Title : The *in vitro* antimicrobial activity of advanced platelet rich fibrin (A-PRF™) against microorganisms of the oral cavity

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Key Words

Antimicrobial

Platelet concentrates

Platelet Rich Fibrin

Bacteria

Surgery

Blood

Chlorhexidine

Growth factors

Serum

A-PRF

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Abstract
The in vitro antimicrobial activity of advanced platelet rich fibrin (A-PRF™) against microorganisms of the oral cavity

Feheem Bhamjee

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In recent years, the development and use of autologous platelet rich concentrates (PC's) has gained traction within the rapidly progressive, multidisciplinary field of regenerative medicine. A PC subtype, marketed as advanced platelet rich fibrin (A- PRF™), is a recent advancement of the original PRF protocol and promoted as a "blood concentrate" containing platelets, leukocytes, circulating stem cells and endothelial cells. A-PRF™ in the form of membranes, plugs, or even shredded particulates are increasingly being used as surgical adjuncts in areas of previous infection or left exposed within the microbial rich oral environment. Although recent literature has noted the biologic benefits of this material within the context of wound healing and regeneration, the antimicrobial potential of A-PRF™ has remained unexplored. The aim of this investigation is to determine if A-PRF™ displays antimicrobial activity against microbes of the oral cavity with a null hypothesis that its activity is no different to a clot of unprocessed venous blood.

Methodology: A-PRF™ and whole blood samples were obtained from consenting individuals and utilised to conduct an *in-vitro* agar disk diffusion investigation to determine their antimicrobial activity. Standardised samples of A-PRF™, unprocessed clotted blood and 0.2% chlorhexidine gluconate (CHX) were tested against organisms cultured from fresh oral rinse samples and pure cultures of *candida albicans, streptococcus mutans, staphylococcus aureus* and *enterococcus faecalis*. The antimicrobial activity was assessed in accordance to the established principles of the agar disk diffusion method and measurement of inhibition zones.

Results: A-PRF™ displayed antimicrobial activity against all of the individual organisms tested within this study following a 24 hour incubation period. However, no significant differences were noted between A-PRF™ and a natural clot of blood when tested against cultures of the oral rinse sample. Finally, the antimicrobial activity of A-PRF™ is significantly inferior to an equal volume of the CHX preparation.

Conclusion: Although A-PRF™ displays antimicrobial activity; its strength, spectrum and biologic activity within a polymicrobial environment requires further investigation.

May 2017

Declaration

I hereby declare that the mini thesis titled "The in vitro antimicrobial activity of advanced platelet rich fibrin (A-PRF™) against microorganisms of the oral cavity" is my own work, that it has not been submitted before for any degree or examination in any university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Feheem Bhamjee

Student number: 2382557   **Date: 19 May 2017**

Signed:,,

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I am also grateful to Dr N Basson and the dedicated staff members of the Oral and Dental Research Institute laboratory, Faculty of Dentistry, University of the Western Cape, for their assistance with the revival, maintenance and culturing of the specimens.

A special thanks to the staff members of the department of oral medicine and periodontology for their excellent patient care and surgical assistance.

Dedication

To my beloved wife and parents whose sacrifice, prayers and support have been fundamental for the completion of this degree.

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List of Abbreviations

Platelet concentrate	PC
Advanced Platelet rich fibrin	A-PRF™
Platelet rich fibrin	PRF
Leukocyte platelet rich fibrin	L-PRF
Injectable-PRF	i-PRF™
Millilitres	mL
Revolutions per minute	rpm
G-force	G
Platelet derived growth factor	PDGF
Transforming growth factors ß1 and ß2	TGF -ß1 and TGF- ß2
Vascular endothelial growth factor	VEGF
Epidermal growth factor	EGF
Insulin like growth factor type 1	IGF-1
Bone morphogenic protein	BMP
Pure platelet rich plasma	P-PRP
Leukocyte- and platelet-rich plasma	L-PRP
mixed cultured plates	MC
Candida albicans	CA
Streptococcus mutans	SM
Staphylococcus aureus	SA
Enterococcus faecalis	EF
Colony forming units	CFU
Microliter	µL
Degrees Celsius	ºC
Brain heart infused	BHI
Grams	g
Millimetre	mm
Chlorhexidine	CHX
Degrees of freedom	d.f
Probability	Pr
Potential of hydrogen	pH

Chapter 1:

Introduction:

In all fields of medicine, infection following surgery is regarded a serious complication as it is detrimental to healing and tissue regeneration (Burnouf *et al.,* 2013).This is especially true within the microbe rich oral environment where postsurgical infection may have devastating local or systemic consequences.

In recent years, the development and use of autologous platelet rich concentrates (PC's) has gained attraction within the rapidly progressive, multidisciplinary field of regenerative medicine (Miron *et al.,* 2016). The term platelet concentrates (PC's) refers to a group of living biomaterials which are derived from the processing of whole blood by various laboratory or chairside protocols (Ehrenfest *et al.,* 2009). These protocols often involve the centrifugation of blood, with or without the use of biochemical additives (Ehrenfest *et al.,* 2009; 2012; 2013; 2014) in order to concentrate activated platelets, growth factors and other bioactive molecules. Platelet activation and vitality is fundamental to all PC's as they are the primary source of the bioactive molecules able to participate in tissue repair and wound healing events (Simonpieri *et al.,* 2012; Dohan *et al.,* 2010; 2006; Davis *et al.,* 2014; Yao Su *et al.,* 2009; Del Corso *et al.,* 2012; Ehrenfest *et al.,* 2014; Kobayashi *et al.,* 2016; Masuki *et al.,* 2016).

Platelet rich fibrin (PRF), also recently termed leukocyte PRF (L-PRF), is a second generation of PC's and is fundamentally a fibrin matrix containing platelets, white blood cells, serum and growth factors (Choukran *et al.,* 2006; Dohan Ehrenfest *et al.,* 2009; Kwase, 2015; Miron *et al.,* 2016; Kobayashi *et al.,* 2016). The simplicity of its processing protocol sets it apart from other derivatives of PC's as it requires only a single centrifugation of whole blood with no need for chemical additives or technique sensitive fractionation steps (Choukran *et al.,* 2006; Dohan Ehrenfest *et al.,* 2009; Fujioka-Kobayashi *et al.,* 2017). Advanced-PRF™ (A-PRF™ PROCESS©, Nice, France) and Injectable-PRF (i-PRF™), represent the latest evolution of the original PRF protocol whereby two specific protocol parameters; i.e. centrifugation speed (G-force) and time; have been reduced in order to enhance growth factor release from the formed A-PRF clot (El Bagdadi *et al.,* 2017; Fujioka-Kobayashi *et al.,* 2017; Ghanaati *et al.,* 2015).

The use of PRF and A-PRF™ has been rapidly adopted within the surgical fields of dentistry and is currently applied as an adjunct to existing techniques or as a solitary biomaterial within surgical sites exposed to the microbial rich oral environment (Del Corso *et al.,* 2012; Lekovic *et al.,* 2012; Davis *et al.,* 2014; Wade *et al.,* 2013; Moraschini *et al.,* 2016; Kotsakis *et al.,* 2016). Previous research has highlighted the antimicrobial potential of different types of PC's and even hypothesised the biologic mechanisms involved (Bielecki *et al.,* 2007; El-Sharkawy *et al.,* 2007; Cieslik-Bielecka *et al.,* 2012; Drago *et al.,* 2013; Anitua *et al.,* 2011;

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Del Fabbro *et al.,* 2016). However, the study design, the subtype of PC used, the microorganisms tested and the techniques used to assess antimicrobial activity were highly variable (Del Fabbro *et al.,* 2016). Research specific to the antimicrobial activity of PRF or A-PRF™ remains sparse.

Chapter 2: Literature review

2.1 PRF and A- PRF™:

Developed by Choukroun (2001), PRF is a simple, quick, inexpensive and open access technique that results in the formation of a fibrin clot that is rich in entrapped platelets and leukocytes (Dohan *et al*., 2006). PRF differs from other forms of PC's as its fabrication protocol takes advantage of the coagulation properties inherent to whole blood without the need for biochemical modification. Thus it negates the use of additives such as anticoagulants, thrombin, calcium chloride or synthetic preservatives (Dohan *et al.,* 2006; 2010; Ehrenfest *et al.,* 2009; 2013; Davis *et al.,* 2014).

The original PRF protocol requires venous blood to be drawn from the patient and deposited into 10mL dry glass tubes. The tubes are then spun within a table top centrifuge (PC-O2 PROCESS© for PRF, Nice, France) at 3000 rpm (approximately 400G) for 10 minutes (Dohan *et al*., 2006; 2010; Davis, 2014). The protocol results in the formation of three distinct layers within the tubes; the base layer comprised of red blood cell (RBC), the PRF clot in the middle and the acellular plasma above (Dohan, 2006; Choukroun, 2006).

The PRF clot is inherently loaded with platelets, leukocytes, and growth factors. These growth factors begin to release from the PRF matrix within 5 to 10 minutes of the clot being formed, and continue to exude between 60 and 100 minutes (Su *et al.,* 2009; Pleumsakunthai *et al.,* 2013). Kobayashi *et al.* (2016) further indicated that PRF displayed a continual and steady release of growth factors over a 10-day period. Thus, the PRF clot is a reservoir of inactive and active cells able to interact with the cells and molecules native to the site upon which it is applied. This cocktail of cells and biochemical factors accelerate healing and promote tissue regeneration (Ehrenfest *et al.,* 2012; Pleumsakunthai *et al.,* 2013). The growth factors isolated from standard PRF protocols include: (Su *et al.,* 2009; Ehrenfest *et al.,* 2012; Cieslik-Bielecka *et al.,* 2012; Pleumsakunthai *et al.,* 2013; Tunali *et al.,* 2014; Davis *et al.,* 2014; Kobayashi *et al.,* 2016)

- Platelet derived growth factor (PDGF) (PDGF-AA; PDGF-AB;PDGF-BB)
- Transforming growth factors ß1 and ß2 (TGF -ß1 and TGF- ß2)
- Vascular endothelial growth factor (VEGF)
- Epidermal growth factor (EGF)
- Insulin like growth factor type 1 (IGF-1)

Dohan Ehrenfest *et al.* (2009), whose classification of PC's is widely used within recent literature, had classified the original PRF protocol as leukocyte and platelet-rich fibrin (L-PRF). However, as the protocol was open source, many publications investigating PRF have demonstrated alterations to the original protocol within their materials and methods. These publications often use non standardised centrifuges and commonly refer to centrifugation time rather than relative centrifugation force (Peck *et al.,* 2016). Dohan Ehrenfest *et al.* (2014) demonstrated that centrifuge type and vibration during processing, significantly

affected the quality and quantity of the PRF clot produced. A common protocol adopted for the fabrication of L-PRF is centrifugation at 2700rpm for 12 minutes (Dohan Ehrenfest *et al.,* 2014; Ghanaati *et al.,* 2014; Kumar *et al.,* 2015; Kobayashi *et al.,* 2016; Badade *et al.,* 2016; Fujioka-Kobayashi *et al.,* 2017) . The current literature involving PRF appears to have a critical shortcoming as the morphological features and the biologic properties of the formed PRF clot may be highly variable. This conundrum is due to non-standardisation of the processing protocol and the instrumentation involved (Peck *et al.,* 2016; Dohan Ehrenfest *et al.,* 2014)

A-PRF™ is a relatively recent development based upon a hypothesis stating that a reduction in the relative centrifugation force (G-force), by reducing centrifugation speed, would increase leukocyte numbers within the PRF matrix (Ghanaati *et al.,* 2014; Kobayashi *et al.,* 2016; Fujioka-Kobayashi *et al.,* 2017). Choukroun (2014) indicates that A-PRF™ has been developed primarily in an attempt to include monocytes within the fibrin network as these cells play an essential role in the growth of bone, blood vessels and the production of two chemokines , vascular endothelial growth factor (VEGF) and bone morphogenic protein (BMP-2). Ghanaati *et al.* (2014) investigated this concept and found that A-PRF™ displays significantly more neutrophilic granulocytes and with a wider distribution throughout the fibrin clot when compared to L-PRF. The group also found that histologically, the platelet distribution was more homogenous throughout the A-PRF™ clot than the L-PRF clot. This finding was further corroborated by El Bagdadi *et al.* (2017). It has also been demonstrated that A-PRF™ releases significantly higher total quantities of growth factors when compared to L-PRF prepared at 2700 rpm (325G) for 12 minutes (Kobayashi et al., 2016; Fujioka-Kobayashi et al., 2017). Furthermore, the growth factor release over time is significantly greater for A-PRF™ (Kobayashi et al., 2016; Fujioka-Kobayashi et al., 2017). In contrast, Dohan Ehrenfest *et al.* (2014) detected no BMP-2 released from A-PRF™ (1500 rpm, 14 minutes, G-force unknown) and indicated that the slow release of growth factors (TGFβ-1, PDGF-AB and VEGF) from L-PRF (2700 rpm, 400G, 12 minutes) was significantly stronger at all experimental times than the release from A-PRF membranes.

The processing protocol of A-PRF™ appears to have evolved within the literature. Earlier studies display a centrifugation protocol of 1500 rpm (100G) for 14 minutes (Ghanaati *et al.,* 2014; Dohan Ehrenfest *et al*., 2014; Kobayashi *et al.,* 2016) while more recent studies (Fujioka-Kobayashi *et al.,* 2017; El Bagdadi *et al.,* 2017) have used a centrifuge speed of 1300 rpm (200G) for 14 minutes for production of A-PRF™ and 1300 rpm (200G) for 8 minutes to produce A-PRF+. The current A-PRF™ processing protocol involves the use of a new preprogramed centrifuge (PRF DUO, PROCESS© for PRF, Nice, France), a standardised blood collection kit and a patented 10 mL glass-based vacuum tube (*Figure 3*). The specified table top centrifuge is capable of producing classic L-PRF, A-PRF™ and the latest i-PRF™ (injectable PRF™). The centrifuge setting labelled as A-PRF™ utilises a centrifugation protocol of 1300rpm for 8 minutes at 200G.

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2.2. Rationale for using A- PRF™ in this study:

Several protocols exist for the production of PC's, the products of which may present with significantly different physical and biological characteristics. According to Dohan Ehrenfest *et al.* (2009; 2012), PC's may be classified into four main categories, depending on their leukocyte and fibrin content. These include pure platelet rich plasma (P-PRP), leukocyte- and platelet-rich plasma (L-PRP), pure PRF and leukocyte and platelet-rich fibrin (L-PRF). However, as this classification had not been universally adopted within early literature, the specific properties a PC used within a study may not be adequately defined. This lack of standardisation may negatively influence the quality of comparative literature based upon the use of PC's.

Since its inception, PRF has become a common feature within dental implant and surgical periodontal literature and its popularity as a clinical tool grants significance to further research its biologic and clinical capabilities (*Table 1*). It is inexpensive, autologous to the patient, and according to its proponents, has a profound impact upon treatment quality and patient acceptance (Choukroun *et al.,* 2006; 2014; Simonpieri *et al.,* 2013).The PRF clot closely mimics the clinical feel of natural tissues as it is firm, versatile and easy to manipulate and suture (Kobayashi, 2012; Del Corso *et al.,* 2012; Simonpieri *et al.,* 2012).

Although the original protocol remains a clearly defined, reproducible and comparable product, the literature based upon PRF has been tainted by very subtle standardisation errors relating to its production (Dohan Ehrenfest et al., 2014; Pinto et al., 2014; Peck et al., 2015; 2016; Ehrenfest et al., 2016). These include; 1) the type of centrifuge used; 2) details regarding the blood collection tubes; 3) the volume of blood collected; 4) the storage time and temperature prior to centrifugation; 5) the relative centrifugation force; 4) centrifugation speed and 6) centrifugation time. Minor alterations to any of these variables may have a profound effect upon the quality of the product which may ultimately affect the outcomes of a study and its reproducibility. A-PRF™ is the latest advancement of PRF technology and, as all instrumentation required for its production have been commercialised, its production is more amenable to standardisation. Thus, the use of the A-PRF™ system within this study ensures that many of the variables mentioned above are standardised and easily reproducible.

The adoption of PRF membranes, plugs, or even shredded particulates into clinical practice also means that they may be applied into areas of previous infection or left exposed within the microbial rich oral environment (Del Corso, 2012; Lekovic, 2012; Davis *et al.,* 2014). Although PRF has shown positive effects on wound healing (Miron *et al.,* 2016), the effects of PRF within microbe contaminated or exposed sites has been largely unexplored.

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Table 1: Clinical indications for the use of PRF Oral surgery	
Maxillary sinus lift techniques	Choukroun *et al* 2006
Alveolar Socket preservation	Del Corso *et al* 2012
Bone and tissue regenerative techniques (GTR & GBR)	Del Corso *et al* 2012, Simonpieri *et al* 2012, Lekovic *et al* 2012
Periodontal plastic surgery	Chandran *et al* 2014, Lekovic *et al* 2012, Moraschini *et al* 2016
As an additive to all types of particulate bone grafting procedures	Del Corso *et al* 2012, Simonpieri *et al* 2012
To function as a membrane between hard and soft tissues	Choukroun *et al* 2006 ,Ehrenfrest *et al* 2009
As a local haemostatic agent	Simonpieri *et al* 2012
To enhance the healing of local sites in medically compromised patients	Simonpieri *et al* 2012

2.3. Previous research referring to the antimicrobial activity of PC's:

Previous research has highlighted the antimicrobial potential of different types of PC's and even hypothesised the biologic mechanisms involved (Bielecki et al., 2007; El-Sharkawy et al., 2007; Cieslik-Bielecka et al., 2012; Drago et al., 2013; Anitua et al., 2011; Del Fabbro et al., 2016). However, the study design, the subtype of PC used, the microorganisms tested and the techniques used to assess antimicrobial activity were highly variable (Del Fabbro et al., 2016). At present, only a single article has investigated the antimicrobial activity of a PRF membrane and none has investigated the use of A-PRF™.

A recent systematic review (Del Fabbro *et al.,* 2016) summarised the available preclinical evidence regarding the antimicrobial efficacy of PC's based upon 24 *in vitro* and animal studies. With regards to the *in- vitro* studies, the authors noted that although PC's reduced the growth of microorganisms during the first hours of incubation, they failed to break down the microbial load. Furthermore, recovery of bacterial growth was observed over time suggesting that PC's possess bacteriostatic rather than microbicidal properties. The animal studies indicated that although locally injected PC's were able to reduce infections caused by different microorganisms, this was to a lesser extent than locally applied antibiotics. Although the type of PC's studied varied extensively between the included publications, none had adopted the use of PRF and it is unknown if the derived evidence may be applied to the use of PRF matrices.

Badade et al. (2017) evaluated the antimicrobial effects of PRF and PRP against *porphyromonas gingivalis* and *aggregatibacter actinomycetemcomitans*. The group indicated that PRP dramatically inhibited the growth of both organism*s* whereas PRF displayed no activity against these pathogens. The results of the publication should be

interpreted with caution as the PRF production protocol deviates from the original as well as from those used within recent literature (Dohan Ehrenfest et al., 2014; Ghanaati et al., 2015; Kumar et al., 2015; Kobayashi et al., 2016; Fujioka-Kobayashi et al., 2017). The protocol made use of 5 mL of intravenous blood collected in a plain bulb and centrifuged (Manual Centrifugation Machine, e-Tek) at 3000 rpm for 10 min. The produced PRF clot was then formed into a membrane after being squeezed dry in an undefined manner. It may thus be argued that the PRF used within this study may not be biologically representative of the original PRF protocol and the comparison made between this version of PRF and PRP may not be adequately standardised.

Chapter 3: Aim and Objectives

3.1. Aim:
To determine if A-PRF™ possesses antimicrobial activity against microbes of the oral cavity.

3.1.2. Objectives:
To evaluate the antimicrobial activity of A-PRF™

Against:

1. Aerobic and facultative anaerobic microorganisms prepared from oral rinse samples.
2. Pure cultures of *Streptococcus mutans* (ATCC 25175)
3. Pure cultures *of Enterococcus faecalis (ATCC 29212)*
4. Pure cultures of *Candida albicans* (ATCC 36810).
5. Pure cultures of *Staphylococcus aureus* (ATCC 25923)

3.1.3. Null hypothesis:
The antimicrobial activity of A-PRF™ is equal to that of unprocessed blood.

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Chapter 4: Methodology

4.1. Study design:

An *in-vitro* disk-diffusion assay of an exploratory nature was carried out. The test material, A-PRF™, was compared against normal blood and a positive control.

4.2. Study site:

The Department of Oral medicine and Periodontology and the Oral and Dental Research Institute laboratory, Faculty of Dentistry, University of the Western Cape, Tygerberg campus.

4.3. Study participants:

A-PRF™ and blood samples were obtained from 21 consenting individuals who met the inclusion criteria and underwent surgical procedures within the Department of Oral Medicine and Periodontology. Only pristine samples left unused within the surgery and sealed within their collection tubes were allocated for the laboratory tests. The allocated material was sufficient to perform laboratory based tests upon a total of 115 Brain Heart Infused (BHI) agar plates. All aspects of this project were discussed with the potential participants and information sheets were provided prior to attaining their consent (*Appendix 1*).

Fresh oral rinse samples, used for the preparation of mixed microbial cultures, were obtained from 21 faculty staff volunteers within the Department of Oral Medicine and Periodontology. The volunteers were orally and systematically healthy, dentate or partially dentate and without the use of prosthetic or orthodontic appliances. Exclusion criteria included edentulous individuals, smokers, snuff dippers and those who have made use of antibiotics, immunosuppressive or chemotoxic drugs within the past three months prior to sample collection.

4.4. Inclusion and exclusion criteria:

Inclusion criteria for A-PRF™ collection:

1. Only patients undergoing surgical procedures involving the use of A-PRF™ were eligible for inclusion.
2. Sufficient A-PRF™ available for use after completion of the surgical intervention
3. Participants were at least 18 years of age
4. Systemically healthy.
5. Informed consent to the use of blood and blood derived A-PRF™

Exclusion criteria for A-PRF™ collection:

1. Smokers, irrespective of the nature of smoking and inclusive of electronic cigarettes

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2. Use of antibiotics, anticoagulants, immunosuppressive or cytotoxic medications within the past three months prior to sample collection.
3. Lack of material after surgery
4. A-PRF™ samples removed from the sealed blood collection tubes
5. A-PRF™ samples older than 30 minutes from time of complete centrifugation

Inclusion criteria for oral rinse samples:

1. Participants should be aged 18 years or older
2. Dentate or partially dentate
3. Systemically healthy
4. Orally healthy

Exclusion criteria for oral rinse samples:

1. Individuals without a natural dentition
2. Smoking of any and all substances
3. Use of antibiotics, anticoagulants, immunosuppressive or cytotoxic medications within the past three months prior to sample collection

4.5. Laboratory groups and sample size

A total of 115 standard BHI agar plates were inoculated in accordance to the following 5 groups for disk diffusion tests:

Group A: 21 mixed cultured (MC) plates prepared from oral rinse samples

Group B: 21 plates of purely cultured *streptococcus mutans* (*SM*) (ATCC 25175)

Group C: 21plates of purely cultured *enterococcus faecalis (EF)* (ATCC 29212)

Group D: 21 plates of purely cultured *candida albicans (CA)* (ATCC 36810)

Group E: 21 plates of purely cultured methicillin sensitive *staphylococcus aureus (SA)* (ATCC 25923)

4.6. Collection and preparation of samples

4.6.1. Preparation of mixed cultures from oral rinse samples:

Volunteers were provided with 10 mL of sterile saline stored within a sterile universal container. Under the supervision of the principle investigator, each volunteer rinsed thoroughly for 60 seconds and thereafter returned the rinse back into the container (Samaranayake *et al*, 1986). No meals were consumed within two hours of the oral rinse sampling procedure.

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A total of 21 fresh oral rinse samples were utilised for the inoculation of 21 standard BHI agar plates within the MC group. The preparation of the mixed culture plates was well coordinated and occurred simultaneously with the preparation of the pure culture groups. This ensured that all culture groups were ready for disk diffusion tests upon the arrival of the A-PRF™ and coagulated blood.

The McFarland standard 1 turbidity tube, corresponding approximately to 3 X 10^8 colony forming units per millilitre (CFU/mL), was used to standardise each of the oral rinse samples. Bacterial suspensions with similar turbidity to a particular McFarland Standard (Table 2) are expected to produce approximate cell count densities. This method is used in a variety of identification or susceptibility kits (Borges *et al.,* 2010; Sutton *et al.,* 2011).

Table 2: McFarland turbidity standards	
McFarland Standard	Approximate Cell Count Density (x108cells)
0.5	1.5
1	3.0
2	6.0
3	9.0
4	12.0
5	15.0
6	18.0
7	21.0

For each oral rinse sample, one Brain Heart Infusion (BHI) agar plate was prepared for culture. 0.5 mL of the oral rinse sample was transferred to the BHI plate and spread over the agar surface using a sterile cotton swab. Each inoculated BHI plate was incubated (Memmert 854 incubator, Memmert GmbH Co. KG, Schwabach, Germany) for 24 hours at 37ºC during the disk diffusion test. The nature of the oral rinse sample coupled to the incubation media allows the growth of aerobic and facultative anaerobic organisms present within the oral rinse sample (Sedgley and Samaranayake, 1994).

4.6.2 Preparation of Pure cultures:

American type culture collection (ATCC) strains of *candida albicans* (ATCC 36810) , *streptococcus mutans (*ATCC 25175*), staphylococcus aureus* (ATCC 25923) and *enterococcus faecalis* (ATCC 29212) were selected based upon their established involvement within the oral microbiome in both health and disease (Ghannoum *et al.,* 2010; Wade *et al*., 2013; Pinheiro *et al.,* 2014; Koukosa *et al.,* 2015; Komiyama *et al.,* 2016). Revival and preparation of the pure cultures were performed by a single microbiologist within the Oral and Dental Research Institute laboratory, Faculty of Dentistry, University of the Western Cape, Tygerberg campus (**Figure 1**). Once prepared and delivered upon their respective BHI agar plates, a separate standardized inoculum of each culture was prepared in accordance to the

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direct colony suspension method and the McFarland Scale. This process involved the transfer of some of the newly revived organisms from their BHI agar plates into 100 μL of sterile saline in order to form a suspension. Each suspension was then calibrated by comparison against the McFarland 1 turbidity tube which equates to approximately 3 X 10^8 CFU/ml (**Figure 2**). Once calibrated, 0.5 mL of each standardised culture suspension was inoculated onto and spread across its designated BHI agar plate.

Figure 1: Revived ATCC cultures as delivered

Figure 2: Calibration of suspensions to McFarland 1 turbidity

4.6.3 Collection of blood and preparation of A-PRF:

The A-PRF™ samples were produced according to prescribed protocol utilizing equipment and materials specified by the company PROCESS© (Nice, France) (**Figure 3**). All phlebotomy procedures were performed by an experienced nurse and collected into the specified A-PRF™+ (PROCESS©, Nice, France) 10 mL sterile plain glass-based vacuum tubes. The number of tubes processed varied according to the estimated surgical requirements as well as the need to balance the tubes within the centrifuge. The blood was then immediately processed according to the manufacturers A-PRF™ settings (1300 rpm for 8 minutes) within the PRF

DUO (PROCESS©, Nice , France) table top centrifuge. Fresh and complete A-PRF™ specimens not used within the surgical interventions were utilised for laboratory procedures (**Figure 4**).

Figure 3 : Instrumentation used for the processing of A-PRF™. A) Phlebotomy materials. B) A-PRF+ blood collection tubes. C) PRF DUO centrifuge displaying the A-PRF protocol. D) Sterile Instrumentation used

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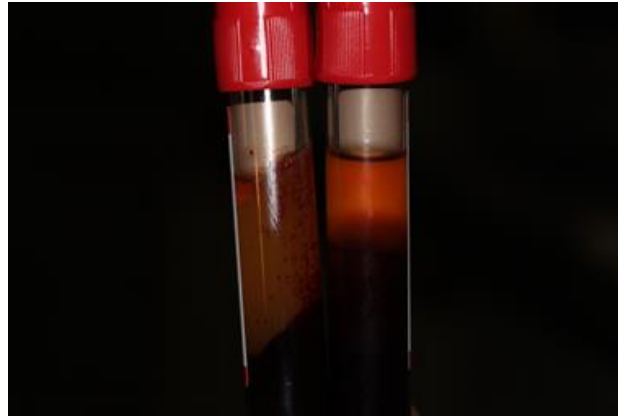
Figure 4: A processed A-PRF™ clot (left) compared to a failed sample (Right)

Once processed, each A-PRF™ clot was removed from the tube and separated from the underlying red blood cell layer under sterile conditions. Each clot was then weighed and divided lengthwise from the buffy coat toward the coronal end of the clot using a 15C surgical blade. Each clot allowed for 3 approximately equal divisions, each of which was then placed into a sterile 5x5 mm stainless steel cylinder lying atop a sterile flat stainless steel tray. Orientation of each sample ensured that the cell rich buffy coat region (Ghanaati et al., 2015) made contact with the flat tray. Thereafter, each specimen was gently compressed with a sterile glass rod to form a PRF plug shaped according to the dimensions of the steel cylinder. Each plug was then weighed and trimmed until standardised to equal 0.1 grams (g), thus effectively forming an A-PRF™ disk suitable for disk diffusion testing (**Figure 5**). All laboratory procedures were completed within one hour from the time of A-PRF™ production.

Figure 5: Formation of an A-PRF™ disk. A) A-PRF™ clot after removal from the collection tube. B) Sterile stainless steel cylinder. C) Gentle compression of the clot into the cylinder. D) Miniature A-PRF™ plug formed within the steel cylinder

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4.6.4 Collection of unprocessed blood:

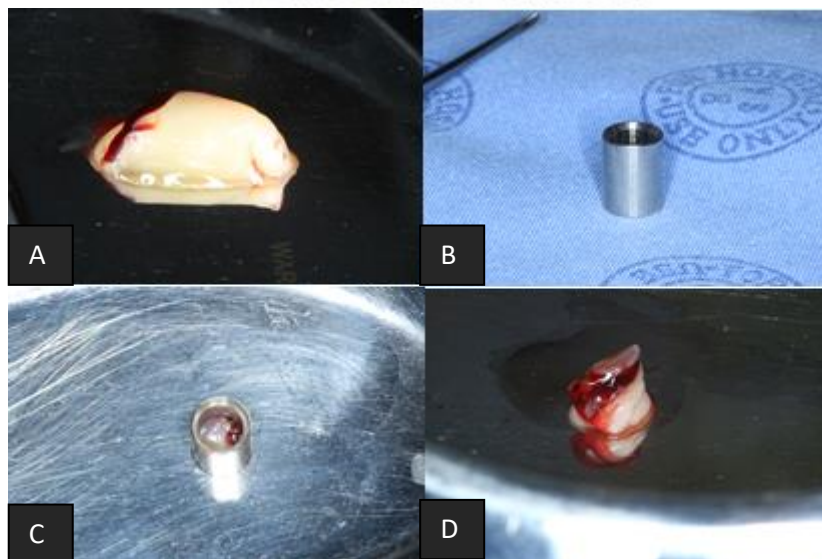An extra 2 mL of blood was drawn from each of the patients included within this study in the manner described above. However, standard blood collection tubes were used and the blood was allowed to clot naturally within the tube. A Sterile instrument was then used to harvest and measure 0.1 g of clotted blood which was then laid onto a sterile 5mm assay disk. The blood laden disks were then placed onto the designated agar plates with the clotted blood against the agar.

4.6.5. Chlorhexidine (CHX) as a positive control

Chlorhexidine remains the most thoroughly investigated antimicrobial compound in dentistry with a firm establishment within the field for over 40 years (Jones, 1997; Supranoto et al., 2015). CHX has also been recognized by the pharmaceutical industry as the positive control against which the efficacy of alternative antiplaque agents should be measured (Jones, 1997). The compound displays a wide spectrum of activity against gram-positive and gram-negative bacteria, yeasts, dermatophytes and some lipophilic viruses (Jones, 1997). The inclusion of CHX as part of disk diffusion tests within this investigation was solely to gauge the efficacy of A-PRF™. For this purpose, a 0.2% CHX gluconate mouthrinse (Resmed, Fulvicare Ltd. South Africa) had been selected as it is freely available within the South African public health sector.

4.7. Disk Diffusion Test to measure inhibition zones:

Three 5 mm disks were placed onto different zones of each inoculated BHI agar plate. One disk being the standardised A-PRF™ disk weighing 0.1 g and the second disk consisting of 0.1 g of clotted blood as described above. The A-PRF™ and blood specimens used within each test group were derived from the same individual. The third disk involved soaking a sterile 5 mm assay disk with 0.1 mL of Resmed chlorhexidine gluconate 0.2% mouthrinse® (Fulvicare Ltd. South Africa), as a positive control. The plates were then incubated (Memmert 854 incubator, Memmert GmbH Co. KG, Schwabach, Germany) for 24 hours at 37ºC.

The antimicrobial effects were determined by measuring the dimensions of the growth inhibition zone found around each disk after 24 hours of incubation. A specimen with sufficient antimicrobial activity creates a zone of bacterial inhibition as it diffuses through the agar (Driscol *et al.,* 2012). The diameter of the inhibition zones were measured at three points, using a calibrated digital calliper in millimetres (mm) (**Figure 6**), and viewed against an illuminated background. All measurements were performed by the principle investigator under the supervision of a microbiologist (Dr N Basson). A zero value was awarded to disks which displayed no inhibition zone even under magnification (5x).

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Figure 6: Digital calliper used during the investigation

Chapter 5: Data processing and analysis

All laboratory samples were categorized and coded to maintain participant anonymity as well as to facilitate data capture within a Microsoft Excel© (Microsoft Corporation, Redmond, WA, USA) spreadsheet. Further analysis of the data was performed utilising the 'R software package' (R Core Team 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

Chapter 6: Ethical approval

Ethical approval was obtained from the UWC health research ethics committee (project registration number 15/7/30). All aspects of the study have been compliant to the UWC research ethics policy as well as the guidelines set forth by the Helsinki Declaration for biomedical research involving human subjects.

Due to the voluntary nature of the project, details regarding the study were discussed with all potential participants prior to attaining their written and informed consent (*Appendix 1, 2 and 3*). The primary investigators personal contact details were made available to all participants for further information regarding the study or its outcome. All biologic samples were incinerated upon the completion of the laboratory phase of the investigation.

6.1. Conflict of interest:

The author declares no conflict of interest. No external funding was obtained for this study.

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Chapter 7: Results

7.1. Demographic characteristics of patient participants:

A-PRF™ and blood specimens obtained for the laboratory investigations were obtained from the 21 patient participants included within this study. Males comprised the majority of the sample population (57.14%) with females making up (42.86%). The age range for patient participants was 22-62 years with a mean age of 42.28 and a standard deviation of 12.51.

7.2. Descriptive analysis

After 24 hours of incubation, only the positive control (CHX) displayed consistent and measurable zones of inhibition. The test group (A-PRF™) displayed variable inhibition against the organisms within each group (**Figure 7**). Furthermore, the inhibition zones created by the test group were not uniformly circumferential in the majority of the samples. The negative control group (Blood) also appeared to produce inhibition zones within some of the sample groups. Specimens producing a non measurable zone of inhibition were recorded as a zero value within the data capture sheet.
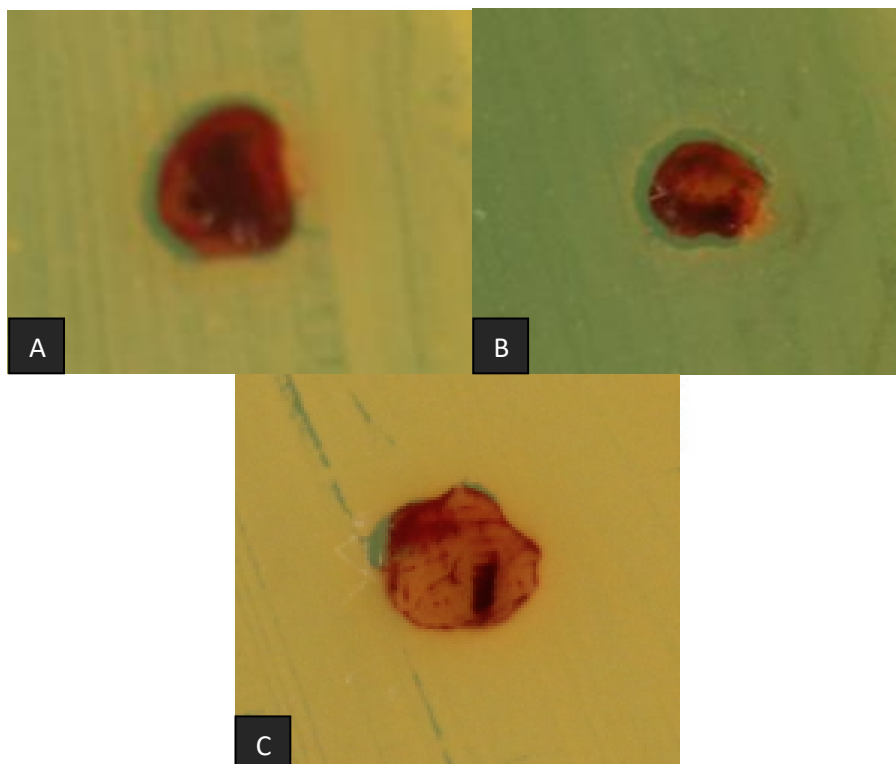

Figure 7: A-PRF™ displaying variably shaped inhibition zones. A) Group CA. B) Group EF. C) Group SA
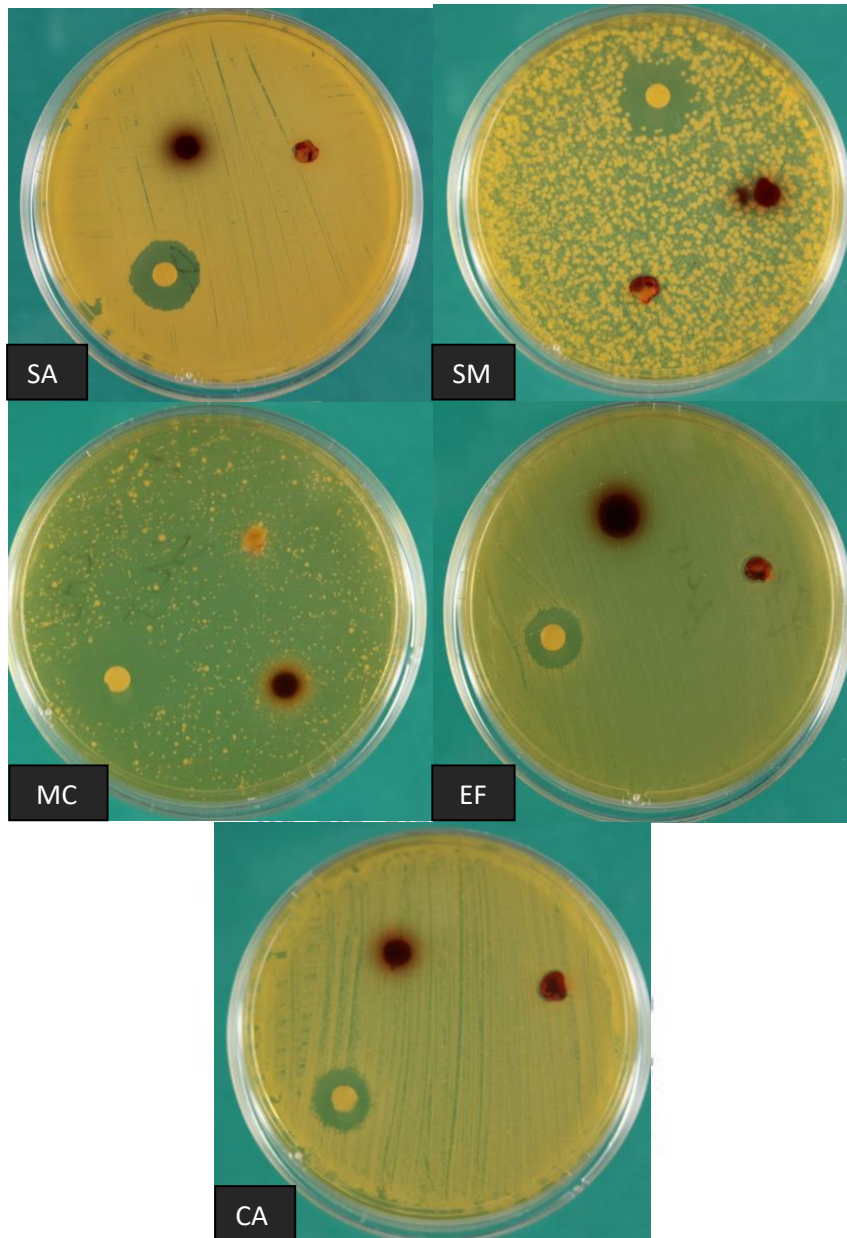
Figure 8: A complete sample set after incubation.

7.3. Statistical Analysis
7.3.1. McNemar's test comparing A-PRF™ to blood.

When comparing the recorded inhibition zones between A-PRF™ and blood, a number of specimens produced no measurable inhibition zones and were recorded as a zero measurement. The McNemar's test was applied in order to analyse the proportions of zero recordings between A-PRF™ and blood within each group. Thereafter the means of A-PRF™ and blood were compared in those instances were non-zero observations were recorded for both variables.

Table 3: McNemar's test for Group A representing the MC group			
Group A = MC; A-PRF-A vs Blood-A			
	A-PRF-A =0	A-PRF-A >0	**Total**
Blood-A =0	15	2	17
Blood-A >0	0	4	4
Total	15	6	21

Table 3 shows that Blood-A and A-PRF-A agree in 19 of the 21 cases. Of the remaining 2 cases, 2 have A-PRF-A >0 and none has Blood-A >0. According to the McNemar test the proportions 2/2 and 0/2 are not significantly different. The McNemar's chi-squared = 0.5, degree of freedom (d.f) = 1, p-value = 0.4795 and the exact p-value is 0.5. The conclusion is that the proportions of Blood-A >0 (4/21) and A-PRF-A >0 (6/21) are not significantly different.

Table 4: McNemar's test for Group B representing the SM group			
Group B = SM; A-PRF-B vs Blood-B			
	A-PRF-B =0	**A-PRF-B >0**	**Total**
Blood-B =0	4	7	11
Blood-B >0	0	10	10
Total	4	17	21

From Table 4: McNemar's chi-squared = 5.1429, d.f = 1, p-value = 0.0233; exact p value = 0.0156. In conclusion, the proportion of A-PRF-B >0 (17/21) is significantly greater than proportion Blood-B >0 (10/21).

Table 5: McNemar's test for Group C representing the EF group			
Group C = EF; A-PRF-C vs Blood-C			
	A-PRF-C =0	A-PRF-C >0	**Total**
Blood-C =0	6	12	18
Blood-C >0	0	3	3
Total	6	15	21

From Table 5: McNemar's chi-squared = 10.0833, d.f = 1, p-value = 0.00150; exact p value = 0.000488. In conclusion, the proportion of A-PRF-C >0 (15/21) is significantly greater than the proportion Blood-C >0 (3/21).

Table 6: McNemar's test for Group D representing the CA group			
Group D = CA; A-PRF-D vs Blood-D			
	A-PRF-D =0	A-PRF-D >0	Total
Blood-D =0	4	12	16
Blood-D >0	0	5	5
Total	4	17	21

From Table 6: McNemar's chi-squared = 10.0833, d.f = 1, p-value = 0.00150; exact p-value = 0.000488. In conclusion, the proportion of A-PRF-D >0 (17/21) is significantly greater than the proportion Blood-D >0 (5/21).

Table 7: McNemar's test for Group E representing the SA group			
Group E = SA; A-PRF-E vs Blood-E			
	A-PRF-E =0	A-PRF-E >0	Total
Blood-E =0	7	14	21
Blood-E >0	0	0	0
Total	7	14	21

From Table 7: McNemar's chi-squared = 12.0714, d.f = 1, p-value = 0.000512; exact p-value = 0.000122. In conclusion, the proportion of A-PRF-E >0 (14/21) is significantly greater than proportion Blood-E >0 (0/21).

Table 8 summarises the results comparing the mean inhibition zones between A-PRF™ and blood in those observations recorded as non-zero values. Figure 9 below provides a graphical representation of Table 8. The total number of observations used is too small to draw any conclusions; however it is notable that all A-PRF™ means are greater than the corresponding blood means. It is interesting to note that blood did not produce a non-zero observation within the SA group.

Table 8: Means of inhibition zones in non- zero observations					
Group	Blood	A-PRF™	T	d.f	p-value
A (MC)	6.475	8.425	3.067	3	0.0547
B (SM)	6.71	8.47	3.616	9	0.0056
C (EF)	7.17	8.29	3.875	2	0.0606
D (CA)	7.30	7.90	1.684	4	0.1675
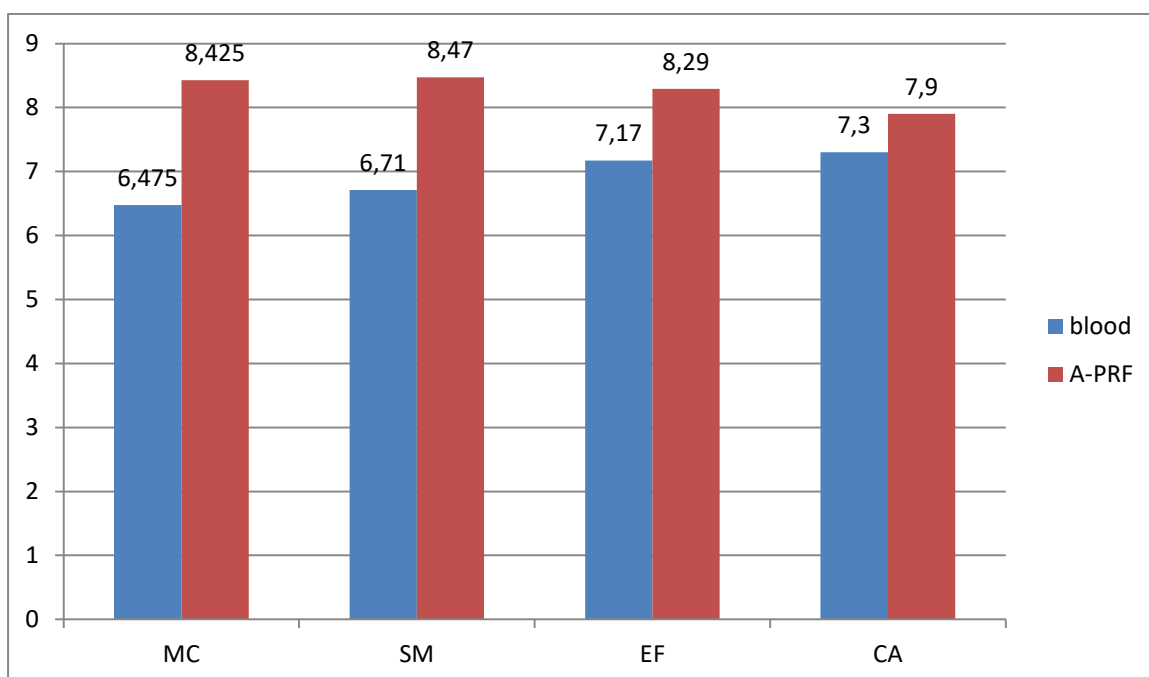
Figure 9 : Mean diameter (in millimetres) of inhibition zones in the non-zero observations.

7.3.2. Probability of forming inhibition zones

The aim of the current investigation was to determine if A-PRF possesses antimicrobial activity against microbes of the oral cavity. Since the possession of antimicrobial activity is interpreted as the ability to form measurable inhibition zones, the probability of A-PRF™ and blood to form these zones was analysed. The data summarised within Table 9 indicates that A-PRF™ displays a non-zero probability to form inhibition zones. The conclusion is that A-PRF™ indeed possesses antimicrobial activity thus satisfying the aim of the investigation. However, it appears that normal unprocessed blood also possesses antimicrobial activity against microbes of the oral cavity. The last column of Table 9 compares the probability of A-PRF™ and blood to produce inhibition zones. In every case except for the mixed culture group, the probability of A-PRF™ to form inhibition zones is significantly greater than the probability of blood.

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Table 9: Probability of inhibition zone formation by A-PRF™ and blood			
Group	Pr-A-PRF (95% confidence limits)	Pr-Blood (95% confidence limits)	Pr-A-PRF > Pr-Blood (p-value)
A (MC)	**0.286** (0.113, 0.522)	**0.190** (0.054, 0.419)	**0.5000**
B (SM)	**0.810** (0.581, 0.946)	**0.476** (0.257, 0.702)	**0.0156**
C (EF)	**0.714** (0.478, 0.887)	**0.143** (0.030, 0.363)	**0.0005**
D (CA)	**0.810** (0.581, 0.946)	**0.238** (0.082, 0.472)	**0.0005**
E (SA)	**0.667** (0.430, 0.854)	**0.000** (0.000,0.161)	**0.0001**
Pr-A-PRF = probability of A-PRF™ to form inhibition zones			
Pr-Blood = probability of blood to form inhibition zones			
Pr-A-PRF > Pr-Blood - p-values from testing the hypotheses of Pr-A-PRF=Pr-Blood			
*Values in brackets = (upper and lower 95% confidence limits for true probability)			

7.3.3. Comparison of inhibition zones between A-PRF™ and chlorhexidine (CHX).

Since A-PRF™ displayed antimicrobial activity; those positive zones of inhibition were then compared to the corresponding zones of CHX. The results indicate that the antimicrobial activity of chlorhexidine is significantly greater than that of A-PRF™ with p-values well below 0.05 (Table 10). Figure 10 graphically compares the mean zones of inhibition between A-PRF™ and CHX in those instances in which A-PRF™ recorded positive antimicrobial activity.

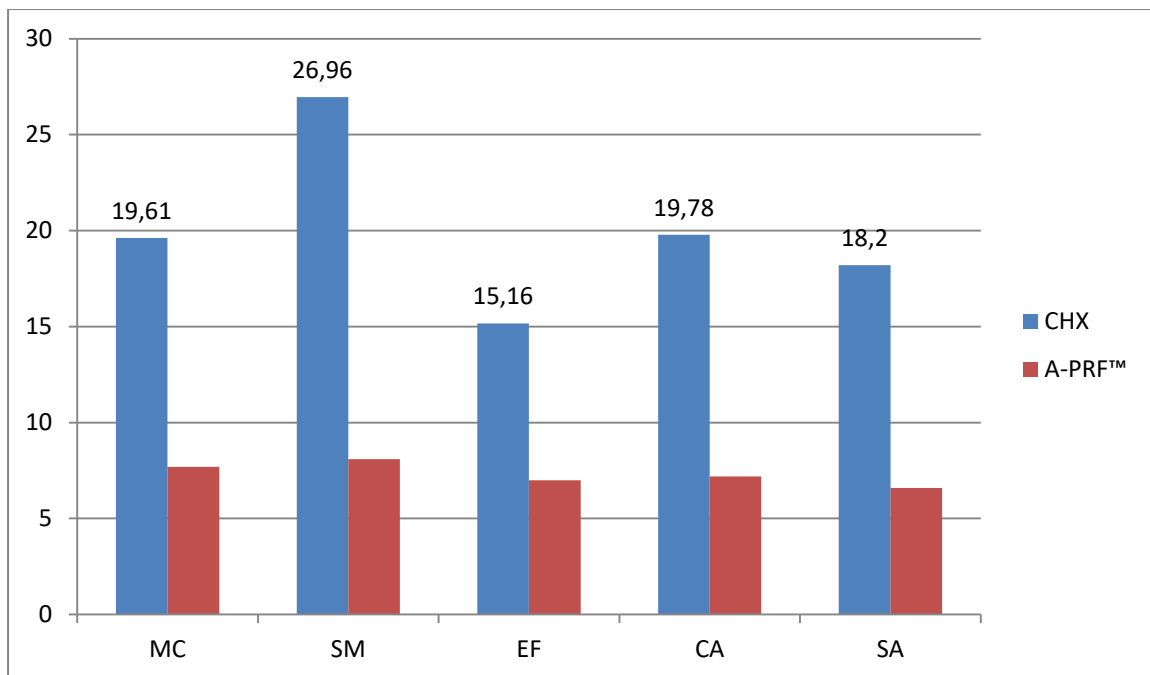| Table 10: Mean difference of inhibition zones of A-PRF™ and CHX						
Group	A-PRF™	CHX	Mean difference	t	d.f	p-value
A (MC)	7.7	19.61667	11.91667	4.5916	5	0.005885
B (SM)	8.094118	26.95882	18.86471	22.0418	16	<0.0001
C (EF)	6.993333	15.15333	8.16	13.4884	14	<0.0001
D (CA)	7.205882	19.77059	12.56471	9.9599	16	<0.0001
E (SA)	6.759286	18.15	11.39071	16.2705	13	<0.0001

Figure 10 Mean inhibition zones of A-PRF™ and CHX in millimetres (mm)

Chapter 8: Discussion

The regenerative capabilities of PRF as a surgical adjunct in dentistry have received considerable attention since its introduction in the early years of the new millennia. In contrast, there remains no clear evidence to elucidate the antimicrobial potential of this particular biomaterial which differs both structurally and biologically to other forms of PC's. Ghanaati *et al.* (2014) histologically described A-PRF™ as a fibrin based, cell seeded matrix containing a variety of blood cells including; platelets, lymphocytes (B and T), monocytes, stem cells and neutrophilic granulocytes capable of releasing a host of growth factors (Kobayashi et al., 2016; Fujioka-Kobayashi et al., 2017)

In theory, the biologic components and the physiologic mechanisms to exert antimicrobial activity are similar within various types of PC's and even clotted blood. However, these autologous biomaterials differ with regards to: 1) the variable mix of cell types; 2) the viability of the contained cells; 3) their manner of activation, natural or chemical; 4) the density of the fibrin network; 5) interactions between the cellular and extracellular components; 6) and the release of a variety of proteins. These differences may significantly impact their respective anti-inflammatory and antimicrobial properties (Del Fabbro *et al.,* 2016; Burnouf *et al.,* 2013; Cieslik-Bielecka *et al.,* 2012; Anitua *et al.,* 2011; Dohan Ehrenfest *et al.,* 2009; El-Sharkawy *et al.,* 2007).Furthermore, the mechanisms and dynamics of the individual antimicrobial components contained within these biomaterials is poorly understood.

The purpose of this investigation was to determine if A-PRF™ possesses antimicrobial activity against microorganisms of the oral cavity. From the results of this investigation, we may conclude that A-PRF™ displays antimicrobial activity against all of the individual organisms tested within this study over a 24 hour time period. These findings are consistent with that of previous studies evaluating the antimicrobial properties of other preparations of PC's (Del Fabbro *et al.,* 2016; Burnouf *et al.,* 2013; Cieslik-Bielecka *et al.,* 2012; Anitua *et al.,* 2011; Bielecki *et al.,* 2007), however in contrast to the findings of Badade *et al.* (2017) who tested their version PRF against anaerobic pathogens associated with periodontal disease.

Since A-PRF™ displays antimicrobial properties; the need arose to determine if this activity is significantly greater than that of a naturally occurring blood clot. Once again, A-PRF™ displayed a significantly greater probability to inhibit the growth of all the individual organisms tested within this study. Unfortunately definitive conclusions regarding the potency of this biomaterial in comparison to a clot of blood could not be drawn.

Another important finding from this investigation is that no significant differences were noted between A-PRF™ and a natural clot of blood when tested against the variable mix of aerobic and facultative anaerobic organisms derived from the oral rinse sample (Sedgley and Samaranayake, 1994). This result infers that the spectrum of antimicrobial activity is limited within the polymicrobial environment native to a healthy oral cavity. It is possible

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that within this environment, many organisms may not be susceptible to the antimicrobial factors present within blood, A-PRF™ or even other derivatives of PC's. Furthermore, the inhibition of organisms sensitive to the antimicrobial components may allow those with resistance to thrive. Another possible phenomenon is that PC's may induce the growth of certain organisms (Bielecki et al., 2012; 2007) and inadvertently induce a flare-up of infection. Future investigations are required to explore the antimicrobial spectrum of A-PRF™ and explore the possibility that it may act as a substrate to facilitate the growth of specific organisms.

From all the organisms tested within this investigation, SA displayed inhibition by A-PRF™ (mean zone of inhibition of 6.71mm) but never by blood. This particular organism is of importance as it is an established pathogen associated with a wide spectrum of diseases, ranging from mild and usually self-limiting conditions, such as impetigo, to severe and potentially life-threatening diseases, such as pneumonia, endocarditis, and sepsis (Sause *et al.,* 2015). Of particular relevance to the oral surgeon is that SA is a major cause of hospital acquired infections, infections related with indwelling medical devices and infection of surgical wounds (Zalavras et al., 2004). Significant research is focussed upon alternative treatment strategies in SA driven infections in order to reduce the risk of developing antibiotic resistant strains (Sause *et al.,* 2015; Anitua *et al.,* 2011). For this reason SA remains the most frequently tested organism within the literature examining the antimicrobial activity of PC's (Del Fabbro et al., 2016). Many different preparations of PC's have demonstrated antimicrobial activity to both methicillin resistant and methicillin sensitive strains of SA (Del Fabbro *et al.,* 2016; Anitua *et al.,* 2011; Bielecki *et al.,* 2007). Further investigations are required to determine if A-PRF™ possesses antimicrobial activity against methicillin resistant SA as well as the potential of sensitive strains to develop resistance.

EF remains the most commonly isolated enterococcus species from human oral samples (Komiyama et al., 2016) and implicated in oral diseases such as caries, endodontic infections, periodontitis, and peri-implantitis (Kouidhi *et al.,* 2011; Rams *et al.,* 2013).The organism is also frequently implicated with endodontic treatment failures, supposedly due to its high resistance to endodontic medicaments and its persistent involvement in biofilm formation (Duggan *et al.,* 2007). Furthermore, infection by resistant strains of EF may not respond to antibiotics such as tetracycline, penicillin, cephalosporin and aminoglycosides (Komiyama et al., 2016).Within this investigation; EF displayed susceptibility to the antimicrobial activity of A-PRF™ to a greater extent than blood. The literature examining the susceptibility of this organism to other types of PC's is contradictory. These conflicting results may be due to differences related to the techniques applied to test antimicrobial sensitivity, the biologic or processing differences between the type of PC's tested or the strain of the organism sampled (Del Fabbro et al., 2016; Drago et al., 2014; Bielecki et al., 2007).

CA is the most frequently isolated of the fungal species within the oral microbiome and asymptomatically colonises the oral cavity of healthy individuals (Ghannoum *et al.,* 2010). Despite this commensal relationship, impairment of an individual's immune response, barrier function, or disturbances to the local microenvironment (including altered composition of local microbiota) can allow these opportunistic fungi to cause infection (Jabra-Rizk *et al.,* 2016; Tang *et al.,* 2016; Marsh *et al.,* 2017). The majority of these infections are as a result of its capacity to form biofilms upon host tissue or abiotic surfaces. CA is also able to co-aggregate with oral *streptococci* forming a synergistic partnership whereby the yeast promotes streptococcal biofilm formation and the streptococci enhance the invasive property of CA (Xu et al., 2017). The results of this investigation indicate that A-PRF™ possessed a greater ability to consistently inhibit the growth of CA when compared to a clot of normal blood. CA was only tested in a single *in-vitro* study (Drago *et al.,* 2013) which assessed the antimicrobial activity of pure platelet rich plasma (P-PRP) (Del Fabbro *et al.,* 2016; Drago *et al.,* 2013). The study noted that CA was sensitive to the antimicrobial activity of P-PRP at higher platelet concentration ranges than compared to the bacteria. This implied that CA was less susceptible to the antimicrobial components of platelets and corroborated the findings of Tang *et al.* (2002) who noticed that the antimicrobial peptides of human platelets were more potent against bacteria than fungi.

SM is last of the bacterial species investigated within this study due to its established involvement with biofilm formation and the pathogenesis of caries (Marsh *et al.,* 2017; Jenkinson, 2011; Nobbs *et al.,* 2009). *Streptococci* are well known primary colonisers of salivary pellicle coated oral surfaces and are integral to oral biofilm development (Jenkinson, 2011). These organisms are well adapted to colonize multiple niches within the oral cavity including pellicle coated surfaces of teeth, dentures and implants (Nobbs *et al.,* 2009). Repeated conditions of low pH within the oral environment favour the establishment of acidogenic microbiota, including SM, within the biofilm (Bradshaw *et al.,* 1998). When the composition of the biofilm is dominated by these saccharolytic and acid tolerant species, the risk of caries development is enhanced (Marsh et al., 2017; Jenkinson, 2011; Nobbs et al., 2009). This study has revealed that A-PRF™ displays a greater potential to inhibit SM when compared to a natural clot of blood. However, as no other PC has been tested against this organism the mechanism of its inhibition and clinical potential requires further exploration.

Since A-PRF™ had displayed antimicrobial activity within this investigation; the final component was to compare its inherent activity to that of a commonly utilised antimicrobial oral rinse (0.2% CHX gluconate mouthrinse, Resmed, Fulvicare Ltd. South Africa). The use of CHX oral rinse or gel preparations during the postoperative phase have been found to enhance the outcomes of wound healing following periodontal and implant surgery (Heitz *et al.,* 2004; Westfelt *et al.,* 1983). The results indicate that the antimicrobial activity of A-PRF™ is significantly inferior to an equal volume of the CHX preparation. As A-PRF™ and CHX may both participate in postsurgical healing; the clinical relevance of this comparison is yet to be explored. It is unknown if CHX may potentiate the antimicrobial activity of A-PRF™ or if the

potential cytotoxic effects of CHX (Giannelli *et al.,* 2008) may be detrimental to the biologic activity of A-PRF™.

Chapter 9: Limitations

Although the results of this investigation indicate that A-PRF™ displayed antimicrobial activity, several limitations have become apparent. Firstly, the *in-vitro* investigation does not mimic a clinical situation whereby A-PRF™ will be placed into an environment surrounded by tissues responding to a surgical event. Within this scenario, A-PRF™ may interact with a host of cells and cytokines involved in the process of wound healing and modify initial immune response and healing events (Miron *et al.,* 2016; Bernouf *et al.,* 2013; El-Sharkawy *et al.,* 2007). The release of growth factors by activated platelets within the fibrin matrix may also modify the expression of antimicrobial peptides from surrounding tissues (Bayer *et al.,* 2016).

It is possible that numerous patient factors may influence the quality of A-PRF™. None of the participants within this investigation were subjected to hematologic investigations to determine their respective coagulation profiles or cell counts. From a clinical perspective, these natural variations amongst patients will exist and the current literature has failed to examine this important aspect of PC's. Yajamanya *et al.* (2016) have demonstrated that the fibrin matrix formed by their version of PRF in elderly patients was more loosely arranged than fibrin matrix of younger individuals. The impact of this finding is yet to be determined.

The very nature of any PRF clot makes standardisation near impossible. Within this investigation, variations were noted relating to the size and mass of the individual A-PRF™ clots. This phenomenon may be related to a host of factors including; individual variations between patients, variables related to the processing protocol (Dohan Ehrenfest et al., 2014), storage time within the vacuum tube (Peck *et al.,* 2016) or the laboratory separation of the A-PRF™ clot from the red blood cells.

During the laboratory phase of this study, great care was taken to ensure that the mass, diameter and slice configuration of each A-PRF™ disk used for testing was similar. However as cell type, cell numbers and concentration of plasma components differ within each clot and between each clot (El Bagdadi et al., 2017; Ghanaati *et al.,* 2014), each sample disk cannot be identical. The formed disks were also subjected to compression during their fabrication, thus leading to a loss of an unknown volume of serum which by itself may possess antimicrobial activity.

The agar disk-diffusion method employed within this investigation is not suited to determine the minimum inhibitory concentration (MIC) of the tested material. This is because it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium (Balouiri *et al.,* 2016). A second problem with this mode of testing is that it is unable to determine if the tested material is bactericidal or bacteriostatic. Irrespective of these drawbacks, the disk diffusion method was sufficient to prove that A-PRF™ displays antimicrobial activity.

The overall laboratory sample size was insufficient to draw significant conclusions relating to the mean differences between the inhibition zones produced by A-PRF™ and blood. This result could in no way be predicted as it relied purely on the ability of both blood and A-PRF™ to possess antimicrobial activity. Future studies should increase both patient variation and sample sizes all future studies based on PC's.

Chapter 10: Conclusion

The use of A-PRF™ in clinical practice has displayed great potential to enhance healing and improve surgical outcomes as it serves as an autologous scaffold harbouring cells and bioactive compounds (Castro *et al.,* 2017; Miron *et al.,* 2016; Moraschini *et al.,* 2016; Del Corso *et al.,* 2012). However, the antimicrobial potential of the material has largely been neglected and may be an important property contributing to the accelerated and uncomplicated healing events noted clinically. The results of this study indicate that A-PRF™ displays antimicrobial activity against *staphylococcus aureus*, *streptococcus mutans, enterococcus faecalis* and *candida albicans* however; no significant activity was detected against organisms cultured from saliva. Furthermore the spectrum and power as an antimicrobial agent is far inferior to that of an established antimicrobial mouthrinse (CHX). Future investigations involving A-PRF™ are required to determine the full spectrum of its antimicrobial activity *in-vitro*, its participation *in-vivo,* and the influence of patient characteristics upon its biologic activity. Furthermore, its clinical potential as a vehicle for local drug delivery within infected sites should be explored (Del Fabbro et al., 2016).

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Appendix 1

Information Sheet for A-PRF™ and Blood Collection

The following document serves to provide the relevant information regarding the proposed study. Details of the principle investigator are provided should you have any further queries. If you wish, the presence of a translator can be made available to assist in understanding the information contained within this document.

Title: The in vitro antimicrobial activity of advanced platelet rich fibrin (A-PRF™) against microorganisms of the oral cavity

Principle Investigator: Dr Feheem Bhamjee

Position: Postgraduate student within the Department of Oral Medicine and Periodontology

Contact details:

Office number: (021) 937-3167   Cell Number: 0725193722

Email address: 2382557@myuwc.ac.za

I, Dr Feheem Bhamjee, hereby invite you to participate in a research study. Please take the time to read the following information regarding the research and what would be required of you as a participant. Feel free to ask questions if you require any further information or clarify any concerns you may have.

What is the purpose of this research?

To investigate the antimicrobial activity of a product formed from your own blood and used to improve healing following surgery in your mouth. The product is called Advanced Platelet Rich Fibrin (A-PRF™). It is formed after collecting a minimum of 40ml of blood (depends on the amount you require in your specific surgery) and processing it in a machine in order to concentrate specific cells and molecules which naturally enhance tissue healing. The process is all natural with zero chemicals added to the blood.

What would be required from you?

A small quantity of this product (A-PRF™), left over after your surgery, will be taken for immediate laboratory testing.

A small quantity of unprocessed blood, drawn at the same procedure will also be required. Therefore a total of 12ml of blood drawn from your arm will be used for research purposes. There is nothing further required of you and any excess material will be immediately destroyed. No blood or blood products will be stored or used for other research.

Why have you been invited?

You are invited to participate as you meet all the necessary criteria which are:

1. You are scheduled to undergo a surgical procedure in which A-PRF™ will be used
2. Aged 18 years or older,
3. You are systematically healthy.

If you currently **smoke**; or use any of the following **medications** then you are not eligible to participate in this research study:

- anticoagulants, (Blood thinning medication)
- antibiotics, used within the last 3 months
- immunosuppressive or cytotoxic medications, used within the past 3 months

Kindly inform Dr F Bhamjee of any specific medical conditions and medications you are currently taking if you are interested in participating.

Your decision to participate:

The decision to participate is entirely up to you and will in no way affect the quality of treatment that you have been scheduled for. If you decide to participate, you would be asked to sign a consent form in order to record that you have chosen to take part. You will still be free to withdraw at any time at no consequence to you. There is no binding agreement to your participation.

Are there any disadvantages to your participation?

The product will be used as part of your surgery to improve your healing independent of your choice to participate. The only disadvantage over your normal surgical risk is that 2ml of additional blood is required for the study.

Are there any benefits to you taking part?

The information obtained from this research will not benefit you; however it will increase our understanding of the products abilities and possibly its future development and use.

Confidentiality:

All personal and medical information obtained from you during the course of this research will be kept strictly confidential and protected. All samples taken and data collected will have no information pertaining to your identity. However, your personal information may be given out if required by law.

How will your data be collected?

Samples collected from you as a participant will be coded according to a master list known only to the principle researcher before being sent for laboratory testing. The master list, linking the participants to the research codes, and all electronic data will be held on a password protected computer with restricted access. Hard paper will be stored in a secure location accessed only by the researcher. Your data will be accessible only to authorized persons such as researchers within the team, supervisors, and regulatory authorities. Your data will be retained for a period of 3 years before it will be disposed of securely.

What will happen on completion of this research study?

The results of this research will be submitted as a thesis for a specialist degree in Periodontics and Oral Medicine. If approved by the university senate, the research will be submitted for publication within a medical/dental scientific journal. The outcome of the study will be made available to you if you request.

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Appendix 2

Informed consent

I, (Name……………………………….) have been informed about the study entitled **The in vitro antimicrobial activity of advanced platelet rich fibrin (A-PRF™) against microorganisms of the oral cavity**, by Dr. F. Bhamjee

I understand the purpose and procedures of the study as explained by Dr. Bhamjee and contained within the information sheet provided.

I understand that **a blood derived product** (A-PRF™) used as part of my surgery will be used for laboratory analysis.

I understand that **an additional 2millilitres of blood** will be drawn during the procedure and be used for laboratory analysis.

I have been given an opportunity to ask questions about the study and have been answered to my satisfaction.

I declare that my participation in this study is entirely voluntary and that all samples will be destroyed appropriately at the end of the study.

If I have any further questions/concerns or queries related to the study I understand that I may contact the researcher at the phone number **(021) 937-3167** or via e-mail **2382557@myuwc.ac.za**

If I have any questions or concerns about my rights as a study participant, or if I am concerned about an aspect of the study or the researchers then I may contact:

DENTISTRY RESEARCH ETHICS COMMMITTEE

Research Office, Tygerberg Campus

Francie van Zyl Drive

Private Bag X1

Tygerberg 7505

Cape Town, SOUTH AFRICA

_____   _____

Signature of Participant   **Date References**

Data collection sheets

Sample of data collection sheet per plate:

Plate 1 Group A				
	Measurement 1	Measurement 2	Measurement 3	Average
PRF				
Blood				

Sample of data collection sheet:

Record number	Age	Gender	A Blood	A-PRF	B Blood	A-PRF	C Blood	A-PRF	D Blood	A-PRF	E Blood	A-PRF
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