclusion criteria and addition of 1 article discovered by review of references, 11 papers were included into the final analysis (figure 2.1 and table 2.2).

Figure 2.1. Study flow diagram

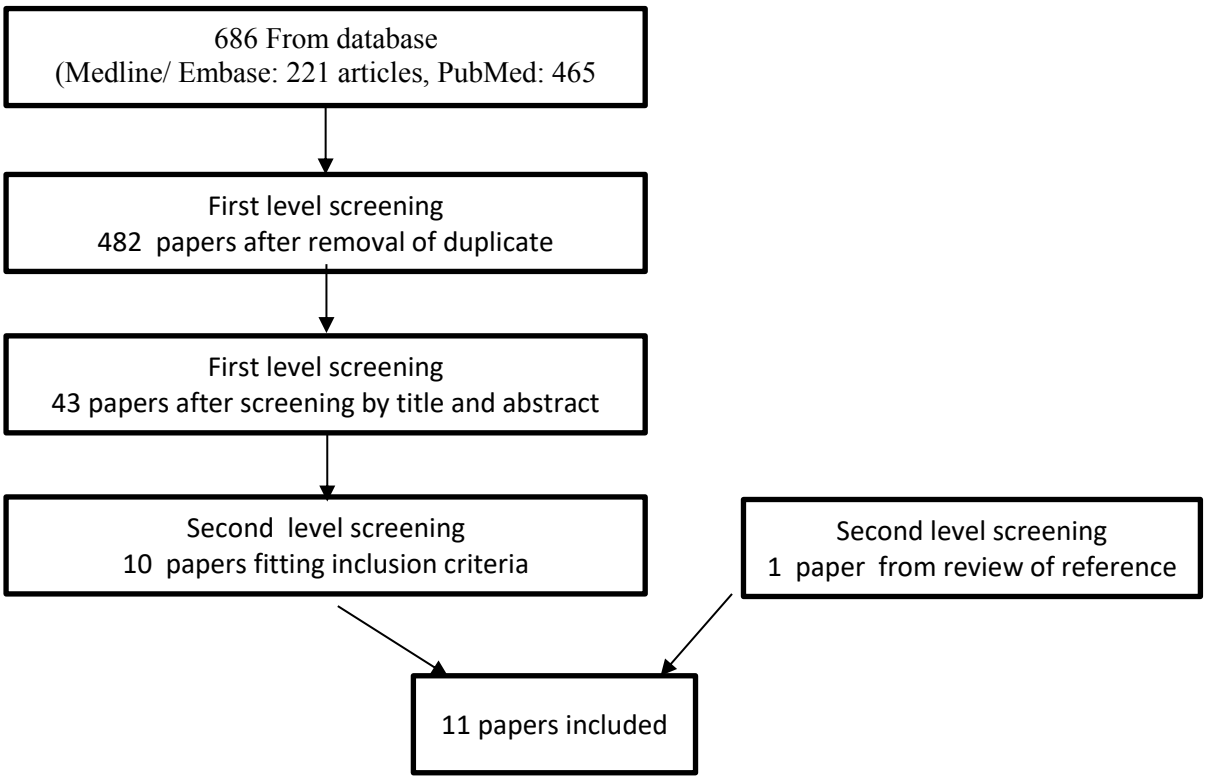


Table 2.2. Outcomes

Paper	Preparation method of PRP	Cellular composition (Compared to whole blood)		Study design and Micro assay	Outcomes Antimicrobial effect: + No antimicrobial effect: -				
					Parameter tested	Comparators			
In vitro studies									
Bielecki TM *et al.*	GPS I Platelet Concentration System (Biomet, Warsaw, Indiana) 3200 rpm for 12 min	PRP	PLT: increased by 760% WBC: increased by 790%	In vitro Blood from 20 healthy human volunteers Kirby-Bauer disk-diffusion method		PRG[1]	Thrombin[2]		
					MSSA	+	-		
					MRSA	++	-		
					E. coli (ESBL and no ESBL)	++	-		
					E. faecalis	-	-		
					K. pneumoniae	-	-		
					P. aeruginosa	- (evident growth)	-		
	1. PRG: Platelet Rich Gel: PRP activated with bovine thrombin (1600 U/ml bovine thrombin in a 10% calcium chloride solution, Trombina 400: Biomed, Lublin, Poland), 2. Trombina 400: Biomed, Lublin, Poland								
Moojen DJ *et al.*	Angel Whole Blood Processing System TM (AWBPS; Sorin Group, Mirandola, Italy) 3200 rpm for 19 min	PRP	PLT: 6 fold increase WBC: 3 fold increase	In vitro Blood from 6 healthy human volunteers Bacterial kill assay		PLG-AT[1]	PLG-BT[2]	PRP	PPP	PBS
					S. aureus (Wood 46 ATCC 10832)	++ Peak after 4 h	+ Peak after 4h	+ Peak after 8 h	+ Peak after 8 h	-
		PPP	PLT: 14 fold decrease WBC: 0			For the 24-h period PLG-AT larger antimicrobial activity compared to PRP and PPP, but not PLG-BT. After 4 hour bacterial regrowth, reaching after 24 hours the stationary phase				
	1.PLG-A: Platelet-leukocyte gel with 50 IU/ml autologous thrombin (from PPP + activAT™ system ethanol reagent solution (Sorin Group, Mirandola, Italy), 2. PLG-BT: Platelet-leukocyte gel activated with 500 IU/ml bovine thrombin								

<table>
<tr><td>Anitua E et al.</td><td>BTI System (IV, Vitoria Spain) 580 g for 8 min. After centrifugation, plasma drawn off into three fractions: F1[1], F2 (discarded), and F3[2]</td><td>F1[1]</td><td>PLT: 1 fold increase WBC: neglectable</td><td>In vitro Blood from 5 healthy human volunteers

Bacterial kill assay</td><td colspan="5"><table>
<tr><th></th><th>F1[1]</th><th>F3[2]</th><th>F3+Leucocyte[3]</th><th>Control[4]</th></tr>
<tr><td>MSSA</td><td>+</td><td>+</td><td>+</td><td>-</td></tr>
<tr><td>MRSA</td><td>++</td><td>+</td><td>++</td><td>-</td></tr>
<tr><td>MSSE</td><td>+/-</td><td>+/-</td><td>+</td><td>-</td></tr>
<tr><td>MRSE</td><td>+</td><td>+</td><td>+</td><td>-</td></tr>
</table></td></tr>
<tr><td></td><td></td><td>F2[2]</td><td>PLT: 2.5 fold increase WBC: neglectable</td><td></td><td colspan="5" rowspan="2">All preparations resulted in decreases in bacterial numbers, with maximum decreases seen after 4 hours of incubation. The effect was bacteriostatic, with rebound and growth for all bacterial strains after 8 hours.</td></tr>
<tr><td></td><td></td><td>F3+ leuc[3]</td><td>PLT: 1.8 fold increase WBC: 3.9 fold increase</td><td></td></tr>
<tr><td></td><td colspan="6">1:F1: plasma, 2: F3: PRP, 3: F3+Leucocyte: PRP + buffy coat. Fractions activated with Calcium chloride, 4: control not stated</td></tr>
<tr><td>Burnouf T et al.</td><td>PRP: Apheresis machine (MCS+, Haemonetics corp., Braintree, MA). Kept frozen at -20°C until use, and all other preparations made from this

PPP: 6000g for 30 min then kept frozen at -20°C until use.</td><td>PRP</td><td>PLT: 6 fold increase WBC: 1 fold increase</td><td>In vitro Blood from 2 healthy human volunteers

Bacterial kill assay

Kirby-Bauer disk-diffusion method</td><td colspan="5"><table>
<tr><th></th><th>PPP</th><th>PRP</th><th>PG[1]</th><th>S/D-PL[2]</th><th>Broth</th><th>PBS</th><th>HI[3]</th></tr>
<tr><td>E. coli (ATCC 8739)</td><td>++</td><td>++</td><td>++</td><td>++</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td></td><td colspan="4">No colony available for 48h
Zone of inhibition detectable</td><td colspan="3"></td></tr>
<tr><td>S. epidermidis (ATCC 12228)</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>P. aeruginosa (ATCC 10147)</td><td>D1[7]++
D2[8] +</td><td>D1[7]++
D2[8] +</td><td>D1[7]+/-
D2[8] +/-</td><td>D1[7]++
D2[8] +</td><td rowspan="2">-</td><td rowspan="2">-</td><td rowspan="2">-</td></tr>
<tr><td>K. pneumoniae (ATCC 13883)</td><td>D1[7]++
D2[8] +</td><td>D1[7]++
D2[8] ++</td><td>D1[7]+
D2[8] -</td><td>D1[7]++
D2[8] ++</td></tr>
<tr><td></td><td colspan="4">Zone of inhibition detectable</td><td colspan="3"></td></tr>
<tr><td>S. aureus (ATCC 29213)</td><td>D1[7]+
D2[8] +</td><td>D1[7]+
D2[8] +</td><td>D1[7]+/-
D2[8] -</td><td>D1[7]+
D2[8] +</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>E. cloacae</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
</table></td></tr>
</table>

					(ATCC 13047)							
					B. cereus (ATCC B 11778)	-	-	-	-	-	-	-
					B. subtilis (ATCC 21778)	-	-	-	-	-	-	-
	1: PG: Platelet gel , PRP activated with Calcium chloride 23mmol/L/L for 30 minutes at 22°C, 2. S/D-PL (solvent/detergent-treated virally inactivated PLT concentrate lysate)., 3 HI Heat inactivated preparations: PRP, PPP, PG and S/D-PL inactivated through heat treatment at 56°C for 30 minutes. The supernatants were then kept frozen at -20°C until use. 7. D1: donor 1, 8. D2: donor 2											
Chen L *et al*.	Centrifugation system: not stated two-step process to produce PRP and PPP: 313 g for 4 min then 1252 g for 6 min.	PRP	PLT: 8.1 fold increase WBC: 1 fold increase	In vitro Blood from 16 volunteers with diabetic dermal ulcers Bacterial kill assay		PRP	APG[1]	APG-APO[2]	PPP	PBS		
					S. aureus (ATCC 6538)	+/-	++	++	+/-	-		
						Reduced bacterial counts observed in the APG and APG-APO groups in the first four hours, when compared to the PBS group. Counts in APG and APG-APO lower than PRP and PPP in the 24 hours.						
					E. coli (ATCC8099)	+	+	+	+	-		
					P. aeruginosa (ATCC 15422)	+	+	+	+	-		
					The 'antibacterial rate' varied between bacteria: *S. aureus* (61-77%), *E. coli* (61-91%), and *P. aeruginosa* (<20%).							
	1.APG: Autologous platelet-rich gel, PRP activated with thrombin and calcium gluconate, 2. APG-APO: APG combined with apocyanin											
Wu X *et al*.	Centrifugation system: not stated two centrifuge spins at 1740 rpm for 6 min. PPP obtained through an additional spin of part of the	PRP	PLT: 1.5 fold decrease WBC: 5.8 fold decrease	In vitro Blood from 14 healthy human volunteers Kirby-Bauer disc diffusion method		PRP	PLF[1]	PPP	Bioseal[2]	PBS		
		PLF[1]	PLT: 11 fold increase WBC: 3.9		*E.coli* (ATCC 25922)	++ max decrease at 0-4 h	+ max decrease at 0-4 h	-	-	-		
						Zones of inhibition:	Zones of inhibition:	Zones of inhibition:	Zones of inhibition:	Zones of inhibition:		

	PRP (10 000 rpm for 10 min).		fold decrease	Bacterial kill assay		yes	yes	no	no	no
		PPP	PLT: 54 fold decrease WBC: 0		*P. aeruginosa* (ATCC 27853)	+ + max decrease at 0-4 h	+ max decrease at 0-4 h	-	-	-
						Zones of inhibition: yes	Zones of inhibition: yes Dose dependent	Zones of inhibition: no	Zones of inhibition: no	Zones of inhibition: no
					K.pneumoniae (ATCC 700603)	++ max decrease at 0-4 h	+ max decrease at 0-4 h	-	-	-
						Zones of inhibition: yes	Zones of inhibition: yes	Zones of inhibition: no	Zones of inhibition: no	Zones of inhibition: no
	1.PLF: Platelet-leucocyte fibrin obtained by preparing platelet-rich thrombin (through adjusting the pH), and cryoprecipitate (through freezing/thawing of PRP), and activated by thrombin from the same donor 2. Bioseal® (commercial fibrin glue)									
Intravia *et al*.	GPS III Platelet Concentrate System (Biomet Biologics, Warsaw, IN) PRPHP[1]: centrifuged for 15 min at 3200 rpm . PRPLP[2]: centrifuged for 5 min at 1500 rpm	PRPHP[1]	PLT:6.4 fold increase WBC: 2.2 fold increase	In vitro Blood from 2 healthy human volunteers Bacterial kill assay		PRPHP[1]	PRPLP[2]	Whole Blood[3]	PBS	Cefazolin
					MSSA	+	+	-	-	+
					MRSA	+	+	-	-	+
		PRPLP[2]	PLT:2 fold increase WBC: 9 fold decrease		MSSE	+	+	-	-	+
					P. acnes	+	+	-	-	-
					Both PRPLP and PRPHP: Significant decrease in bacterial growth for MSSA, *S. epidermidis*, MRSA and *P. acnes* compared to the whole blood control group at 8 hours. After 24 hours, there was still a significant decrease in bacterial growth for *S. epidermidis*, MRSA, and *P. acnes*.					
	1.PRPHP: PRP with high platelets and WBC, 2. PRPLP: PRP with low platelets and WBC, Pre-treated with autologous conditioned plasma (ACP) Double Syringes (Arthrex Inc., Naples, FL)									
Lopez C *et al.*	Centrifugation system: not stated two centrifuge	PRP	PLT: 1.6 fold increase WBC: 2	In vitro Blood from 18 healthy horses	At 6 hours	PRP	PRG[1]	LPP[2]	LPG[3]	IP[4]	Positive control[5]	Negative control[6]

	spins, one at 120g for 5 min, and then at 240g for 5 min. Plasma:3500g spin for 5 min		fold increase	Bacterial kill assay									
		LPP[2]	PLT: 1.2 fold increase WBC: 5 fold decrease		MSSA (ATCC 29213)	+++	+++	+++	+++	-	-	+++
					MRSA (ATCC 43300)	++	+	+	+	-	-	+++
					At 24 h MSSA: growth "inhibited in order of importance by PRP, plasma, LPP, LPG and PRG" At 24 h MRSA: LPG and plasma better bacteriostatic than IP. growth similar in PRP, PRG and LPP when compared to IP							
	1.PRG, pure platelet-rich gel (activated with calcium gluconate (in a 1:10 ratio with 9.3 mg/ml),2. LPP leukocyte-poor plasma, 3. LPG leucocyte-poor plasma gel (activated with calcium gluconate (in a 1:10 ratio with 9.3 mg/ml), 4.IP: heat-inactivated plasma, prepared by heating the plasma at 65ºC for 30 minutes to denature its complement, 5. Positive control group (Muller-Hinton broth with bacteria), 6. Negative control group (composition not stated)												
Mariani E *et al.*	Centrifugation system: not stated L-PRP two-step process centrifuged at 730 g for 15 min, and then at 3800 g for 10 min P-PRP one-step process of centrifugation at 460 g for 8 min	L-PRP[1]	PLT: 3 fold increase WBC: 1 fold increase	In vitro Blood from 10 healthy human volunteers Bacterial kill assay		L-PRP[1]	P-PRP[2]	L-PRP cryo[3]	Negative control[4]				
		P-PRP[2]	PLT: 1 fold increase WBC: neglectable		*E. coli (ATCC 25922)*	+	+	+	-				
		L-PRP cryo[3]	PLT: 1 fold increase WBC: neglectable		*`S. aureus (ATCC 29213)*	+	+	+	-				
					P. aeruginosa (ATCC 27853)	+	+	+	-				
					K. pneumoniae (ATCC 700603)	+	+	+	-				
					E. faecalis (ATCC 29213)	+	+	+	-				
	1.L-PRP: Leucocyte and platelet rich plasma,2. P-PRP: pure platelet rich plasma,3. L-PRP cryo: prepared from L-PRP which was frozen at −30 °C for 2 h and then thawed to obtain the cryopreserved fraction, Negative control: TSB broth. P-PRP, L-PRP and L-PRP cryo were activated with 10 % calcium chloride (final concentration of 22.8mM)												
Thomsen *et al.*	Blood into LeucoPatchVR	Leucopatch	PLT: $3528x10^6$/p	In vitro Blood from		Leucopatch			Control[1]				
					P. aeruginosa	+			-				

	devices (Reapplix Aps) two-step centrifugation at 3000 g for 8 min and 3000 g for 2 min.		atch WBC: 55x10^6/patch	healthy 7 human volunteers Bactericidal assay	5x 10^8 CFU/ml		
					P. aeruginosa 5x 10^7 CFU/ml	++	-
					P. aeruginosa Alginate embedded model in beads	+	-
					P. aeruginosa Alginate embedded model in disc	++	-
				Burst assay, phagocytosis assay, migration assay, biofilm killing assay and fluorescence in-situ hybridization (FISH) assay described in text			
	1.Control: buffer + *P. aeruginosa*						
In vitro and *in vivo* studies							
Li H *et al.*	Centrifugation system: not stated two centrifuge spins, one at 300g for 10 min, and the second at 3,000g for 15 min. Supernatant used as PPP	PRP	PLT:10 fold increase WBC: 4 fold increase	In vivo study animal 18 rabbit for spinal implant-associated infection model inoculated with 100µl of 10^2 CFU/ml of S. aureus In vitro 6 rabbits		PRP gel[1]	PPP gel	*In vivo study: one group treated with PRP gel, and the other with PBS (control). Samples from around the implant collected at euthanasia for microbiological assessment. Compared to the control, there were significantly fewer bacterial colonies in all the samples in the PRP gel group.*
					MSSA	+ (200 IU/ml thrombin)	-	
					MRSA	+	-	
					Group A Strept	+	-	
					N. gonorrhoeae	+ (only with 200 IU/ml thrombin)	-	
					Pseudomonas	-	-	
					E. coli	-	-	
					For all bacteria: CFU counts increase after 4 h and reach a plateau			

			Bacterial kill assay	at 12-24 h	
	1.PRP gel: Activated using different concentrations of Bovine thrombin (20, 100, 200 IU/ml) and 10% calcium chloride				

Legend:
PBS: phosphate buffered saline , MSSA: Staphylococcus aureus both methicillin-sensitive, MRSA: Staphylococcus aureus both methicillin-resistant (MRSA), E. Coli: Escherichia coli- ESBL: Extended Spectrum Beta Lactamase, and E. coli non-ESBL, E. faecalis: Enterococcus faecalis, E. cloacae: Enterobacter cloacae, K. Penumoniae: Klebsiella pneumoniae, P. aeruginosa: Pseudomonas aeruginosa, MSSE: Staphylococcus epidermidis, B. cereus: Bacillus cereus, B: subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, N. gonorrhoeae: Neisseria gonorrhoeae, P. acnes: Propionibacterium acnes, Group A Strep: Group A Streptococcus

2.3.2 Study design

All 11 papers included in the review were *in vitro* studies where various amounts of blood were drawn according to the requirements of the individual L-PRP preparation method (varying from 53 ml to 300 ml of blood from each donor). Blood was drawn from healthy human volunteers in seven studies [86, 91-93, 97, 110-113], from patients with diabetic ulcers in one study [114], from horses in one study [115], and from rabbits in one study [116] There was one study that included both *in vitro* and animal study. As for inclusion criteria, only the *in vitro* studies have been further analysed [116].

2.3.3 Parameters tested

The antimicrobial activity of L-PRP was tested against different bacterial strains including *Staphylococcus aureus,* both methicillin-sensitive (MSSA), and resistant (MRSA), *Escherichia coli*, *Enterococcus faecalis, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus epidermidis*, *Bacillus cereus, Bacillus subtilis, Streptococcus pyogenes, Neisseria gonorrhoeae*, and *Propionibacterium acnes*. In addition to the antimicrobial properties of the biomaterial, other leucocyte and platelet parameters were also included in some studies. Moojen *et al*. [92] for example aimed to evaluate the contribution of leucocytes with their oxidative killing action, and therefore included in their study measurements of myeloperoxidase (MPO) activity and MPO release. The authors found that MPO was gradually released by thrombin activated PRP and not activated PRP preparations in the first hours. No significant differences were detected in the MPO activity between the different preparations, nor was there a correlation between MPO concentration, activity and bacterial killing. To test the antimicrobial contribution of leucocytes in the preparation, Chen *et al*., [114] used apocynin, an inhibitor of NADPH oxidase activity, to block the oxidative burst action of leucocytes. The authors did not find differences in the

antimicrobial activity of the preparation where leucocyte function was inhibited compared to an activated L-PRP preparation. Release of different growth factors at various incubation times were also measured in one study. PF-4, TGF-β1, and PDGF-BB were measured [115] in order to test the possible antimicrobial contribution of different concentration of growth factors within PRP preparations and the ability of bacteria to denature or reduce growth factors levels. Measurement of complement and antibody levels were also included in the study conducted by Wu *et al*. [110]. Using immunoassay kits, Mariani *et al.* [113] measured proteins released by PRP preparations, such as 'Macrophage Inflammatory Protein' (MIP)-1α (CCL3), 'Regulated on Activation Normal T Expressed and Secreted protein'(RANTES), GRO-α, Interleukin (IL)-8, Interleukin (IL)-6, neutrophil-activating protein (NAP)-2, and stromal cell-derived factor (SDF)-1α. When the concentration of the proteins and the bacteria growth inhibition was tested, the proteins showed strong antimicrobial potential. Other parameters tested were the contribution to the antimicrobial effect of activated (with different activators) and not activated PRP [97, 110, 114, 115]. In Li *et al.* [116] various concentrations of bovine thrombin were used to activate L-PRP and used to assess the role of thrombin in the antimicrobial properties of PRP.

2.3.4 Method of preparation of L-PRP and its influence of the haematological values of leucocytes and platelets

The methods of L-PRP preparation used in the selected studies were considerably different, as shown in Table 2.2: single or double centrifugation, different platforms, different spin and centrifugation values ('g' standing for multiples of earth gravitational field and 'rpm' standing for rotation per minute) and different centrifugation duration times. Not surprisingly the variation within the methods of preparation caused variations in the quantities of the haematological components of the blood products. In all the included

studies the processing methods resulted in enrichment of platelet concentration of the PRP, ranging from as low as two-fold [91, 115, 117] up to ten and eleven-fold in Li *et al.* [116] and Wu *et al.* [110].[116] There was also a wide variation in the concentration of leucocytes in the L-PRP after processing, varying from a minimal concentration of leucocytes used in an *in vitro* study (3.9-fold decrease from whole blood) [110] to a maximal concentration of 4-fold enrichment tested in Li *et al*. None of the studies specified the differential white blood counts, so the relative percentage of each type of white blood cell in the L-PRP preparations is unknown. The viability and function of leucocytes after the PRP processing was assessed in the study conducted by Moojen *et al.* [92]. Here the authors measured MPO concentrations after a single step centrifugation method used to prepare L-PRP, and found that neutrophils and monocytes were not only viable but biologically active as shown by the rapid increase in MPO concentration detected shortly after the addition of L-PRP to the bacterial culture.

2.3.5 Activation of L-PRP

Of the 11 studies, 9 prepared L-PRP as a gel form by activating platelets to release their granule components. L-PRP was activated by different activators including a combination of calcium chloride and bovine thrombin, or calcium chloride alone, or autologous thrombin alone or calcium gluconate alone (Table 2.2). Only one study evaluated the antimicrobial effect of L-PRP in its pure, inactivated form [111].

2.3.6 Microbiology assay

The 11 studies included were very similar in terms of the methodologies used to test the biomaterials. Nine of the 11 (Table 2.2) used a bacterial killing assay, whereby bacterial cultures and the biomaterials were mixed together, incubated under agitation, and aliquots removed at certain time points (up to 24 hours) for serial dilution, plating and subsequent

assessment of bacterial counts (in terms of CFU/ml). The evaluation of the zone of inhibition (Kirby-Bauer disk-diffusion method) was used in 3 studies [93, 97, 110]. As well as the methodologies being similar, there was good concordance in terms of the bacterial isolates tested, with 9 of the 11 papers testing S. aureus, and half of the papers testing Gram positive organisms only.

2.3.7 Antimicrobial outcomes

Outcome of in vitro studies

The first paper addressing the antimicrobial activity of a PRP preparation with a clear recognition of the inclusion of leucocytes, was the study published in 2007 by Bielecki *et al.* [93]. The authors conducted an *in vitro* study drawing blood from healthy human volunteers to evaluate the antimicrobial activity of PRP activated with bovine thrombin in a 10% calcium chloride solution (PRG, Platelet Rich Gel) against the most frequent bacteria responsible of wound and bone infections: MSSA, MRSA, *E. coli* (Extended Spectrum Beta Lactamase, ESBL) and non-ESBL, *K. pneumoniae* (ESBL), *E. faecalis* and *P. aeruginosa*. High concentrations of both platelets and leucocytes were obtained (increased by 760% and 790% respectively) using a single centrifugation method. The antimicrobial activity of the L-PRP preparation was determined using a Kirby-Bauer disc-diffusion method, and showed different effects for different strains, with strong antimicrobial activity detected against MSSA, MRSA and *E. coli*, whilst no bactericidal activity was found against *E. faecalis*, *K. pneumoniae* and *P. aeruginosa*. Alarmingly the addition of L-PRP to *P. aeruginosa* led to an actual increase in growth. Even though the objective of the study was not specifically to evaluate the antibacterial activity of different concentrations of platelets or leucocytes, the authors stated that within the 20 blood samples studied, no correlation between antibacterial

activity and the value of platelets and leucocytes in the blood and the platelet-rich plasma was detected.

The study conducted by Moojen *et al*. [92] was the first attempt in recognising the specific contribution of leucocytes in L-PRP preparations. The authors used a semi-automated table top centrifuge to obtain PRP, reaching high concentrations of both platelets and leucocytes (platelets and leucocytes more than 7-fold and 3-fold enrichment, respectively). L-PRP was activated with either autologous or bovine thrombin, resulting in a preparation referred by the authors as PLG-AT or PLG-BT (Platelet-leucocyte gel autologous (AT) or bovine thrombin (BT). Other comparators were PPP (Platelet Poor Plasma), PRP (Platelet Rich Plasma) and PBS (phosphate buffered saline), the latter which acted as a control. The different preparations were used to test antimicrobial activity against MSSA using a bacterial killing assay. Even though all the four blood preparations showed antimicrobial activity, the PRP containing high concentrations of both platelets and leucocytes, and activated with autologous thrombin (PLG-AT), proved to be the most antimicrobial (and to give the longest duration of effect) compared to PRP, PPP and PRP-BT. In this study non-activated PRP and PPP also exhibited some antimicrobial activity, but with a more delayed effect. Autologous activated PRP showed the largest effect for the entire 24 hours. There were no preparations resulting in 100% efficiency of bacterial killing. In order to explore the contribution of leucocytes to the antimicrobial properties shown by the L-PRP preparation, myeloperoxidase (MPO) activity and MPO release at different time points of incubation were measured. MPO was gradually released in PLG-AT, PLG-BT and PRP preparations in the first few hours. Interestingly though, no significant differences were detected in the MPO activity between the four different preparations, nor was there a correlation between MPO concentration, activity and efficiency of bacterial killing. The authors speculated that

the stronger antimicrobial effect found with the PLG-AT preparation is likely due to the effect of thrombin acting on the antimicrobial peptides released by platelets rather than effect of leucocyte activation.

Different preparations including low and high concentrations of platelets, and inclusion or exclusion of leucocytes, were used by Anitua *et al*. [91] to evaluate the bactericidal effect of the different blood products against four bacterial strains. Here a bacterial killing assay was performed to test the antimicrobial activity of PRP products against MSSA, MRSA, methicillin-sensitive *S. epidermidis* (MSSE) and resistant *S. epidermidis* (MRSE). The different fractions obtained from plasma after a single centrifugation step were: F1 (plasma), which contained a 1-fold enrichment of platelets and no leucocytes compared to whole blood, F3 (PRP) containing 2.5-foldenriched platelets and no leucocytes, and F3+leucocytes:1.8-fold enrichment of platelets and 3.9-fold enrichment of leucocytes. The authors found that after four hours all the fractions revealed bacteriostatic properties against MSSA and MRSA and MRSE. The preparation including leucocytes was revealed to be superior against MSSE, where it was the only fraction able to reduce the growth in the experiment.

In order to explore the nature of the antimicrobial activity of PRP and specifically to investigate which components exert this effect, Burnouf *et al*. [97] tested the effect of different blood preparations against eight strains of wound bacteria (four Gram positive and four Gram negative bacteria). The authors compared PRP (containing leucocytes), calcium chloride activated PRP (PG: Platelet Gel), PPP (platelet poor plasma), solvent/detergent-treated PLT lysate (S/D P-L) and complement inactivated preparations . All the preparations were immediately frozen and kept frozen at ≤-20°C until use. PRP was processed by apheresis for the blood samples from the two donors obtaining a 6-fold enrichment for

platelets and 1-fold for leucocytes. The authors performed a bacterial plate assay followed by colony counting, and assessed log reductions at 3 hours when the test sample was compared to the PBS controls. *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* all showed an initial log reduction in bacterial numbers 3 hours after spiking into the different preparations, however the reductions were lowest with the PG, and bacterial regrowth was seen at 48 hours with all preparations. The preparations were most effective against *E. coli*, where there was a large inactivation of bacteria (7.51 to >9.01 log), and no viable colonies for 48 hours after spiking. Furthermore, similar antimicrobial activity was seen in different preparations regardless of the concentrations of the platelets and leucocytes. As the PG preparation appeared to have the lowest antimicrobial effect on these strains, it seems that the use of calcium chloride for activation of coagulation could have decreased the bactericidal property of the PRP. Although there were some antimicrobial effects, none of preparations were able to inhibit, *B. cereus, B. subtilis*, and *S. epidermidis*. Reassuringly, complement-inactivated control preparations did not show any antimicrobial activities. Having taken all into account, the authors concluded that plasma complement, rather than specifically platelets or leucocytes, are the elements responsible for the antimicrobial activity seen against *E. coli, K. pneumoniae*, and *S. aureus*.

Chen *et al.* [114] investigated the *in vitro* antimicrobial activity of APG (autologous platelet-rich gel) extracted from the blood of patients with diabetic dermal ulcers, against the most common bacteria found in diabetic chronic wounds: *S. aureus*, *E. coli* and *P. aeruginosa*. To mitigate any confounding effects of previous IV antibiotics that the patients may have received as part of routine care, the blood for the PRP preparation was drawn from patients 8-12 hours after antibiotic administration. PRP was obtained through a double step centrifugation procedure and activated with thrombin and calcium gluconate. Comparator

preparations were: APG, PRP (inactivated), PPP, and APG-APO (autologous platelet gel with apocyanin). Apocyanin is an inhibitor of NADPH oxidase activity, and is thus a way to study L-PRP antimicrobial activity, whilst excluding the possible antimicrobial contribution of leucocytes producing superoxide. PBS was used as control. Both PLG and APG-APO were effective in reducing the bacterial counts of *S. aureus* compared to the PBS control in the first four hours, and were more effective during 24 hours compared to PRP and PPP. Since the APG, APG-APO and PRP preparations showed antibacterial effects against *E. coli* and *P. aeruginosa* compared to PBS, but there was no effect with PPP, the authors attribute the antimicrobial effects seen to the prior IV antibiotics administered to the patients rather than the biomaterial preparations. The authors concluded that the antimicrobial activity seen against *S. aureus* is likely not due to the inclusion of leucocytes in PRP preparation, as APG and APG-APO showed a similar antibacterial effect. It is thought that the activity may be attributable to the thrombin and calcium gluconate used to activate the platelets.

In order to test the antimicrobial activity against Gram negative bacteria that are commonly present in enterocutaneous fistula tracts (including *E. coli*, *P. aeruginosa* and *K. pneumoniae*), Wu *et al*. [110] compared three different biomaterials, and a commercial fibrin glue called Bioseal®. Blood drawn from 14 healthy volunteers was centrifuged and processed to obtain: PLF (platelet-leucocyte fibrin) which is a preparation containing a high concentration of platelets (10-fold enrichment from whole blood) and low leucocytes (3.9 fold decrease from the baseline) which is activated with thrombin; PRP (similar concentrations of platelets to whole blood and 3.9 fold decrease in leucocytes from the baseline) and PPP (poor platelet concentration and no leucocytes). The Kirby-Bauer disc diffusion test and bacterial killing assays were used to compare the antibacterial activity of the different preparations. Also, the levels of complement and antibodies (IgA, IgG, IgM,

C3, C4) were measured in PLF, PRP and PPP and no significant difference was found between the different preparations. For the microbiological assays a greater antibacterial effect was found in with PRP and PLF compared to PPP. However, it was noted over time that PLF seemed to lose its antimicrobial effect more than PRP (although this was not statistically significant). The commercial fibrin glue (Bioseal®) did not show any antimicrobial effect. For the bacterial killing assay, the effect seemed only bacteriostatic, with the maximum killing of bacteria observed in the first four hours, followed by regrowth up to peak numbers at 24 hours. The antimicrobial activity against *P. aeruginosa* was dose-dependent, requiring higher concentrations of the PLF preparation than those that were effective for the other two bacterial species. In the *in vitro* study conducted by Intravia *et al*. [111], different PRP preparations with low and high concentrations of platelets and leucocytes were tested to verify the antimicrobial properties against bacteria commonly found in arthroplastic surgery (MSSA, MRSA, *S. epidermidis* (MSSA) and *Propionibacterium acnes*). Two different preparations were obtained using different g values and centrifugation duration, along with commercial systems (Table 2). The preparations contained low (PRPLP), or high (PRPHP) concentrations of platelets and leucocytes, and both showed a significant decrease in bacterial growth at 8 hours for all of the bacterial samples when compared to the controls. PRP rich in leucocytes and platelets (PRPHP) seemed to give a superior decrease of *S.epidermidis* and *P.acnes* at 8 hours compared to the preparation with negligible inclusion of leucocytes and lower platelets (PHPLP). Also, at 1 and 4 hours after MRSA incubation, the preparation enriched with leucocytes (PRPHP) showed a stronger growth inhibition than the leucodepleted biomaterial (PRPLP). After 24 hours, significant inhibition was still seen for MSSE, MRSA, and *P. acnes* with both preparations.

Lopez *et al*. [115], compared different preparations of PRP, (with low and high levels of leucocytes, activated or in their pure form), in terms of their antimicrobial properties against MSSA and MRSA. Blood was drawn from 18 healthy horses, and using double centrifugation, the authors obtained a range of preparations containing different concentrations of platelets and leucocytes. These were enriched respectively by 1.64 and 2-fold from the blood baseline in the PRP preparation whereas in the LPP (leucocyte poor plasma) preparation, the leucocyte count was negligible. The pure form of these two products was also compared to the activated form (activated via calcium gluconate), and to plasma and to heat-inactivated plasma (IP). With the exception of IP, at six hours, all blood components, significantly inhibited bacterial growth. The non-activated form of PRP showed a better bacteriostatic activity against MRSA when compared to the activated form (LPG: leucocytes-poor plasma gel) and plasma. MSSA showed higher sensitivity to the treatments than MRSA, and PRP against MSSA showed the highest bactericidal effect followed by plasma, then LPP, LPG, and PRG. Against MRSA, at 6 hours PRP was again the preparation showing stronger antimicrobial effect, whilst at 24 hours LPG and plasma had stronger bacteriostatic effect against MRSA. The release of growth factors (PF-4, TGF-β1, PDGF-BB) by the different preparations at different incubation times were also measured, but no correlation between bacterial counts and leucocytes, platelets, PF-4, TGB-β1, PDGF-BB was recorded suggesting that bacteria were not able to denature the growth factors, and that plasma components could be responsible for the antimicrobial effects observed.

A recent publication by Mariani *et al.* [113] specifically investigated the possible contribution of leucocytes in a PRP preparation against different bacteria of relevance to bone, soft tissue and wound infections (including MSSA, *E. coli*, *P. aeruginosa*, *K.*

pneumoniae, and *E. faecalis*). Two different PRP preparations (including or excluding leucocytes) were obtained from 150 ml of whole blood, drawn from 10 healthy donors. The preparation involved different centrifugation steps as shown in table 2, and resulted in L-PRP: Leucocyte and platelet rich plasma and P-PRP: and a pure platelet rich plasma preparation. Moreover, to investigate whether or not the cryo-preserved blood product influenced the antimicrobial effect, a third preparation was obtained by freezing the L-PRP preparation at −30 °C for 2 h, L-PRP-cryo preparation. All the products were then used in their activated form for the experiment. As the three different preparations showed similar antimicrobial results (with bacterial growth inhibition effective for up to 4 hours for all the preparations), the authors concluded that the inclusion of leucocytes does not contribute significantly to the microbicidal activity of PRP. The inhibition varied between 1-4 log, according to the bacteria strain and experimental conditions tested. After treatment with the three plasma fractions a time-dependent inhibition of bacterial growth (for up to 4 hours) and at low bacterial count for *E. coli*, and *P. aeruginosa*, and at higher numbers for *S. aureus*, and *Enterococcus faecalis* was detected. In contrast, there was not a time-dependent inhibition of growth with *K. pneumoniae*. Generally, the quantities and abundance of the microbicidal proteins correlated well with growth inhibition, where higher quantities of proteins correspond with greater inhibition. Thomsen *et al.* [86] have recently published a study where the phagocytic fitness and bactericidal activity of leucocytes included in a multilayer matrix of fibrin and platelets were analysed. In the patch a high concentration of leucocytes is included (55 x 10^6/patch). The authors observed that the neutrophils included in this preparation are active and capable of chemotaxis, phagocytic activity and respiratory burst. When P. aeruginosa was mixed with leucopatch, the production of ROS of PMNs included in leucopatch, measured by chemiluminescence assay, was substantial. The

response was bacteria concentration-dependent. The production of ROS during phagocytosis of *P. aeruginosa* was also tested in isolated polymorphonuclear neutrophils (PMNs: 10^7cells/ml) was tested. The resulting chemiluminescence signal was twice as high compared to the signal from leucopatch. When ROS production was tested for a longer time period of 7 days, ROS production was still observed at day 4. The chemotactic leucocyte migration was also investigated using transwell chambers. The authors found that the PMNs included in leucopatch were capable of migration towards P. aeruginosa. Moreover, bactericidal assays tested Pseudomonas aeruginosa in both planktonic and in an alginate-embedded model, simulating the biofilm mode of bacteria growth. Serum-opsonized P. aeruginosa was exposed to leucopatch, diluted samples were plated and after 24 hours CFU (Colony-forming unit) were counted. Compared to control (Buffer with P. aeruginosa), strong reduction of colonies was observed after 20 minutes of leucopatch exposure and reduced further reduced after 90 minutes. Two concentration of bacteria were tested (5 x 10^7 CFU/ml and 5 x 10^8 CFU/ml). The loss of bacteria availability was more evident (99% loss of bacteria with respect to the initial inoculum) when the lower concentration of bacteria was exposed to the leucopatch. When bacterial growth was tested in alginate beads and disc, mimicking a biofilm model, also antimicrobial activities of the patch were detected. The alginate beads and discs with Pseudomonas were exposed to leucopatch for 2 hours, transferred to tubes containing PBS (phosphate-buffered saline), homogenized, serially diluted, plated, and the next day the CFU were counted. After 2 hours, colonies count was reduced by 70% when simulating biofilm in form of disc when exposed to leucopatch and leucopatch inhibited bacterial growth completely compared to non-leucopatch control in the alginate beads model.

Outcome of in vitro and in vivo studies

In vivo and *in vitro* studies were conducted by Li *et al*. [116] to test the antibacterial effect of PRP gel and the ideal concentration of bovine thrombin for activation of the PRP. For the *in vitro* study, a killing curve assay was used to test the effect of PRP and PPP gels against MSSA, MRSA, *Group A Streptococcus, N. gonorrhoeae*, *Pseudomonas* spp. and *E. coli*. PRP gel was obtained from rabbit blood, through a two-stage centrifugation process and activated using a range of different concentrations of thrombin in calcium chloride. Results were compared to PBS controls and to PPP-gel. The concentration of platelets and leucocytes in the PRP gel preparation was extremely high (approximately 10-fold and 4-fold increase from the baseline respectively), and this preparation was shown to inhibit MSSA, MRSA, *Group A streptococcus* and *N. gonorrhoeae* growth in the first 4 hours, after which an increase of growth was identified. Only high concentrations of thrombin produced sufficient antimicrobial activity of the PRP gel against *N. gonorrhoeae*. There was no antimicrobial activity of the PRP gel against *Pseudomonas* spp. and *E.coli*, and the PPP gel was ineffective for all. The *in vivo* study tested the antimicrobial activity of PRP gel against rabbits with a spinal implant-associated infection with *S.* aureus. The injection of the PRP gel increased bone formation, and decreased the bacterial counts found in the bone at different time points.

2.4 Discussion

2.4.1 L-PRP versus PRP

Only 4 studies have specifically compared the antibacterial properties of autologous PRP in the presence and absence of leucocytes or L-PRP [91, 111, 113, 115]. The overall conclusion of the authors was that no significant difference between the two preparations was found. Most of the authors suggested that the temporal bacteriostatic properties of L-

PRP seem to be caused by either plasma and/or platelet components rather than the leucocytes themselves [97, 114, 115]. However, looking for results on specific bacteria strain, Anitua *et al.* [91], showed that the fraction containing a very high concentration of leucocytes (almost 4 times the baseline) was the only preparation able to effectively reduce MSSE. Furthermore Intravia *et al.* [111], reported that the PRP preparations with a high concentration of leucocytes were superior to PRP with no leucocytes against MRSA, MSSE and *P.acnes*. Also compared to whole blood, both preparations limited bacterial growth, but for MSSA the preparation with leucocytes showed a longer (24 hours) inhibition of MSSA. Moreover, Lopez *et al*. [115] showed that the non-activated preparation enriched with leucocytes gave a better 6 hours and 24 hours bacteriostatic effect against MSSA compared to preparations poor in leucocytes. In contrast, against MRSA, the activated form of the preparation with poor leucocytes and plasma seemed to perform better than the other preparations tested at 24 hours. In contrast Mariani *et al.* [113], concluded that 'Leucocyte presence does not increase microbicidal activity of Platelet-rich Plasma *in vitro*' in a study where the quantity of leucocytes included in the L-PRP preparation was low, no dissimilar from the whole blood. Whole blood was used as a positive control by Intravia *et al.* [111], and in their study the authors showed the poor antimicrobial activity of the whole blood compared to the biomaterial enriched with platelets and leucocytes. Moreover, a significant antimicrobial effect of PRP was seen when leucocytes were at least 2-fold enriched from baseline [116]. To evaluate the potential antimicrobial contribution of leucocytes, Chen et al. [114] used apocyanin in L-PRP preparation to exclude the possible contribution of leucocytes producing superoxide. The authors showed similar antimicrobial activity regardless of apocyanin, concluding that the antibacterial effect of the biomaterial was not due to the inclusion of leucocytes.

2.4.2 Activation of PRP preparations

Results are controversial regarding the activation of the PRP preparations. Burnouf *et al*. [97], suggested that the activation of platelets by calcium chloride decreased antimicrobial properties against selected bacterial strains. Similarly Wu *et al*. [110] showed thrombin activated PRP preparations have less antimicrobial activity against *E.coli, P. aeruginosa* and *K. pneumoniae* compared to the inactivated preparations. These findings are thought to be due to consumption of complement during the coagulation activation. Also Lopez *et al*. [115] indicated that after 24 hours of incubation, calcium gluconate activated PRP preparations exhibited less antimicrobial activity against MSSA compared to inactivated preparations. When the preparations where tested against MRSA, at 6 hours, the strongest antimicrobial effect against MRSA was again exerted by the non-activated form of the leucocyte enriched preparation, whilst at 24 hours the activated form of leucocytes poor plasma showed stronger antimicrobial effect compared to the other preparations tested. Controversially, other studies indicate that strongest antibacterial activity is reached activating PRP preparation [114] and when different concentration of thrombin were compared, with the highest concentration of thrombin tested resulted exerting the strongest antimicrobial effect, with autologous thrombin performing better than bovine thrombin [116].

2.4.3 L-PRP/PRP preparation methods and microbiology assays

Due to the heterogeneity of the preparations and the multiple variables in the study settings, it is difficult to compare results of studies and reach definitive conclusions regarding the relevance of the leucocytes in antimicrobial activity of L-PRP preparations. The proportion of the different components included in the biomaterials that exerted the strongest antimicrobial properties clearly still remains to be identified. It is important to remember

that the *in vitro* studies may not reflect the clinical scenario, since this environment may not mimic the dynamic condition of an *in vivo* setting where the antimicrobial properties of each individual components of the biomaterial may be increased, prolonged or even reduced by the complex interaction of cellular signals, and dynamic fluid exchange. An example of different results of platelet gel effects against bacteria obtained *in vitro* and in an *ex vivo* skin model is clearly shown in a recent published study. Edelblute *et al*. [72] demonstrated different antimicrobial performance against three strains of bacteria in different settings. No inhibition was seen for *Pseudomonas* spp. in the *in vitro* experiment, while inactivation of the same strain was detected in the *ex vivo* model. Moreover, as suggested by Burnouf *et al*.[97], ATCC (American Type Culture Collection) bacterial strains used in most of the studies, may not reflect the actual bacterial behaviour of clinical isolates. Also, different strains showed different responses to diverse blood products. This might due to the antimicrobial activity of AMPs directly related to the intrinsic AMP susceptibility phenotype of the infecting bacteria strain, reflecting the point that different bacteria strains have dissimilar susceptibility to inhibition by platelet AMPs [118].

2.4.5 Are the included leucocytes viable and active?

The methodology described in the studies to centrifuge and prepare blood products should not theoretically damage leucocytes. It is likely that in Burnouf *et al.* [97], leucocytes included in the PRP preparation were no longer viable (Prof J. Lord, personal communication) (as the preparations were kept frozen until use, therefore the contribution to the antimicrobial effect showed by the PRP preparation is likely not due to leucocyte inclusion.

In the study conducted by Moojen *et al*. [92] the authors found that neutrophils and monocytes were viable and active as showed by the rise of MPO concentration detected

shortly after the addition of L-PRP to the bacterial culture. Thomsen *et al.* [86] investigated the phagocytic fitness of leucocytes included in a multilayer matrix of fibrin and platelets. The authors observed that the neutrophils included in this preparation were active and capable of chemotaxis, phagocytic activity and respiratory burst. Interestingly, the authors contradict the general assumption that neutrophils have a short lifespan of less than 24 hours. Neutrophils in fact showed reduced but oxidative burst activity even after 7 days, confirming previous published revealing a neutrophil lifespan of more than 5 days [87].

2.4.6 L-PRP in wound healing: concerns for scarring

Mindful of all the limitations of the studies included in this review, the overall conclusion seems to be that the contribution of leucocytes in a PRP preparation is still poorly understood or at least not fully appreciated in the studies so far conducted. On the other hand, it is possible to state that none of the papers included in this review strongly suggest a remarkable antimicrobial effect specifically due to the leucocytes included in PRP preparation. Leucocyte inclusion in the biomaterial should be carefully further evaluated especially when the biomaterial is used for wound repair and when scar formation is a major concern. Among all the factors that affect scar quality, the one that seems to have the greatest impact is the time that it takes a wound to heal [119]. A large body of literature supports the assertion that achieving wound healing within two weeks, will minimize the scarring. Therefore, a major focus in burns and wound healing research is to clarify the pathophysiology of the healing process of a wound, the risk factors related to the scarring process, and the conversion of this knowledge into therapeutic solutions. The use of PRP in wound healing as an accelerator of wound repair seems to justify its use. Several studies are now evaluating the role of leucocytes in wound repair and mediators and growth factors (especially TGF-β1) released by leucocytes have been correlated to promote scarring [59]. Their inclusion for wound healing and scar formation should therefore be carefully considered. Especially it should be further investigated which subpopulations

of leucocytes are included in the preparations and which effects individually they might have in both antimicrobial and immune-metabolic activities.

2.5 Conclusion

In this review despite a number of studies showing that preparations including leukocytes have antimicrobial properties, there is not enough evidence to attribute this bactericidal effect to the presence of leucocytes in the biomaterial. PRP preparations, with or without leucocytes demonstrated bacteriostatic properties against the majority of the bacterial strains tested. Diverse strains of bacteria respond differently to PRP and L-PRP, some of them requiring the presence of leucocytes (MSSE), and some being dose dependent (*Pseudomonas* spp.). This review should form a groundwork for future studies to further explore the contribution of leucocytes in PRP preparation in order to obtain an optimal preparation to both fight infection and effectively promote wound healing.

CHAPTER 3. METHODS OF PREPARATION OF PRP PRODUCTS

3.1 Introduction

Many different procedures have been suggested to prepare PRP products and used in clinical settings as topical biomaterial for wound healing. PRP can be prepared using several different methods and this variability leads to a wide inconsistency of the final product. It has extensively been shown that several factors contribute to the efficacy of PRP products [12]: the presence and concentration of the different components included in PRP preparation, the methodology used to prepare the product and the methodology used to activate the platelets will all contribute to the antimicrobial and wound healing effect of the products.

The aim of this chapter was primarily to try different methods described in the published literature to prepare PRP, and L-PRP and ultimately, to define a method that produces consistent results.

3.2 Methods

Taking from previously published works [8, 12, 18, 120] and from our laboratory expertise, different methods to prepare PRP products have been tested: single and double-step centrifugation techniques, different spins and different activation methods. The methodology that allows reproducing consistent results is here reported.

3.2.1 Blood collection

To obtain PPP, PRP and L-PRP, fresh blood was drawn from healthy volunteers. All participants provided informed consent. All the procedures were conducted under sterile

condition under a laminar flow hood (Esco, Class II Biological Safety Cabinet, Singapore). A large bore needle was used to prevent activation/damage of platelets due to mechanical stress. In order to prevent early platelet activation and clot formation, a blood collection bottle pre-filled with sodium citrate was used (BD Vacutainer, New Jersey, USA). Once the blood was collected, the bottle was gently inverted to allow an adequate mixing of the anticoagulant and the blood.

3.2.2 Determination of components in PPP-PRP- L-PRP products

A sample of 120 µl of whole blood, PPP, PRP, L-PRP was extracted and analysed using an automated analyser XN-1000 whole blood counter (Sysmex UK, Milton Keynes), in order to obtain the measurement of platelets, leucocytes and differential counts included in each preparation. The analyser has a set of unique platelet parameters including three platelet counts: platelet impedance (PLT-I), platelet optical (PLT-O) and platelet fluorescence (PLT-F) counts, platelet mean volume (MPV) and the immature platelet fraction (IPF). For our study, platelet count was recorded as PLT-F. where platelets are specifically stained by a fluorescent dye. It has been showed that this method had improved accuracy in platelet counting [121].

3.2.3 Preparation of blood preparations

- for all our experiments, PPP and L-PRP preparations were obtained using a single step method as described below

- for our experiments, 2 different PRP products have been used. A standard single-step method that allows to obtain a PRP product with a 2-fold platelet enrichment from the whole blood (standard method used in our laboratory) has been used to test the antimicrobial properties against Gram positive bacteria. With the progression of the study, in order to

target the working definition of PRP suggested [12] and to achieve similar concentration of cells obtain in our L-PRP preparation, we decided to aim to a higher concentration of platelets included in our PRP preparation with a minimal concentration of cells of between 4-fold higher than the circulating blood platelet. Therefore, when Gram negative bacteria were tested, we adopted the double-step method for PRP production. The two different methodologies used, single-step method and double-step method are described below.

PPP and L-PRP preparation

High g centrifugation (2000 g) of anticoagulated blood for 20 minutes at 21°C was used to obtain L-PRP and PPP. The centrifuge used was an Eppendorf Centrifuge (5804/5804 R, Hamburg, Germany). Under these conditions, centrifugation results in the separation of whole blood into 3 layers, as shown in Figure 3.1.

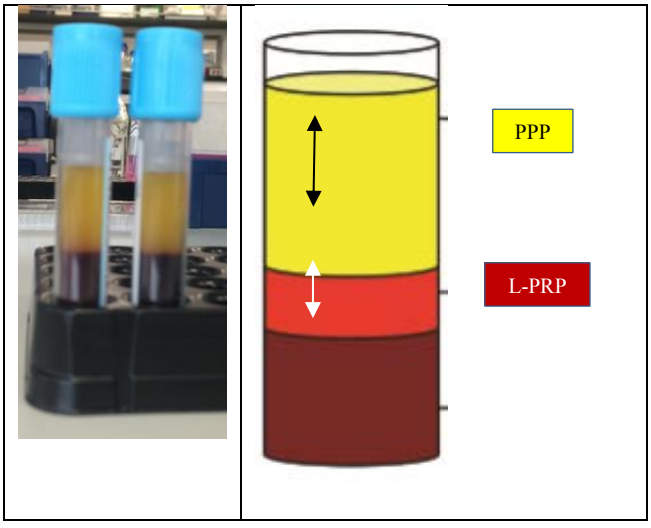


Figure 3.1. Separation of whole blood after centrifugation

The lower layer is composed of red cells. An intermediate very thin layer contains leucocytes and platelets, also known as buffy coat, which represents the **L-PRP preparation** and finally an upper layer that con tains plasma and a poor concentration of platelets, the **PPP preparation**.

Firstly, the upper layer was transferred under sterile condition into an empty sterile tube (Corning™ Falcon™ 15 ml Conical Centrifuge Tubes, Sigma-Aldrich, Saint Louis, USA). Once the upper layer had been removed, the intermediate thin layer was harvested and transferred in an empty 15 ml sterile Corning™ Falcon™. To prevent cellular damage caused by mechanical stress, large bore sterile micropipettes were used to harvest the preparations. 120 µl of each preparation were also extracted and components measured by the Sysmex analyser.

L-PRP volume yield was approximately 10% the initial volume of WB. The average ± standard deviation platelet count of whole blood was 211±41.8 x 10^6/ml, white blood count was 7.29±1.8 x 10^6/ml, neutrophils 4.76±1.8 x 10^6/ml, monocytes 0.6±0.2 x 10^6/ml, lymphocytes 1.77±0.29 x 10^6/ml, and red cells 4.63±0.98 x 10^9/ml.

With the single-step centrifugation method described, the average ± standard deviation platelet count of L-PRP preparation was 899±398 x 10^6/ml, white blood count was 28±12 x 10^6/ml, neutrophils 16.84±9 x 10^6/ml, monocytes 2.3±0.8 x 10^6/ml, lymphocytes 8.5±3.6 x 10^6/ml, and red cells 3.37±0.9 x 10^9/ml.

The average yield of platelets and white cells was 43% and 41% respectively

With this method, it was possible to obtain an L-PRP preparation that contained a platelet count with a minimal enrichment of 4-fold (average 4.26) from the whole blood and a leucocyte count with a minimal enrichment of 3-fold (average 3.89) (table 3.1) and a typical

differential count of neutrophils 61%, monocytes 8%, and lymphocytes 31%. (Type 2B according to Mishra PRP classification, [16]). PPP preparations contained a very low concentration of platelets (< 10 x 10^6/ml) and no other cellular components.

PREPARATION	PLT-F	WBC	NEUTR	MONOCY	LYMPH	RBC
L-PRP donor 1	796	37.31	26.39	2.45	7.77	5.33
WB donor 1	224	9.2	6.15	0.8	2.02	4.48
L-PRP donor 2	815	16.17	7.01	1.43	7.3	3.88
WB donor 2	159	6.41	3.79	0.53	193	3.75
L-PRP donor 3	798	37.18	25.97	3.72	6.91	2.42
WB dnor 3	165	9.3	6.66	0.91	1.58	4.75
L-PRP dnor 4	600	19.17	11.8	1.7	5.3	3.68
WB donor 4	209	5.04	3.43	0.32	1.23	6.72
L-PRP donor 5	1770	48.7	27.07	2.81	16.52	2.65
WB donor 5	212	5.61	2.99	0.41	1.93	4.27
L-PRP donor 6	1165	33.27	18.38	3.11	11.25	2.72
WB donor 6	219	6.18	3.59	0.53	1.95	3.9
L-PRP donor 7	715	12.89	4.09	1.84	6.74	3.18
WB donor 7	284	5.9	3.7	0.37	1.68	4.4
L-PRPdonor 8	536	22.45	14.04	1.6	6.58	3.17
WB donor 8	223	9.01	6.04	0.75	2.04	4.46
Whole Blood						
Mean	211.8571	7.291429	4.765714	0.601429	1.775714	4.637143
SD	41.88249	1.80959	1.43658	0.22349	0.296921	0.982662
L-PRP						
Mean	899.375	28.3925	16.84375	2.3325	8.54625	3.37875
SD	398.6205	12.55281	9.055518	0.825067	3.652607	0.934779
L-PRP: Leucocyte-Platelet Rich Plasma, WB: Whole Blood						

Table 3.1. Cellular composition of L-PRP preparation analysed in 8 donors

PRP preparation

Single-step method: Low g centrifugation (200 g) of anticoagulated blood for 10 minutes at 21°C was used. Under this condition, the centrifugation allows the separation of red cells

(lower layer) from the remaining whole blood components. The upper layer contains platelets and plasma, the desired PRP product. In order to prevent contamination of cells present in the lower portion, only the upper 2/3 of this solution was transferred under sterile condition into an empty sterile tube (Corning™ Falcon™ 15ml Conical Tubes, sigma-Aldrich, Saint Louis, USA). The 1/3 of the upper solution and the lower layer were discarded. To prevent cellular damage caused by mechanical stress, large bore sterile micropipettes were used to harvest the preparation. 120 µl of the preparation was extracted and components measured by the Sysmex analyser.

PRP volume yielded was approximately one-third the initial volume of WB, with an overall platelet yield of 64%. With this method, a 2-fold platelet enrichment from the whole blood was consistently obtained. (Average PLT-F in whole blood: 211.8±41.8 x 10^6/ml, PLT-F in PRP: 378±57 x 10^6/ml). Leucocytes and red cells were consistently negligible (Type 4B according to Mishra PRP classification, [16]).

PREPARATION	PLT-F	WBC	NEUTR	MONOCY	LYMPH	RBC
PRP donor1	472	0.02	0	0	0	0
PRP donor 2	314	0.17	0	0	0	0
PRP donor 3	321	0.1	0	0	0	0
PRP donor 4	406	0.02	0	0	0	0
PRP donor 5	406	0.01	0	0	0	0
PRP donor 6	407	0.02	0	0	0	0
PRP donor 7	389	0.01	0	0	0	0
PRP donor 8	314	0.37	0.23	0.04	0.1	0
Whole Blood						
Mean	211.8571	7.291429	4.765714	0.60142857	1.775714	4.637143
SD	41.88249	1.80959	1.43658	0.22348964	0.296921	0.982662
PRP						
Mean	378.625	0.09	0.02875	0.005	0.0125	0
SD	57.07623	0.126717	0.081317	0.01414214	0.035355	0
PRP: Platelet Rich Plasma, WB: Whole Blood						

Table 3.2. Cellular composition of PRP preparation analysed in 8 donors

Double-step method: Platelet pellet re-suspended in plasma

This method consists of a first 'soft' spin centrifugation, low g centrifugation (200 g) of anticoagulated blood for 10 minutes at 21 C. The obtained supernatant plasma was transferred into another sterile tube. In order to prevent platelet aggregation 5 µl of Prostaglandin (PGI_2, 1 mg/ml Sigma-Aldrich, Saint Louis, USA) was added. A second 'high' speed centrifugation was performed at a high g centrifugation (1000 g) for 10 min. The platelet pellet at the bottom of the tube was obtained and re-suspended in a calculated volume of double spun plasma to obtain the desired final platelet concentration.

Double spun plasma preparation: Following blood collection, a portion of whole blood was divided in 1.5 ml microtubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 2000 g for 20 min. The upper layer was further transferred into 1.5 ml microtubes and a second centrifugation at 13,000g for 2 min was performed. The obtained supernatant was transferred into a Corning™ Falcon™ (Sigma-Aldrich, Saint Louis, USA) 15 ml tube and a sample was analysed by Sysmex. The pellet obtained from the second centrifugation was discarded and this double spun plasma was used to adjust platelet concentration.

With this method, the desired platelet count was obtained.
The average PLT-F harvested in the L-PRP preparations was 899.4x 10^6/ml. In order to compare PRP and L-PRP preparations with similar concentration of platelets, the desired platelet count target was 900 x 10^6/ml. Leucocytes and red cells were consistently negligible. Repeated analysis of the double spun plasma showed the purity of the preparation (with no cellular presence).

CHAPTER 4. ANTIMICROBIAL PROPERTIES OF PRP PRODUCTS

4.1 Introduction

Local infections and sepsis related to burn wounds raise great concerns in the management of burn patients. In patients with severe burns (where over 40% of the Total Body Surface Area is burned) 75% of mortality is reported to be related to sepsis from infection of wounds or other organs and/or inhalation injuries [122, 123]

Gram-positive bacteria *Staphylococcus Aureus* [124] and Gram-negative bacteria, *Acinetobacter Baumannii* [125-127] and *Pseudomonas Aeruginosa* [127-129] are the most frequent bacteria strains found in burns wounds. It also has been shown that they are the most relevant pathogens of biofilms deep in wounds [130, 131]. Several dressings with antimicrobial properties are used do prevent or treat wound colonisation and infection [132]. The great concern is in regards the emergence of multidrug resistant organisms and the difficulty of eradicate bacteria growing in sessile biofilms. There is thus a need to discover novel antimicrobial products that can be effective particularly against these resistant strains of bacteria [131].

PRP products have been safely used in clinical setting to encourage wound healing. The alluring antimicrobial properties of these biomaterials suggested in published studies, directs our study to further investigate the bactericidal effects of these products. The aim of this chapter was to evaluate *in vitro* antimicrobial effects of different platelet-rich plasma preparations (with or without leukocytes) against the most frequent, resistant bacteria present in burn wounds by testing their capability to reduce bacteria growth and biofilm formation.

4.2 Methods

A series of *in vitro* experiments were conducted to define the efficacy of the biomaterials in terms of their ability to control bacteria growth and biofilm formation. (Appendix A).

4.2.1 Donors

The study group comprised 8 healthy volunteers examined between October 2015 and August 2016. All donors were free from infection and none had taken any medication during the previous two months. From each 50 ml of whole blood was collected. Written informed consent for participation in the study was obtained from each donor with the approval of the university research ethics committee.

4.2.2 Preparation of PPP, PRP, L-PRP

PPP, PRP, and L-PRP were prepared as described in Chapter 3. The antimicrobial effect of these biomaterials was tested with and without platelet activation prior to use. Different activators have been suggested by different authors, including calcium chloride, autologous and bovine thrombin. Calcium chloride is a safe option especially when used in a clinical setting as it has been shown that bovine thrombin can cause coagulopathy (ref 26,27). Autologus thrombin is another safe and faster alterative compared to calcium chloride, particularly relevant if used in clinical setting to minimise time of application. Autologus thrombin preparation kits are now available but the drawback compared to calcium chloride is the cost. Therefore, the activator used in our experiments was sterile calcium chloride ($CaCl_2$) at a 22.8 mM (3.4mg/mL) concentration [113], which required 20-30 minutes incubation for clot formation to occur.

4.2.3 Neutrophil isolation

Neutrophils were isolated from whole blood using a standard density gradient separation method using Percoll® (Sigma-Aldrich, Saint Louis, USA) [133]. All the procedures were

conducted under sterile conditions under a laminar flow hood and the solutions used were filter sterilised using a 33mm filter unit (Miller- GP, Sigma-Aldrich, Saint Louis, USA). 2% Dextran solution was used to permit separation of buffy coat from red cells. The solution was obtained by adding 1g of Dextran T500 (Pharmacosmos AS, Holbæk, Denmark) to a filter sterilised 50 ml of 0.9% normal saline (NaCl) solution. In order to create a gradient, 80% Percoll and 56% Percoll solutions were required. 6 ml of whole blood was collected into anticoagulated lithium heparin tubes (BD Vacutainer Tubes, New Jersey, USA) and transferred to 50 ml Falcon centrifuge tubes (Corning CentriStar, Sigma-Aldrich, Saint Louis, USA). 2% Dextran was added to a ratio of 1ml of 2% Dextran to 6 ml of whole blood. Separation of the buffy coat from the red cells occurred over 40 minutes. The buffy coat was layered on to the Percoll solution and centrifuged at 110 rpm (Jouan B4i Bench-top Centrifuge, Thermo Scientific, Wilmington, DE, USA) for 20 min (no brake). After centrifugation, the blood separated into clear bands: from the bottom to the top: red cells, isolation media, neutrophils, isolation media, peripheral blood mononuclear cell (PBMC) and plasma. Using a pipette the neutrophils were transferred into a 50 ml Falcon filled with 50 ml of RPMI 1640 (Sigma-Aldrich, Saint Louis, USA) medium and centrifuged at 1600 rpm ((Jouan B4i Bench-top Centrifuge, Thermo Scientific, Wilmington, DE, USA) for 10 min (no brake). After centrifugation neutrophils were resuspended in RPMI 1640. Neutrophil count was assessed using haemocytometer and confirmed by automatic count by Countess II Automated Cell Counter (Thermo Fisher Scientific, Waltham, USA). Neutrophil count was adjusted to a range of values: $0.5x10^6$/ml to $10x10^6$/ml.

Neutrophils were either primed, using 10 ng/ml TNFα [134] or not primed.

4.2.4 Bacteria stocks and preparation

The bacterial strains were obtained from the Laboratory T102, Institute of Microbiology and Infection, University of Birmingham. As described in table 4.1, the Gram-positive bacteria used was *Staphylococcus aureus* (MSSA_F77), the Gram-negative bacteria were *Pseudomonas aeruginosa* (PS_PA01) and *Acinetobacter Baumannii* (AB_AYE).

Study identifier	Organism	Description
MSSA_F77	*Staphylococcus aureus*	[NCTC_8532]
PS_PA01	*Pseudomonas aeruginosa*	[ATCC_15692] originally isolated from an infected wound
AB_AYE	*Acinetobacter baumannii*	MPR Clinical Isolate (unique)

Table 4.1 List of the control and clinical isolates used in this study

Bead frozen stocks were removed from the freezer and one bead was streaked across the surface on an agar plate. The plate was kept in incubator at 37°C overnight. Bacterial liquid stock was prepared as for the following protocol: overnight broth of each organism were prepared in duplicate by picking with a loop and inoculating 2-3 fresh colonies into 5 ml of LB (lysogeny broth) media in a universal. Additionally, a universal with only broth was prepared and used as negative control. Preparations were incubated overnight at 37°C with gentle agitation. On the day of the experiment, fresh MH (Mueller Hinton) broth was prepared and submitted for autoclaving. (10.5 g of MH broth powder in 500 ml of water). The bacterial count was assessed by optical density (OD) at 600 nm using the Jenway 6300 spectrophotometer (Bibby Scientific, Stone, Staffordshire, UK). The calibration control used was 1000 µl MH broth. For the bacterial preparation, 100 µl of culture were added to 900 µl MH broth in a cuvette. MH broth was used to dilute the culture in order to obtain 0.1 OD

following the formula: Actual OD/ desired OD = (OD reading x 10)/0.1 = fold dilution needed.

4.2.5 Positive and negative controls

Positive controls: P. aeruginosa in sterile water or *A. baumannii* and *S. aureus* in MH broth were used as positive control. Experiments were performed using at least six technical replicates of each isolate.

Negative controls: Acetic acid (AA) with a concentration of 5% was included as negative control. It has been previously reported that AA has an excellent bactericidal effect against Gram-negative bacteria [135] and it is effective at preventing biofilm formation at different concentration, from 0.31% up to 5% against *P. aeruginosa* and *A. baumannii* [105, 135]. In the preparation including *S. Aureus,* Ciprofloxacin solution (40 mg/ml) was used as negative control (10 ml of Ciprofloxacin 40 µg/ml, 0.1 g Ciprofloxacin powder, 1 ml 5% AA, 9 ml sterile water). Hanks' Balanced Salt Solution (HSSB), MH broth, sterile water was also used as negative controls.

4.2.6 Antimicrobial activity assay

The antimicrobial activity of blood preparations was tested using the Kirby-Bauer disc-diffusion method, a growth kinetic assay and a crystal violet biofilm formation assay.

Kirby-Bauer disk diffusion

The antimicrobial activity of blood preparations was tested using the Kirby-Bauer disc-diffusion method [136]. Lysogeny Broth (LB) agar plates were coated with methicillin-sensitive *S. Aureus* MSSA_F77 and *P. Aeruginosa* PS_PA01. 75 µl of PPP, L-PRP, and PRP, all previously activated with CaCl2 (solution of 180 µl + 20 µl CaCl2) were coated in the MSSA_F77 plate. Ciprofloxacin was used as negative control. 75 µl of L-PRP

previously activated with $CaCl_2$ (solution of 180 µl + 20 µl $CaCl_2$) were coated in the MSSA_F77 plate. Ciprofloxacin (40 mg/ml) was used as negative control.

The agar plates were incubated at 37˚C and the antimicrobial activity was visually assessed after 18- 24 hours by measuring the zones of inhibition. Biomaterials were considered to exhibit antimicrobial properties if a zone of growth inhibition is observed and recorded as the diameter in millimetres across the centre of the coated discs.

Growth kinetics assay

The growth kinetics of all strains were determined by measuring the optical density (OD) of cultures automatically in a FLUOstar Optima (BMG LABTECH, UK), as previously described [137]. The growth kinetics of all cultured biomaterials and controls included in a 96-well plate were examined by determining optical density (OD) using a spectrophotometer. Growth data were obtained in 8 different donors, 3 different bacteria strains, and each containing a minimum of four technical replicates. The rate of growth was analysed over a 12 h and 20 min period at 37°C. The FLUOstar instrument settings are listed in table 4.2. Increases in the optical density readings over time are caused by increased turbidity indicating bacterial growth. The line graph (figure 4.1) illustrates a typical change in OD of both positive and negative controls over 12 hours In the preparation including MSSA_F77 and broth (positive control) OD increased over time as expected from the increased turbidity of the preparation. The OD in the preparation with Ciprofloxacin maintains a flat curve as the antibiotic inhibits bacteria growth.

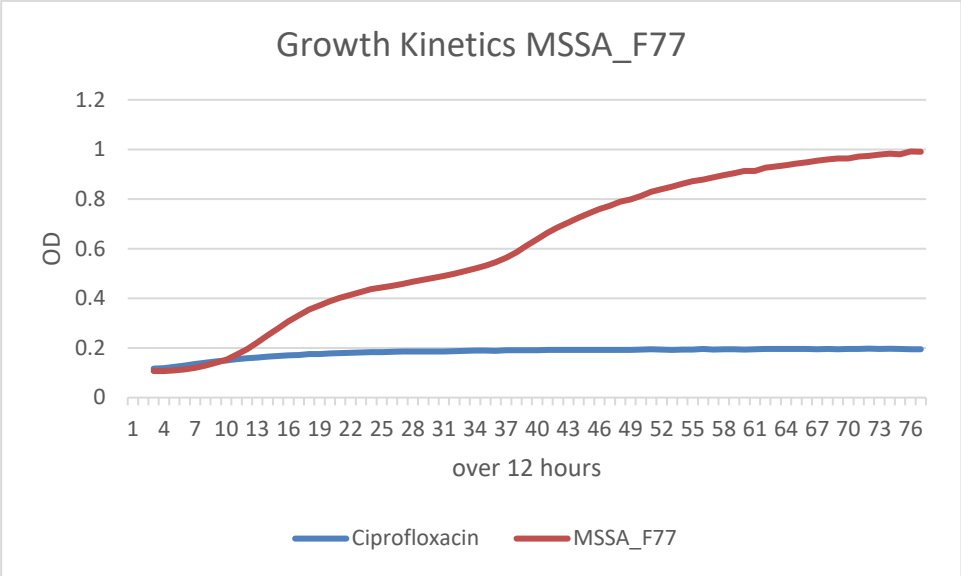


Figure 4.1 Growth kinetics of MSSA_F77 and negative control assessed by optical density

Modality	Absorbance - Growth
Time	12 h 20 min
Excitation filter	600 nm
Emission filter	600nm
Numb circle	75
Shaking time before each circle	4 sec

Table 4.2 FLUOstar OPTIMA settings for GK

Growth Kinetics: MSSA_F77 spiking

In order to evaluate the antimicrobial effects of PPP, PRP and L-PRP and isolated neutrophils against MSSA_F77, a 96-well MTT [Corning, New York] was seeded as for the following protocol:

For each donor a total volume of 1ml of PPP, PRP, L-PRP and isolated neutrophils was required.1ml of each preparation contained:

100 µl of biomaterial [primed/un-primed isolated neutrophils, activated PPP/PRP/L-PRP (10 µl $CaCl_2$)] + 800 µl MH broth + 100 µl of MSSA_F77 (table 4.3).

	1	2	3	4	5	6	7	8	9	10	11	12
A	PPP				PPP				PPP			
B	L-PRP				L-PRP				L-PRP			
C	PRP				PRP				PRP			
D	Neutrophils (primed)				Neutrophils (primed)				Neutrophils (primed)			
E	Neutrophils (unprimed)				Neutrophils (unprimed)				Neutrophils (unprimed)			
F	POS (MHB+MSSA_F77)				POS (MHB+MSSA_F77)				POS (MHB+MSSA_F77)			
G	Control (Cipro)				Control (Cipro)				Control (Cipro)			
H	Control (MHB)				Control (MHB)				Control (MHB)			
	DONOR 1				DONOR 2				DONOR 3			

Table 4.3 Growth Kinetics: MSSA_F77. Sample of designed template for 96-well MTT

Growth Kinetics: AB_AYE and PA01_PA01 spiking

To evaluate the antimicrobial effects of PPP, PRP, L-PRP and isolated neutrophils against AB_AYE and PA01_PA01, a 96-well MTT [Corning, New York] was seeded as for the following protocol: In each well of a 96-well MTT, 100µl of the AB_AYE or PA01_PA01 and 100µl of PPP, L-PRP or PRP preparations were seeded (table 4.4).

		1	2	3	4	5	6	7	8	9	10	11	12
AYE	A	100 µl PPP + 100 µl AYE						100 µl PPP + 100 µl AYE					
	B	100 µl PRP + 100 µl AYE						100 µl PRP + 100 µl AYE					
	C	100 µl AYE+ 100 µl MHB						100 µl AYE+ 100 µl MHB					
	D	100 µl AA+ 100 µl AYE						100 µl AA+ 100 µl AYE					
PA01	E	100 µl PPP + 100 µl PA01						100 µl PPP + 100 µl PA01					
	F	100 µl PRP + 100 µl PA01						100 µl PRP + 100 µl PA01					
	G	100 µl AYE+ 100 µl WATER						100 µl AYE+ 100 µl WATER					
	H	100 µl AA+ 100 µl PA01						100 µl AA+ 100 µl PA01					
		DONOR 1						DONOR 2					

Table 4.4 Growth Kinetics: AB_AYE and PS_PA01. Sample of designed template for 96-well MTT

Biofilm formation and detection

The ability of the biomaterials to prevent biofilm formation was evaluated using a crystal violet biofilm formation assay as described by Baugh *et al.* [138]. The following bacteria strains have been tested:

- PS_PA01 Pseudomonas aeruginosa ATCC_15692 (10,2,2/4,2/5,5,3,3,8)
- PS_919 Pseudomonas aeruginosa QEHB Clinical burn isolate (10,2,5,4,6,2,-,4)
- AB_AYE Acinetobacter baumannii MPR Clinical Isolate (unique)
- AB_53 Acinetobacter baumannii QEHB Clinical burn isolate (QUEE13AC-27)

The ability of biofilm production of *S. aureus* has been previously studied by our group [105] and demonstrated that biofilm production was both poor and unreliable.

In each well of a 96-well MTT [Corning, New York], 100 µl of the test strains and 100µl of PPP, L-PRP or PRP were seeded. Suitable controls were included in each assay. Negative controls were: 200 µl of MH broth and 100 µl of the test strains and 100 µl of 5% AA. Positive controls were: 100µl AB_AYE in 100 µl of MH broth and 100 µl PS_PA01 in 100 µl of sterile water (table 4.5). Plates were sealed and statically incubated at (37°C) for 3 hours allowing biofilm formation. The liquid was removed from the wells and thoroughly the plates were rinsed in tap water in order to remove any unbound cells. Existing formed biofilms were visualised through staining with 1% crystal violet (CV) [Sigma Aldrich, Poole, UK] solution for 15 minutes and subsequently rinsed with tap water to remove any unbound CV. The bound dye was then solubilised by the addition of 70% ethanol. After 15 minutes incubation the biomass of the biofilm of the solubilised CV solution was measured using a FLUOstar Optima [BMG Labtech]. All biofilm assays were performed twice with six technical replicates per repeat.

	1	2	3	4	5	6	7	8	9	10	11	12
A	L-PRP	L-PRP	L-PRP	L-PRP	L-PRP	L-PRP	L-PRP	L-PRP	L-PRP	L-PRP	L-PRP	L-PRP
B	PRP	PRP	PRP	PRP	PRP	PRP	PRP	PRP	PRP	PRP	PRP	PRP
C	PPP	PPP	PPP	PPP	PPP	PPP	PPP	PPP	PPP	PPP	PPP	PPP
D	MBH											
E	MBH											
F	MBH											
G	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
H	POS (in broth)						POS(in water)					

AB-AYE PS_PA01

Table 4.5 Biofilm formation. Sample of 96-well MTT seeded with PPP, L-PRP, PRP products and controls

4.2.7 Statistical analysis

Kinetics Analysis: The kinetics were modelled over time using linear mixed models that can account for the variability in measurements between donors and the repeated measures structure of the data. Models incorporating three different random effects structures were explored and we found that the model that included both random slopes and intercepts with no correlation between them provided the best fit to the data, as measured using AIC (Akaike information criterion). We allowed the time of the observation to be modelled using a restricted cubic spline to allow for flexible non-linear models to be fit to the data. The number of knots in the spline was determined using AIC. ANOVA was used to assess the overall contribution of time and group as main effects, and the interaction between time and group.

Biofilm analysis: Preliminary tests of the biofilm forming ability of different bacteria were studied using Kruskal-Wallis tests (section 4.3.3). Poor biofilm forming bacteria were excluded for further analysis. The effect of group on the biomass level for selected bacteria was then assessed using one-way analysis of variance (ANOVA) (section 4.3.4). Visual inspection and descriptive summaries of the data suggested that the groups exhibited different means and standard deviations. Levene's test was used to formally assess the equality of variances across the groups and Welch's statistic provided a robust test of equality of means corrected for heteroscedasticity. Games-Howell adjustments were used to account for the unequal variances in the post-hoc tests of mean differences between groups.

Statistically significance was assessed using a threshold of $p<0.05$. All analysis was performed using IBM SPSS Statistics software (v.22).

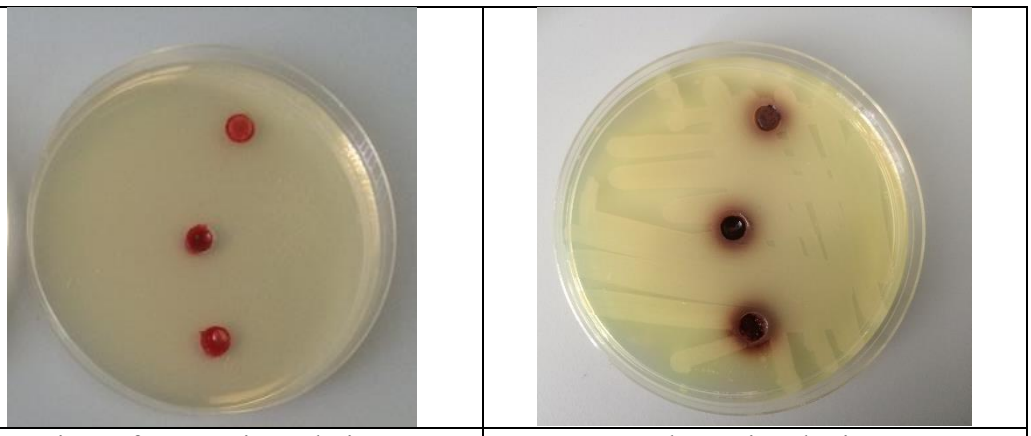
4.3 Results

4.3.1 Kirby-Bauer disk diffusion

PPP, L-PRP, PRP preparations all failed to show any MSSA_F77 growth inhibition. (Figure 4.2). After 18 hours incubation, no inhibition zone was detected in the plates where the biomaterials were inoculated. As expected a large zone of inhibition was observed with the MSSA_F77 coated plate in the presence of Ciprofloxacin. An undefined inhibition zone was observed for the PS_PA01 coated plate in the presence of activated L-PRP after 18 hours incubation (figure 4.3).

Time of L-PRP inoculation	18 hours incubation

Figure 4.2 Agar plate MSSA_F77 coated: no inhibition zone detected after L-PRP inoculation



Time of L-PRP inoculation	12 hours incubation

Figure 4.3 Agar plate PA_01 coated: poor inhibition zone detected after L-PRP inoculation

4.3.2 Bacteria growth after 24 h incubation

All the preparations were further incubated overnight at 37˚C and inspected the next day to visually assess the turbidity (figure 4.4). PPP, PRP, L-PRP, isolated neutrophils inoculated with MSSA_F77, AB_AYE, PS_PA01 and positive control were turbid. Negative controls remain clear after 24 hours.

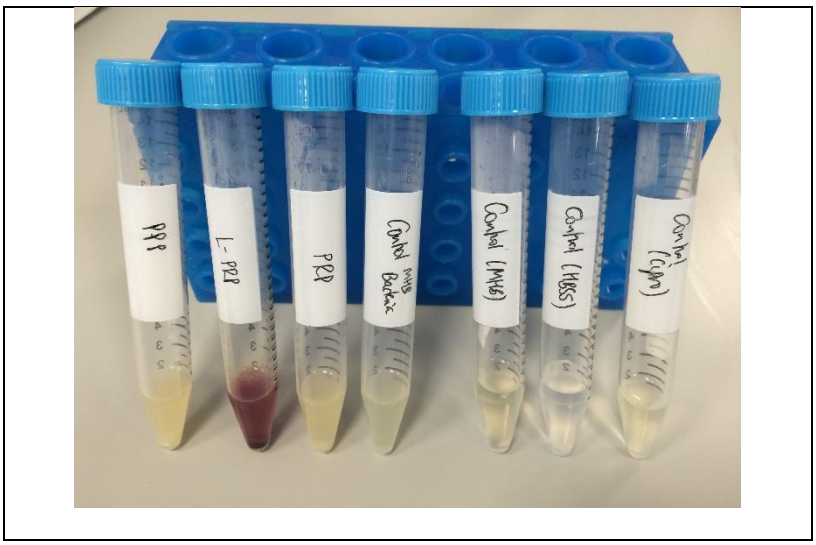


Figure 4.4 Visual assessment of turbidity of the preparation

4.3.3 Bacteria ability to form biofilm

A spectrum of biofilm formation was consistently seen for PS_PA01 and AB_AYE and therefore included in the study for the further analysis of anti-biofilm effects of blood products (figure 4.5). PS_919 and AB_53 were poor at producing biofilms. Isolates where the average biofilm formation (as measured by OD) after crystal violet staining was not significantly different to the broth or controls, were excluded from further analysis. In figure 4.4, the optical density on the y axis refers to the average biofilm biomass for all the isolates shown on the x axis. It is apparent that AB_53 and PS_919 demonstrated poor ability to form biofilms ability when compared to their other species counterparts. Using Kruskal-Wallis test, no significant difference was seen when AB_53 and AB_AYE groups where

compared. In contrast, PS_PA01 and AB_AYE showed excellent biofilming capability compared to theirs controls (p-value <0.001).

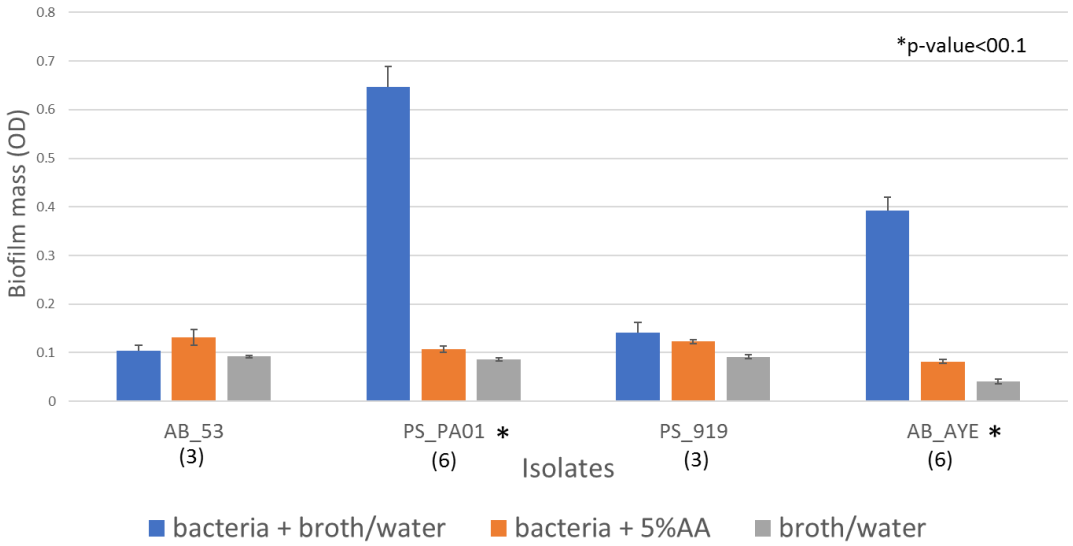


Figure 4.5. Biomass of the biofilms produced by the isolates tested. Optical density on the y axis refers to the mean biofilm biomass for all the isolates shown on the x axis. Error bars: standard error of the mean for each value. *: values statistically significantly different between the 3 groups (p-value<0.001). (n): numbers of replicates for each isolate tested

4.3.4 Effects of Blood Products in preventing biofilm formation

Blood was drawn from 2 healthy donors and blood products were prepared as described in Chapter 3.2.4. The composition of blood products for each donor is shown in table 4.6. In donor 1, in the L-PRP preparation PLT-F was 4-fold increase from the whole blood, WBC 3.9-fold, neutrophils 3.3-fold, lymphocytes 4.6-fold, and monocytes 3.3-fold and red cell count similar to whole blood. In the PRP preparation, using the double spin method, PLT-F was 3.8-fold increased than whole blood.

In donor 2, in the L-PRP preparation PLT-F was 4.4-fold increase from the whole blood, WBC 4.6-fold, neutrophils 4.4-fold, lymphocytes 4.6-fold, and monocytes 5.7-fold and red

cell count was decreased from whole blood by 2-fold. In the PRP preparation, using the double spin method, PLT-F was 4.3-fold increased than whole blood.

	PLT-F	WBC	RBC
DONOR 1			
Whole blood	232 x10^6/ml	3.60 x10^6/ml neutr:0.92 x10^6/ml lymph:1.88 x10^6/ml mono:0.51 x10^6/ml	4.37 x10^9/ml
L-PRP	944 x10^6/ml	14.24x10^6/ml neutr:3.04 x10^6/ml lymph:8.66 x10^6/ml mono:1.69 x10^6/ml	4.08 x10^9/ml
PRP	889x10^6/ml	0	0
DONOR 2			
Whole blood	204 x10^6/ml	8.81 x10^6/ml neutr:6.15 lymph:2.02 mono:0.80	4.48 x10^9/ml
L-PRP	910 x10^6/ml	41.92 x10^6/ml neutr:27.57 x10^6/ml lymph:9.21 x10^6/ml mono:4.62 x10^6/ml	2.87 x10^9/ml
PRP	890x10^6/ml	0	0

Table 4.6 Platelets and Leucocytes composition in different Blood-Derived products

Different blood preparations such as activated L-PRP, non-activated L-PRP, PPP, PRP, negative control (PA01 + 5% AA and broth) and positive control (PA01 + water) were tested against *Pseudomonas Aeruginosa PS_PA01* and *Acinetobacter Baumannii AB_AYE*. The statistical analysis was run separately for the 2 donors.

Analysis of Donor 1

Pseudomonas Aeruginosa PS_PA01

The observed data for each group are plotted (figure 4.6). For each group, data of the 6 replicates are plotted.

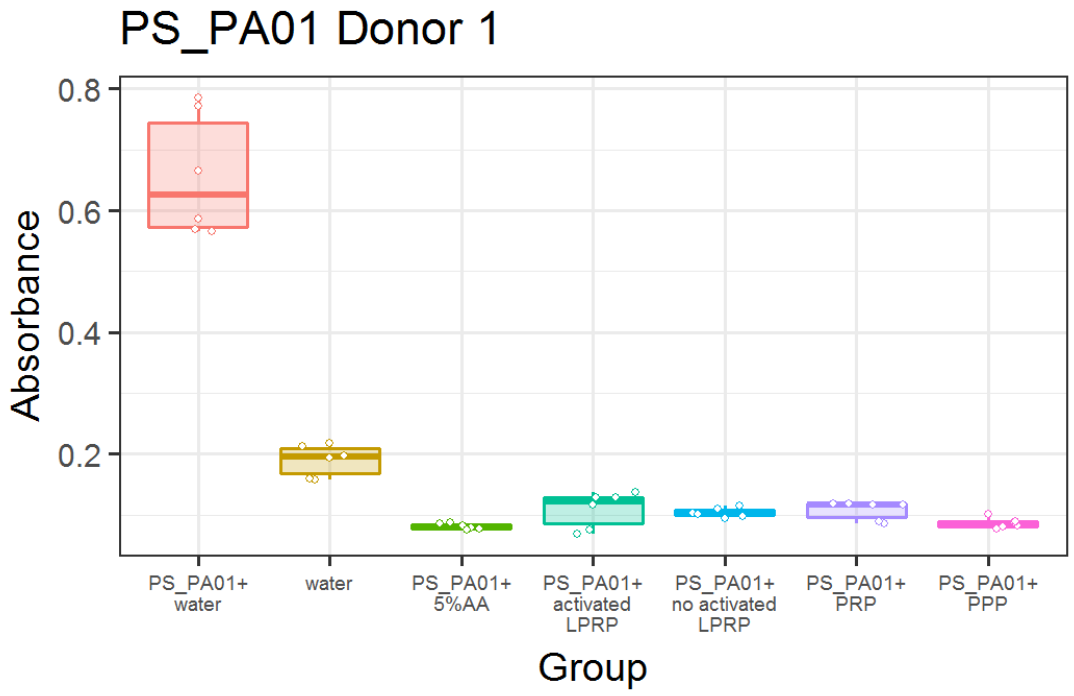


Figure 4.6 Donor 1 PS_PA01 biomass reading

The plot clearly shows that the levels of biofilm mass (expressed as levels of absorbance) were minimal in the following preparations:

- Preparations including PS_PA01+activated LPRP, PS_PA01+no activated LPRP, PS_PA01+PRP, and PS_PA01+PPP.
- Negative controls: sterile water, and PS_PA01+ 5% Acetic Acid.

Positive control (PS_PA01+Water group) had elevated levels of biofilm biomass relative to the other groups indicating significant inhibition of bacterial growth by all test conditions.

A test of homogeneity of variances returns a Levene's test statistic of 15.347 (6,35 degrees of freedom; p-value: <0.001) suggesting there is strong evidence in our data to reject the assumption of equal variances. Accounting for the unequal variances, an ANOVA using Welch's robust test of equality of means returns a statistic of 44.71 (6, 14.991 degrees of freedom; p-value: <0.001) giving convincing evidence for statistically significant mean differences between groups. Post-hoc tests of mean differences between groups are carried out using Games-Howell adjustments, and we find the following pairs with a statistically significant difference in means using a threshold of p<0.05 (Table 4.7):

Group I	Group J	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PA01 + water	**activated LPRP**	0.468	0.042	0.000	0.287	0.648
PA01 + water	**no activated LPRP**	0.576	0.041	0.000	0.392	0.760
PA01 + water	**PPP**	0.554	0.041	0.000	0.370	0.737
PA01 + water	**PA01 + 5%AA**	0.550	0.042	0.000	0.367	0.732
PA01 + water	**broth**	0.571	0.041	0.000	0.387	0.754
PA01 + water	**PRP**	0.548	0.043	0.000	0.368	0.729
activated LPRP	**no activated LPRP**	0.109	0.011	0.001	0.062	0.155
activated LPRP	**PPP**	0.086	0.011	0.002	0.040	0.132
activated LPRP	**PA01 + 5%AA**	0.082	0.012	0.002	0.036	0.128
activated LPRP	**broth**	0.103	0.011	0.001	0.057	0.149
activated LPRP	**PRP**	0.081	0.016	0.006	0.023	0.139
PPP	**no activated LPRP**	0.023	0.004	0.003	0.009	0.036
PPP	**broth**	0.017	0.005	0.043	0.000	0.034

Table 4.7 - Post-hoc comparisons of mean absorbance for PS_PA01 Donor 1

Acinetobacter baumanii AB_AYE

The observed data for each group are plotted (figure 4.7). For each group, data of the 6 replicates are plotted.

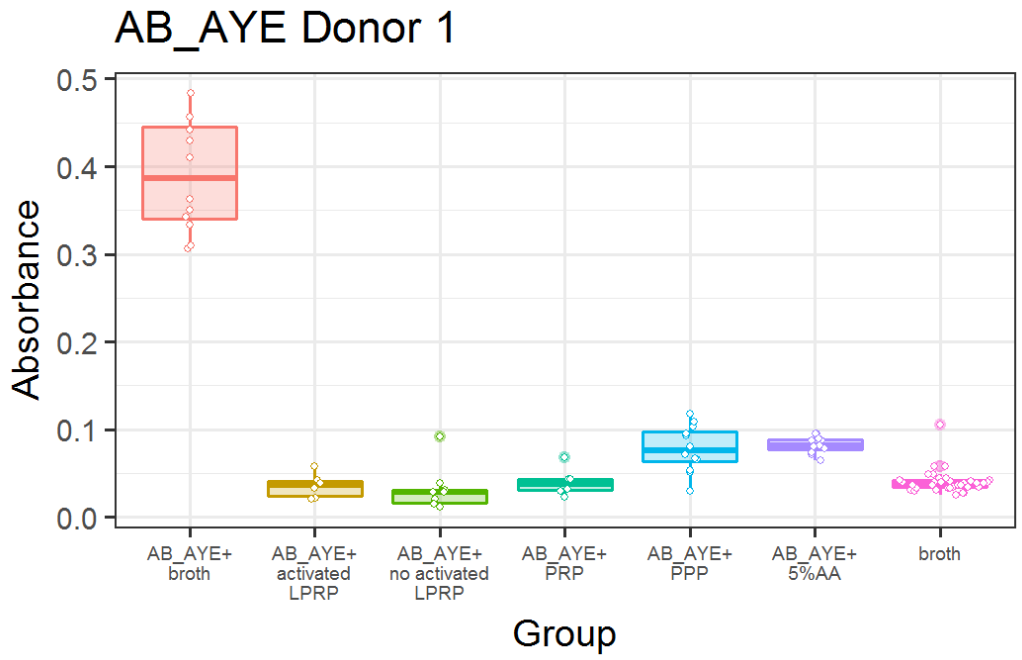


Figure 4.7 Donor 1 AB_AYE biomass reading

The plot clearly shows that the levels of biofilm mass (expressed as levels of absorbance) were minimal in the following preparations:

- Preparations including AB_AYE+activated LPRP, AB_AYE +no activated LPRP, AB_AYE +PRP, and AB_AYE +PPP.
- Negative controls: broth, and AB_AYE + 5% Acetic Acid.

Positive control group (AB_AYE + broth) had elevated levels of biofilm biomass relative to the other groups suggesting a significant effect of all preparations on bacterial growth.

A test of homogeneity of variances returns a Levene's test statistic of 20.865 (6,84 degrees of freedom; p-value: <0.001) suggesting there is convincing evidence in our data to reject

the assumption of equal variances. Accounting for the unequal variances, an ANOVA using Welch's robust test of equality of means returns a statistic of 70.80 (6, 23.288 degrees of freedom; p-value: <0.001) giving robust evidence for statistically significant mean differences between groups. Post-hoc tests of mean differences between groups are carried out using Games-Howell adjustments and we find the following pairs with a statistically significant difference in means using a threshold of $p<0.05$ (Table 4.8):

Group I	Group J	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AYE + broth	**activated LPRP**	0.357	0.020	0.000	0.288	0.426
AYE + broth	**no activated LPRP**	0.361	0.021	0.000	0.291	0.432
AYE + broth	**PRP**	0.353	0.020	0.000	0.284	0.422
AYE + broth	**PPP**	0.315	0.020	0.000	0.245	0.384
AYE + broth	**AYE+ 5%AA**	0.311	0.019	0.000	0.243	0.378
AYE + broth	**broth**	0.352	0.019	0.000	0.285	0.420
activated LPRP	**PPP**	-0.042	0.010	0.006	-0.074	-0.010
activated LPRP	**AYE+ 5%AA**	-0.046	0.006	0.002	-0.071	-0.021
no activated LPRP	**PPP**	-0.047	0.011	0.008	-0.083	-0.010
no activated LPRP	**AYE+ 5%AA**	-0.051	0.009	0.002	-0.082	-0.020
PRP	**PPP**	-0.038	0.010	0.022	-0.072	-0.004
PRP	**AYE+ 5%AA**	-0.042	0.007	0.007	-0.071	-0.014
PPP	**broth**	0.038	0.008	0.005	0.010	0.065

Table 4.8 - Post-hoc comparisons of mean absorbance for AB_AYE Donor 1

Analysis of Donor 2

Pseudomonas Aeruginosa PS_PA01

The observed data for each group are plotted (figure 4.8). For each group, data of the 6 replicates are plotted.

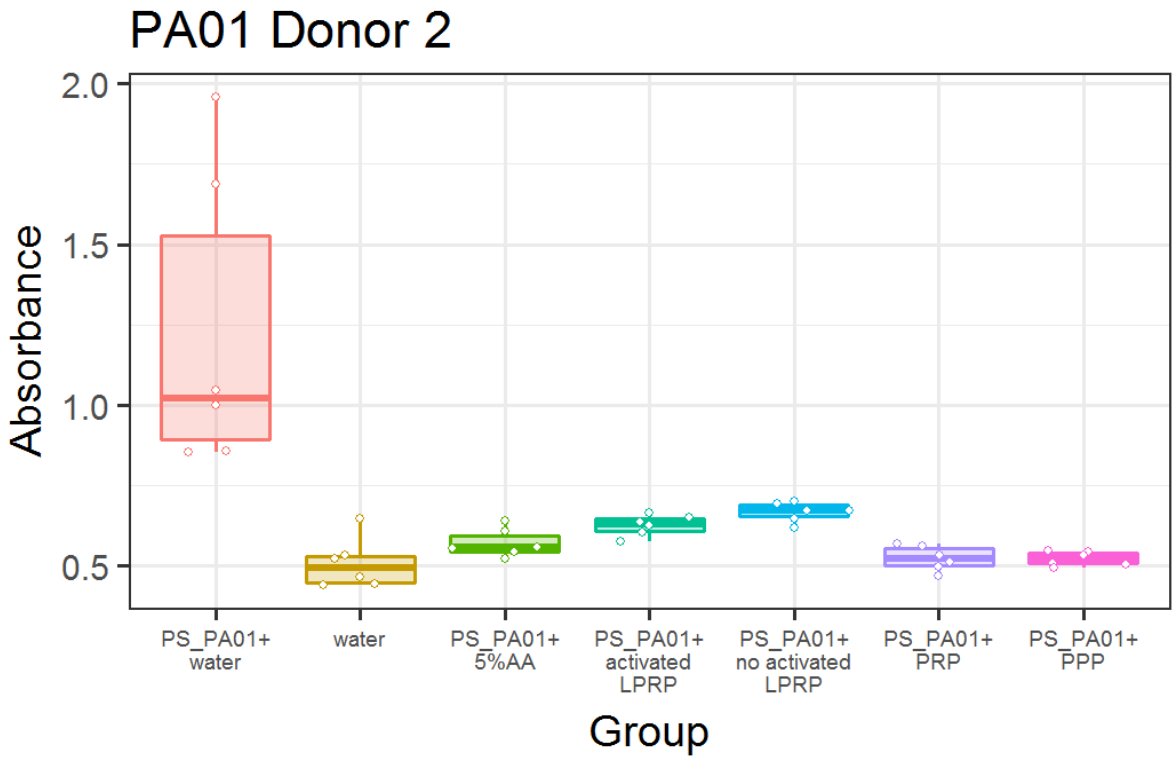


Figure 4.8 Donor 2 PS_PA01 biomass reading

Positive control (PS_PA01+Water group) had elevated levels of biofilm biomass relative to the other groups with a wider range of values than seen for donor 1, though an effect of all preparations was again suggested. The large variability in the observed values meant there were no statistically significant differences between the mean absorbance value of the positive control (PS_PA01+Water group) and the other groups.

A test of homogeneity of variances returns a Levene's test statistic of 19.912 (6,35 degrees of freedom; p-value: <0.001) suggesting there is strong evidence in our data to reject the

assumption of equal variances. Accounting for the unequal variances, an ANOVA using Welch's robust test of equality of means returns a statistic of 18.670 (6, 15.259 degrees of freedom; p-value: <0.001) giving robust evidence for statistically significant mean differences between groups. Post-hoc tests of mean differences between groups are carried out using Games-Howell adjustments and we find the following pairs with a statistically significant difference in means using a threshold of $p<0.05$ (Table 4.9):

Group I	Group J	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
activated LPRP	PPP	-0.159	0.034	0.027	-0.298	-0.019
no activated LPRP	PPP	-0.097	0.022	0.019	-0.178	-0.015
PPP	PA01 + 5%AA	0.143	0.020	0.001	0.069	0.217
PPP	broth	0.146	0.015	0.000	0.090	0.201
PA01 + 5%AA	PRP	-0.102	0.021	0.008	-0.177	-0.027
broth	PRP	-0.104	0.016	0.001	-0.162	-0.046

Table 4.9 - Post-hoc comparisons of mean absorbance for PS_PA01 Donor 2

Acinetobacter baumanii AB_AYE

The observed data for each group are plotted (figure 4.9). For each group, data of the 6 replicates are plotted.

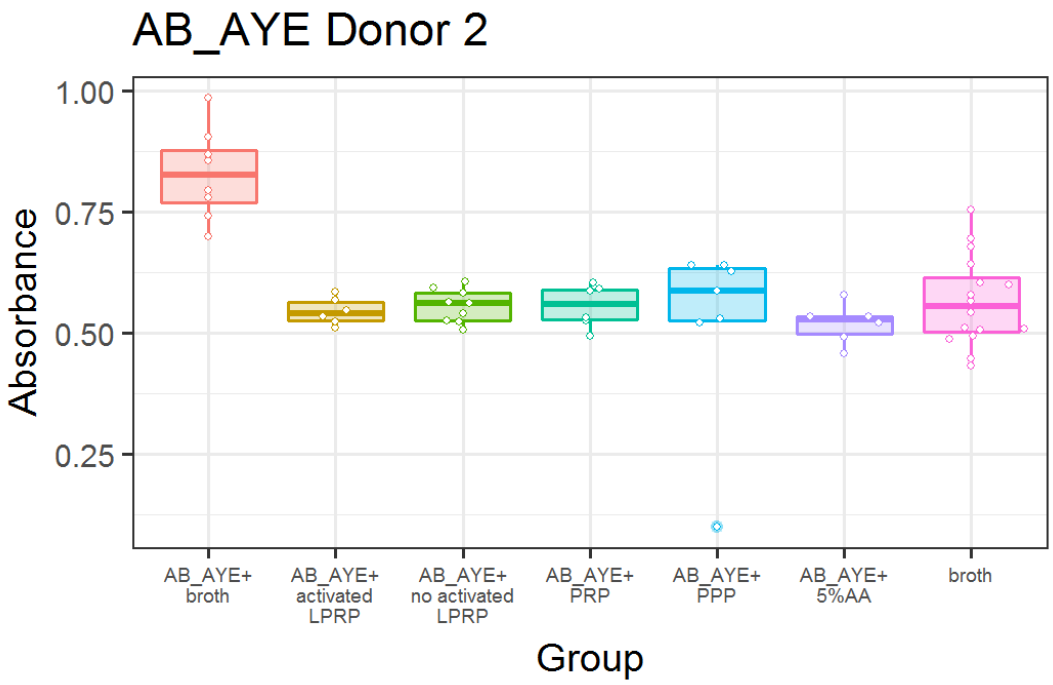


Figure 4.9 Donor AB_AYE biomass reading

Positive control group (AB_AYE + broth) had elevated levels of biofilm biomass relative to the other groups, suggesting and inhibitory effect of the various preparations on bacterial growth.

A test of homogeneity of variances returns a Levene's test statistic of 2.638 (6,51 degrees of freedom; p-value: 0.026) suggesting there is strong evidence in our data to reject the assumption of equal variances. Accounting for the unequal variances, an ANOVA using Welch's robust test of equality of means returns a statistic of 10.626 (6, 19.744 degrees of freedom; p-value: <0.001) giving strong evidence for statistically significant mean differences between groups. Post-hoc tests of mean differences between groups are carried

out using Games-Howell adjustments and we find the following pairs with a statistically significant difference in means using a threshold of $p<0.05$ (Table 1.10):

Group I	Group J	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AYE + broth	activated LPRP	0.284	0.035	0.000	0.153	0.414
AYE + broth	no activated LPRP	-0.273	0.035	0.000	0.143	0.404
AYE + broth	PRP	-0.273	0.038	0.000	0.139	0.408
AYE + broth	PPP	-0.308	0.080	0.045	0.007	0.609
AYE + broth	AYE+ 5%AA	0.309	0.037	0.000	0.176	0.443
AYE + broth	broth	0.263	0.040	0.000	0.126	0.401

Table 1.10- Post-hoc comparisons of mean absorbance for AB_AYE Donor 2

4.3.5 Growth kinetic essay

Acinetobacter baumanii AB_AYE

Bacterial growth (expressed as absorbance) over 12 hours and 20 min for the 8 donors is shown in figure 4.10. The dashed line corresponds to the control samples. For each donor, separate lines for PPP and PRP are displayed.

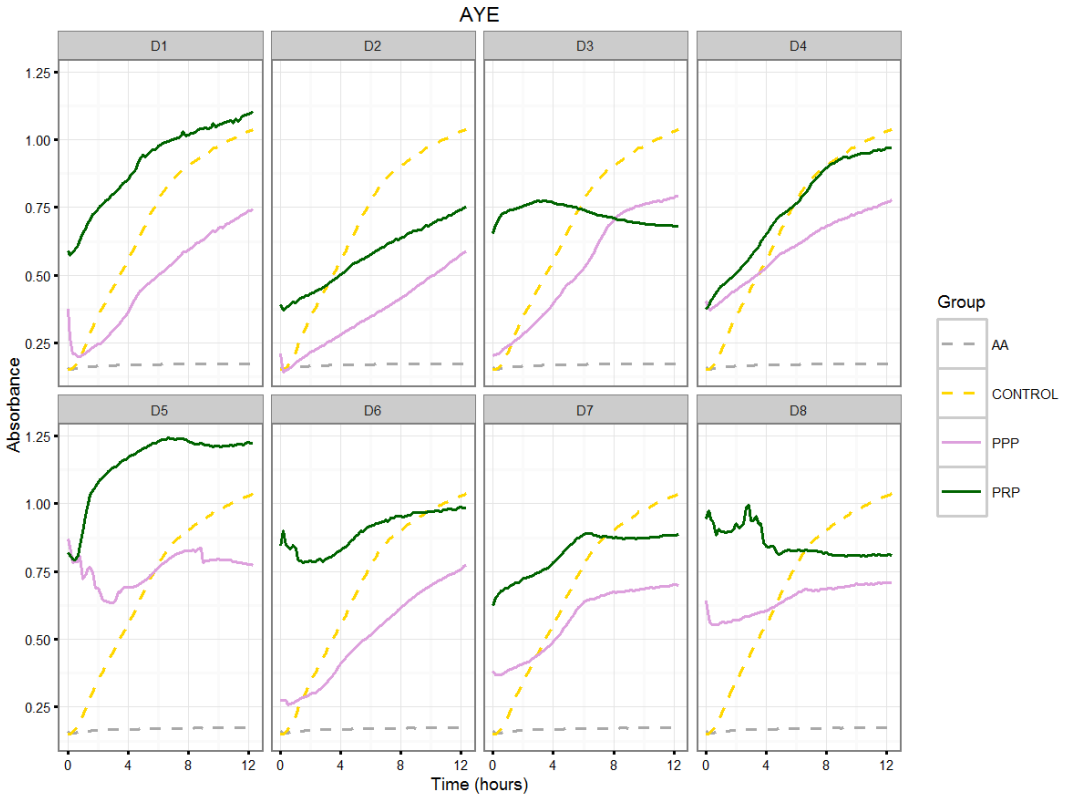


Figure 4.10 AB_AYE growth (expressed as absorbance) over time plotted for the 8 donors

The level of absorbance was different at time 0 for the four different groups (PPP, PRP, AA, and control) and changed over time with different kinetics for each preparation varied by time. Some donor preparations clearly inhibited growth compared to the control (PPP for donors 1, 2, and 6) whereas others showed little effect of the PRP from donors 1, 4, and 5). Figure 4.11 shows the model fitted values of absorbance for the four groups over time using the data from the 8 donors.

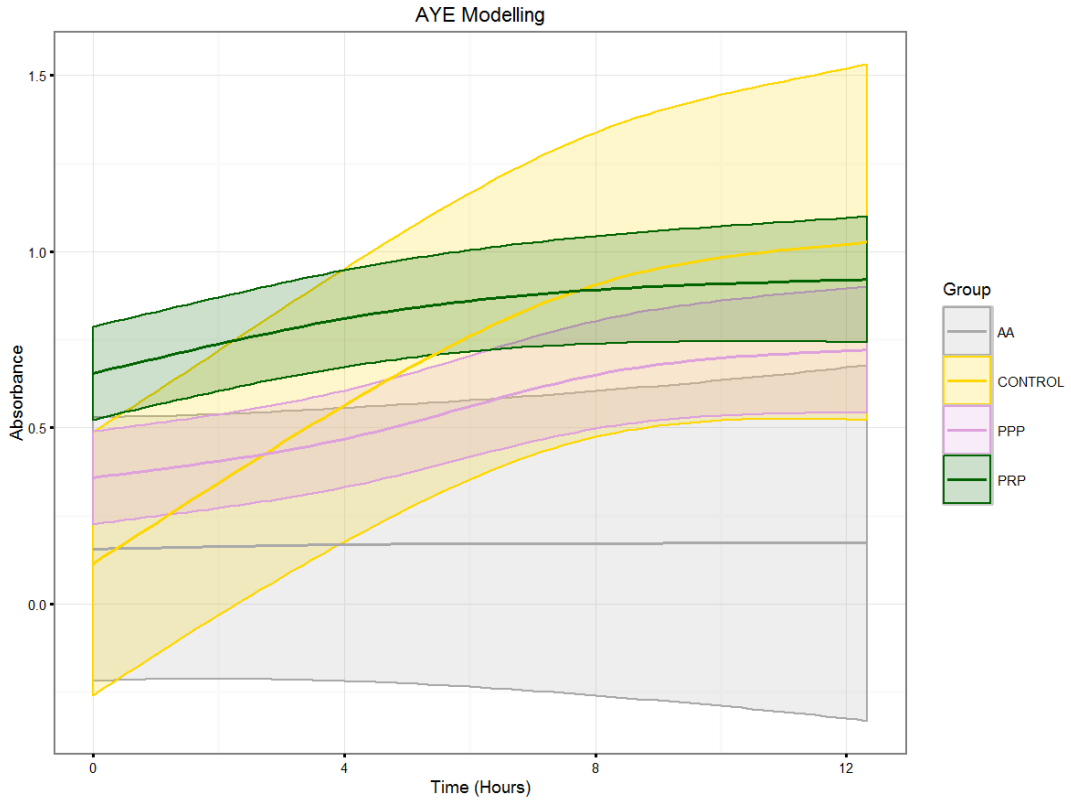


Figure 4.11 AB_AYE growth over time. Model fitted values of absorbance for the AA, Control, PPP and PRP groups

Interested in the relative change over time, rather than the absolute change, we scaled the data so that the absorbance at time 0 for each donor and control was scaled to 0.

The 'Group' main effect is now effectively zero, but the main effect of time and the interaction between time and group are both statistically significant. The model fitted output for these four groups is plotted in figure 4.12:

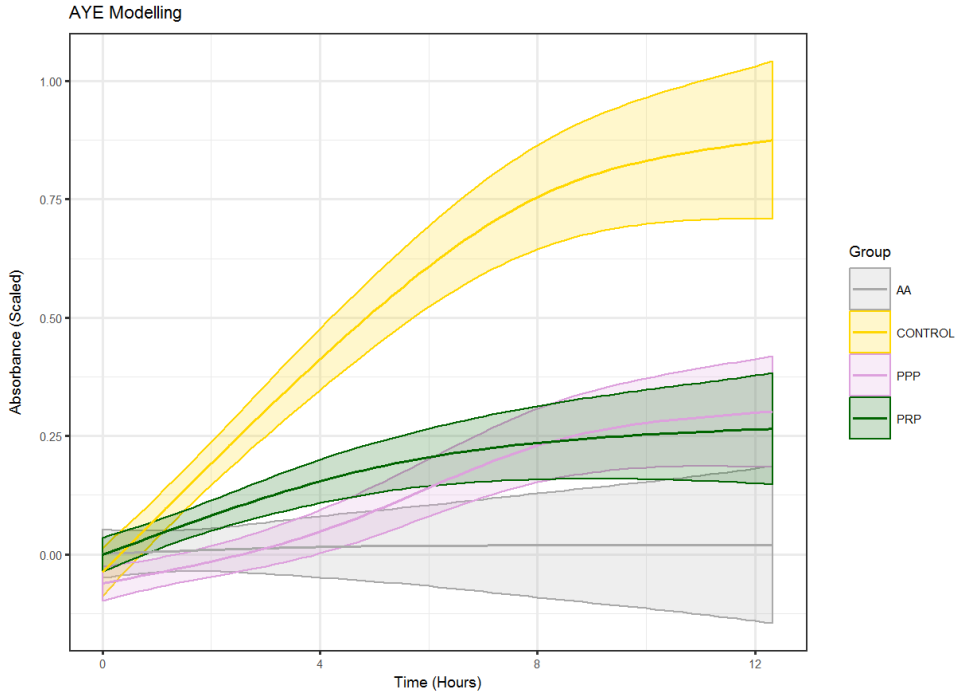


Figure 4.12 AB_AYE growth over time. The absorbance at time 0 for each donor and control is scaled to 0

There is now a clear difference in the behaviour of the Control sample over time compared with the other three groups and all three test groups showed lower absorbance than the control group. There is little difference between the PPP and PRP groups by the end of the experiment. Although the AA group is not distinguishable from the PPP group, this is likely to be a result of insufficient data to provide a suitably precise estimate of the absorbance in the AA group over time. We can observe this by examining the estimated levels of absorbance at 0 hours and at 12 hours 20 minutes. These estimates are presented, along with 95% CIs, in the following table:

Group	Time = 0 minutes		Time =12hrs 20 minutes	
	Est	95%CI	Est	95%CI
AA	0.002	(-0.049, 0.053)	0.020	(-0.146, 0.187)
Control	-0.037	(-0.088, 0.014)	0.876	(0.709, 1.042)
PPP	-0.062	(-0.098, -0.026)	0.302	(0.184, 0.419)
PRP	-0.001	(-0.037, 0.035)	0.266	(0.148, 0.383)

When the control group is compared to the PPP group, the p-value of the interaction term is <0.0001, suggesting there is a highly statistically significant difference between the groups. Similarly, comparing the control group to the PRP group, the p-value for the interaction term is <0.0001.

Analysing the data at 12h and 20 min post incubation using pairwise tests comparing each group with all the others, we can see that PPP and PRP preparation are both significantly different to control group (p-value <0.0001) whilst there is no significant difference between the 2 preparations and the preparation including AB_AYE+5%Acid Acid.

Therefore, we can state that the preparations including AB_AYE+5%Acid Acid (negative control), AB_AYE+PRP, and AB_AYE+PPP significantly reduced AB_AYE growth compared to the positive control (AB_AYE+broth), overtime and 12 hours post incubation.

Group 1	Group 2	p-value
AA	CONTROL	<0.0001
AA	PPP	0.0078
AA	PRP	0.0241
CONTROL	PPP	<0.0001
CONTROL	PRP	<0.0001
PPP	PRP	0.7308

Pseudomonas Aeruginosa PS_PA01

The absorbance over time for the 8 donors is plotted in figure 4.13. The dashed lines correspond to the control samples. For each donor, separate lines corresponding to PPP and PRP are displayed.

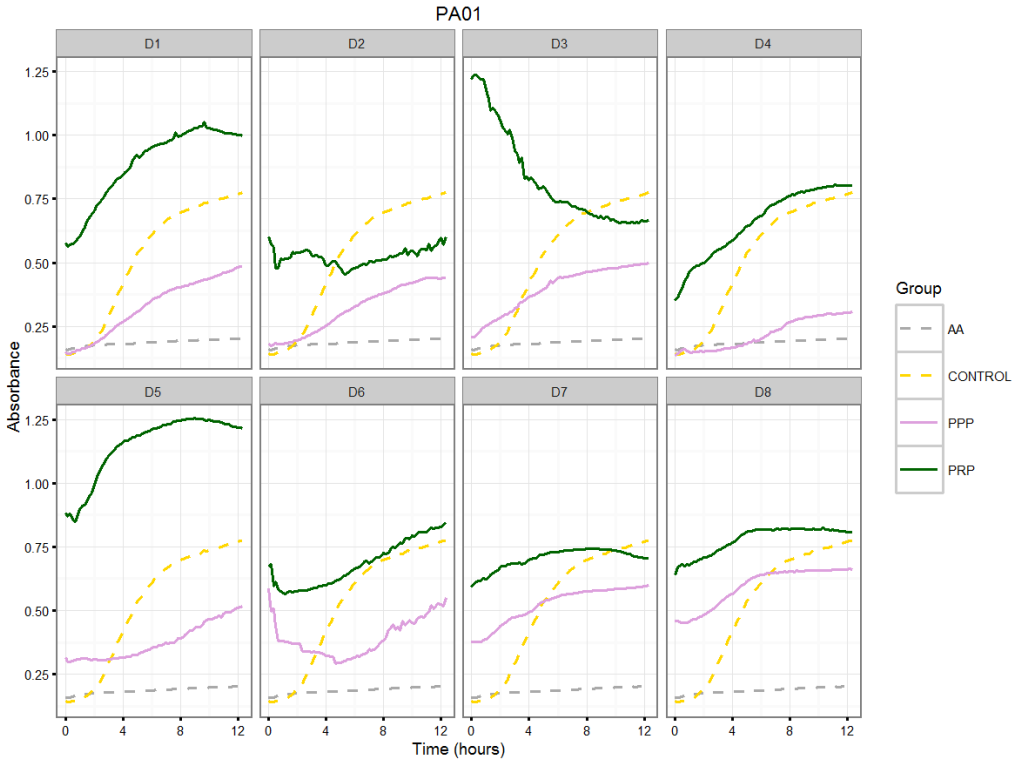


Figure 4.13 PS_PA01 (expressed as absorbance) over time plotted for the 8 donors

The level of absorbance was again different at time 0 for the four distinct groups (PPP, PRP, AA and control), varied by time and did so in a different manner depending on the group. Figure 4.14 shows the model fitted values of absorbance for the four groups over time.

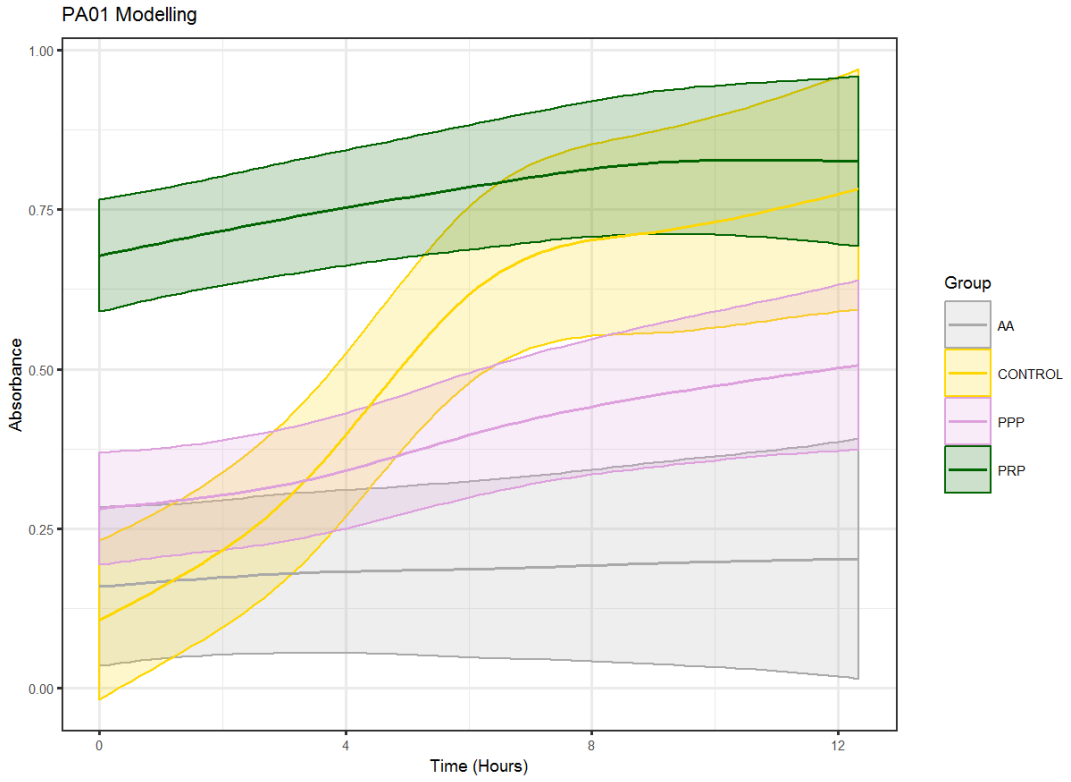


Figure 4.14 PS_PA01 growth over time. Model fitted values of absorbance for the AA, Control, PPP and PRP groups

To obtain a clearer estimate of the relative changes over time by group, data were scaled so that the absorbance at time 0 for each donor and control is scaled to 0.

The 'Group' main effect is now effectively zero, but the main effect of time and the interaction between time and group are both statistically significant. The model fitted output for these four groups is plotted in figure 4.15:

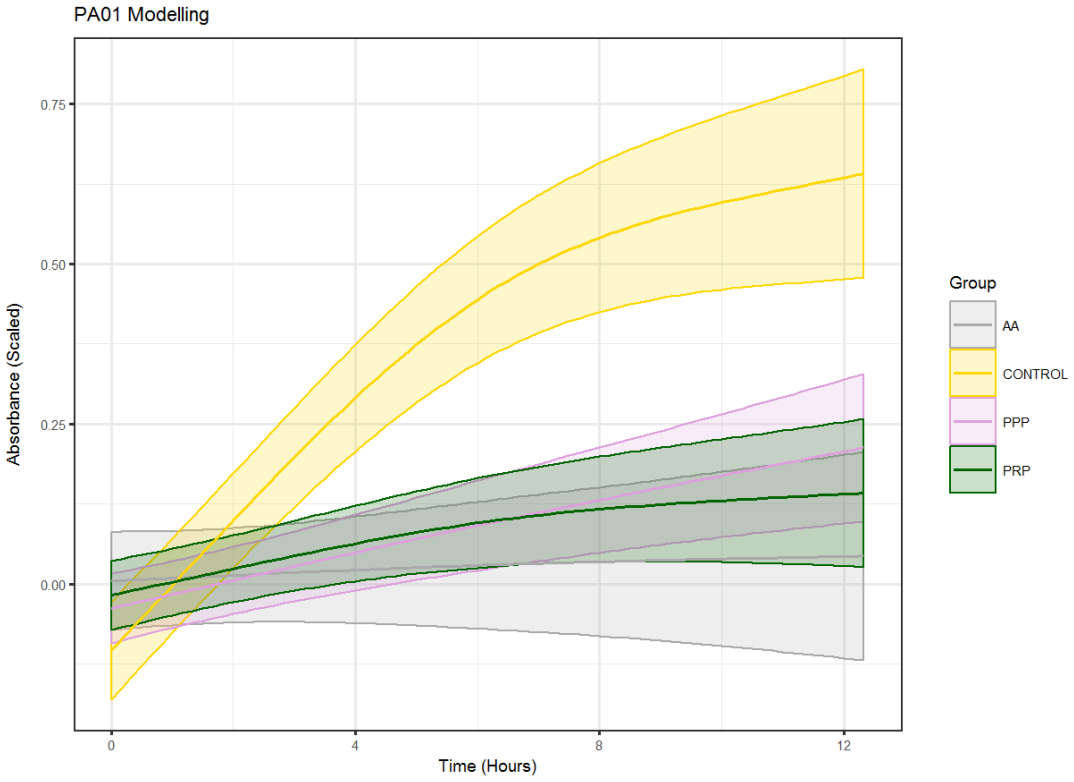


Figure 4.15 PS_PA01 growth over time. The absorbance at time 0 for each donor and control is scaled to 0

We now see a clear difference in the behaviour of the control sample over time compared with the other three groups and the PPP and PRP were similarly effective.

As for the AYE analysis, we now see a clear difference in the behaviour of the Control sample over time compared with the other three groups. There is little difference between the PPP and PRP groups by the end of the experiment, although the trajectories of the lines do suggest potential divergence were the experiment to be run for a longer period of time. Again, the AA group is not distinguishable from the PPP or PRP groups and this is likely to be a result of insufficient data to provide a suitably precise estimate of the absorbance in the AA group over time. We can observe this by examining the estimated levels of absorbance

at 0 hours and at 12 hours 20 minutes. These estimates are presented, along with 95% CIs, in the following table:

Group	Time = 0 minutes		Time =12hrs 20 minutes	
	Est	95%CI	Est	95%CI
AA	0.005	(-0.072, 0.082)	0.044	(-0.119, 0.207)
Control	-0.104	(-0.181, -0.027)	0.641	(0.478, 0.804)
PPP	-0.038	(-0.092, 0.016)	0.213	(0.098, 0.328)
PRP	-0.017	(-0.072, 0.037)	0.142	(0.027, 0.258)

The interaction terms between the control group and both the PPP and PRP group are statistically significant, suggesting there is a difference between the groups over time.

When the control group is compared to the PPP group, the p-value of the interaction term is <0.0001, suggesting there is a highly statistically significant difference between the groups. Similarly, comparing the control group to the PRP group, the p-value for the interaction term is <0.0001.

Analysing the data at 12h and 20 min post incubation using pairwise tests comparing each group with all the others, we can see that PPP and PRP preparation are both significantly different to control group (Control versus PPP: p-value <0.0001 and Control versus PRP: p-value: 0.027) whilst there is no significant difference between the 2 preparation and the preparation including PA_01+5%Acid Acid.

Group 1	Group 2	p-value
AA	CONTROL	<0.0001
AA	PPP	0.0042
AA	PRP	0.4463
CONTROL	PPP	<0.0001
CONTROL	PRP	0.0027
PPP	PRP	0.5818

Therefore, we can state that the preparations including PA_01+5%Acid Acid (negative control), PA_01+PRP, and PA_01+PPP significantly reduced PA_01 growth compared to the positive control (PA_01+broth), overtime and 12 hours post incubation.

Effect of removing PA01 Donor 3 removed

The behaviour in the PRP measurements for donor 3 (D3) was noticeably different from other preparations and thus we re-ran the above analysis on the data with D3 removed. However, we obtained a similar result to the previous analyses (data not shown) and thus these data remained in the analysis.

Staphylococcus aureus MSSA_F77

The absorbance over time for the 8 donors is plotted in figure 4.16. The dashed lines correspond to the control samples (CIPRO stands for Ciprofloxacin and POS for Positive control, F77 in broth).

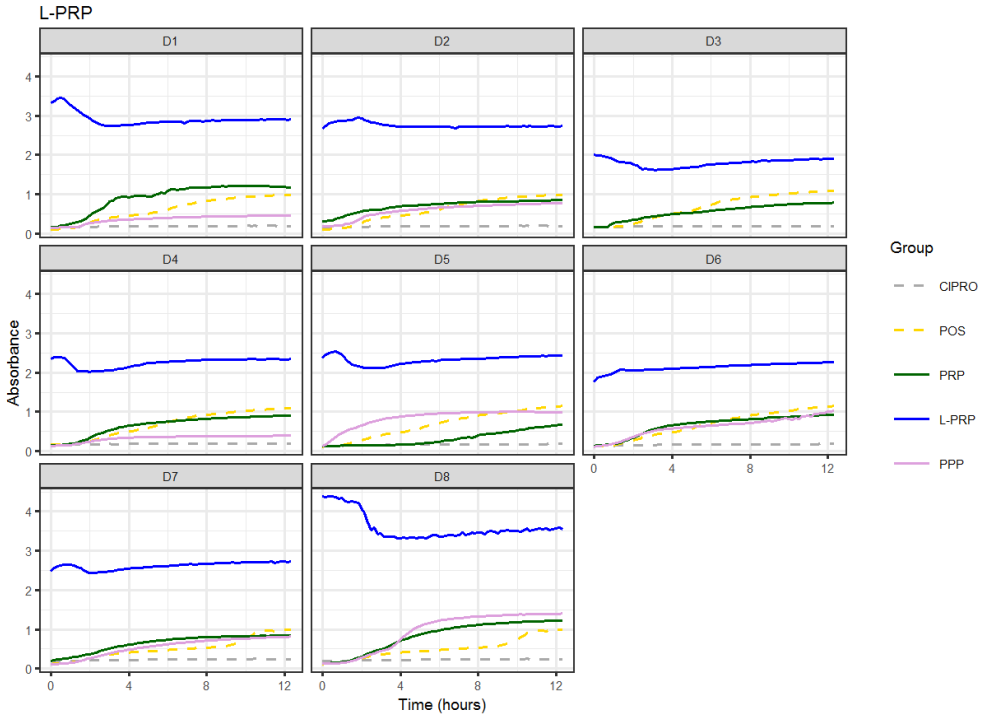


Figure 4.16 MSSA_F77 (expressed as absorbance) over time plotted for the 8 donors

All three model terms - main effect of time, main effect of group, and the interaction between group and time - were statistically significant, but the main effect of group was not. From this we can infer that the level of absorbance was different between the five different groups (CIPRO, Control, PRP, L-PRP, and PPP) at time 0. Figure 4.17 shows quite clearly that the values of L-PRP are considerably higher than for any other group over all time points and that none of the other treatments with the exception of ciprofloxacin differed from the control.

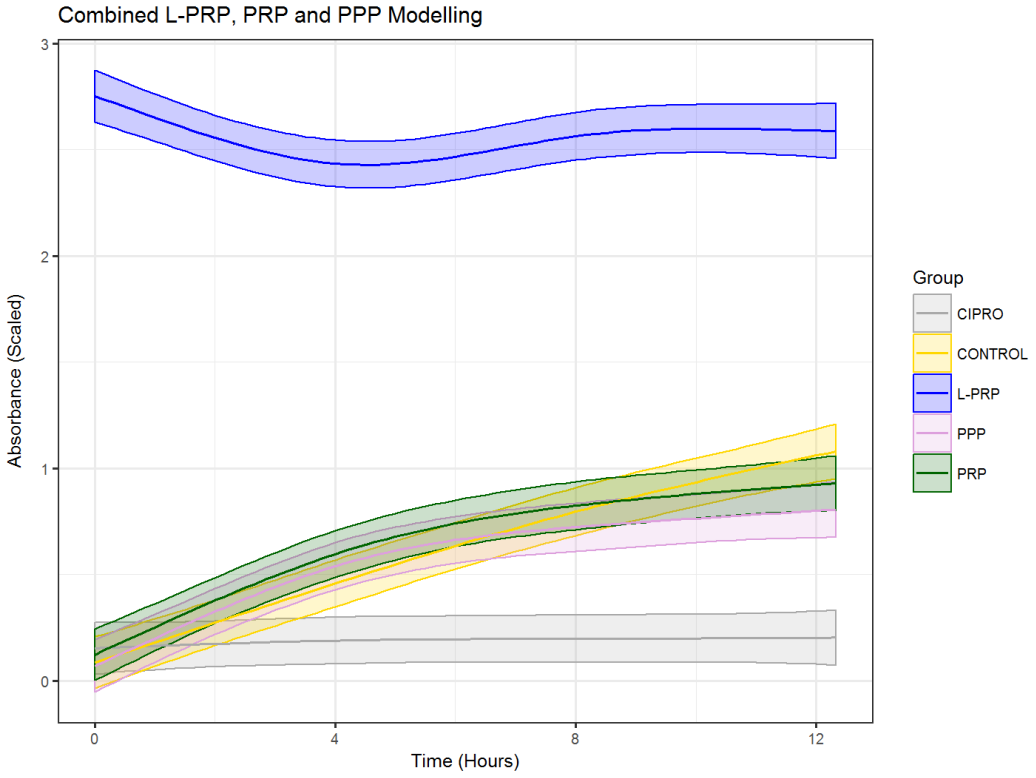


Figure 4.17 MSSA_F77 growth over time. Model fitted values of absorbance for the Ciprofloxacin, Control, PPP, PRP and L-PRP groups

Interested in the relative change over time, rather than the absolute change, we scaled the data so that the absorbance at time 0 for each donor and control is scaled to 0. The model fitted output for these groups is plotted in figure 4.1.

We can examine the estimated levels of absorbance at 0 hours and at 12 hours 20 minutes. These estimates are presented, along with 95% CIs, in the following table:

Group	Time = 0 minutes		Time =12hrs 20 minutes	
	Est	95%CI	Est	95%CI
CIPRO	0.002	(-0.103, 0.107)	0.052	(-0.094, 0.197)
L-PRP	0.080	(0.006, 0.154)	-0.085	(-0.188, 0.018)
POS	-0.042	(-0.147, 0.063)	0.953	(0.808, 1.099)
PPP	-0.046	(-0.124, 0.031)	0.685	(0.579, 0.790)
PRP	-0.050	(-0.124, 0.024)	0.757	(0.655, 0.860)

If we compare the CIPRO group to the PPP group, the p-value of the interaction term is <0.0001, suggesting there is a highly statistically significant difference between the groups. Similarly, comparing the POS group to the PPP group, the p-value for the interaction term is <0.0001. There is a large degree of overlap between the groups by 12 hours 20 minutes with no evidence in the data to distinguish between the three groups at that time. The trajectories of all three groups are different, but the lack of precision around the estimated absorbance means that we cannot conclude that there are significant differences between the groups. Looking at predicted averages alone, there is only a 0.3 unit difference between the absorbance of the POS and PPP groups by 12 hours 20 minutes.

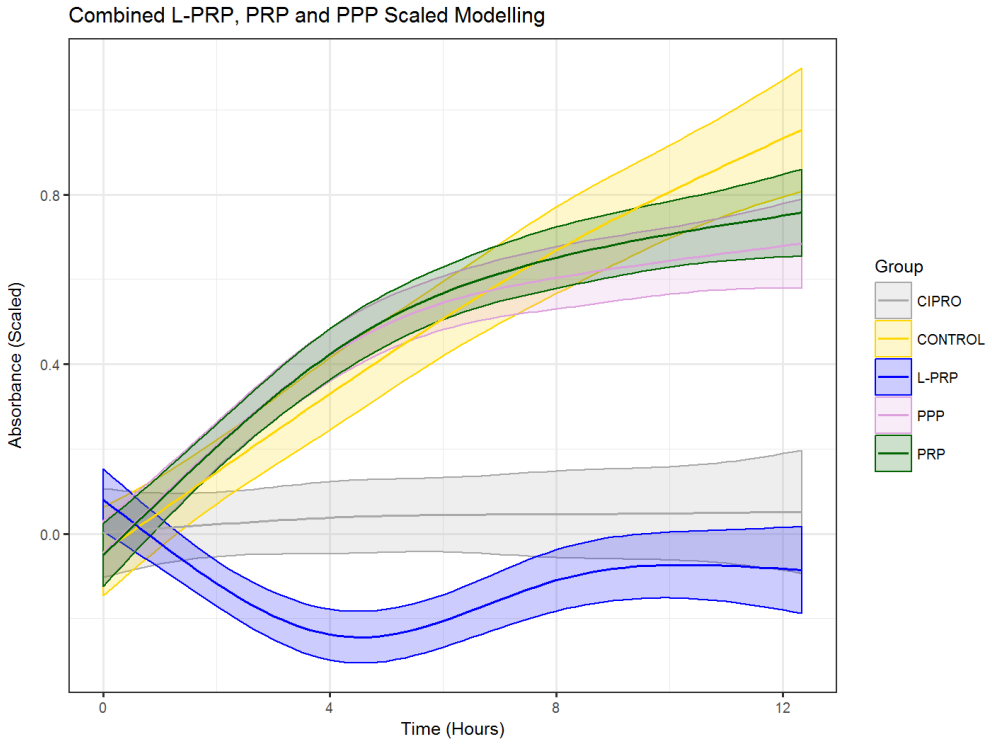


Figure 4.18 MSSA_F77 growth over time. The absorbance at time 0 for each donor and control is scaled to 0

In these adjusted data, whilst PRP and PPP preparations did not show growth control of MSSA_F77, whilst the preparation including leucocytes (L-PRP) does have a major effect on bacterial growth, being able to control MSSA_F77 growth and being significantly different to control at 12 hours post incubation (p- value <0.0001).

Group 1	Group 2	p-value
CIPRO	CONTROL	<0.0001
CIPRO	L-PRP	<0.0001
CIPRO	PPP	0.0026
CIPRO	PRP	<0.0001
CONTROL	L-PRP	<0.0001
CONTROL	PPP	0.1537
CONTROL	PRP	0.1126
L-PRP	PPP	<0.0001
L-PRP	PRP	<0.0001
PPP	PRP	0.5884

L-PRP Data Adjusted for Covariates

Each donor gave a different baseline blood count, thus leading to different platelet and white blood cell counts, in the final L-PRP preparation, as reported in Chapter 3, table 3.1. The value of platelets, WBC, neutrophils, monocytes, and lymphocytes are expressed as 10^6/ml.

We considered each potential covariate in turn as we did not have sufficient data to include them all in one model. The following analyses should be regarded as exploratory due to the limited amount of data available on the covariates in the model. As in the previous analyses we allowed the time of the observation to be modelled using a restricted cubic spline to allow for flexible non-linear models to be fit to the data.

Platelets (PLT): We fitted a model including Platelets and Time both as main effects and as an interaction term. The best fitting model produced the output reported in figure 4.19.

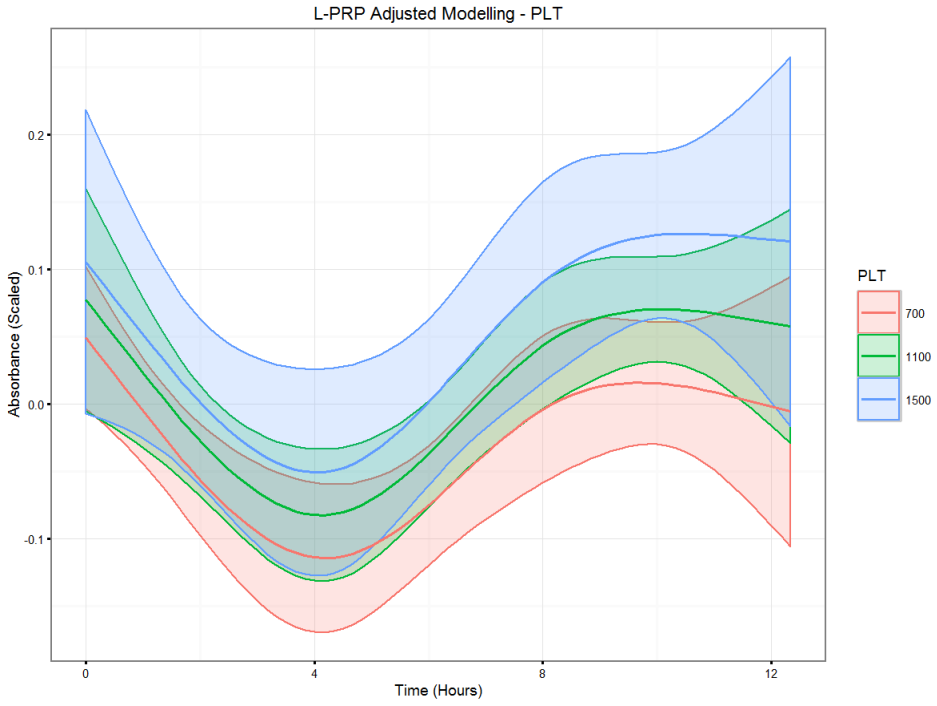


Figure 4.19 MSSA_F77 growth over time. L-PRP group adjusted modelling for platelets value

This suggests that the higher the value of PLT, the higher the initial (t=0) and final (t = 12 hours 20 minutes) levels of absorbance. There are statistically significant main effects of time (p=0.0004) and PLT (p=0.0002) but no significant interaction term (p=0.72), suggesting that the value of PLT does not affect the trajectory of absorbance over time.

White Blood Count (WBC): We fitted a model including WBC and time both as main effects and as an interaction term. The best fitting model produced the output reported in figure 4.20.

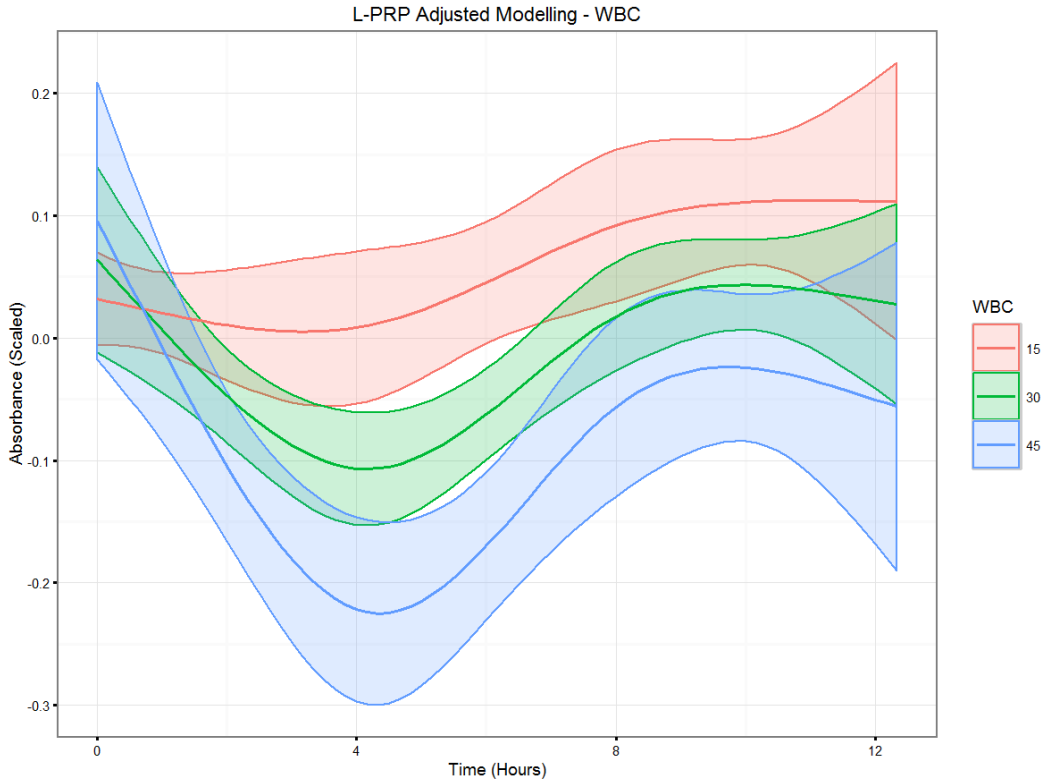


Figure 4.20 MSSA_F77 growth over time. L-PRP group adjusted modelling for WBC value

There are statistically significant main effects of time (p=0.0003) and WBC (p=0.0003) and a significant interaction term (p<0.0001), suggesting that the value of WBC does affect the

trajectory of absorbance over time. The fitted model output suggests that a higher WBC value results in a greater dip in absorbance by 4 hours before returning to a value by the end of the experiment that is reduced relative to the initial values.

Neutrophils: We fitted a model including neutrophils (NEUTR) and time both as main effects and as an interaction term. The best fitting model produced the output reported in figure 4.21.

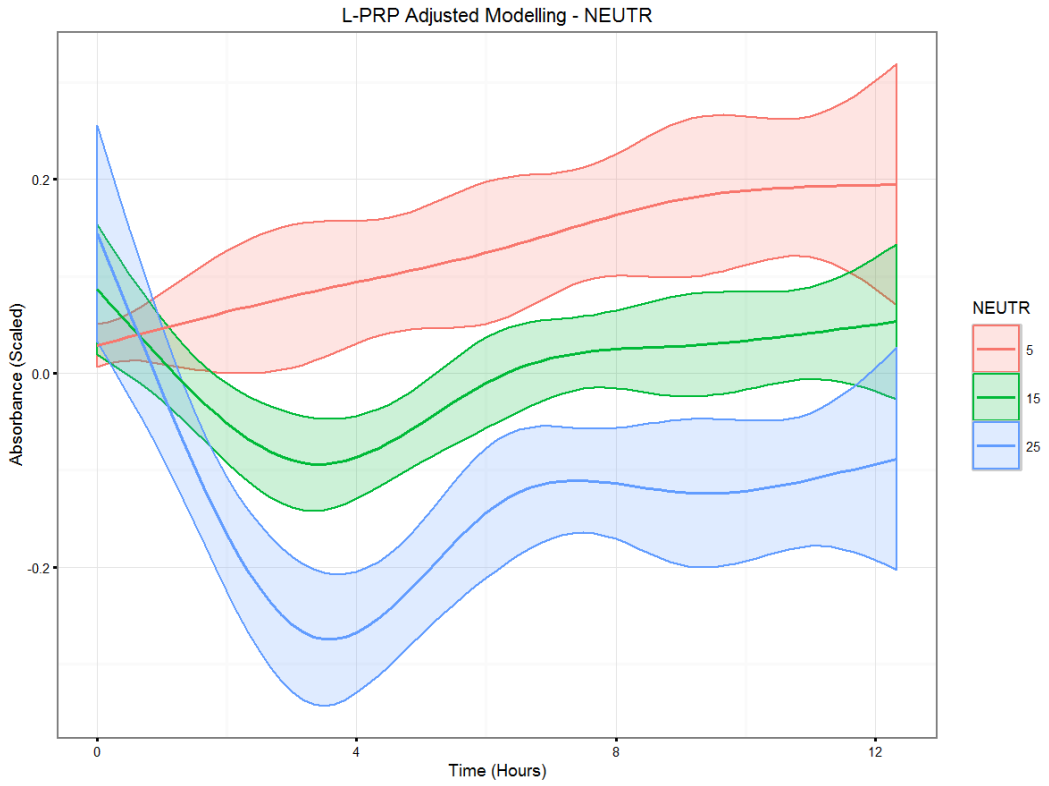


Figure 4.21 MSSA_F77 growth over time. L-PRP group adjusted modelling for neutrophils value

There are statistically significant main effects of time (p=0.0001) and neutrophil value (p<0.0001) and a significant interaction term (p<0.0001), suggesting that the value of neutrophils does affect the trajectory of absorbance over time. The fitted model output suggests that a higher neutrophil value results in a greater dip in absorbance by 3.5 hours.

There is considerable separation in the trajectories for the three values of neutrophils, suggesting that the trajectories are quite sensitive to the recorded neutrophil value.

Monocytes: We fitted a model including monocyte and time both as main effects and as an interaction term. The best fitting model produced the output reported in figure 4.22.

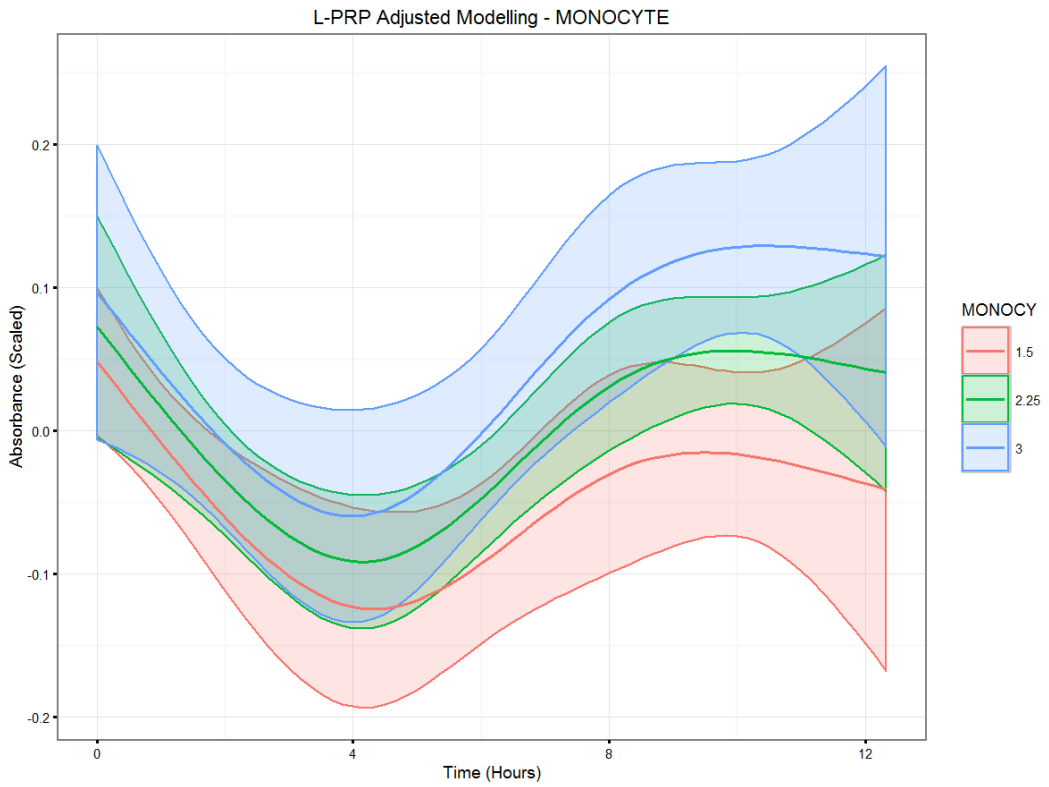


Figure 4.22 MSSA_F77 growth over time. L-PRP group adjusted modelling for monocytes value

We find that the higher the monocyte value, the higher the initial (t=0) and final (t = 12 hours 20 minutes) levels of absorbance. There are statistically significant main effects of time (p=0.0004) and monocyte level (p=0.0004) but no significant interaction term (p=0.33), suggesting that the monocyte level does not affect the trajectory of absorbance over time.

Lymphocytes: We fitted a model including lymphocyte and time both as main effects and as an interaction term. The best fitting model produced the output reported in figure 4.23.

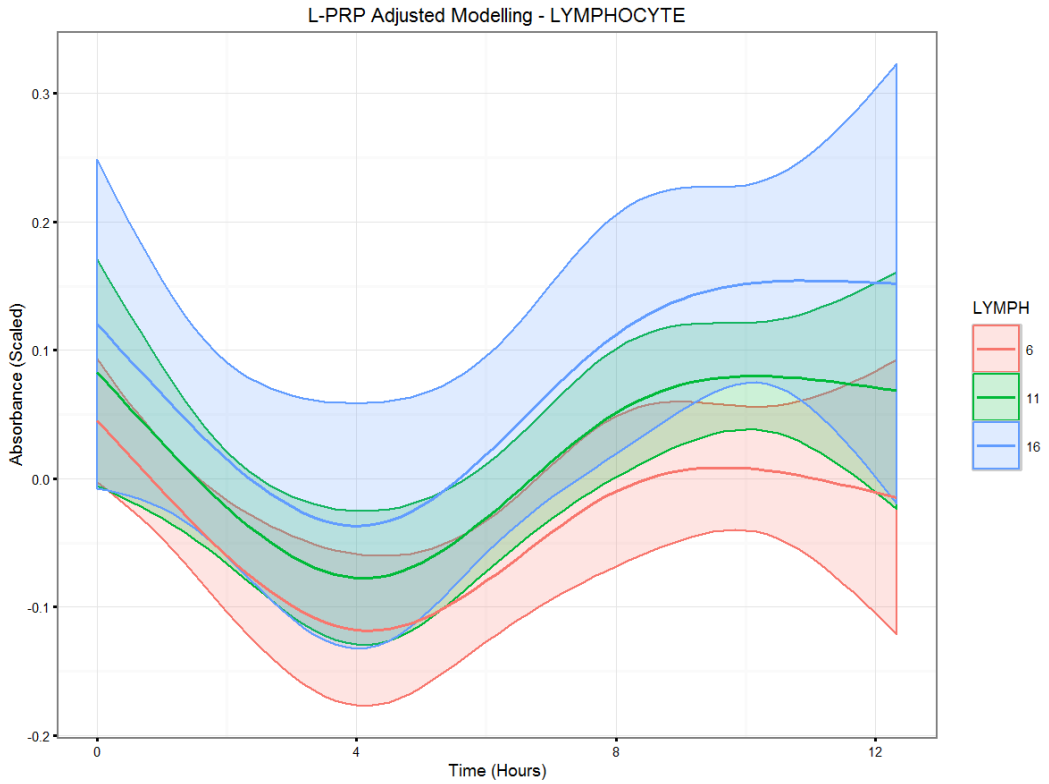


Figure 4.23 MSSA_F77 growth over time. L-PRP group adjusted modelling for lymphocytes value

We find that the higher the lymphocyte value, the higher the initial (t=0) and final (t = 12 hours 20 minutes) levels of absorbance. For a given lymphocyte value the initial and final absorbance values also seem similar, although there is considerable uncertainty associated with these estimated values. There are statistically significant main effects of time (p=0.0004) and lymphocyte level (p=0.0002) but no significant interaction term (p=0.73), suggesting that the lymphocyte level does not affect the trajectory of absorbance over time.

Isolated Neutrophils

The absorbance over time for the different concentrations of isolated neutrophils tested is plotted below (figure 4.24). The dashed lines correspond to the control samples (POS, as Positive control indicating the preparation including MSSA_F77 in broth and CIPRO, indicating the preparation including MSSA_F77 and ciprofloxacin) and are the same in each panel.

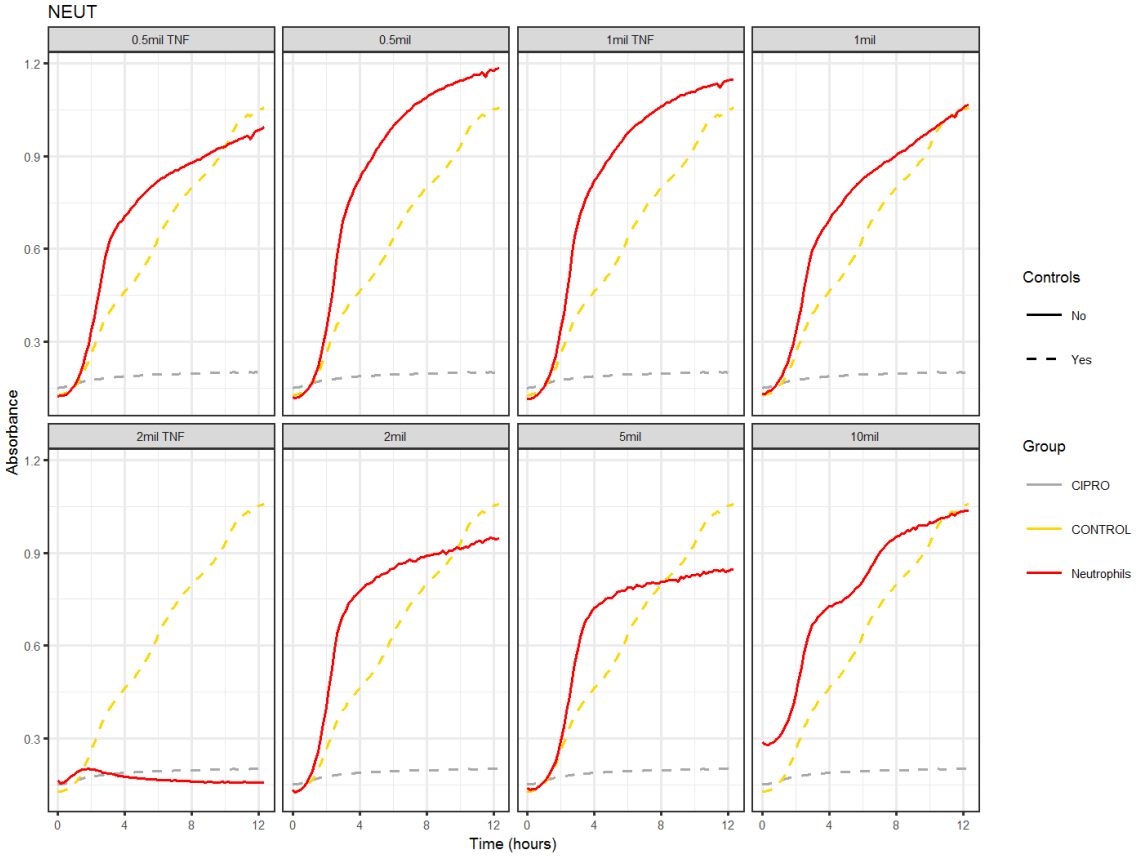


Figure 4.24 MSSA_F77 (expressed as absorbance) over time plotted for different concentration of isolated neutrophils

Overall, the plot clearly shows that preparation with isolated neutrophils do not have bacterial growth control. Primed neutrophils at the $2x10^6$/mls concentration were tested, a great bacterial control was detected. This data has not been further confirmed in repeated experiments (data not shown).

CHAPTER 5. DISCUSSION

The antimicrobial property of PRP preparations is unquestionably an attractive addition to the known wound healing effect of these biological blood-derived products. From the literature research, we established that although the inclusion of leucocytes in PRP preparations was previously neglected and few studies have been conducted to assess the specific antimicrobial contribution of the leucocytes. The limited numbers of studies so far conducted, the lack of standardisation of the methodology of blood-derived products' preparation, and the wide variability of comparators evaluated did not provide enough evidence to determine the specific contribution of leucocytes to the bactericidal effect of PRP preparation. However, overall PRP preparations, whether containing or excluding leucocytes demonstrated bacteriostatic properties against the majority of the bacterial strains tested.

With the intention of testing either wound healing or bactericidal properties of PRP products, it is crucial to firstly standardise the methodology of PRP preparations, showing the ability to reproduce consistent results in the preparation of the biomaterials. Therefore, the first part of our experimental study was dedicated to the standardisation of the methodology of the preparation of PRP.

Araki et al. [139] had previously demonstrated that a maximum platelet yield using low g for 10 minutes can be obtained using this a single low spin step method. This methodology, is the standard method used in our laboratory to obtain PRP and allows to obtain a PRP product that consistently yields 2-fold platelet enrichment from the whole blood, with negligible leucocytes and red cells content (Type 4B according to Mishra PRP classification

[16]. The PRP product obtained with this method has been used to test the antimicrobial properties of the blood product against Gram positive bacteria (MSSA F_77).

For all our experiments, PPP and L-PRP preparations were obtained using a single high-speed method, which allows to obtain a L-PRP product with a platelets concentration with minimal 4-fold enrichment from whole blood, and a minimal leucocyte count with a minimal enrichment of 3-fold (Type 2B according to Mishra PRP classification [16].

As with the L-PRP, the PRP preparation that included leucocytes, we could reach a higher concentration of platelets (4-fold enrichment from whole blood), with the progression of our study, we decided to aim to a higher concentration of platelets included in our PRP preparation [11] with a minimal 4-fold platelet enrichment from the whole blood (still Type 4B according to Mishra PRP classification [16]. Therefore, when Gram negative bacteria (Acinetobacter Baumannii and Pseudomonas Aeruginosa) were tested, we adopted the double-step method for PRP production. Our research aim was to test if PRP products have an effect on controlling bacteria growth and/or prevent biofilm formation. We chose to evaluate the antibacterial effect of these biomaterials against two Gram-negative bacteria (*P. aeruginosa* and *A. Baumanii*) and one Gram-positive bacteria (*S. aureus*) as these pathogens have been shown to be significant contributors to the delay of wound healing and relevant biofilm producers [105, 140, 141].

In summary, our present study revealed some interesting preliminary findings:

- The preparation that only includes isolated neutrophils did not inhibit bacterial growth for MSSA_F77;
- PRP and PPP preparations reduced AB_AYE and PS_PA01 bacterial growth;
- PRP and PPP preparations did not show growth control of MSSA_F77;

- L-PRP preparation was able to control MSSA_F77 growth.
- PRP, PPP, and L-PRP preparations prevented both AB_AYE and PS_PA01 biofilm formation.

To the best of our knowledge, this is the first study assessing the ability of blood products to prevent biofilm formation against *P. aeruginosa* and *A. baumannii*.

Interestingly, our findings show that all the biomaterials tested had the ability to reduce levels of biofilm biomass.

Against *P. aeruginosa*, some differences were found when we replicated the experiment for Donor 2. Whilst in the first test the positive control had significant elevated levels of biofilm biomass relative to the various biomaterials tested suggesting a bacteriostatic effect of the preparations, in the replicate experiment with Donor 2 blood products the positive control showed elevated levels of biofilm biomass but showed wide variation in the 6 replicates such that the biomaterial effect did not reach statistical significance. Increasing the sample size should help to decide if the effect is real for the various PRP preparations. Moreover, against *P. aeruginosa* for donor 1 the pre-Calcium Chloride-activated form of L-PRP showed a greater ability in controlling biofilm formation compared to the identical un-activated L-PRP form ($p<0.01$). This finding was not confirmed for donor 2.

Looking at the growth of *P. aeruginosa* in its planktonic state we confirmed findings previously reported by other others [97, 113, 114] in that PRP and PPP preparations significantly reduced bacterial growth. We found comparable results against *A. baumannii* and these are important data as few studies have tested the antimicrobial properties of blood-derived biomaterials against this multi-drug resistant, biofilm-forming pathogen [73, 142] and our data confirms these reports.

The results reported here support the previous findings that the antimicrobial effect of PRP is independent of platelet concentration [91, 93, 95], at least for these Gram negative bacteria strains Complement itself has been suggested to be one of the major contributors to the antimicrobial effect of PRP preparations [96] and Complement will be present in both our PRP and PPP preparations and future studies should perhaps measure its concentration in each preparation. Some authors have even suggested that the plasma Complement rather than platelets or leucocytes contributes to the antibacterial effect [95, 97]. This could explain the failure to inhibit bacterial growth when only isolated neutrophils were included in the preparation, with complement and microbicidal peptides and proteins that are particularly abundant in PRP preparations then absent. Another possible explanation could also be that the preparations including only isolated neutrophils were too dilute, not reaching an effective concentration of neutrophils.

According to these results it could be advocated that with the topical use of whole blood similar antimicrobial effects could be obtained. The mechanisms explaining the antimicrobial properties of blood products are not fully understood and still under debate [84]. It is well established though, that platelets need activation in order to release their antimicrobial polypeptides [66]and also even when only PPP is used, it is relevant to specify that during centrifugation active substances are released[85]. In fact, as suggested by Cieslik-Bielecka *et al.*[84], the PPP name is not entirely accurate, as plasma is the part of blood with all of its clotting mechanisms intact. The authors suggest instead the term “cell-poor serum” (CPS), fluid part of blood without platelets and clotting factors, but with the inclusion of active substances.

Looking at the growth of *S. aureus* we did not show bacteria growth control by the preparations that did not include leucocytes (PRP and PPP). These data are in disagreement with previous reports [91, 92]. The only preparation that showed the ability to control MSSA_F77 growth was the L-PRP preparation. Interestingly, the white blood count (WBC) and the neutrophil value included in L-PRP preparation, significantly affected the trajectory of bacterial growth inhibition. The fitted model suggested that a higher WBC and neutrophil value resulted in a greater dip in absorbance by 3.5 hours for the neutrophils and by 4 hours for WBC. Platelet, monocyte or lymphocyte numbers did not affect the bacteria growth.

Even with minor variation in timing for different bacteria strains, our literature review showed that 4 hours is the optimal time of incubation when the maximum decrease of bacteria is achieved by the PRP biomaterials. All studies agreed that the preparations are bacteriostatic, resulting in regrowth, but recognise that PRP preparations are a helpful addition more likely to be used in a clinical setting for prophylaxis rather than therapeutically for the treatment of established infection. Some authors suggest using formulations containing leucocytes and platelets in combination during surgical debridement to reduce both the bacterial load and stimulate healing [86, 110, 143, 144].

Limtations of the study

The main limitations of the study are the *in vitro* conditions of the experiments and the small size sample.

The preparations used in the experiments are similar to the conditions used in previous published study, where PRP products are diluted in Muller-Hinton broth. It is relevant to remind that in clinical setting PRP products are used alone and not diluted in broth. Moreover, *in vitro* studies may not reflect the clinical scenario, since this environment may

not mimic the dynamic condition of an *in vivo* setting where the antimicrobial properties of each individual components of the biomaterial may be increased, prolonged or even reduced by the complex interaction of cellular signals, and dynamic fluid exchange.

The study is limited to the use of samples from 8 healthy volunteers to test the microbicidal properties of the biomaterials against planktonic bacteria and only bloods from 2 donors to test the capability of the blood product to prevent biofilm formation. Some difference in results have been detected in between donors in our experiments. For instance, we noticed some strange behaviour in the PRP measurements for donor 3 when tested against P. *Aeruginosa*, but when re-run the analysis removing donor 3 data, we obtained a similar result. It is paramount to mention that the donor's variability may exist.

The only preparation that showed the ability to control MSSA_F77 growth was the L-PRP preparation and we found that white blood count and the neutrophil value included in L-PRP preparation significantly affected the trajectory of bacterial growth inhibition, whilst platelet, monocyte or lymphocyte numbers did not affect the bacteria growth. Many of the similar patterns in the preceding analyses are likely to be due to the limited covariate data incorporated into the model. We only used biomarkers measured at a single time point on each of the eight donors. Hence, the model fit is likely to be very sensitive to those specific values. A larger sample over a considerably larger number of donors would allow for much better estimation of the effect on absorbance of these biomarkers.

The limited number of samples and donors used to test the ability of the preparations to prevent biofilm formation need to be strongly addressed. To test the capability to prevent biofilm, we used the experimental model previously described by Baugh et al. [138] and Halstead [145]. The limitation of this model is clearly the *in vitro* setting, which unlikely

mimics biofilm formation in the *in vivo* conditions. It would be interesting to conduct experiments testing the antimicrobial properities of the blood products in a preformed biofilm either usinga Cristal Violet assay as described by Halstead *et al* [105] or mimicking the biofilm mode of growth alginate biofilm bactericidal assay as described by Thomsen *et al.* [86].

The study could be extended and replicated to confirm the findings by:

- Testing the ability to control MSSA_F77 growth using a PRP product with a higher concentration of platelets (double-step method)
- Measuring the release of different growth factors, complements and antibodies levels
- Evaluating the contribution of leucocytes in the L-PRP preparation by measuring myeloperoxidase (MPO) activity and MPO release, and investigate the activity and capability of chemotaxis, phagocytic activity and respiratory burst of the neutrophils (chemiluminescence assay) included in L-PRP preparation.
- Increasing the sample size, particularly to assess the antimicrobial property of the biomaterial to prevent biofilm formation

Conclusion

Blood-derived products showed different antimicrobial properties according to the bacterial target and the cellular composition of the product tested.

Whilst PRP products showed significant control of Gram negative bacteria in both their planktonic and biofilm growth, only the preparation including leucocytes seems to exert anti-bacterial effects against planktonic *S. Aureus*.

This *in vitro* study provides preliminary evidence that blood-derived products can inhibit growth of key burn wound pathogens, both in their planktonic and biofilm forms.

Taking all these findings together we suggest that the biomaterials can have a clinical benefit in all the circumstances where the promotion of wound healing is desired and PRP products can contribute to a substantial infection prophylaxis. These preliminary results should be further explored: additional research should investigate the antimicrobial ability of these bioproducts compared to antibiotics, study the behaviour of the products against a preformed established biofilm and ultimately confirm their antimicrobial efficacy *in vivo* settings.

REFERENCES

1. Anitua, E., M. Sánchez, and G. Orive, *Potential of endogenous regenerative technology for in situ regenerative medicine.* Advanced Drug Delivery Reviews, 2010. **62**(7–8): p. 741-752.
2. Posnett, J. and P.J. Franks, *The burden of chronic wounds in the UK.* Nurs Times, 2008. **104**(3): p. 44-5.
3. Rawlins, J.M., et al., *Epidemiology and outcome analysis of 208 children with burns attending an emergency department.* Pediatr Emerg Care, 2007. **23**(5): p. 289-93.
4. Robson, M.C., *Wound infection: a failure of wound healing caused by an imbalance of bacteria.* Surgical Clinics of North America, 1997. **77**(3): p. 637-650.
5. Guo, S. and L.A. DiPietro, *Factors affecting wound healing.* Journal of dental research, 2010. **89**(3): p. 219-229.
6. Organization, W.H., *Antimicrobial resistance: global report on surveillance*. 2014: World Health Organization.
7. Hancock, R.E., *Peptide antibiotics.* Lancet, 1997. **349**(9049): p. 418-22.
8. Davis, V.L., et al., *Platelet-rich preparations to improve healing. Part II: platelet activation and enrichment, leukocyte inclusion, and other selection criteria.* J Oral Implantol, 2014. **40**(4): p. 511-21.
9. Martínez-Zapata, M.J., et al., *Efficacy and safety of the use of autologous plasma rich in platelets for tissue regeneration: a systematic review.* Transfusion, 2009. **49**(1): p. 44-56.
10. Anitua, E., et al., *Autologous platelets as a source of proteins for healing and tissue regeneration.* THROMBOSIS AND HAEMOSTASIS-STUTTGART-, 2004. **91**(1): p. 4-15.
11. Marx, R.E., *Platelet-rich plasma: evidence to support its use.* Journal of oral and maxillofacial surgery, 2004. **62**(4): p. 489-496.
12. Dhurat, R. and M. Sukesh, *Principles and Methods of Preparation of Platelet-Rich Plasma: A Review and Author's Perspective.* J Cutan Aesthet Surg, 2014. **7**(4): p. 189-97.
13. Everts, P.A., et al., *What do we use: platelet-rich plasma or platelet-leukocyte gel?* J Biomed Mater Res A, 2008. **85**(4): p. 1135-6.
14. Martinez-Zapata, M.J., et al., *Autologous platelet-rich plasma for treating chronic wounds.* Cochrane Database Syst Rev, 2012. **10**: p. Cd006899.
15. Dohan Ehrenfest, D.M., L. Rasmusson, and T. Albrektsson, *Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF).* Trends Biotechnol, 2009. **27**(3): p. 158-67.
16. Mishra, A., et al., *Sports medicine applications of platelet rich plasma.* Curr Pharm Biotechnol, 2012. **13**(7): p. 1185-95.
17. Alsousou, J., et al., *The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery A REVIEW OF THE LITERATURE.* Journal of Bone & Joint Surgery, British Volume, 2009. **91**(8): p. 987-996.

18. Anitua, E., et al., *Platelet-rich plasma: preparation and formulation.* Operative Techniques in Orthopaedics, 2012. **22**(1): p. 25-32.
19. Cattaneo, M., et al., *Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH.* Journal of Thrombosis and Haemostasis, 2013. **11**(6): p. 1183-1189.
20. Harrison, P., et al., *Guidelines for the laboratory investigation of heritable disorders of platelet function.* British journal of haematology, 2011. **155**(1): p. 30-44.
21. Dohan Ehrenfest, D.M., et al., *In search of a consensus terminology in the field of platelet concentrates for surgical use: platelet-rich plasma (PRP), platelet-rich fibrin (PRF), fibrin gel polymerization and leukocytes.* Curr Pharm Biotechnol, 2012. **13**(7): p. 1131-7.
22. Marlovits, S., et al., *A new simplified technique for producing platelet-rich plasma: a short technical note.* European Spine Journal, 2004. **13**(1): p. S102-S106.
23. Harrison, S., et al., *Platelet activation by collagen provides sustained release of anabolic cytokines.* The American journal of sports medicine, 2011. **39**(4): p. 729-734.
24. Fufa, D., et al., *Activation of platelet-rich plasma using soluble type I collagen.* Journal of Oral and Maxillofacial Surgery, 2008. **66**(4): p. 684-690.
25. Miron, R.J., et al., *Injectable platelet rich fibrin (i-PRF): opportunities in regenerative dentistry?* Clin Oral Investig, 2017.
26. Landesberg, R., M. Moses, and M. Karpatkin, *Risks of using platelet rich plasma gel.* Journal of Oral and Maxillofacial Surgery, 1998. **56**(9): p. 1116-1117.
27. Cmolik, B., et al., *Redo cardiac surgery: late bleeding complications from topical thrombin-induced factor V deficiency.* The Journal of thoracic and cardiovascular surgery, 1993. **105**(2): p. 222-7; discussion 227-8.
28. Man, D., H. Plosker, and J.E. Winland-Brown, *The use of autologous platelet-rich plasma (platelet gel) and autologous platelet-poor plasma (fibrin glue) in cosmetic surgery.* Plastic and reconstructive surgery, 2001. **107**(1): p. 229-37; discussion 238-9.
29. Altmeyer, P., et al., *Wound healing and skin physiology*. 2012: Springer Science & Business Media.
30. Proksch, E., J.M. Brandner, and J.M. Jensen, *The skin: an indispensable barrier.* Experimental dermatology, 2008. **17**(12): p. 1063-1072.
31. Bikle, D.D., et al., *1, 25-Dihydroxyvitamin D3 production by human keratinocytes. Kinetics and regulation.* Journal of Clinical Investigation, 1986. **78**(2): p. 557.
32. Bullard, K.M., M.T. Longaker, and H.P. Lorenz, *Fetal wound healing: current biology.* World journal of surgery, 2003. **27**(1): p. 54-61.
33. Martin, P., *Wound healing--aiming for perfect skin regeneration.* Science, 1997. **276**(5309): p. 75-81.
34. Li, J., J. Chen, and R. Kirsner, *Pathophysiology of acute wound healing.* Clin Dermatol, 2007. **25**(1): p. 9-18.
35. YAREDHEROUY, P.M., et al., *Autologous platelet-derived wound healing factor promotes angiogenesis via avß3-integrin expression in chronic wounds.* International journal of molecular medicine, 2000. **6**: p. 515-519.

36. Kakudo, N., et al., *Proliferation-promoting effect of platelet-rich plasma on human adipose–derived stem cells and human dermal fibroblasts.* Plastic and reconstructive surgery, 2008. **122**(5): p. 1352-1360.
37. Hom, D.B., B.M. Linzie, and T.C. Huang, *The healing effects of autologous platelet gel on acute human skin wounds.* Archives of facial plastic surgery, 2007. **9**(3): p. 174-183.
38. Kazakos, K., et al., *The use of autologous PRP gel as an aid in the management of acute trauma wounds.* Injury, 2009. **40**(8): p. 801-805.
39. Frykberg, R.G., et al., *Chronic wounds treated with a physiologically relevant concentration of platelet-rich plasma gel: a prospective case series.* Ostomy/wound management, 2010. **56**(6): p. 36.
40. Danielsen, P., et al., *Effect of topical autologous platelet-rich fibrin versus no intervention on epithelialization of donor sites and meshed split-thickness skin autografts: a randomized clinical trial.* Plastic and reconstructive surgery, 2008. **122**(5): p. 1431-1440.
41. AM Everts, P., et al., *Is the use of autologous platelet-rich plasma gels in gynecologic, cardiac, and general, reconstructive surgery beneficial?* Current pharmaceutical biotechnology, 2012. **13**(7): p. 1163-1172.
42. Carter, M.J., C.P. Fylling, and L.K. Parnell, *Use of platelet rich plasma gel on wound healing: a systematic review and meta-analysis.* Eplasty, 2011. **11**: p. e38.
43. Marck, R.E., E. Middelkoop, and R.S. Breederveld, *Considerations on the use of platelet-rich plasma, specifically for burn treatment.* J Burn Care Res, 2014. **35**(3): p. 219-27.
44. Maghsoudi, H., N. Nezami, and M. Mirzajanzadeh, *Enhancement of burn wounds healing by platelet dressing.* International journal of burns and trauma, 2013. **3**(2): p. 96.
45. Ching, Y.H., et al., *The use of growth factors and other humoral agents to accelerate and enhance burn wound healing.* Eplasty, 2011. **11**: p. e41.
46. Danilenko, D.M., et al., *Growth factors in porcine full and partial thickness burn repair. Differing targets and effects of keratinocyte growth factor, platelet-derived growth factor-BB, epidermal growth factor, and neu differentiation factor.* The American journal of pathology, 1995. **147**(5): p. 1261.
47. Akita, S., et al., *Basic fibroblast growth factor is beneficial for postoperative color uniformity in split-thickness skin grafting.* Wound repair and regeneration, 2010. **18**(6): p. 560-566.
48. Akita, S., et al., *Basic fibroblast growth factor accelerates and improves second-degree burn wound healing.* Wound repair and regeneration, 2008. **16**(5): p. 635-641.
49. Bing, M., et al., *Randomized, multicenter, double-blind, and placebo-controlled trial using topical recombinant human acidic fibroblast growth factor for deep partial-thickness burns and skin graft donor site.* Wound repair and regeneration, 2007. **15**(6): p. 795-799.
50. Marck, R.E., et al., *The application of platelet-rich plasma in the treatment of deep dermal burns: A randomized, double-blind, intra-patient controlled study.* Wound Repair Regen, 2016. **24**(4): p. 712-20.

51. Lupia, E., et al., *Elevated thrombopoietin in plasma of burned patients without and with sepsis enhances platelet activation.* Journal of Thrombosis and Haemostasis, 2009. **7**(6): p. 1000-1008.
52. Warner, P., et al., *Thrombocytopenia in the pediatric burn patient.* Journal of Burn Care & Research, 2011. **32**(3): p. 410-414.
53. Marck, R.E., et al., *Time course of thrombocytes in burn patients and its predictive value for outcome.* Burns, 2013. **39**(4): p. 714-722.
54. Anitua, E., et al., *Leukocyte Inclusion within a Platelet Rich Plasma-Derived Fibrin Scaffold Stimulates a More Pro-Inflammatory Environment and Alters Fibrin Properties.* PLoS One, 2015. **10**(3): p. e0121713.
55. Assoian, R., et al., *Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization.* Journal of Biological Chemistry, 1983. **258**(11): p. 7155-7160.
56. Grotendorst, G.R., et al., *Stimulation of granulation tissue formation by platelet-derived growth factor in normal and diabetic rats.* Journal of Clinical Investigation, 1985. **76**(6): p. 2323.
57. Maciel, F.B., et al., *Scanning electron microscopy and microbiological evaluation of equine burn wound repair after platelet-rich plasma gel treatment.* Burns, 2012. **38**(7): p. 1058-65.
58. HOSGOOD, G., *Wound Healing: The Role of Platelet-Derived Growth Factor and Transforming Growth Factor Beta.* Veterinary Surgery, 1993. **22**(6): p. 490-495.
59. Shah, M., D.M. Foreman, and M. Ferguson, *Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring.* Journal of cell science, 1995. **108**(3): p. 985-1002.
60. Douglas, H.E., *TGF-ss in wound healing: a review.* J Wound Care, 2010. **19**(9): p. 403-6.
61. Niessen, F.B., et al., *Keratinocyte-derived growth factors play a role in the formation of hypertrophic scars.* The Journal of pathology, 2001. **194**(2): p. 207-216.
62. Berman, B., A. Maderal, and B. Raphael, *Keloids and Hypertrophic Scars: Pathophysiology, Classification, and Treatment.* Dermatol Surg, 2016.
63. Lichtman, M.K., M. Otero-Vinas, and V. Falanga, *Transforming growth factor beta (TGF-beta) isoforms in wound healing and fibrosis.* Wound Repair Regen, 2016. **24**(2): p. 215-22.
64. Penn, J.W., A.O. Grobbelaar, and K.J. Rolfe, *The role of the TGF-beta family in wound healing, burns and scarring: a review.* Int J Burns Trauma, 2012. **2**(1): p. 18-28.
65. Cieslik-Bielecka, A., et al., *Microbicidal properties of Leukocyte- and Platelet-Rich Plasma/Fibrin (L-PRP/L-PRF): new perspectives.* J Biol Regul Homeost Agents, 2012. **26**(2 Suppl 1): p. 43s-52s.
66. Yeaman, M.R., *Platelets: at the nexus of antimicrobial defence.* Nat Rev Microbiol, 2014. **12**(6): p. 426-37.
67. Tang, Y.Q., M.R. Yeaman, and M.E. Selsted, *Antimicrobial peptides from human platelets.* Infect Immun, 2002. **70**(12): p. 6524-33.
68. Yeaman, M.R., *The role of platelets in antimicrobial host defense.* Clin Infect Dis, 1997. **25**(5): p. 951-68; quiz 969-70.

69. Sadek, M.I., et al., *Chemokines induced by infection of mononuclear phagocytes with mycobacteria and present in lung alveoli during active pulmonary tuberculosis.* American journal of respiratory cell and molecular biology, 1998. **19**(3): p. 513-521.
70. Lopes-Pires, M.E., et al., *Lipopolysaccharide treatment reduces rat platelet aggregation independent of intracellular reactive-oxygen species generation.* Platelets, 2012. **23**(3): p. 195-201.
71. Youssefian, T., et al., *Host defense role of platelets: engulfment of HIV andStaphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation.* Blood, 2002. **99**(11): p. 4021-4029.
72. Edelblute, C.M., et al., *Human platelet gel supernatant inactivates opportunistic wound pathogens on skin.* Platelets, 2015. **26**(1): p. 13-6.
73. Edelblute, C.M., et al., *Donor platelet plasma components inactivate sensitive and multidrug resistant Acinetobacter baumannii isolates.* Pathog Dis, 2015. **73**(9): p. ftv095.
74. Edelblute, C.M., et al., *Human platelet gel supernatant inactivates opportunistic wound pathogens on skin.* Platelets, 2015. **26**(1): p. 13-16.
75. J., L.J.B.A.T.M.P.T.G.R.H., *Platelet-derived growth factors induce the expression of the antimicrobial peptide human beta-defensin-2 in primary keratinocytes.* Experimental Dermatology. Conference: 42nd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung, ADF 2015 Ulm Germany. Conference Start: 20150305 Conference End: 20150307. Conference Publication: (var.pagings). 24 (3) (pp E4), 2015. Date of Publication: March 2015., 2015.
76. Tohidnezhad, M., et al., *Platelets display potent antimicrobial activity and release human beta-defensin 2.* Platelets, 2012. **23**(3): p. 217-23.
77. Yeaman, M.R., *Bacterial-platelet interactions: virulence meets host defense.* Future Microbiol, 2010. **5**(3): p. 471-506.
78. Zielinski, T., et al., *The generation of superoxide anion in blood platelets in response to different forms of Proteus mirabilis lipopolysaccharide: effects of staurosporin, wortmannin, and indomethacin.* Thrombosis research, 2001. **103**(2): p. 149-155.
79. Aktan, I., B. Dunkel, and F.M. Cunningham, *Equine platelets inhibit E. coli growth and can be activated by bacterial lipopolysaccharide and lipoteichoic acid although superoxide anion production does not occur and platelet activation is not associated with enhanced production by neutrophils.* Vet Immunol Immunopathol, 2013. **152**(3-4): p. 209-17.
80. Zalavary, S., et al., *Platelets enhance Fc (gamma) receptor-mediated phagocytosis and respiratory burst in neutrophils: the role of purinergic modulation and actin polymerization.* Journal of leukocyte biology, 1996. **60**(1): p. 58-68.
81. Bengtsson, T., et al., *Platelets enhance neutrophil locomotion: evidence for a role of P-selectin.* Scandinavian journal of clinical and laboratory investigation, 1999. **59**(6): p. 439-449.
82. Bengtsson, T., et al., *Release of oxygen metabolites from chemoattractant-stimulated neutrophils is inhibited by resting platelets: role of extracellular adenosine and actin polymerization.* Blood, 1996. **87**(10): p. 4411-4423.
83. Bielecki, T., et al., *The role of leukocytes from L-PRP/L-PRF in wound healing and immune defense: new perspectives.* Curr Pharm Biotechnol, 2012. **13**(7): p. 1153-62.

84. Cieslik-Bielecka, A., et al., *Why the platelet-rich gel has antimicrobial activity?* Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics, 2007. **103**(3): p. 303-305.
85. Bielecki, T., et al., *The analysis of leukocytes for CD3, CD14, CD15, CD16, CD19, CD45, CD56 with progenitor cells (CD34+) and platelet-derived growth factors in blood and platelet-leukocyte rich plasma/gel-preliminary report.* Pol. J. Environ. Stud, 2009. **18**(1A): p. 649-652.
86. Thomsen, K., et al., *The phagocytic fitness of Leucopatches may impact the healing of chronic wounds.* Clinical & Experimental Immunology, 2016.
87. Pillay, J., et al., *In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days.* Blood, 2010. **116**(4): p. 625-627.
88. Klebanoff, S.J., *Myeloperoxidase: contribution to the microbicidal activity of intact leukocytes.* Science, 1970. **169**(3950): p. 1095-1097.
89. Yang, L.C., et al., *Antimicrobial activity of platelet-rich plasma and other plasma preparations against periodontal pathogens.* J Periodontol, 2015. **86**(2): p. 310-8.
90. Drago, L., et al., *Antimicrobial activity of pure platelet-rich plasma against microorganisms isolated from oral cavity.* BMC Microbiol, 2013. **13**: p. 47.
91. Anitua, E., et al., *Antibacterial effect of plasma rich in growth factors (PRGF(R)-Endoret(R)) against Staphylococcus aureus and Staphylococcus epidermidis strains*, in *Clin Exp Dermatol*. 2012. p. 652-7.
92. Moojen, D.J., et al., *Antimicrobial activity of platelet-leukocyte gel against Staphylococcus aureus.* J Orthop Res, 2008. **26**(3): p. 404-10.
93. Bielecki, T.M., et al., *Antibacterial effect of autologous platelet gel enriched with growth factors and other active substances: an in vitro study.* J Bone Joint Surg Br, 2007. **89**(3): p. 417-20.
94. Mariani, E., et al., *Platelet-rich plasma affects bacterial growth in vitro.* Cytotherapy, 2014. **16**(9): p. 1294-304.
95. Drago, L., et al., *Plasma components and platelet activation are essential for the antimicrobial properties of autologous platelet-rich plasma: an in vitro study.* PLoS One, 2014. **9**(9): p. e107813.
96. Daha, M.R., *Role of complement in innate immunity and infections.* Critical Reviews™ in Immunology, 2010. **30**(1).
97. Burnouf, T., et al., *Antimicrobial activity of platelet (PLT)-poor plasma, PLT-rich plasma, PLT gel, and solvent/detergent-treated PLT lysate biomaterials against wound bacteria.* Transfusion, 2013. **53**(1): p. 138-46.
98. Parsek, M.R. and P.K. Singh, *Bacterial biofilms: an emerging link to disease pathogenesis.* Annual Reviews in Microbiology, 2003. **57**(1): p. 677-701.
99. Hall-Stoodley, L. and P. Stoodley, *Biofilm formation and dispersal and the transmission of human pathogens.* Trends in microbiology, 2005. **13**(1): p. 7-10.
100. Karatan, E. and P. Watnick, *Signals, regulatory networks, and materials that build and break bacterial biofilms.* Microbiology and Molecular Biology Reviews, 2009. **73**(2): p. 310-347.
101. Hall-Stoodley, L., et al., *Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media.* Jama, 2006. **296**(2): p. 202-211.

102. Costerton, J.W., *Cystic fibrosis pathogenesis and the role of biofilms in persistent infection.* Trends in microbiology, 2001. **9**(2): p. 50-52.
103. Schaudinn, C., et al., *Periodontitis: an archetypical biofilm disease.* The Journal of the American Dental Association, 2009. **140**(8): p. 978-986.
104. Coenye, T., et al., *Biofilms in skin infections: Propionibacterium acnes and acne vulgaris.* Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders), 2008. **8**(3): p. 156-159.
105. Halstead, F.D., et al., *The Antibacterial Activity of Acetic Acid against Biofilm-Producing Pathogens of Relevance to Burns Patients.* PloS one, 2015. **10**(9): p. e0136190.
106. Bjarnsholt, T., et al., *Why chronic wounds will not heal: a novel hypothesis.* Wound repair and regeneration, 2008. **16**(1): p. 2-10.
107. James, G.A., et al., *Biofilms in chronic wounds.* Wound Repair and regeneration, 2008. **16**(1): p. 37-44.
108. Rozalski, M.I., et al., *Antimicrobial/anti-biofilm activity of expired blood platelets and their released products.* Postepy Hig Med Dosw (Online), 2013. **67**: p. 321-5.
109. Atkins, D., et al., *Assessing the applicability of studies when comparing medical interventions.* 2010.
110. Wu, X., et al., *Antimicrobial properties of single-donor-derived, platelet-leukocyte fibrin for fistula occlusion: An in vitro study.* Platelets, 2013. **24**(8): p. 632-6.
111. Intravia, J., et al., *In vitro evaluation of the anti-bacterial effect of two preparations of platelet rich plasma compared with cefazolin and whole blood.* Muscles Ligaments Tendons J, 2014. **4**(1): p. 79-84.
112. Cavallo, C., et al., *Comparison of Platelet-Rich Plasma Formulations for Cartilage Healing*. An in Vitro Study. Vol. 96. 2014. 423-429.
113. Mariani, E., et al., *Leukocyte presence does not increase microbicidal activity of Platelet-rich Plasma in vitro.* BMC Microbiol, 2015. **15**: p. 149.
114. Chen, L., et al., *Antibacterial effect of autologous platelet-rich gel derived from subjects with diabetic dermal ulcers in vitro.* J Diabetes Res, 2013. **2013**: p. 269527.
115. Lopez, C., M.E. Alvarez, and J.U. Carmona, *Temporal Bacteriostatic Effect and Growth Factor Loss in Equine Platelet Components and Plasma Cultured with Methicillin-Sensitive and Methicillin-Resistant Staphylococcus aureus: A Comparative In Vitro Study.* 2014. **2014**: p. 525826.
116. Li, H., et al., *Unique antimicrobial effects of platelet-rich plasma and its efficacy as a prophylaxis to prevent implant-associated spinal infection.* Adv Healthc Mater, 2013. **2**(9): p. 1277-84.
117. Lopez, C., et al., *Bacteriostatic effect of equine pure platelet-rich plasma and other blood products against methicillin-sensitive Staphylococcus aureus. An in vitro study.* Vet Comp Orthop Traumatol, 2014. **27**(5): p. 372-8.
118. Bayer, A.S., et al., *In vitro resistance of Staphylococcus aureus to thrombin-induced platelet microbicidal protein is associated with alterations in cytoplasmic membrane fluidity.* Infection and immunity, 2000. **68**(6): p. 3548-3553.
119. Cubison, T., S.A. Pape, and N. Parkhouse, *Evidence for the link between healing time and the development of hypertrophic scars (HTS) in paediatric burns due to scald injury.* Burns, 2006. **32**(8): p. 992-999.

120. Akhundov, K., et al., *Development of a cost-effective method for platelet-rich plasma (PRP) preparation for topical wound healing.* Ann Burns Fire Disasters, 2012. **25**(4): p. 207-13.
121. Tanaka, Y., et al., *Performance evaluation of platelet counting by novel fluorescent dye staining in the XN-series automated hematology analyzers.* J Clin Lab Anal, 2014. **28**(5): p. 341-8.
122. Brown, T.P.L.H., et al., *Survival benefit conferred by topical antimicrobial preparations in burn patients: a historical perspective.* Journal of Trauma and Acute Care Surgery, 2004. **56**(4): p. 863-866.
123. Church, D., et al., *Burn wound infections.* Clin Microbiol Rev, 2006. **19**(2): p. 403-34.
124. David, M.Z. and R.S. Daum, *Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic.* Clinical microbiology reviews, 2010. **23**(3): p. 616-687.
125. Perez, F., et al., *Global challenge of multidrug-resistant Acinetobacter baumannii.* Antimicrobial agents and chemotherapy, 2007. **51**(10): p. 3471-3484.
126. Peleg, A.Y., H. Seifert, and D.L. Paterson, *Acinetobacter baumannii: emergence of a successful pathogen.* Clinical microbiology reviews, 2008. **21**(3): p. 538-582.
127. Kunz, A.N. and I. Brook, *Emerging resistant Gram-negative aerobic bacilli in hospital-acquired infections.* Chemotherapy, 2010. **56**(6): p. 492-500.
128. Moore, N.M. and M.L. Flaws, *Epidemiology and pathogenesis of Pseudomonas aeruginosa infections.* Clin Lab Sci, 2011. **24**(1): p. 43-6.
129. Lister, P.D., D.J. Wolter, and N.D. Hanson, *Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms.* Clinical microbiology reviews, 2009. **22**(4): p. 582-610.
130. Fazli, M., et al., *Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds.* Journal of clinical microbiology, 2009. **47**(12): p. 4084-4089.
131. Rajpaul, K., *Biofilm in wound care.* Br J Community Nurs, 2015. **Suppl Wound Care**: p. S6, s8, s10-1.
132. Norman, G., et al., *Antibiotics and antiseptics for surgical wounds healing by secondary intention.* Cochrane Database Syst Rev, 2016. **3**: p. Cd011712.
133. Uyesaka, N., A. Tajiri, and K. Arai, *Separation of human neutrophils in self-generated continuous density gradients of Percoll.* Jpn J Physiol, 1989. **39**(4): p. 629-34.
134. Wewers, M.D., et al., *Tumor necrosis factor infusions in humans prime neutrophils for hypochlorous acid production.* Am J Physiol, 1990. **259**(4 Pt 1): p. L276-82.
135. Ryssel, H., et al., *The antimicrobial effect of acetic acid—an alternative to common local antiseptics?* Burns, 2009. **35**(5): p. 695-700.
136. Ferraro, M.J., *Performance standards for antimicrobial disk susceptibility tests*. 2000: NCCLS.
137. Webber, M.A., et al., *The global consequence of disruption of the AcrAB-TolC efflux pump in Salmonella enterica includes reduced expression of SPI-1 and other attributes required to infect the host.* J Bacteriol, 2009. **191**(13): p. 4276-85.
138. Baugh, S., et al., *Loss of or inhibition of all multidrug resistance efflux pumps of Salmonella enterica serovar Typhimurium results in impaired ability to form a biofilm.* J Antimicrob Chemother, 2012. **67**(10): p. 2409-17.

139. Araki, J., et al., *Optimized preparation method of platelet-concentrated plasma and noncoagulating platelet-derived factor concentrates: maximization of platelet concentration and removal of fibrinogen.* Tissue Engineering Part C: Methods, 2011. **18**(3): p. 176-185.
140. Gjødsbøl, K., et al., *Multiple bacterial species reside in chronic wounds: a longitudinal study.* International wound journal, 2006. **3**(3): p. 225-231.
141. Krom, B.P. and J. Oskam, *Microbial biofilms and wound healing: an ecological hypothesis.* Phlebology, 2014. **29**(1 suppl): p. 168-173.
142. Sun, S., et al., *Combating Superbug Without Antibiotic on a Postamputation Wound in a Patient with Diabetic Foot.* Int J Low Extrem Wounds, 2016. **15**(1): p. 74-7.
143. Jia, W.T., et al., *The prophylactic effects of platelet-leucocyte gel in osteomyelitis: an experimental study in a rabbit model.* J Bone Joint Surg Br, 2010. **92**(2): p. 304-10.
144. Li, G.Y., et al., *Efficacy of leukocyte- and platelet-rich plasma gel (L-PRP gel) in treating osteomyelitis in a rabbit model.* J Orthop Res, 2013. **31**(6): p. 949-56.
145. Halstead, F.D., et al., *Antimicrobial dressings: Comparison of the ability of a panel of dressings to prevent biofilm formation by key burn wound pathogens.* Burns, 2015. **41**(8): p. 1683-94.

Appendix A

***In vitro* studies testing the ability of blood product to control bacteria growth**

Testing Gram positive MSSA_F77

Laboratory of Immunology and Infection Blood products preparation Blood from 8 healthy donors - **PPP** - **PRP (single- step method)** - **L-PRP** - **Isolated Neutrophils** (at different concentration Neutrophil count was adjusted to a range of values: $0.5x10^6$/ml to $10x10^6$/ml. - Neutrophils were either primed, using 10 ng/ml TNFα primed and not primed	→	**Laboratory T102, Institute of Microbiology and Infection** Bacteria preparation MSSA_F77 Controls preparation: MH broth, Ciprofloxacin *Kirby Bauer disk diffusion* LB agar plate coated with MSSA_F77 and PPP, PRP, L-PRP *Growth Kinetics assay* 4 replicates for each donor MSSA-F77 with PPP MSSA-F77 with PRP MSSA-F77 with L-PRP MSSA_F77 with isolated neutrophils MSSA_F77 with MH broth MSSA_F77 with Ciprofloxacin *Bacteria growth after 24h* Visual assessment of turbidity All preparations used for growth kinetic assay assessed

Testing Gram negative PS_PA01 and AB_AYE

Laboratory of Immunology and Infection Blood products preparation Blood from 8 healthy donors - **PPP** - **PRP (double-step method)**	→	**Laboratory T102, Institute of Microbiology and Infection** Bacteria preparation PS_PA01 and AB_AYE Controls preparation: sterile water, acetic acid, MH broth *Kirby Bauer disk diffusion* LB agar plate coated with PS_PA01 and PPP, PRP and L-PRP *Growth Kinetics assay* 6 replicates for each donor PS_PA01 and AB_AYE with PPP PS_PA01 and AB_AYE with PRP AB_AYE with MH broth PS_PA01 with sterile water PS_PA01 and AB_AYE with 5% acetic acid *Bacteria growth after 24h* Visual assessment of turbidity All preparations used for growth kinetic assay assessed

***In vitro* studies testing the ability of blood product to prevent biofilm formation**

Laboratory of Immunology and Infection
Blood product preparations
Blood from 2 healthy donors
- **PPP**
- **PRP (double- step method)**
- **L-PRP (activated and no-activated)**

→

Laboratory T102, Institute of Microbiology and Infection
Bacteria preparation PS_PA01 and AB_AYE
Controls preparation: MH broth, sterile water, 5% acetic acid

Cristal Violet assay
6 replicates for each donor
PS_PA01 with sterile water
PS_PA01 with activated L-PRP
PS_PA01 with no-activated L-PRP
PS_PA01 with PRP
PS_PA01 with PPP
PS_PA01 with 5% acetic acid
MH Broth

AB_AYE with MH Broth
AB_AYE with activated L-PRP
AB_AYE with no-activated L-PRP
AB_AYE with PRP
AB_AYE with PPP
AB_AYE with 5% acetic acid
MH broth

Bacteria growth after 24h
Visual assessment of turbidity of all preparations