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“Laboratorial trial regarding the antibacterial minimum inhibitory concentration in bacteria isolated from the oral cavity, such as *Pseudomonas aeruginosa* and *Enterococcus faecalis*, when interacting with PRF and its by-products.”

In vitro study

Laura Manuela Martins da Silva

Dissertação conducente ao **Grau de Mestre** em **Medicina Dentária (Ciclo Integrado)**

—

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Subtítulo

Trabalho realizado sob a Orientação de
Orientador: Prof. Doutor Paulo Miller
Co-Orientador: Especialista António Ferraz
Co-Orientador: Prof. Doutora Cristina Coelho

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DECLARAÇÃO DE INTEGRIDADE

Eu, Laura Manuela Martins da Silva, declaro ter atuado com absoluta integridade na elaboração deste trabalho, confirmo que em todo o trabalho conducente à sua elaboração não recorri a qualquer forma de falsificação de resultados ou à prática de plágio (ato pelo qual um indivíduo, mesmo por omissão, assume a autoria do trabalho intelectual pertencente a outrem, na sua totalidade ou em partes dele). Mais declaro que todas as frases que retirei de trabalhos anteriores pertencentes a outros autores foram referenciadas ou redigidas com novas palavras, tendo neste caso colocado a citação da fonte bibliográfica.

DEDICATÓRIA

Dedico esta dissertação às mulheres da minha família, em especial às minhas avós, pelo exemplo de coragem e sabedoria que sempre me mostraram.

COMUNICAÇÕES CIENTÍFICAS

Subordinadas ao tema da dissertação foram apresentadas duas comunicações científicas em forma de Poster.

Nas Jornadas Científicas de Ciências Dentárias de 2023 sob a forma de E-poster intitulado "Avaliação da interação de concentrados plaquetários autólogos com as bactérias *Pseudomonas aeruginosa* e *Enterococcus faecalis* por meio da concentração mínima inibitória (MIC)".

No II INTERNATIONAL CONGRESS of UNIPRO RESEARCH UNIT de 2023 com o Poster "*Pseudomonas aeruginosa* and *Enterococcus faecalis'* interaction with platelet concentrates" com publicação do "Abstract" no jornal *Scientific Letters*.

AGRADECIMENTOS/ACKNOWLEDGMENTS

À minha mãe eterna gratidão pelo apoio incondicional e sacrifícios em prol dos meus estudos. Que o seu exemplo me sirva de modelo para o futuro.

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RESUMO

Objetivos

O PRF (Fibrina rica em Plaquetas) e seus subprodutos têm sido usados devido ao seu potencial regenerativo. Este estudo experimental tem como objetivo explorar os efeitos antimicrobianos do L-PRF em membrana e em exsudado frente a bactérias como *Pseudomonas aeruginosa* e *Enterococcus faecalis*.

Materiais e Métodos

Foi conduzido um estudo dividido em três estadios com protocolos diferentes entre si, permitindo-nos averiguar qual protocolo demonstrava melhor a ação antibacteriana do L-PRF.

Todos os protocolos implicaram a cultura das estirpes bacterianas ATCC e a colheita de sangue de dadores voluntários. Após a incubação das bactérias com L-PRF, os resultados foram documentados ao fim de pelo menos 24 horas.

Uma pesquisa adicional da literatura permitiu-nos contextualizar os resultados obtidos em laboratório.

Resultados e Discussão

Ambos membrana e exsudado foram eficazes a inibir o crescimento de ambas as bactérias estudadas. No entanto, não se observou qualquer inibição por parte do exsudado quando testado numa placa de poços.

Em agar Muller-Hinton o halo de inibição para ambas as bactérias foi de 12mm em média com a membrana e com o 20 µL de exsudado não diluído de 10,5mm para a *Pseudomonas* e de 10,0mm para o *Enterococcus*, em média.

Conclusão

Ambas os compostos testados têm potencial no combate às infeções bacterianas, sendo de interesse que estudos futuros investiguem qual o protocolo e método de aplicação mais vantajosos para o tratamento ou profilaxia de doenças infeciosas periodontais e endodônticas. Deve também ser explorada a comparação dos efeitos inibitórios entre estirpes ATCC e estirpes isoladas de casos clínicos.

Palavras-Chave

PRF, membrane, exudate, antibacterial activity, E.faecalis, P.aeruginosa.

ABSTRACT

Aim

Platelet rich fibrin (PRF) and its by-products have been extensively used due to their regenerative potential. This experiment aimed to further study the immunological properties of L-PRF's membrane and exudate when in the presence of bacterial threats like *Pseudomonas aeruginosa* and *Enterococcus faecalis.*

Materials and Methods

A three-stage trial was conducted, each stage with a different protocol. This made it possible to assess which protocol and *in vitro* factors were the most effective when investigating PRF's immune properties.

All the protocols involved the culture of ATCC bacterial strains and the blood collection from volunteer donors. The bacteria were cultured with L-PRF, and results were noted after 24 hours.

Additional research of the literature allowed the results obtained experimentally to be contextualised.

Results and Discussion

Both the membrane and exudate had an inhibitory effect on both *Pseudomonas aeruginosa* and *Enterococcus faecalis*. No inhibition was observed in either bacterium by the exudate when tested with the use of well plates.

In Muller-Hinton agar the halo of inhibition for *Pseudomonas* and *Enterococcus* measured 12mm on average with the membrane and 10,5mm and 10mm with 20 µL of undiluted exudate, respectively.

Conclusion

Both products have potential when it comes to fighting bacterial infections and it is suggested that future studies should investigate the ideal preparation and application method in periodontal and endodontic infectious diseases. In such future studies more tests should be run and the comparison between the inhibitory effects on ATCC strains and on patient's isolated bacterial stripes should be performed.

Keywords

PRF, membrane, exudate, antibacterial activity, E.faecalis, P.aeruginosa.

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LISTA DE ABREVIATURAS, SIGLAS E ACRÓNIMOS

AMC: Amoxicillin/Clavulanic Acid

ATCC: American Type Culture Collection

GRO- α: Growth-Regulated Oncogene-alpha

H-PRF: Horizontal Platelet Rich Fibrin

IL-6: Interleukin 6

IL-8: Interleukin 8

I-PRF/i-PRF: Injectable Platelet Rich Fibrin

LAB-PRF: Local Antibiotic Platelet Rich Fibrin

L-PRF: Leukocyte and Platelet Rich Fibrin

L-PRP: Leukocyte and Platelet Rich Plasma

MIC: Minimum Inhibitory Concentration

MIP-1α: Macrophage Inflammatory Protein

MHB: Muller-Hinton Broth

NAP-2: Neutrophil-Activating Protein

PC: Platelet Concentrate

PRF: Platelet Rich Fibrin

P-PRF: Pure Platelet Rich Fibrin

PPP: Platelet Poor Plasma

PRP: Platelet Rich Plasma

P-PRP: Pure Platelet Rich Plasma

PSLs: Platelet Storage Lesions

RANTES: Regulated on Activation Normal T Expressed and Secreted Protein

RBC: Red Blood Cell

RCF: Relative Centrifugal Force

SAB-PRF: Systemic Antibiotic Platelet Rich Fibrin

SAM: Ampicillin/Sulbactam

SDF-1 α: Stromal-Cell Derived Factor

T-PRF: Titanium Platelet Rich Fibrin

1- Introduction

Within the oral cavity a wide variety of microorganisms can be found as a part of the natural microbiota of the mouth. When this symbiotic balance is disrupted, bacterial infections can take place causing harm to the host and resulting in great damage.

Enterococcus faecalis and *Pseudomonas aeruginosa* can be found within the oral cavity and are responsible for several endodontic and periodontal diseases. *E. faecalis* is the primary microorganism associated with endodontic failure.(1)

Pseudomonas Aeruginosa is a Gram-negative pathogen, a very frequent cause of infections in humans. It is able to adhere to a wide variety of surfaces, living and non-living, and thus its infections can involve any part of the body. It is also one of the many bacteria which can be found in the oral biofilm.(2)

Enterococcus Faecalis is a major Gram-positive pathogen, one of the most common species of Enterococci. It is frequently present in failed root canal treatments, therefore surviving endodontic treatment, and is associated with various forms of periradicular disease.(2)

Natural platelet concentrates (PCs) can be sourced from a patients' blood. These appear as a response to bacterial proliferation in already existing lesions, allowing the body to recover and preventing re-infection.(3)

Platelet concentrates have shown great potential when it comes to accelerating the healing process and controlling inflammation, utilizing the platelets' ability to recognize and neutralize pathogens and their immune cells indirect influence, amongst other mechanisms.(1)

Autologous platelet concentrates have been extensively used to promote soft and hard tissue regeneration. Several different techniques have already been developed resulting in different PCs with distinct biologic characteristics. These can be classified into four major categories according to their leucocyte and fibrin content: pure platelet rich plasma (P-PRP), pure platelet rich fibrin (P-PRF), leucocyte and platelet rich plasma (L-PRP) and leucocyte and platelet rich fibrin (L-PRF). The leucocyte-based PCs are believed to contain a substantial concentration of white blood cells.(4)

The mechanism governing the antibacterial effect is not yet fully understood, although current evidence suggests an important role is played by platelets in the generation of oxygen metabolites, such as superoxide, hydrogen peroxide and hydroxyl free radicals. The platelets' known interactions with growth factors and other molecules that modulate the wound healing response in both hard and soft tissue are the properties which recommend them for medical use.(2)(3)

The MIC (minimum inhibitory concentration) is the lowest concentration of an antimicrobial substance which is able to inhibit the visible growth of bacteria, in this case, *E.faecalis* and *P.aeruginosa*. Measuring this parameter enables us to determine the minimum concentration of PRF and its by-products needed to eradicate or stop the proliferation of each bacterium.(2)

2- Goals and Hypotheses

This study aimed to evaluate the *in vitro* sensitivity of the ATCC strains *Pseudomonas aeruginosa* (27853) and *Enterococcus faecalis* (29212) to PRF and its derivatives (i-PRF, L-PRF membrane and L-PRF exudate).

These will be the hypotheses tested:

1. PRF and its by-products inhibit the bacterial growth of ATCC strain *E.faecalis*.
2. PRF and its by-products have an antimicrobial effect on the bacterial ATCC strain *P.aeruginosa.*

Null Hypotheses:

1. PRF and its by-products do not inhibit the bacterial growth of ATCC strain *E.faecalis.*
2. PRF and its by-products do not have an antimicrobial effect on the bacterial ATCC strain *P.aeruginosa.*

3- Methodology

3.1 Experimental Protocol

3.1.1 Blood Collection and L-PRF preparation

Blood was sourced from 3 healthy volunteer donors in total, using a tourniquet and 19- gauge needle. Blood was drawn from an antecubital vein of each donor. The first 2.5 mL was discarded and an average of four tubes of 9mL per donor was collected into blood collection tubes. This step was repeated for all three stages of the experiment. The mapping of the donors to the usage of their blood in each stage is shown in Table1.

Stage	Donors Used
1	C
2	A & B
3	B

Table 1-Donors' distribution according to stages

In order to prepare the L-PRF, the whole blood samples - collected into silica coated plastic vacuum blood collection tubes without anticoagulant- were quickly centrifuged at 2700 RPM for 12 min (IntraSpin® System -Intra-Lock International – Boca Raton, FL USA).

Upon the completion of the cycle, the blood in the tubes showed three distinct layers – an upper layer consisting of platelet poor plasma, a middle layer with PRF and a lower layer of blood corpuscles (RBC).

The PRF was retrieved from the tube and the RBC base carefully removed in order to retain part of it in the blood clot.

The blood clot was then compressed using a Xpression™ Box (IntraSpin® System -Intra-Lock International – Boca Raton, FL USA) which allowed L-PRF membrane and L-PRF exudate to be obtained.

First Stage (13-16/12/2023)

1. Defrosting of the ATCC strain *Pseudomonas aeruginosa* (27853).
2. Culture of the ATCC strain in Soy Trypticase Agar.
3. Incubation of the respective stripe for 18-24h at 35-37ºC.
4. Inoculation of 3-4 colonies in Muller-Hinton broth and correction of the optical density of the suspensions to 0,5 on the McFarland scale ($1x10^8$ UFC/mL) using a bioMérieux France densitometer.

5. Distribution of the bacterial suspensions across 11 out of the 12 wells used.
   The maximum volume defined for all the wells was 1000 µL. The fifth well was filled with 1000 µL of the bacterial solution (positive control) and the sixth well with 1000 µL of Muller-Hinton broth (negative control) – these were the only two wells in which there was no exudate. The remaining 10 wells were filled by subtracting the different exudate volumes to the maximum volume of 1000 µL and filling the remaining volume with the bacterial solution. The contemplated exudate volumes are represented in Figure 1.Figure 1-Distribution of the exudate in the wells
6. Distribution of the non-control volumes of exudate into 5 Petri dishes cultured with *Pseudomonas* in Muller-Hinton Agar and incubation of both the wells plate and the petri dishes for 18-24h at 35-37ºC. Each Petri dish hosted 2 different volumes of exudate.

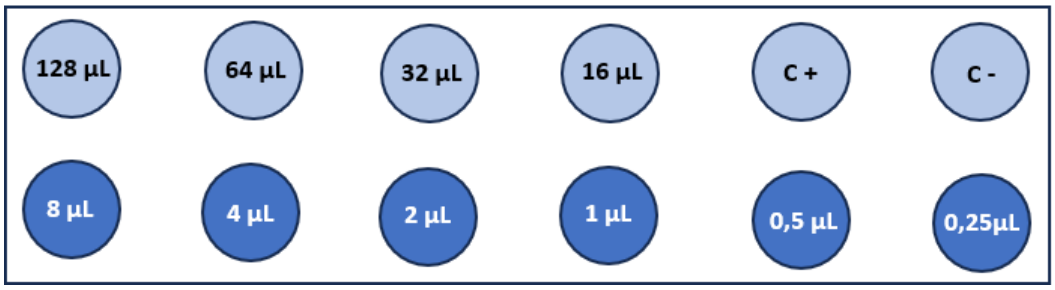

Figure 1-Distribution of the exudate in the wells

Second Stage (03-06/01/2024)

1. Defrosting of the ATCC strains *Pseudomonas aeruginosa* (27853) and *Enterococcus faecalis* (29212).
2. Culture of the ATCC strains in Soy Trypticase Agar and Muller-Hinton broth.
3. Incubation of the respective stripes for 18-24h at 35-37ºC.
4. Inoculation of 3-4 colonies in saline solution and correction of the optical density of the suspensions to 0,5 on the McFarland scale ($1x10^8$ UFC/mL) using a bioMérieux France densitometer.
5. Dilution of the obtained exudate in Muller-Hinton broth by adding to the first tube 1 mL of exudate and 1 mL of MHB (1:1). Dilution of the second tube was prepared using 100 µL of the first tube and 900 µL of MHB (1:2). The same process (addition of 100 µL of the previous tube to the next tube along with 900 µL of MHB) was repeated until 10 tubes of diluted exudate were obtained with the dilution of the final tube reaching a ratio of 1:512.

6. In a plate of 96 wells, 12 wells were used for each of the bacterial stripes. In the first well, 20 µL of the first dilution tube was added along with 180 µL of the bacterial suspension. The following wells were filled using the same process: 20 µL of the corresponding dilution in a progressive way, meaning that no dilution was used twice, and 180 µL of the bacterial solution. The last two wells were the positive and negative controls.
7. Placement of three membranes from two different donors in two petri dishes culture with *Pseudomonas* and *Enterococcus* in Muller-Hinton Agar. The dish cultured with Pseudomonas hosted two membranes from different donors and the dish cultured with Enterococcus hosted one membrane.
8. Incubation for 18-24h at 35-37ºC.
9. Interpretation of the results.

Third Stage (25-26/01/2024)

1. Defrosting of the ATCC strains *Pseudomonas aeruginosa* (27853) *and Enterococcus faecalis* (29212).
2. Culture of the ATCC strains in Soy Trypticase Agar and Muller-Hinton broth.
3. Incubation of the respective stripes for 18-24h at 35-37ºC.
4. Inoculation of 3-4 colonies in saline solution and correction of the optical density of the suspensions to 0,5 on the McFarland scale ($1x10^8$ UFC/mL) using a bioMérieux France densitometer.
5. Inoculation of the Muller-Hinto agar with a cotton swab soaked in the bacterial suspension. This was smeared in three different directions in order to promote a uniform growth of the microorganisms.
6. Placement of 6 white paper discs of 6 mm diameter (Oxoid) equidistant to each other in Muller-Hinton agar. The prepared serum was then dripped onto each disc and the positive and negative controls as shown in Figure 2.
7. This test was run in triplicate for each tested ATCC strain.
8. Two membranes of the same donor were used, one for each bacterium under study. These were placed on two different petri dishes, each cultured with the respective bacterial stripe in Muller-Hinton agar.
9. Incubation for 18-24h at 35-37ºC.
10. Measuring of the inhibition halos with a digital calliper graduated in 1mm steps.

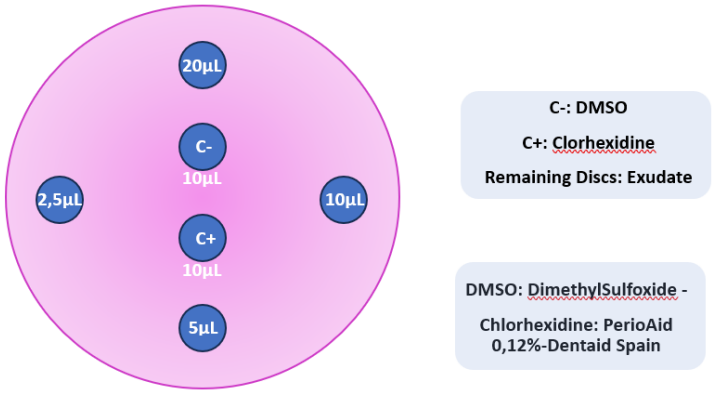

Figure 2- Exudate distribution on the paper discs.

3.2 Bibliographic Research Methodology

The literature search was conducted using the search engines Pubmed, Science Direct and EBSCOhost. The main keywords were "Antimicrobial activity", "PRF", "Platelet concentrates", "Enterococcus faecalis", "Pseudomonas aeruginosa" and "oral biofilm" used under the following search expressions "PRF and antimicrobial properties", "Platelet concentrates and enterococcus faecalis", "Platelet concentrates and thrombocytes and antimicrobial activity" and "Pseudomonas aeruginosa and enterococcus faecalis and oral biofilm".

To better ascertain the goals for this research, a PICO research strategy was developed as illustrated in the following table.

Population	Patients with persistent root canal infections post treatment or periodontal infections; patients in need of prophylaxis therapy preceding a surgical procedure.
Intervention	To use PRF and its by-products as an aiding alternative towards asepsis and improved regeneration of oral tissues.
Comparison	Conventional antibiotics and aseptic agents used in dentistry; Different types of platelet concentrates.
Outcome	To better determine the efficacy and antimicrobial spectrum of platelet concentrates.
Do patients with persistent infections benefit from having blood drawn as to reuse it as platelet concentrates in order to better fight an oral state of sepsis.	

Table 2 - PICO Strategy

The inclusion criteria consisted of papers from **2013 to actuality**, ***in vitro*** **or** ***in vivo*** studies involving only **human cells** and falling under the categories **Medicine and Dentistry**, prioritizing those falling under **dentistry**.

All papers not falling under the above criteria or deemed as not relevant were excluded and all those initially selected were screened for eligibility. Duplicates were removed as illustrated by the following flowchart.

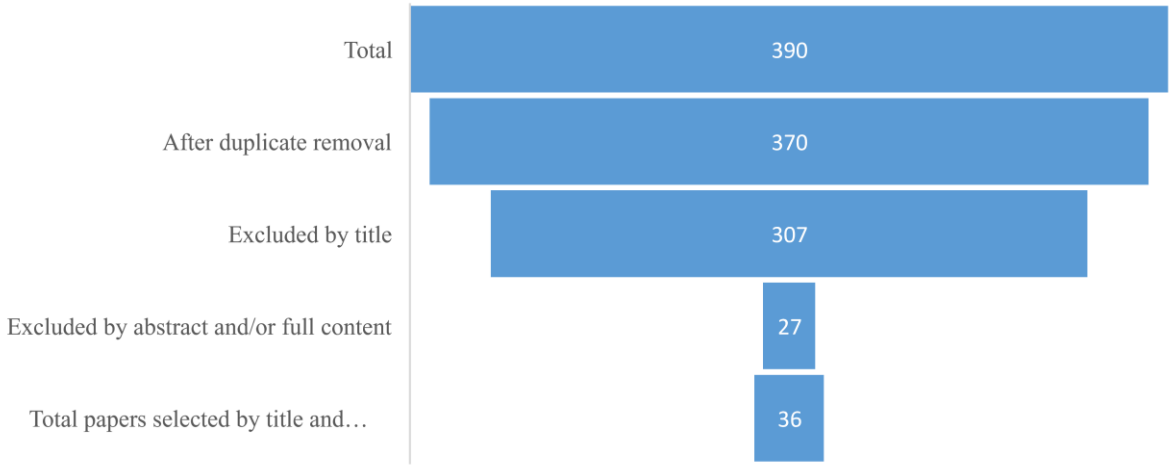

Figure 3- Bibliographic Research Flowchart

4- Results

4.1 Bibliographic Search Results

Table of Search Results (Final Selection)

Title	Authors	PCs	Results and Conclusion
Platelet activation and antimicrobial activity of L-PRF: a preliminary study. (1)	*Melo-Ferraz et al*	L-PRF	L-PRF inhibits the growth of P.aeruginosa, E.faecalis and C.albicans and L-PRF exudate is effective in activating platelets from whole blood. L-PRF is advantageous in the healing process for its ability of activating platelets and its antimicrobial activity.
Antimicrobial activity of pure platelet-rich plasma against microorganisms isolated from oral cavity. (2)	*Drago et al*	P-PRP	P-PRP inhibited the growth of all bacteria tested except for *P.aeruginosa.* P-PRP could be used to fight or prevent postoperative infections.
Leukocyte presence does not increase microbicidal activity of Platelet-rich Plasma in vitro. (3)	*Mariani et al*	L-PRP, L-PRP cryo and P-PRP	Both L-PRP and P-PRP showed comparable antimicrobial activity against all bacteria tested. L-PRP-cryo also obtained similar results to L-PRP meaning that its cryopreservation is achievable without compromising its antibacterial properties.
Can Our Blood Help Ensure Antimicrobial and Anti-Inflammatory Properties in Oral and Maxillofacial Surgery? (4)	*Micko et al*	PRF	The protocol and preparation method highly influence the biological properties of PRF. If prepared correctly it could be a great way to ensure anti-inflammatory and antibacterial effects in oral and maxillofacial surgery.
Antibacterial and Antifungal Efficacy of Platelet-Rich Fibrin and Platelet-Rich Fibrin Matrix against Root Canal Microflora. (5)	*Singh et al*	PRF and PRFM	Both PRF and PRFM were tested on root canal microflora and showed antibacterial activity. PRF could be useful for endodontic regenerative procedures.
Antimicrobial effect of platelet-rich plasma and platelet-rich fibrin. Indian Journal of Dental Research. (6)	*Badade et al*	PRP and PRF	PRF did not inhibit the growth of *P.gingivalis* nor *A.actinomycetemcomitans*. PRP inhibited the growth of both bacteria. PRP seems to be more effective against periopathogens than PRF.
Comparison of Antimicrobial Activity of Injectable Platelet-Rich Fibrin (i-PRF) and Leukocyte and Platelet-Rich Fibrin (l-PRF) Against Oral Microbes: An In Vitro Study. (7)	*Sindhusha et al*	i-PRF and L-PRF	i-PRF outperformed the antimicrobial effects of metronidazole and L-PRF, reducing the bacterial load of *Porphyromonas gingivalis*.
Study of antibacterial and antifungal efficacy of platelet-rich fibrin and platelet-rich fibrin matrix. (8)	*Nagaraja et al*	PRF and PRFM	PRF inhibited root canal microbiota effectively but was not effective on *C.ablicans*. PRFM did not show any antibactererial or antifungal activity. It was concluded that PRF could aid the revascularization process of immature necrotic teeth.

Platelet-rich plasma affects bacterial growth in vitro. (9)	*Mariani et al*	PPP and P-PRP	PPP exhibited a short - term inhibition against bacteria tested whereas P-PRP presented a much more effective inhibition starting at the first hour of incubation. *K.pneumoniae* and *E.coli* were less susceptible to inhibition.
Impact of different platelet-rich fibrin (PRF) procurement methods on the platelet count, antimicrobial efficacy, and fibrin network pattern in different age groups: an in vitro study. (10)	*Mamajiwala et al*	PRF	The PRF of the youngest patient group showed higher platelet concentration, higher antimicrobial activity and denser fibrin network. The protocol/preparation method and the patient's age influences greatly the properties of PRF.
Comparative evaluation of platelet count and antimicrobial efficacy of injectable platelet-rich fibrin with other platelet concentrates: An in vitro study. (11)	*Karde et al*	i-PRF, PRF and PRP	i-PRF performed better than PRF, PRP and whole blood inhibiting bacterial growth. It seems i-PRF shows greater regenerative and antimicrobial potential in comparison to other PCs.
Comparison of Antimicrobial Activity against Porphyromonas gingivalis between Advanced Platelet-Rich Fibrin and Injectable Platelet-Rich Fibrin. (12)	*Pham et al*	A-PRF+ and i-PRF	Both A-PRF+ and i-PRF inhibit *P.gingivalis* despite i-PRF being the most effective. Both PRFs were most effective when sourced from patients with periodontites than from patients with gingivitis. PRF from healthy patients had the worst performance.
Diverse bacterial profile in extraradicular biofilms and periradicular lesions associated with persistent apical periodontitis. (13)	*Zhang et al*		*E.faecalis* was mostly presentin both extraradicular biofilm and periapical lesions while *P.aeruginosa* was predominantly found in extraradicular biofilm. The bacterial profile from extraradicular biofilm differs from that of periapical lesions and a great variety of bacteria are associated with the formation of the extraradicular biofilm.
Enterococcus faecalis Antagonizes Pseudomonas aeruginosa Growth in Mixed-Species Interactions. (14)	*Tan et al*		When in nutritionally poor environments, E.faecalis inhibits P.aeruginosa by decreasing the pH. The microenvironment plays an important role in the growth and trajectory of biofilms and bacterial communities.
Insights into Platelet Storage and the Need for Multiple Approaches. (15)	*Handigund et al*		The storage of platelet concentrates is doable for up to 5 days and new methods should be explored. The more commonly used method is preservation at room temperature.
Six hours of resting platelet concentrates stored at 22-24 ºC for 48 hours in permeable bags preserved pH, swirling and lactate dehydrogenase better and caused less platelet activation. (16)	*Naghadeh*		PCs stored for 42h under agitation at room temperature and then rested for 6h preserved better its pH and showed less platelet activation than PCs stored under continuous agitation.
L-carnitine effectively improves the metabolism and quality of platelet concentrates during storage. (17)	*Deyhim et al*		L-carnitine reduced the glucose consumption in stored PCs at the expense of an increased oxygen consumption. It seems it would be a good addictive to reduce PSL and to improve the quality and metabolism of stored PCs.
Evaluation of in vitro storage characteristics of cold stored platelet concentrates with N acetylcysteine (NAC). (18)	*Handigund et al*		Adding NAC to platelet concentrates could prevent platelet activation and reduce metabolism. It could be a solution for cold storage of PCs.

N-acetylcysteine reduce the stress induced by cold storage of platelets: A potential way to extend shelf life of platelets. (19)	*Handigund et al*		NAC protected refrigerated PCs in long term storage while retaining the platelet concentrates' integrity. It seems that preserving PCs for longer than 10 days could be viable.
Antimicrobial/anti-biofilm activity of expired blood platelets and their released products. (20)	*Różalski et al*	PRP	Expired platelet concentrates showed significant bacterial inhibition meaning that they retain their antimicrobial activity even after expiration date.
Antimicrobial effect against Aggregatibacter actinomycetemcomitans of advanced and injectable platelet-rich fibrin from patients with periodontal diseases versus periodontally healthy subjects. (21)	*Pham et al*	A-PRF+ and i-PRF	i-PRF performed better than A-PRF+. The i-PRF from periodontitis and gingivitis patients had greater bacterial inhibition than that of the same groups of patients for A-PRF+. Both i-PRF and A-PRF+ inhibited *A.actinomycetemcomitans*.
Effect of advanced and injectable platelet-rich fibrins against Aggregatibacter actinomycetemcomitans in subjects with or without periodontal diseases. (22)	*Tran et al*	A-PRF + and i-PRF	i-PRF had better antibacterial activity than A-PRF on *A.actinomycetemcomitans* in the gingivitis and periodontitis group. Overall, the PRF from the periodontitis group performed better than the gingivitis and healthy groups against Aa.
Antimicrobial capacity of Leucocyte- and Platelet Rich Fibrin against periodontal pathogens. (23)	*Castro et al*	L-PRF	L-PRF membrane inhibited the growth of periodontal pathogens, especially of *P.gingivalis*. L-PRF exudate inhibited *P.gingivalis*, stimulated the growth of *A.actinomycetemcomitans* and failed to inhibit all other bacterial tested. L-PRF membrane performed better than the exudate.
Plasma Components and Platelet Activation Are Essential for the Antimicrobial Properties of Autologous Platelet-Rich Plasma: An In Vitro Study. (24)	*Drago et al*	PRP	The antibacterial activity of PRP seems to be related to a co-operation between plasma and platelets where the activating the coagulation plays an important role.
Antibacterial effects of platelet-rich fibrin produced by horizontal centrifugation. (25)	*Feng et al*	L-PRF and H-PRF	Both L-PRF and H-PRF showed great antibacterial activity against *S.aureus* and *E.coli*, despite H-PRF having outperformed L-PRF. H-PRF contained more leukocytes than L-PRF which could explain the difference regarding growth inhibition.
A new aspect of *in vitro* antimicrobial leukocyte- and platelet-rich plasma activity based on flow cytometry assessment. (26)	*Cieślik-Bielecka*	L-PRP	Leukocytes were positively correlated to the antimicrobial activity of L-PRP, unlike platelets. L-PRP inhibited all bacteria tested. Leukocytes are responsible for the antibacterial effects of L-PRP and its subtypes are related to its antimicrobial activity.
Inhibitory Activities of Platelet-Rich and Platelet-Poor Plasma on the Growth of Pathogenic Bacteria. (27)	*Maghsoudi et al*	PRP and PPP	PPP had no inhibitory effect and PRP with 1 and 2 centrifugation steps inhibited different bacteria amongst each other inhibiting in total all bacteria except for *K.pneumoniae*, *P.aeruginosa* and *Serratia* sp. A correlation between platelets and the antibacterial activity of PRP was found.
The impact of local and systemic penicillin on antimicrobial properties and growth factor release in platelet-rich fibrin: In vitro study. (28)	*Ozcan et al*	PRF	The addition of penicillin prior to centrifugation improved the antibacterial activity of PRF. The systemic penicillin performed similarly to regular PRF. Adding localised antibiotics to PRF allows for strong antibacterial effect.

Impact of aminopenicillin administration routes on antimicrobial effects of platelet-rich fibrin: An in-vitro investigation. (29)	*Straub et al*	A-PRF	A double oral dose of amoxicillin/clavulanic acid (AMC) obtained the best results in fresh PRF and in stored PRF. Intravenous ampicillin/sulbactam (SAM) showed better results than the double oral dose of AMC. The use of oral drugs would be more convenient for patients and allow the prevention of infections at surgical sites.
Investigation of three common centrifugation protocols for platelet-rich fibrin (PRF) as a bio-carrier for ampicillin/sulbactam: a prospective trial. (30)	*Straub et al*	A-PRF and A-PRF+	A single intravenous dose of ampicillin/sulbactam is enough to obtain desired concentrations in PRF. The protocol that used the biggest value of rpm and of RCF obtained the best results (larger inhibition zones).
Evaluation of advanced platelet-rich fibrin (PRF) as a bio-carrier for ampicillin/sulbactam. (31)	*Straub et al*	A-PRF+	PRF loaded with antibiotics was able to obtain similar values of ampicillin/sulbactam concentration as those found in plasma. The antibacterial effect of the PRF was also similar to that of SAM only. PRF could act as a carrier for antibiotics.
Titanium platelet–rich fibrin (T-PRF) as high-capacity doxycycline delivery system. (32)	*Ercan et al*	T-PRF	The combination of T-PRF with doxycycline produced the greatest inhibition halos when compared to T-PRF and the association of collagen and doxycycline. T-PRF could help carrying doxycycline prolonging its effect.
Injectable Platelet-Rich Fibrin as a Drug Carrier Increases the Antibacterial Susceptibility of Antibiotic—Clindamycin Phosphate. (33)	*Egle et al*	i-PRF	i-PRF enhanced the antibacterial activity of clindamycin phosphate against *S.aureus* and *S.epidermidis* when compared to clindamycin phosphate on its own. This means i-PRF could present as a more efficient way to deliver antibiotics.
Can platelet-rich fibrin act as a natural carrier for antibiotics delivery? A proof-of-concept study for oral surgical procedures. (34)	*Bennardo et al*	L-PRF	Vancomycin when added to PRF would interfere with its structure. Gentamicin and linezolid were compatible with the protocol and were effective inhibiting all bacteria. PRF allowed the release of the antibiotics in effective concentrations with gentamicin-PRF standing out as the most effective.
Antimicrobial Efficacy of a Novel Antibiotic-Eluting Injectable Platelet-Rich Fibrin Scaffold against a Dual-Species Biofilm in an Infected Immature Root Canal Model. (35)	*Rafiee et al*	i-PRF	PRF with triple antibiotics mixture induced a strong inhibition of *A.naeslundii* while *E.faecalis* was similarly inhibited by the mixture of antibiotics and PRF and the antibiotics mixture alone. Overall, *E.faecalis* was more susceptible to inhibition than *A.naeslundii*.
Incorporating antibiotics into platelet-rich fibrin: A novel antibiotics slow-release biological device. (36)	*Polak et al*	PRF	PRF with antibiotics showed strong antibacterial activity while collagen sponges performed similarly to PRF with saline solution whether they were loaded or not with antibiotics. PRF with antibiotics seemed to have long term inhibitory effect against *F.nucleatum* and *S.aureus*.

Table 3- Search Results

4.2 Laboratorial Results

This laboratorial trial involved the use of both membrane and exudate after blood was collected from volunteers.

4.2.1 First Stage

The first stage, conducted using the blood of one volunteer, showed promising results with an obvious colour change in some of the wells (Figure 4) – this can be attributed to the bacteria in question, *Pseudomonas aeruginosa*, since its growth is accompanied by the dying of the culture media in green.

However, it was seen that the first well remained the same colour, not having changed – this could indicate an inhibition at an undiluted exudate volume of 128 µL.

Besides the green colouring, a biofilm was also observed in the wells which was thicker at volumes 64 µL and 32 µL.

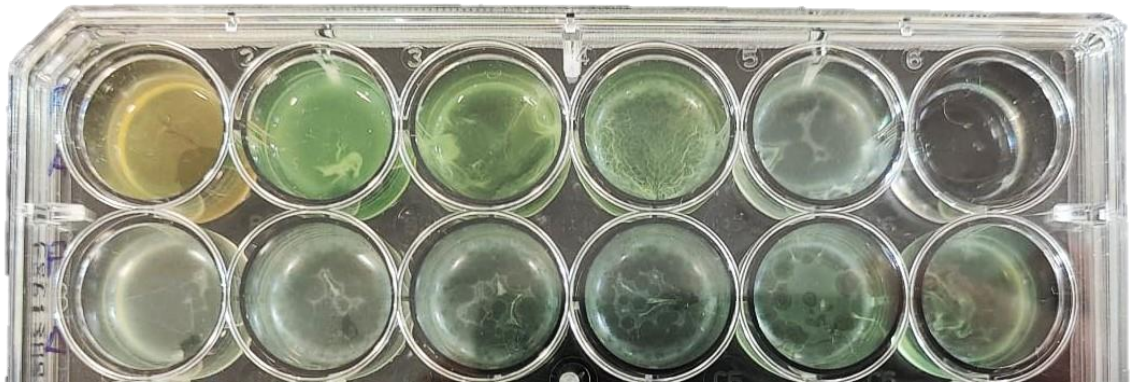

Figure 4-Pseudomonas aeruginosa at Stage 1: Wells

Bacterial Growth was observed for every concentration as seen in Figure 5.Petri dishes filled with Muller-Hinton agar were also cultured with 20 µL of each well with the exception of the positive and negative control wells.

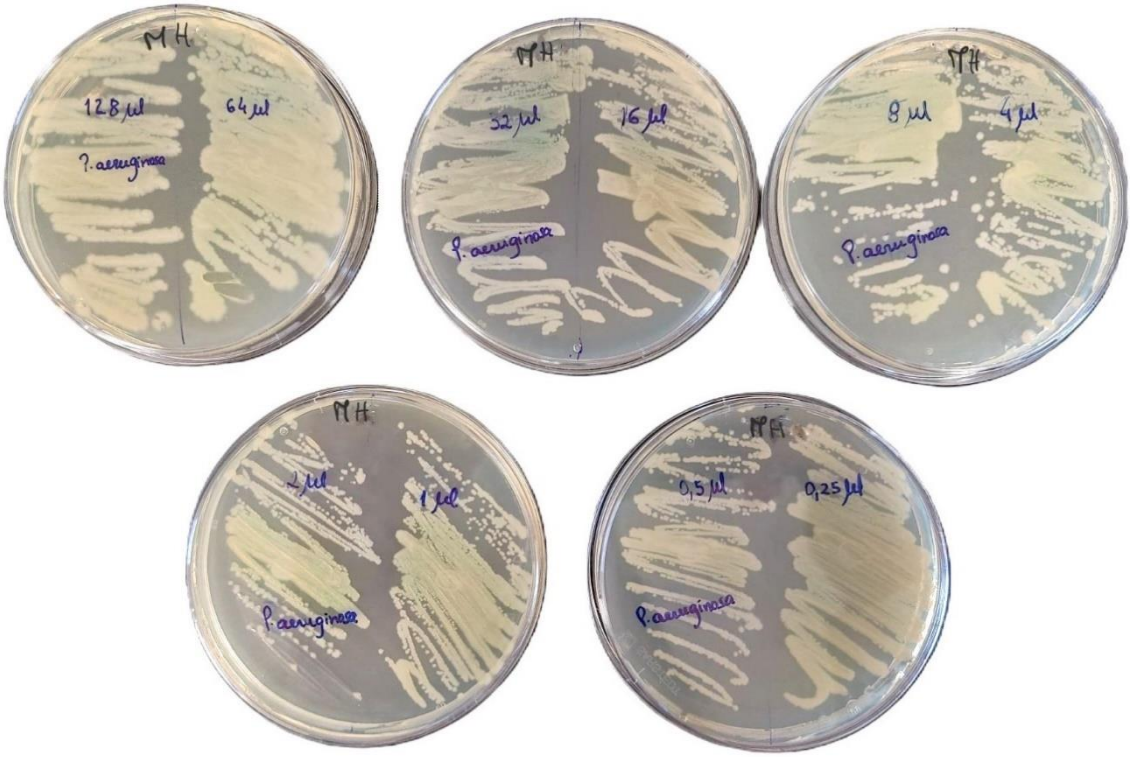

Figure 5- Pseudomonas aeruginosa at Stage 1: Petri dishes

4.2.2 Second Stage

The second stage was conducted using the blood of two donors.

The exudate using donor B's blood, tested in 8 wells at different dilutions showed no promising results regarding the inhibition of *Pseudomonas aeruginosa* and *Enterococcus faecalis*. It can be seen in Figure 6 that all the wells with exudate appear blurred due to the formation of a precipitate, indicating that all sustained bacterial growth.

The control wells behaved as expected.

E.faecalis showed an inhibition halo of 12 mm in the membrane of donor A which was more prominent around the head of the membrane, as shown in Figure 7.

P.aeruginosa was not included in the second stage results due to a contamination prior to the experiment. It was therefore not possible to show any results as the bacterium did not exhibit an expected growth when cultured.

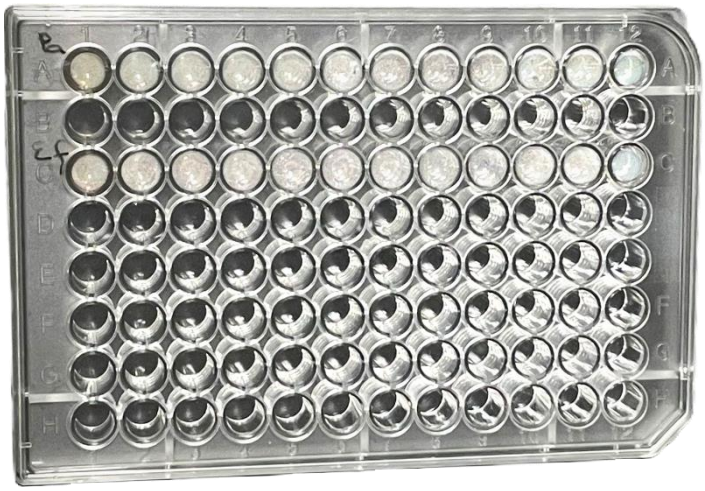

Figure 6- P. aeruginosa e Enterococcus faecalis at Stage 2: Exudate

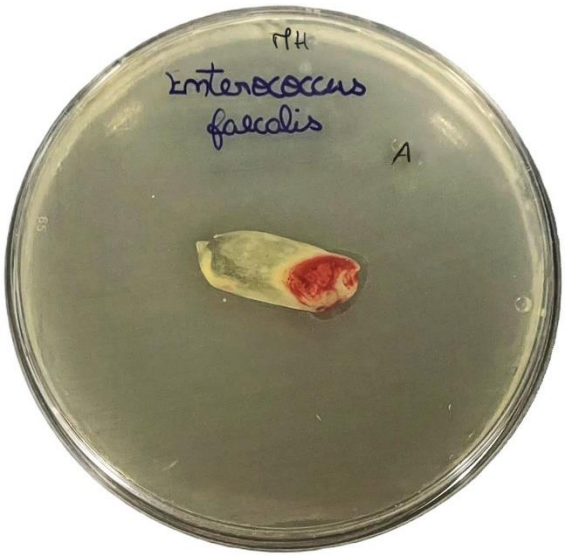

Figure 7- E.faecalis at Stage 2: Membrane

4.2.3 Third Stage

This stage used the blood of donor B, the donor presenting the best results to date. L-PRF exudate resulted in the inhibition of both the bacteria studied.

E. faecalis presented an inhibition halo of (10,0 ± 0,5) mm around the disc with an undiluted volume of 20 µL in Petri dish 3 as shown in Figure 8 while the remaining two petri dishes did not show any inhibition by the exudate.

P.aeruginosa exhibited an inhibition halo of (8,0 ± 0,5) mm at the undiluted volume of 20 µL in Petri dish 1 (Figure 9) and a halo of (13,0 ± 0,5) mm around the disc with 20 µL of undiluted exudate in Petri dish 2 as observed in Figure 10. Petri dish 3 did not present any inhibition with the exception of the disc impregnated with the positive control.

Both the mean and the standard deviation were calculated for the results of L-PRF exudate for both bacteria. Only the observed inhibition halos and their respective measurement were included.

P.aeruginosa with two Petri dishes out of three producing results showed an average of inhibition halo by L-PRF exudate of 10,5 mm with a standard deviation of 3,5 mm.

E.faecalis with only one Petri dish out of three presenting measurable results, showed an inhibition halo by L-PRF exudate of 10,0 mm. With only a single result, the mean and standard deviation are not statistically meaningful.

The inhibition halo of the positive control for *E.*faecalis measured an average of 19,3 mm with a standard deviation of 1,5 mm and for *Pseudomonas* was on average 13,0mm with a standard deviation of 1,0 mm.

The results obtained for the membrane were in accordance with data from previous experiments and the current literature.

The inhibition halo of *E.faecalis* around the L-PRF membrane measured (12,0 ± 0,5) mm (Figure 11) and the inhibition halo of *P.aeruginosa* was also of (12,0 ± 0,5) mm (Figure 12).

Given the fact that only one membrane was tested per bacteria in this stage, the means and standard deviations are not statistically meaningful.

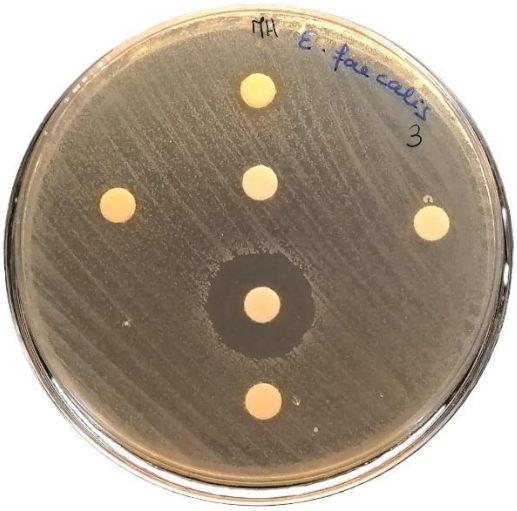

Figure 8-E.faecalis at stage 3: Exudate-Petri dish 3

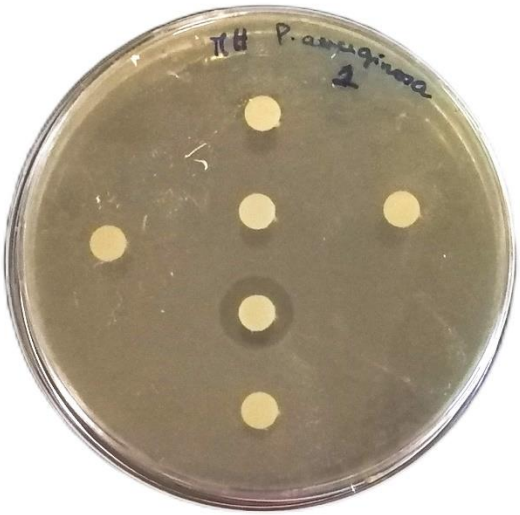

Figure 9- P.aeruginosa at stage 3: Exudate-Petri dish 1

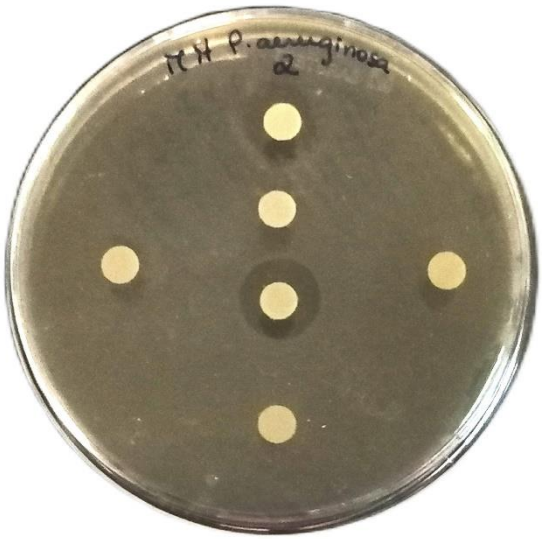

Figure 10- P.aeruginosa at stage 3: Exudate-Petri dish 2

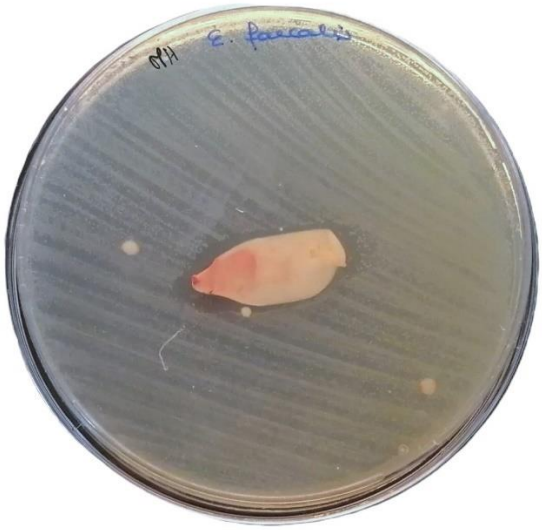

Figure 11- E.faecalis at stage 3: Membrane

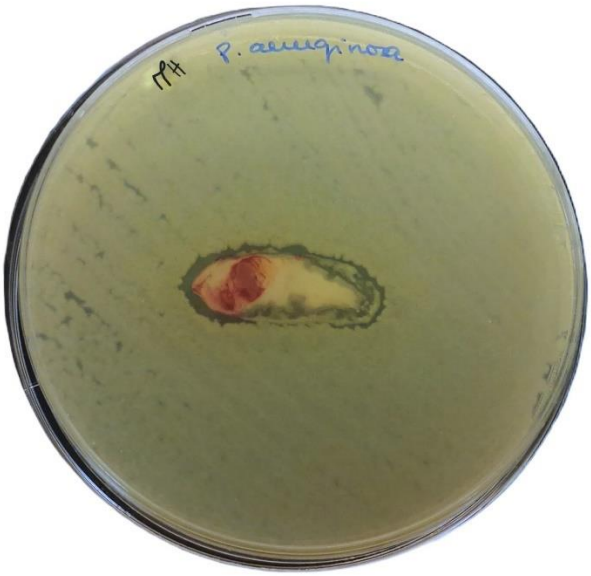

Figure 12- P.aeruginosa at stage 3: Membrane

5- Discussion

5.1 PRF and its precedents and derivatives (evolution)

Platelet concentrates and blood derived products were first used over 40 years ago in the medical field as a way to speed and improve the natural healing process. Over time, their usage for the regeneration of hard and soft tissues increased. According to *Micko et al.*, PRF's potential has been explored more and more over the past twelve years despite the fact that all studies to date have been unable to fully understand and explain its antimicrobial and anti-inflammatory activity.(4)

Although the primary usage of PRF has focused on tissue regeneration and wound healing, due to their main biological components being platelets, leukocytes and growth factors, they have also shown indirect antimicrobial effects.(5)

As its uses are frequent in the oral and maxillofacial medical fields, it is unsurprising that the antimicrobial properties of PRF are being primarily tested in an oral microbiome.(4)

PRF is a fully autologous second-generation platelet concentrate prepared using the patient's own blood. The use of additives such as anticoagulants is not necessary unlike other forms of platelet concentrates.(6)(5)

5.2 Platelet concentrates and antimicrobial effects

Platelet rich fibrin is capable of creating a three-dimensional structure packed with platelet derived growth factors, cytokines and in some cases, white blood cells. This fully autologous material's properties are highly influenced by the preparation protocols and methodologies used, and therefore demonstrate variable efficiency and behaviour.(5)

Additives such as anticoagulants (sodium citrate), coagulation activators (calcium chloride) and gel agents have been used to thicken the fibrin fibres offering supraphysiological mechanical properties to PRF's derived membrane. This comes at the cost of deficient cellular migration and cytokine release, which negatively influences the antimicrobial efficacy of PRF. (4)

These additives can potentially cause adverse reactions and immunological responses in the hosts and should be avoided, as some have an antimicrobial effect on their own and could therefore influence the results of experiments.(6)

There are protocols that enable us to obtain pure platelet concentrates, additive free, such as L-PRF and I-PRF. These PRF byproducts avoid the use of anticoagulants and bovine thrombin and are simply obtained by centrifuging the blood without additives, allowing the slow and

natural polymerization of fibrinogen to fibrin to occur, which requires only the physiological thrombin levels of the host's blood.(7)

Despite the current lack of consensus regarding which blood element causes the antimicrobial properties of platelet concentrates, many authors have proposed their explanations in response to *in vitro* results. While some theorise leukocytes to be relevant, others suggest platelets to be the solo or primary source of bacterial growth inhibition.(3)

Activated platelets are known to release growth factors and microbicidal proteins consisting of a mixture of platelets, leukocytes and plasma, and multiple chemokines playing a role in the antimicrobial activity of PCs.(8)(9)(3) Once released, these proteins and molecules are able to recruit leukocytes- an additional source of cytokines and microbicidal proteins.(3)

Some of the most common chemokines displaying microbicidal activity are Macrophage Inflammatory Protein (MIP)-1α, Regulated on Activation Normal T Expressed and Secreted protein (RANTES), Growth-regulated oncogene-alpha (GRO)- α, Interleukin (IL)-8, neutrophil-activating protein (NAP)-2 (a neutrophil activator) and stromal-cell derived factor (SDF)-1 α (great chemo attractant factor).(3)(9)

Some of these have been proven to correlate with growth inhibition of bacteria. MIP1α, RANTES and (GRO)- α play a role in the inhibition of *E.faecalis* and *P.aeruginosa*. IL-8 is involved in the inhibition of *Pseudomonas* aeruginosa's growth and Interleukin (IL-6) in the growth inhibition of *Enterococcus faecalis*.(3)

5.3 Explaining PRF's quality discrepancy between patients

A study by *Mamajiwala et al.* aimed to evaluate whether variations in centrifugation protocols in terms of the RCF (relative centrifugal force) and the duration could influence PRF's platelet count, antimicrobial efficacy, and the fibrin network pattern in different age groups (20-34 years, 35-49 years and 50-65 years). (10)

A comparison of the platelet count of the obtained PRF membranes with that of whole blood was made and it was found that regardless of the age group, at a relative centrifugation force of 228g for 8 minutes, more than 90% of the platelets were enclosed within the membrane. It was also seen that by prolonging the centrifugation time and increasing its speed, platelet concentration suffered a reduction to 50%. (10)

These results seem to be in agreement with *Sindusha et al.* and *Karde at al.*'s as their protocol featured a low-speed centrifugation in a way of preserving greater number of cells and influencing the overall antimicrobial efficacy. (7)(11)

It was concluded that a reduction in RCF, speed and time of centrifugation allowed an increase of platelet concentrations in all age groups studied.(10)

A dense pattern of the fibrin network is usually preferred in regeneration and wound healing as it provides more mechanical support to the surrounding tissues. This study has observed that increasing speed and time of centrifugation produced a denser fibrin pattern irrespective of the age group;, and that the pattern loosened with increasing donor age. This allowed them to conclude that for older age groups, an increase of speed and time during centrifugation could present advantages. (10)

Pham et al. noted that in older age groups periodontitis and severe periodontitis was more prevalent than healthy gingiva or gingivitis and that PRF from periodontitis patients performed better against *P.gingivalis* than that from the younger groups (with healthy gingiva or with gingivitis).(12)

The discrepancies between not only different age groups and preparation protocols but also between different genders remain to be explored. To investigate whether the periodontal condition of a patient influences their PRF antibacterial efficacy or not is also of interest.

5.4 The influence of *Pseudomonas* and *Enterococcus* on periradicular infections

Zhang et al. identified and categorised the bacterial colonies associated with persistent extraradicular infections and which are often present in periapical lesions and extraradicular biofilms. This classification was done to improve the understanding of the pathologies and thereby improve root canal treatments. It identified *Enterococcus* along with other bacterial species as nearly 30% of the bacteria in periapical lesions and extraradicular biofilms. It also acknowledged the high frequency of detection and abundancy of this bacteria in different studies and its relationship with post-treatment diseases, despite recent studies reporting low abundance rates. (13)

Drago et al. identified *E.faecalis* as being associated with primary and extraradicular infections and post-treatment persistent infections due to its ability to penetrate the dentinal tubules and survive root canal treatment.(2)

Pseudomonas was found in significantly higher levels in the extraradicular biofilms than in the periapical soft tissues. (13)

5.5 *Pseudomonas aeruginosa* and *Enterococcus faecalis* interactions when coexisting in the same biofilm

P.aeruginosa and *E.faecalis* are known to be regularly coisolated in polymicrobial biofilm-associated infections unrestricted to the oral cavity.

A study by *Ai Zhu Tan et al*. investigated the influence of the microenvironment in polymicrobial interactions, and how the growth trajectory of bacterial colonies can be shaped. (14)This study explored the influence of iron-restricted environments on the biofilms in which both these species coexist. (14)

It was shown that in an iron restricted environment such as the human body (where the iron is mostly kept intracellularly and modulated such that very little of it is available to bacteria) *Enterococcus faecalis* increases the productions of L-lactate which fails to pass E. faecalis' membrane freely and is therefore exported as lactic acid with a pKa lower than the pH of the surrounding environments. This lactic acid is later deprotonated back as L-lactate, releasing H^+as a byproduct and resulting in an even more acidic environment.(14)

P. aeruginosa's growth was inhibited when grown in low pH environments, as a result of *E.faecalis'* lactic acid export and the additional L-lactate mediated chelation of iron. No growth was detected when in chelated media at pH of 5,50 and lower. It is of interest to note that both lactic acid with a pH of 2,69 and citric acid with a pH of 2,41 inhibited *P.aeruginosa* when added.(14)

These results suggest possible methods to inhibit the growth of *Pseudomonas* in conjunction with platelet concentrates.

E faecalis' inhibition of *P.aeruginosa* was not strain specific, and antagonistic behaviour between both species was not observed outside nutritionally restricted environments. (14)

5.6 Platelet concentrates' storage implications: Worth doing or contamination hazard?

The storage and preservation of platelet concentrates is currently a controversial topic as it entails a high contamination risk.

Handigund et al., explored the different storage methods described in the literature and found that to date, the most commonly used protocol involves the keeping of the platelet concentrates at room temperature (22ºC) for no longer than 7 days in polyvinylchloride oxygen permeable bags as to maintain aerobic metabolism and thus preventing the decrease in pH.(15)

However, room temperature offers the ideal conditions for bacterial growth and therefore bacterial contamination.

It was highlighted that throughout the time PCs are stored a progressive and cumulative activation of platelets and build up of metabolic waste led to the decline of platelet's viability and function provoking platelet storage lesions (PSLs).(15)

Since room temperature storage heightens the risk of bacterial defiling of the platelet concentrates, lowering the temperature was considered by means of refrigeration (4ºC), cryopreservation and lyophilization.(15)

Handigund et al., cited a different study in which temperature cycling seemed to make all difference in proving that low temperature induced platelet changes can be reversed if the exposure to the cold is shorter than 12 hours.(15) Agitation cycling is also being studied as a viable option for the reduction of PSLs. (16)

Interestingly, a study by *Mariani et al.*, comparing the antimicrobial effect of L-PRP cryo (frozen at -30ºC for 2 hours and then thawed) to that of L-PRP and P-PRP, found that the cryopreserved sample showed similar inhibitory efficacy to those freshly harvested, in agreement with Handigund's previous work citation.(3)

A great number of studies focus on the preservation of the haemostatic characteristics of platelet concentrates for transfusion when stored through the addition of antimicrobial agents or amino acids to regulate metabolism and prevent the proliferation of bacteria. L-carnitine was tested on a sample of PRP kept at room temperature and reduced the glucose and lactate levels and thus, by keeping at bay metabolic disturbances, maintaining platelet viability.(17) N-acetylcysteine addition protects refrigerated platelet concentrates prolonging their storage for up to 10 or more days.(18)(19)

It would be of interest to investigate additives preferably without antimicrobial activity, as these could influence results and, prolong the shelf life of platelet concentrates used for antibacterial purposes. Also; relevant would be to study whether the defined time interval for platelet storage (5-7 days), based on transfusion purposes, would remain the same for PCs used only for antimicrobial activity.

Różalski et al., tested the antimicrobial activity of an expired platelet concentrate (PRP) against the ATCC strain of *S.aureus* and found it to be significant on both planktonic and biofilm cultures. They have also demonstrated that using ADP to activate platelets allowed them to observe a synergistic effect towards platelets agonists aiding the bacterial inhibition.(20)

Another interesting discover to highlight is the synergism showed by antibiotics (vancomycin and oxacillin) with platelet-derived lysates against *S.aureus* which allowed for reduction of the MICs of the antibiotics tested.(20)

It can be derived from this topic that innovation regarding the storage methods of platelets and platelet concentrates is needed as it enables the possibility of a onetime donation per patient preceding the treatment. It could also provide for a greater number of preserved units in the event of a traumatic intervention in a hospital context.

5.7 Comparison of different platelet concentrates and conventional drugs's antimicrobial activity

Sindhusha et al. compared I-PRF's and L-PRF's antimicrobial activity on periodontal pathogens and found that platelet concentrates demonstrate better antimicrobial activity when compared to drugs such as metronidazole. This could be due to their greater amount of oxygen metabolites. It was also concluded that I-PRF seemed to have a superior antimicrobial efficacy against *Porphyromonas gingivalis* when compared to metronidazole and L-PRF. (7)

A study by *Pham et al.* compared the antimicrobial effects of A-PRF+ and I-PRF against *P.gingivalis* and also found I-PRF to be the most effective. (12)

I-PRF's distinct preparation protocol, in which a low-speed centrifugation is featured, results in a higher concentration of platelets and white blood cells prior to the formation of the fibrin clot. This suggests that by retaining a greater number of cells, I-PRF's antimicrobial properties become enhanced. (7)

Despite presenting a significantly wider inhibition halo when compared to L-PRF, I-PRF's and L-PRF's difference regarding their antibacterial effect was not statistically significant.(7)

A different study by *Pham et al.* compared the antimicrobial effects of A-PRF+ and i-PRF sourced from patients with different periodontal conditions on *A.actinomycetemcomitans*. It was observed that although both inhibited the bacterium, the inhibitory effect of i-PRF was greater than that of A-PRF+, and that the i-PRF obtained from patients with periodontitis caused a greater bacterial growth reduction than the one obtained from healthy and gingivitis patients. (21)

A very similar study by *Tran et al.* found that i-PRF performed better than A-PRF+ at inhibiting the growth of *A.actinomycetemcomitans*, having shown a lower MIC value in the periodontitis patients group and overall lower compared to the MIC obtained with A-PRF+.(22)

A study by *Karde et al.* concluded that I-PRF is able to release higher concentrations of growth factors, and to provoke a greater induction of fibroblast migration when compared to PRP, PRF and whole blood. This seems to be due to the low speed and shorter centrifugation protocol that allows the presence of a greater number of cells not only of platelets and leukocytes but also circulating stem cells and endothelial cells. These offer great advantage to the antimicrobial efficacy and regenerative potential.(11)

A study by *Castro et al.* evaluating the antibacterial properties of L-PRF membrane and L-PRF and exudate on periodontal pathogens reported that L-PRF showed strong inhibition of *P.gingivalis* on agar plates and an increased growth of *A.actinomycetemcomitans* when in contact with L-PRF exudate.(23)

Badade et al, compared PRP to PRF regarding the inhibition of periodontal pathogens and it was concluded that PRP was the only one of the two effective against *P.gingivalis* and *A.actinomycetemcomitans*. It was also explained that despite the encouraging results, PRP's efficacy is dependent on the varying of its properties regarding the concentration of platelets, leukocytes, and growth factors as well as other operator related variables.(6)

It must also be noted that PRP's protocol includes the use of additives such as sodium citrate. This has advantages and disadvantages as it is able to enhance the antimicrobial activity of the platelet concentrate but can also cause adverse reactions to the host.

Drago et al. studied the antimicrobial activity of P-PRP and PPP against bacteria isolated from patients' oral cavities and found that it was able to inhibit *E.faecalis* and *S.aureus* at a dilution of 1:8. They have also observed that *S.agalactiae* and *S.oralis* were more susceptible to P-PRP being inhibited at a dilution of 1:16. It was concluded that for these four bacteria, responsible for oral infections and with history of being susceptible to platelet concentrates' antimicrobial activity, both P-PRP and PPP were able to similarly inhibit all of them, apart from *S.agalactiae* and *S.oralis* in which inhibition occurred distinctly with a tendency to greater susceptibility for activated P-PRP in a MIC value difference of 2 dilutions.(24)

Drago et al. did not find any correlation between antimicrobial activity and the concentration of platelets in the blood and P-PRP as it was seen that all dilutions had similar activity in all strains regardless of their platelet concentration.(24)

A different study by *Drago et al.* aimed to determine the antibacterial activity of P-PRP against bacterial species. Two different strains of *E.faecalis* were studied - *E.faecalis* (3 vancomycin-sensitive *enterococcus* (VSE)) and *E.faecalis* (2 vancomycin- resistant *enterococcus* (VRE)) - and the results compared against each other and the ATCC strain used as control. *P.aeruginosa*

collected from patients with oral and dental infections was also one of the bacteria studied and its' ATCC strain used as control.(2)

It was found that *P.aeruginosa* is not inhibited by P-PRP and that no differences were observed between *E.faecalis* VRE and *E.faecalis* VSE.(2)

Our study found that L-PRF both in the form of membrane and exudate is effective at inhibiting both *E.faecalis* and *P.aeruginosa*, meaning that it could be an advantage to use it as opposed to P-PRP when fighting *P.aeruginosa* infections. (2)

Mariani et al. also studied the effects PPP and P-PRP had on the bacterial growth of ATCC strains of oral cavity related species. However different results were reported regarding the inhibition of *Pseudomonas aeruginosa*. It was seen that both P-PRP and PPP induced a significant growth inhibition at the first and second hour of incubation and it was noted that P-PRP's reduction of bacterial growth was more pronounced than PPP's.(9)

E.faecalis had its' growth inhibited by P-PRP and PPP, with a statistically significant difference on the antibacterial effect between both PCs studied. (9)

A study by *Feng et al.* compared the antimicrobial activity of H-PRF, obtained through horizontal centrifugation, to L-PRF's and noted that the former seemed to have a higher concentration of immune cells and a better antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. It appeared that horizontal centrifugation enabled an improved separation of the cell layers by minimising the building of cells on the surfaces of the centrifugation tubes and increasing the total immune cells number tenfold when compared with fixed-angle centrifugation. It was concluded that H-PRF presented a significantly larger inhibition zone for both bacteria when compared to L-PRF exudate and that *E.coli* suffered a greater inhibition than *S.aureus*.(25)

This study by *Feng et al.* allowed us to directly correlate the number of immune cells to the observed antibacterial activity.(25)

Through flow-cytometric analysis, *Feng et al*. observed that two layers of the H-PRF, although similar in terms of the number of immune cells present, presented different antibacterial effect with the most effective one being the fifth layer, particularly in the exudate. It was shown that this layer; had a greater number of T cells compared to the first layer.(25)

This greater antibacterial effect was attributed to the regulatory powers of T cells over other immune cells. It was also seen that both the membrane and exudate from H-PRF were antibacterial, indicating that both cell components and cytokines were the source of the antimicrobial properties observed.(25)

A study by *Melo-Ferraz et al.* analysed, through Kirby-Bauer agar diffusion method, the antimicrobial activity of L-PRF exudate on ATCC strains of *P.aeruginosa*, *E.faecalis* and *C.albicans*. and found that there was inhibition of all. The inhibition halo of *P.aeruginosa* and of *E.faecalis* was on average 18,5mm and 11mm, respectively.(1)

The inhibition halo of *Pseudomonas* by L-PRF exudate was greater than that by chlorhexidine at 0,12%.(1)

In another study, *Cieślik-Bielecka et al.* studied the antimicrobial properties of L-PRP on pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *Staphylococcus aureus* (MSSA), *E.faecalis* and *P.aeruginosa* and found that it was effective inhibiting the bacteria. They were also able to conclude that the bactericidal effect on the bacteria was because of the leukocytes, observing a relationship between leukocyte subtypes and L-PRP's antibacterial activity.(26)

E.faecalis presented an inhibition halo of approximately 11mm with 20 µL of L-PRP and in group 1 an additional 5 µL of autologous thrombin and in group 5 with an additional 5 µL of bovine thrombin.(26)

P.aeruginosa presented an inhibition halo of around 11mm in group 1 and of 10mm in group 5.(26)

Our experiment, using L-PRF's membrane produced inhibition halos of 12mm on average for both *E.faecalis* and *P.aeruginosa*. The exudate impregnated paper discs were surrounded by a halo of on average 10,5mm for *P.aeruginosa* and of 10mm for *E.faecalis*.

The L-PRF of *Melo-Ferraz et al.* obtained better inhibitory results, with greater inhibition halos, than ours when comparing results from both *P.aeruginosa* and *E.faecalis*.(1)

The authors noted that while L-PRP activation with bovine thrombin seemed to be more effective in strains such as MRSA and MSSA, L-PRP activation with autologous thrombin produced better results in *E.faecalis* and *P.aeruginosa* as opposed to bovine.(26)

Interestingly, *Melo-Ferraz et al.* found through a flow cytometry analysis that L-PRF behaved similarly to thrombin regarding the activation of platelets.(1) This could mean that L-PRF could present more advantages than L-PRP against MRSA and MSSA bacterial strains, should its antimicrobial activity against them be proven.

In our study it was observed that L-PRF was not as effective at inhibiting both bacteria as chlorhexidine at 0,12%. It must be noted however, that L-PRF's membrane was almost as effective as 0,12% chlorhexidine when inhibiting *P.aeruginosa* with a difference of around 1mm on average between the inhibition halos.

E.faecalis seemed to be significantly more sensitive to chlorhexidine at 0,12% than to L-PRF as the first produced an inhibition halo of approximately 19mm on average.

When compared to *Cieślik-Bielecka's* L-PRP, our experiment's L-PRF was as effective, if not slightly more against *E.faecalis* and *P.aeruginosa* both in membrane and exudate form.(26)

Curiously, a study by *Mariani et al.* aiming to compare the *in vitro* antimicrobial activity of P-PRP and L-PRP against ATCC bacteria like *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterococcus faecalis*, as well as to assess the contribution of leukocytes to microbicidal properties, found that there was a time dependent inhibition of bacterial growth for up to 4 hours for both *E.coli* and *P.aeruginosa* at low bacterial concentrations and at higher concentrations for *S.aureus* and *E.faecalis*.(3)

The loss of antibacterial effect by all preparations after 18 hours was also reported at the concentrations tested, suggesting that either the PCs used display a short- term and incomplete antimicrobial activity or/and the microbicidal proteins in PRP are insufficient to provide a long-term bacterial inhibition.(3)

Mariani et al., suggests that leukocytes do not play a substantial role in the antibacterial activity of PRP as it was seen in their experiment that both L-PRP and P-PRP showed similar bacterial growth inhibition.(3)

Maghsoudi et al. in a study aiming to test PPP and PRP's antimicrobial activity against relevant bacteria in the oral cavity has also claimed to have found a strong correlation between platelet concentration and the antibacterial activity of PRP which is in agreement with *Mariani et al.*'s study.(27)

It was found that neither PPP nor PRP-1 (prepared using a 1-step centrifugation of the blood) or PRP-2 (prepared using a 2-step centrifugation) inhibited the growth of *P.aeruginosa*. It was also reported that while a disk impregnated with penicillin was effective at inhibiting the growth of Gram-positive bacteria, Chloramphenicol prevented the growth of all tested bacteria except that of *Pseudomonas aeruginosa*. (27)

5.8 The use of Platelet Concentrates as natural vessels for antibiotics

In addition to its regenerative and antimicrobial advantages, platelet concentrates such as PRF have been tested as potential carriers for substances like antibiotics allowing for a more localised action and therefore for less adverse reactions. (28)

Different protocols have been tested as the antibiotics can be mixed with the PRF before or after centrifugation or administered orally or intravenously previously to blood collection.(29)

A study by *Ozcan et al.* compared the antimicrobial effects of P-PRF with no added antibiotics to LAB-PRF, in which the penicillin was added to PRF only before it was centrifuged, and to SAB-PRF prepared 1 hour after the oral administration of penicillin, therefore containing systemic antibiotic. These were all tested against *S.aureus* and *E.coli*.(28)

It was seen that with LAB-PRF, no growth was observed for *S.aureus* or *E.coli* while, for the duration of the experiment, growth was observed with both P-PRF and SAB-PRF. It can be derived from this study that the addition of local penicillin to PRF resulted in complete bacterial inhibition while the systemic administration did not result in any additional inhibitory activity.(28)

Another study tested the antibacterial activity of fresh and stored PRF for 24 and 48 hours, with patients undergoing oral therapy with amoxicillin/clavulanic acid (AMC) in single or double dose and undergoing intravenous therapy with ampicillin/sulbactam (SAM) against *S.aureus*, *E.coli*, *H.influenzae*, *P.gingivalis* and *S.pneumoniae*.(29)

It was found that AMC loaded PRF had no inhibitory effect on *E.coli* and that intravenous SAM showed superior antibacterial activity to the oral antibiotics against all bacterial strains. The double oral dose of AMC performed better than the single dose and the fresh PRF produced the largest inhibition halos. It was also noted that after intravenous administration of antibiotics only a few minutes between the end of the infusion and the blood collection for PRF are needed, while for oral antibiotics the waiting period ranges between one hour to over two hours.(29)

A different study by *Straub et al.* explored different centrifugation protocols for PRF as a carrier of ampicillin/sulbactam (SAM) and observed its antimicrobial activity against five oral microorganisms *in vitro*. It was found that a centrifugation of 2300 rpm for 12 minutes with a maximum RCF of 652g produced the largest inhibition halos. They found that a single dose of SAM was enough to obtain therapeutic concentrations in PRF.(30)

Samples of PRF+SAM stored at 36ºC for 24 hours also showed inhibitory effect on all bacteria despite a reduction in efficacy compared to fresh PRF+SAM.(30)

Another study by *Straub et al.* tested the antimicrobial effect of A-PRF sourced from patients 10 minutes after an intravenous injection of 2g/1g ampicillin/sulbactam and found it to be effective against *S.aureus*, *H.influenzae*, *P.gingivalis* and *S.pneumoniae*. Out of ten samples of PRF from different patients, only 3 showed inhibitory effect against *E.coli* with small halos of inhibition, meaning that the amount of SAM released from PRF was not enough to inhibit this bacterium. PRF from patients without antibiotic therapy did not inhibit any of the bacteria.(31)

An interesting study by *Ercan et al.* compared the potential of T-PRF (Titanium platelet-rich fibrin) as a carrier of doxycycline to the more frequently used collagen loaded with doxycycline.

It was seen that doxycycline loading of T-PRF thickened its fibrin pattern and that the maximum release capacity of T-PRF/Doxy was much greater than that of Collagen/Doxy after one hour. (32)The drug release of T-PRF/Doxy continued up to 72 hours later whereas Collagen/Doxy drug release stopped after 1 hour.(32)

The antibacterial activity was assessed on *P.aeruginosa* and *S.aureus* and it was found that T-PRF on its own inhibited both bacteria and that T-PRF/Doxy showed an even greater inhibition zone for up to 7 days. No inhibition was found for Collagen/Doxy on either bacterium.(32)

Egle et al. investigated whether the combination of clindamycin phosphate with i-PRF influenced its antibacterial activity after hydrolysis when tested against *S.aureus* and *S.epidermidis*. It was shown that clindamycin phosphate did not cause structural changes to PRF until the seventh day of incubation, most likely due to its degradation. It was also seen that the addition of i-PRF to clindamycin phosphate lowered the MIC values, which varied depending on the blood donor.(33)

Bennardo et al. tested L-PRF as a carrier for vancomycin, gentamicin and linezolid at different concentrations and added before centrifugation against *E.coli*, *P.aeruginosa*, *S.mitis*, *H.influenzae*, *S.pneumoniae* and *S.aureus* to assess the antimicrobial effects of PRF. (34)It was observed that vancomycin interfered with the preparation of L-PRF. The authors described the inhibition caused by gentamicin-PRF as massive while the antibacterial activity of linezolid-PRF was similar and not statistically different to that of PRF against *P.aeruginosa* and *E.coli*. L-PRF inhibited all bacteria tested.(34)

Rafiee et al. studied the antibacterial effect of the combination of i-PRF and a triple antibiotic mixture on *A.naeslundii* and *E.faecalis* biofilm. It was observed that the group combining both i-PRF and the antibiotics showed the highest bacterial growth reduction for both the bacteria with a higher antibacterial activity for *E.faecalis*.(35)

A study by *Polak et al.* used PRF clot and PRF membrane as a release device for antibiotics such as metronidazole, penicillin and clindamycin added to fresh PRF prior to centrifugation. The antimicrobial activity was evaluated on *S.aureus* and *F.nucleatum*.(36)

It was seen that all PRF + antibiotics combinations showed significant growth inhibition. While the growth reduction of *S.aureus* was higher with clindamycin and penicillin compared to metronidazole, the inhibition of *F.nucleatum* was more pronounced with clindamycin and metronidazole than with penicillin. No significant differences were observed between the clot and membrane.(36)

In addition to PRF and antibiotics combinations, it was also compared the ability of collagen sponges to behave as carriers for the same antibiotics to the PRF clot and membrane and it was

shown that collagen sponges with antibiotics had similar antibacterial activity for *S.aureus* to PRF. However, sponge carrying clindamycin or metronidazole showed lower results against *F.nucleatum* than PRF carrying the same antibiotics.(36)

Platelet concentrates come as an innovative advantage to the medical field as antibiotic resistant bacterial infections have become more and more common, despite their antimicrobial mechanism remaining relatively unknown. Not only do they present antimicrobial effects on their own, but they also have the possibility of acting as carriers for traditional antibiotics for a more localised and autologous effect.

Depending on the microorganism or microbiota to target it is of interest to search the literature for which PCs are the most effective. For example, PRP and its derivatives have been demonstrated as very effective on a varied array of periodontal pathogens.

Ideally, for each bacterial species, a specific platelet concentrate; or combination of platelet concentrate and antibiotic should be attributed, so as to better fight oral and overall infections.

Additives' usage should be modulated depending on which PRF's property is the most useful and relevant to each case. For example, perhaps orthognathic surgery, cleft lip and palate surgery and sinus lift could benefit mostly from PRF's mechanical properties in detriment to less immunological efficacy. In this case, the use of thickening additives in the preparation protocol could present advantages.

6- Conclusion

Although it was not possible to determine the minimum inhibitory concentration from this experiment, it was shown that L-PRF had an antimicrobial effect on *E.faecalis* and *P.aeruginosa* in the form of both membrane and exudate.

It can be concluded that PRF and its by-products inhibit the bacterial growth of ATCC strain *E.faecalis* and that PRF and its by-products have an antimicrobial effect on the bacterial ATCC strain *P.aeruginosa.* Both hypotheses tested, 1 and 2, were confirmed.

This experimental study is limited by the fact that only ATCC strains were used as opposed to clinical bacterial isolates which are often more pathogenic; only healthy blood donors participated in the blood collection and it is of interest to study how the blood of donors with periodontal or overall inflammatory oral diseases influences the antimicrobial activity of PRF; a very short number of bacteria and samples was tested which makes it difficult to assess the antimicrobial spectrum of L-PRF.

It is of interest to study the sensitivity of patient isolated bacterial stripes to PRF and its by-products and compare it to the results observed with ATCC bacterial strains. Broadening the spectrum of bacteria studied and of samples prepared to gather a greater pool of data would enable more accurate results.

To compare the results between different blood donors could also be relevant as each donor presents a significant variable that should be taken into consideration and intra- and inter-variations could manifest in the results.

Despite all limitations and the fact that no MIC has been derived from this study, with the least supporting literature, L-PRF exudate showed surprising results leading us to believe its application in future dental procedures could be near.

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8- Annexs

8.1 Certificates of Attendance

EVENTOS CIENTÍFICOS IUCS
JORNADAS CIENTÍFICAS AEIUCS
XXXI JORNADAS CIENTÍFICAS DE CIÊNCIAS DENTÁRIAS

DIPLOMA

O Presidente das XXXI Jornadas Científicas de Ciências Dentárias certifica que:
Silva L., Mendes I., Miller P., Coelho C., Monteiro M C., Criado M B.., Melo-Ferraz A.
apresentaram um trabalho científico sob a forma de E-poster intitulado, "Avaliação da interação de concentrados plaquetários autólogos com as bactérias *Pseudomonas aeruginosa e Enteroroccus faecalis* por meio da concentração mínima inibitória (mic)" no âmbito das XXXI Jornadas de Ciências Dentárias, subordinadas ao tema "Inflamação dos tecidos Periimplantares – Soluções Atuais", que decorreram no dia 17 de maio de 2023, nas Instalações do Instituto Superior de Ciências da Saúde - CESPU.

PROF. DOUTOR JOAQUIM MOREIRA
PRESIDENTE DAS XXXI JORNADAS CIENTÍFICAS DE CIÊNCIAS DENTÁRIAS

CESPU · CESPU INSTITUTO UNIVERSITÁRIO DE CIÊNCIAS DA SAÚDE · AEIUCS · NMD AEIUCS NÚCLEO MEDICINA DENTÁRIA AEIUCS

Certificate of Attendance

Laura Manuela Martins Silva

For participation in the II **INTERNATIONAL CONGRESS of UNIPRO RESEARCH UNIT 2023**, including the II **CONFERENCE IN ADVANCES IN RESEARCH ON ORAL CANCER** convened by **UNIPRO** (IUCS/ CESPU) and the **WHO** Collaborating Centre on Oral Cancer (London, UK), which took place in Penafiel (Portugal), in 01-02 June 2023, with the Poster Presentation entitled:

Pseudomonas aeruginosa and Enterococcus faecalis' interaction with platelet concentrates

UNIPRO Oral Pathology and Rehabilitation Research Unit

CESPU INSTITUTO UNIVERSITÁRIO DE CIÊNCIAS DA SAÚDE

Gandra, 01 June 2023

Luís Monteiro

Oral Pathology and Rehabilitation Research Unit **(UNIPRO)** Coordinator

8.2 Ethics Committee Approval

CESPU
INSTITUTO UNIVERSITÁRIO
DE CIÊNCIAS DA SAÚDE

Comissão de Ética

Exmo. Senhor Investigador
Paulo Manuel Cruz Miller

N/Ref.ª: CE/IUCS/CESPU-14/21 **Data:** 2021/junho/21

Assunto: - **Parecer relativo ao Projeto de Investigação:** 16/CE-IUCS/2021
- **Título do Projeto:** *"Ativação plaquetária e atividade antimicrobiana da fibrina rica em plaquetas e Leucócitos (L-PRF)"*
- **Investigador responsável:** Paulo Manuel Cruz Miller

Exmo. Senhor,

Informo V. Exa. que o projeto supracitado foi analisado na reunião da Comissão de Ética do IUCS, da CESPU, CrI, no dia 17/06/2021.

A Comissão de Ética emitiu um parecer **favorável** à realização do projeto tal como apresentado.

Com os melhores cumprimentos,

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