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Laboratory trial regarding the antimicrobial Effect of L-PRF on bacteria and fungi from the oral cavity.

Inês Filipa Santos Mendes

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

—

Gandra, **maio de 2024**

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Trabalho realizado sob a Orientação de
Prof. Doutor Paulo Miller
Especialista António Melo-Ferraz e Prof. Doutora Cristina Coelho, como Co-orientadores.

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DECLARAÇÃO DE INTEGRIDADE

Eu, Inês Filipa Santos Mendes, 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.

Comunicações Científicas em Congressos na Forma de Poster ou Orais

- May 23, 2023: Author and presenter of a Poster at XXXI Jornadas de Medicina Dentária do IUCS/CESPU entitled **"Efeito de L-PRF sobre a *Candida albicans*".** Authors: Inês Mendes, Laura Silva, Paulo Miller, Cristina Coelho, Maria Céu Monteiro, Maria Begoña Criado, António Melo-Ferraz. (Annex 2)

- June 1-2, 2023: Author and presenter of a Poster at II UNIPRO International Congress 2023 entitled **"Antifungal effect of L-PRF on *Candida albicans*".** Authors: Inês Mendes, Laura Silva, Paulo Miller, Cristina Coelho, Maria Céu Monteiro, Maria Begoña Criado, António Melo-Ferraz. (Annex 3)

- April 9, 2024: Author and presenter of an oral comunication at XXXII Jornadas de Medicina Dentária do IUCS/CESPU entitled "**Efeito antimicrobiano do L-PRF sobre a *Candida albicans* e *Escherichia coli*. Estudo in vitro**". Authors: Inês Mendes, Laura Silva, Ana Mendes, António Melo-Ferraz, Paulo Miller. It was awarded the Honourable Mention by the Scientific Committee. (Annex 4)

Publicações Científicas

- Publication of an abstract related to a poster entitled **"Antifungal effect of L-PRF on *Candida albicans*".** Authors: Inês Mendes, Laura Silva, Paulo Miller, Cristina Coelho, Maria Céu Monteiro, Maria Begoña Criado, António Melo-Ferraz. (Annex 5)

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RESUMO

Introdução: A *Candida albicans* e *Escherichia coli* são dois microrganismos presentes na cavidade oral. O L-PRF é um concentrado de plaquetas (PCs) de segunda geração com uma elevada concentração de leucócitos que promovem a proteção dos tecidos contra a inflamação e que aparenta possuir capacidades antibacterianas.

Objetivos: Averiguar o efeito inibitório do L-PRF sobre a *Candida albicans* e *Escherichia coli* (estirpes ATCC).

Materiais e Métodos: Para a fundamentação teórica, foi efetuada a pesquisa na PubMed e na EBSCO, entre 2014-2024. Foi feita a colheita sanguínea de 2 dadores saudáveis. O sangue foi centrifugado segundo o protocolo escolhido, com a posterior recolha das membranas e exsudado. Foi utilizado o método de difusão em agar Mueller-Hinton para as membranas e os testes de microdiluição em caldo e de difusão em meio sólido em discos para o exsudado.

Resultados: A membrana criou halo de inibição de 9mm na *Escherichia coli* e de 11mm, 11mm e 9mm na *Candida albicans*. O exsudado não obteve resultados.

Discussão: O L-PRF torna-se interessante pela presença de leucócitos que participam na resposta imune e no potencial antimicrobiano. Estudos, como o de *Melo-Ferraz et al.* e de *Fenge et al.* obtiveram respostas antimicrobianas pelo L-PRF, na *Candida albicans* e *Escherichia coli*, respetivamente.

Conclusões: Podemos admitir que existe um efeito inibidor por parte do L-PRF, mas são necessários mais estudos com uma maior população de estudo e com o uso de estirpes microbianas retiradas de casos clínicos.

Palavras-chave: "*Candida albicans*", "*Escherichia coli*", "L-PRF", "antimicrobial".

ABSTRACT

Introduction: *Candida albicans* and *Escherichia coli* are two microorganisms that inhabit the oral cavity. L-PRF is a second-generation platelet concentrate (PC) with an elevated count of leucocyte that promote tissue protection against inflammation and it appears possesses antimicrobial properties.

Objectives: Ascertain the inhibitory effect of L-PRF against *Candida albicans* and *Escherichia coli* (ATCC strains).

Materials and Methods: As a theoretical foundation, a Pubmed and EBSCO search was conducted, between 2014-2024. Blood was obtained from 2 healthy donors. The blood was centrifuged according to the selected protocol and the membranes and exudate were collected. The Mueller-Hinton agar diffusion method was used for the membranes and the broth microdilution and solid disc diffusion tests were used for the exudate.

Results: The membrane produced an inhibition halo of 9mm on *Escherichia coli* and 11mm, 11mm and 9mm on *Candida albicans*. No results were attained with the exudate.

Discussion: L-PRF is interesting because of the presence of leukocytes involved in the immune response and its antimicrobial potential. Studies such as those by *Melo-Ferraz et al.* and *Fenge et al.* obtained antimicrobial responses to L-PRF against *Candida albicans* and *Escherichia coli*, respectively.

Conclusion: We can acknowledge that there is an inhibitory effect on the part of L-PRF. However, further studies with a larger study population and with microbial strains from clinical cases are needed.

Keywords: "*Candida albicans*", "*Escherichia coli*", "L-PRF", "antimicrobial".

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LIST OF ABREVIATIONS:

L-PRF: Leucocyte- and Platelet Rich Fibrin

PRF: Platelet Rich Fibrin

H-PRF: Horizontal – Platelet Rich Fibrin

PC: Platelet concentrates

RBC: Red Blood Cells

PRGF: Plasma Rich in Growth Factors

PDGF: Platelet Derived Growth Factor

PRP: Platelet Rich Plasma

PRFM: Platelet Rich Fibrin Matrix

PRGF: Plasma Rich in Growth Factors

TGF-β: Transforming Growth Factor- Beta

BMP-9: Bone Morphogenic Proteins

VEGF: Vascular Endothelial Growth Factor

IGF: Insulin Growth Factor

IL-1: Interleukin 1

IL-4: Interleukin 4

IL-6: Interleukin 6

TNF- α: Tumor Necrosis Factor- Alpha

GPIIbIIIa: Glycoproteins IIb/IIIa

Ca^{++}: Calcium

AMC: Amoxicillin/Clavulanic Acid

SAM: Ampicillin/Sulbactam

1. INTRODUCTION

In the field of dentistry, Leukocyte-Platelet Rich Fibrin (L-PRF) has been employed to enhance tissue healing and bone regeneration due to its three-dimensional fibrin matrix, which is enriched with numerous autologous cells, including leucocytes (macrophages and neutrophils), platelets and a plethora of cytokines and growth factors. These components are continuously released over time, alongside other bioactive substances.(1,2)

It is a second-generation platelet concentrate (PC) developed by *Dr. Joseph Choukroun et al.*, which has been proven effective in regenerative endodontics, implantology, periodontology, maxillofacial surgery, and other related fields.(3) Notably, L-PRF is considered a low-cost, simple, fast, and safe option due to its autologous nature, which mimics the natural biological response, prevents inflammatory reactions, and promotes homeostasis.(4,5) Derived directly from the patient's blood (without anticoagulant), the Platelet Rich Fibrin (PRF) concentrate captures over 90% of the platelets and more than 75% of the leukocytes from the initial blood composition. It can be applied as a membrane and/or exudate, both of which are products of the same PRF. Among these, the L-PRF membrane is the most extensively utilized in surgical applications.(6)

The wound site, especially in the oral cavity, is vulnerable to pathogens invasion, and subsequent infection. It is well established that the oral cavity is a niche environment for pathogens, with *Candida albicans* and *Escherichia coli* representing two prominent examples.(1,3,7)

Candida albicans is a human opportunistic yeast that inhabits the oral cavity and can be the cause of oral candidiasis in the presence of dysbiosis. It may also play a role in the development of carious lesions. It is frequently found within the root canal and has the capacity to colonise and proliferate on dentine surfaces, including the tubules, thereby presenting a significant challenge in its eradication in such instances.(3)

Escherichia coli is a gram-negative, bacillus, anaerobe facultative organism commonly found in the gastrointestinal tract and the oral cavity. It is particularly associated with

aggressive periodontitis and periimplantitis, which may develop following prolonged use of antibiotics and/or antiseptics.(7)

Both pathogens create lesions and precarious conditions in the mouth that can be pre-emptively managed using antimicrobial products.

Several studies have demonstrated the possible antimicrobial effect of L-PRF, attributed to its high quantity of platelets and leucocytes. Upon activation, these platelets release growth factors that facilitates numerous processes, including cell mitosis, angiogenesis, recruitment, and migration of cells to the lesion, cell differentiation and enhanced collagen production. Additionally, platelets secrete proteins with antimicrobial activity, participate in antibody-dependent cell cytotoxicity, and produce reactive oxygen species.(8) They appear to be crucial in the pathogen recognition and neutralisation, as well as indirectly contributing to the recruitment and modulation of leukocyte behaviour, enhancing their phagocytic abilities and microorganism elimination by activating intracellular signalling pathways.(1) Leukocytes produce cytokines with angiogenic and pro-inflammatory functions, with T-cells being particularly important in neutralizing bacteria and their products through the release of cytotoxic granules and enzymes.(9)

The aim of this study is to determine whether L-PRF inhibits the growth of *Candida albicans* or *Escherichia coli* in the forms of membrane and exudate.

2. OBJECTIVE

The aims of this study were:

- Ascertain the antimicrobial effect of L-PRF exudate in different volumes against:
  - *Candida albicans*
  - *Escherichia coli*

- Ascertain the inhibitory effect of L-PRF membrane opposed to:
  - *Candida albicans*
  - *Escherichia coli*

Secondary objective:

- To determine the minimum inhibitory concentration achieved by the exudate on *Candida albicans* and *Escherichia coli*.

Null Hypothesis:

- The L-PRF exudate in different volumes doesn't have an inhibitory effect on *Candida Albicans*.
- The L-PRF exudate in different volumes doesn't have an inhibitory effect on *Escherichia coli*.
- L-PRF membrane had no inhibitory effect on *Candida albicans*.
- L-PRF membrane had no inhibitory effect on *Escherichia coli*.

3. MATERIALS AND METHODS

3.1. Research Methodology

3.1.1. Search Strategy

A comprehensive literature search was conducted at PubMed (via the National Library of Medicine) and EBSCO to establish the theoretical foundation for this study. The search used a combination of the following terms: "L-PRF," "Candida albicans," "Escherichia coli," "antimicrobial." Additionally, a manual search was performed outside the primary search in order to identify individually relevant articles, with the aim of enriching the introduction and discussion sections.

The totality of articles was compiled for each combination of key terms, and duplicates were removed via Zotero.

3.1.2. Inclusion criteria.

Articles published in English.

Between 2014-2024.

Studies conducted on humans.

Studies in the field of dentistry.

3.1.3. Study Selection

After the removal of duplicate, the articles were scanned for relevance by title and abstract. Secondly the non-excluded studies were assessed accordingly with the eligibility criteria and the selected studies were individually and thoroughly analysed concerning the purpose of this study (Figure 1).

A table of results was constructed with the articles relevant for the discussion segment of this work piece (Annex 4).

Table 1 - PICO strategy

Population	Patients undergoing oral surgery or dental procedures.
Intervention	Application of L-PRF as an adjuvant to standard surgical procedures.
Comparation	Standard surgical procedures without L-PRF application.
Outcome	Decreased incidence of post-operative bacterial and fungal infections in the oral cavity.
In patients undergoing oral surgery or dental procedures does the adjunctive use of L-PRF compared to standard procedures alone reduce the incidence of post-operative bacterial and fungal infection in the oral cavity?	

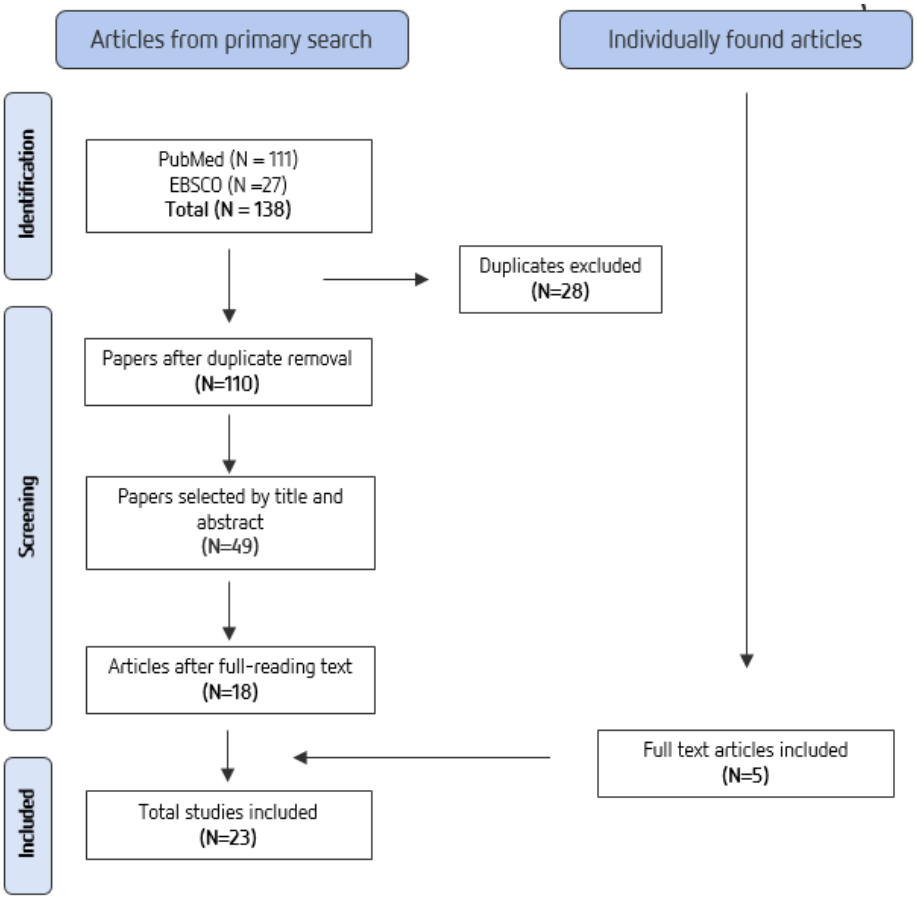

Figure 1 - Flow diagram of the Study Selection

3.2. Investigation Methodology

3.2.1. Study population

Two healthy, non-smoking volunteers, both female young adults born in the same year, were selected for the study. They had no history of antibiotic, anticoagulant, or immunosuppressive therapy at least three months prior.

3.2.2. Ethics

- This study emanates from the project "Platelet activation and antimicrobial activity of Leucocyte-Platelet Rich Fibrin(L-PRF)", (Ativação plaquetária e atividade antimicrobiana da fibrina rica em plaquetas e leucócitos (L-PRF)), which was approved by the IUCS Ethics Committee – CESPU (ref. 16/CE-IUCS/2021). (Annex 1)

3.2.3. Blood collection and L-PRF preparation

From 2 healthy donors, 9 mL of venous blood was drawn into silica-coated plastic vacuum blood collection tubes without anticoagulant. The blood was immediately centrifuged at 2700 rpm for 12 minutes (IntraSpin System - IntraLock International - Boca Raton, Fl USA). A separation of the whole blood into three layers was then observed - red blood cells (RBC) at the bottom, PRF in the middle and platelet poor plasma (PPM) at the top. The PRF was secluded from the tube and the RBC layer was carefully removed, leaving a small portion in the clot. The clot was promptly compressed using a Xpression™ Box (IntraSpin System – Intra Lock International – Boca Raton, FL USA) for 3 minutes to obtain L-PRF membrane and L-PRF exudate (*Figure 2*).

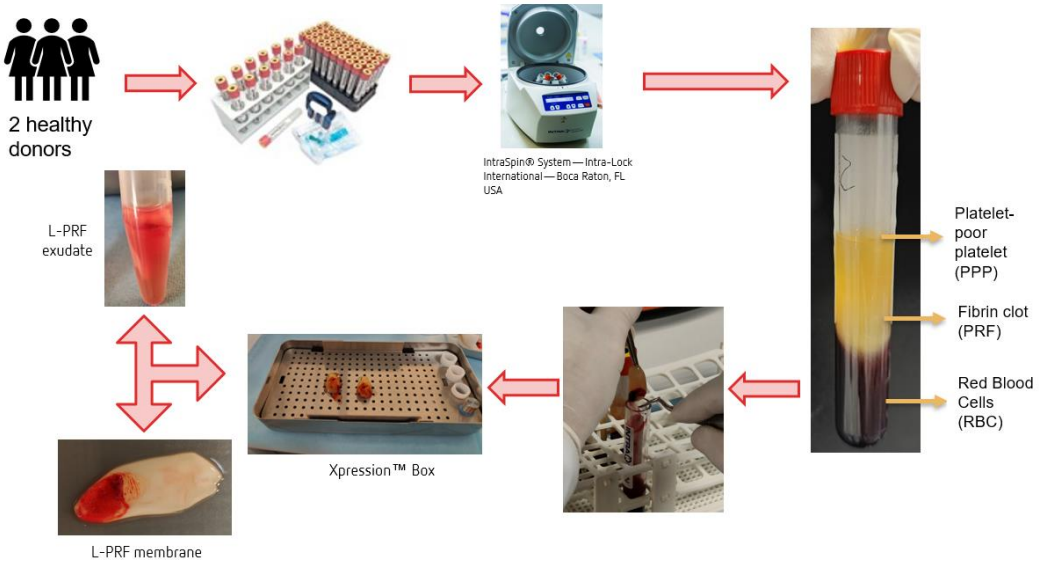

Figure 2 - Schematic representation of the L-PRF membrane and exudate production process.

3.2.4. Bacterial strains

Standard strains of *Candida albicans* (ATCC 90028) and *Escherichia coli* (ATCC 2592) were used to ascertain the antimicrobial effects of L-PRF. They were thawed and seeded on trypticase soy agar (TSA) and incubated at 35-37°C for 24-48 hours. 2-3 colonies of each strain were inoculated onto BioMérieux ATB medium, and the suspension was adjusted to 0.5 in the McFarland standard (1x108UFC/mL) using densitometer (BioMérieux Marcy-L'étoile-France).

3.2.5. Broth microdilution test (L-PRF exudate)

In the first instance, the exudate was diluted in the first 10 wells on a row of a microdilution 96-well plate. The last 2 wells were used for the negative and positive controls, Dimethyl Sulfoxide (Merck – KGaA)) and Chlorhexidine + Cetylpyridinium Chloride (PerioAid 0,12%-Dentaid Spain), respectively. It was added to new wells 20µl of each dilution and controls plus 180µl of the microorganism suspension (Figure 3). After incubation (18-24h, 35-37°),

the contents of the wells were observed for signs of turbidity (pathogen growth) or transparency (inhibition). The procedure was carried out for the 2 micro-organisms.

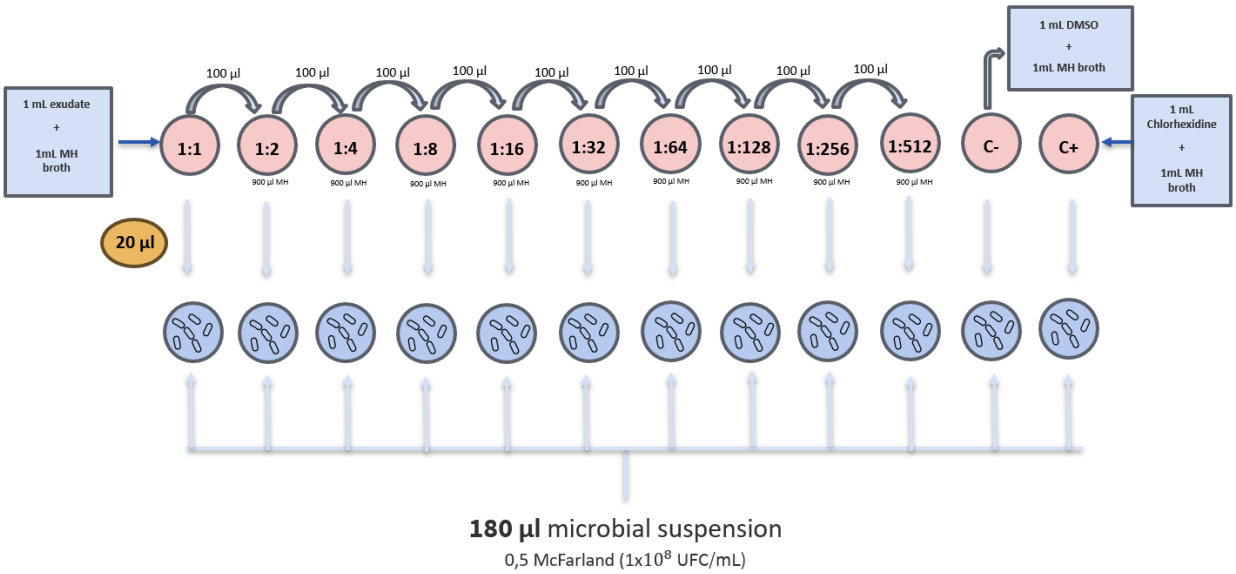

Figure 3 - Diagram representing the steps of the broth microdilution test before incubation.

3.2.6. Disk Diffusion test (L-PRF exudate)

The Müller-Hinton agars were inoculated with the microorganisms using the spread method, and a range of disks (Thermo scientific™ Oxoid™ Diagnostic Disks) were impregnated with the exudate (20 µl, 10 µl,5 µl and 2,5 µl), the positive control chlorhexidine + cetylpyridinium Chloride (PerioAid 0,12%-Dentaid Spain) and the negative control Dimethyl Sulfoxide (Merck – KGaA) (Figure4). After incubation (18-24h 35-37º), the plates were observed for an inhibition zone, which is a circular area relating to the level of

antimicrobial activity upon the bacteria using a digital calliper. The procedure was conducted for both microorganisms.

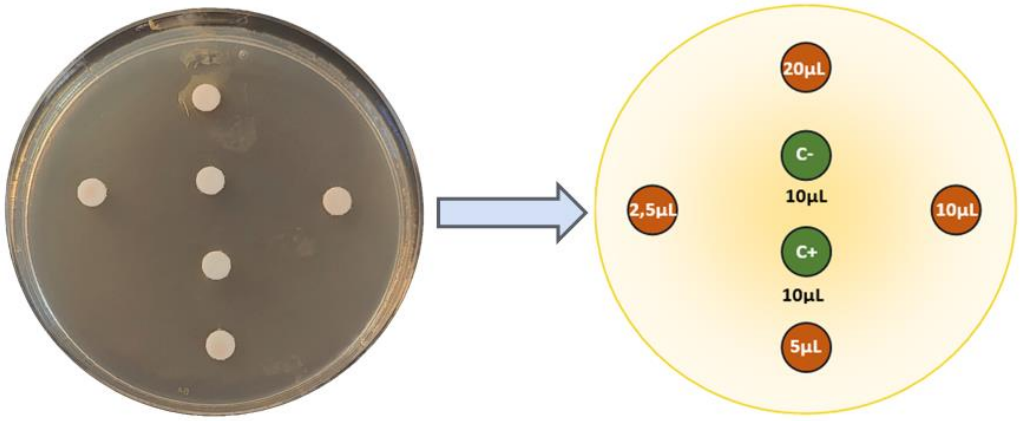

Figure 4 - a) Disks positioned equidistantly; b) The following diagram illustrates the pipetting location of the different volumes of L-PRF exudate and controls.

3.2.7. Agar diffusion test (L-PRF membrane)

The agar was inoculated with the microorganisms using the spread method and the L-PRF membrane was placed on top of the preparation (Figure 5). Incubation for 18-24 hours at 35-37°C. The inhibition zone was measured using a digital calliper. The procedure was repeated for both microorganisms.

Figure 5 - Positioning of the membrane atop the inoculated agar.

4. RESULTS

4.1. Broth microdilution test (L-PRF exudate)

On the first row of Figure 6, it can be observed that the contents of the wells are turbid, with the exception of the last well, which corresponds to the positive control for *Escherichia coli*.

In the case of *Candida albicans*, although microbial growth was observed in all wells, except for the last one, the first two wells exhibited greater growth, with the contents being completely turbid, in contrast to the others, which had a precipitate with less turbid content. (Figure 6)

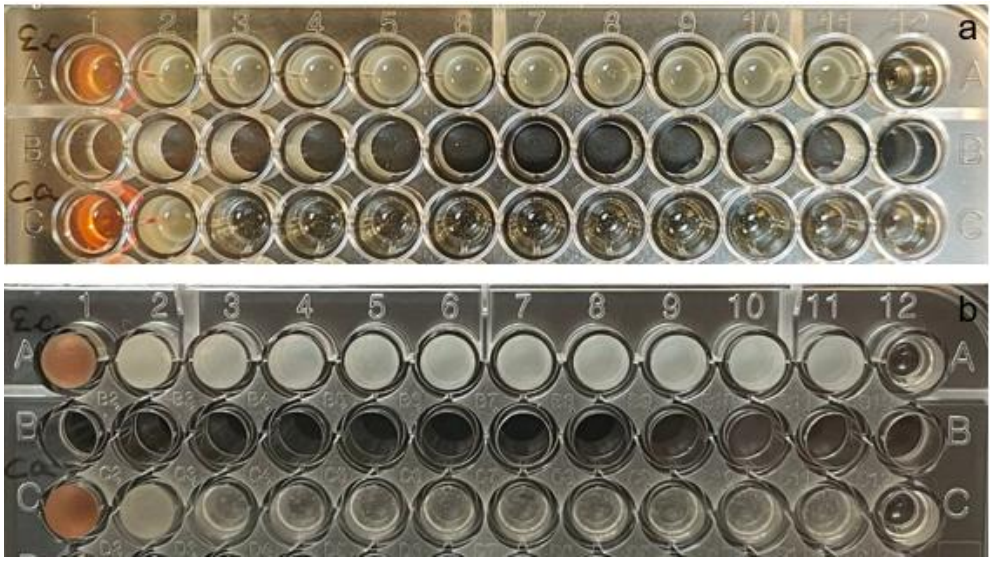

Figure 6 - a) Frontal view of the microdilution plate; B) Back view reversed horizontally for a better observation.

Trypticase soy agars (TSA) were inoculated with the contents of the wells corresponding to the 1:4 (well 4C), 1:8 (well 5C) and 1:16 (well 6C) dilutions to confirm whether the yeast had grown. After incubation for 18-24 hours at 35-37°C, the results were obtained and are shown in Figure 7. The yeast exhibited growth in all Petri dishes, therefore, it was not possible to calculate the minimum inhibitory concentration (MIC).

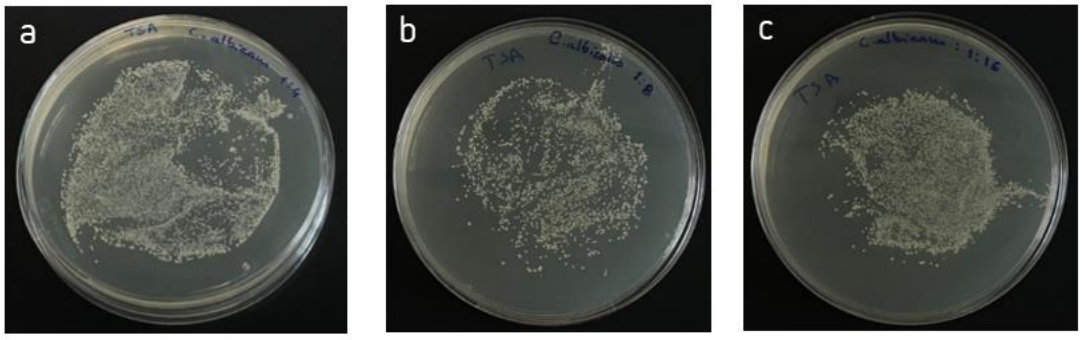

Figure 7- Contents inoculation of different wells to ascertain if there was Candida albicans growth; ***a)*** *well 4C.* ***b)*** *well 5C.* ***c)*** *well 6C. There was yeast growth in the three wells.*

4.2. Diffusion in solid media with disks (L-PRF exudate)

The test was performed in triplicate for both microorganisms in order to validate the results. The only inhibition zone present was that created by the positive control. The embedded discs with L-PRF exudate did not exhibit any inhibitory effect on *Escherichia coli* nor *Candida albicans*, as shown in Figure 8 , Figure 9 and Table 2.

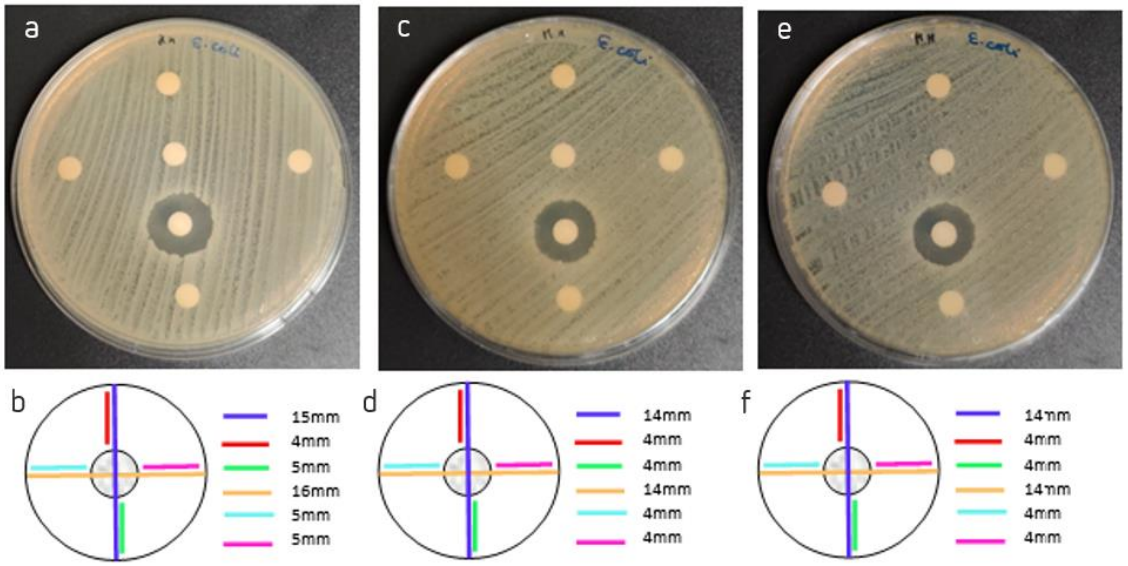

Figure 8 - Antimicrobial properties of different volumes of L-PRF exudate in Mueller-Hinton agar against Escherichia coli in a,b and c. L-PRF exudate didn't have an antimicrobial effect. Representation of the inhibition halo obtained by chlorhexidine with different measurements in b,d,f. Image matching: a-b; c-d; e-f.

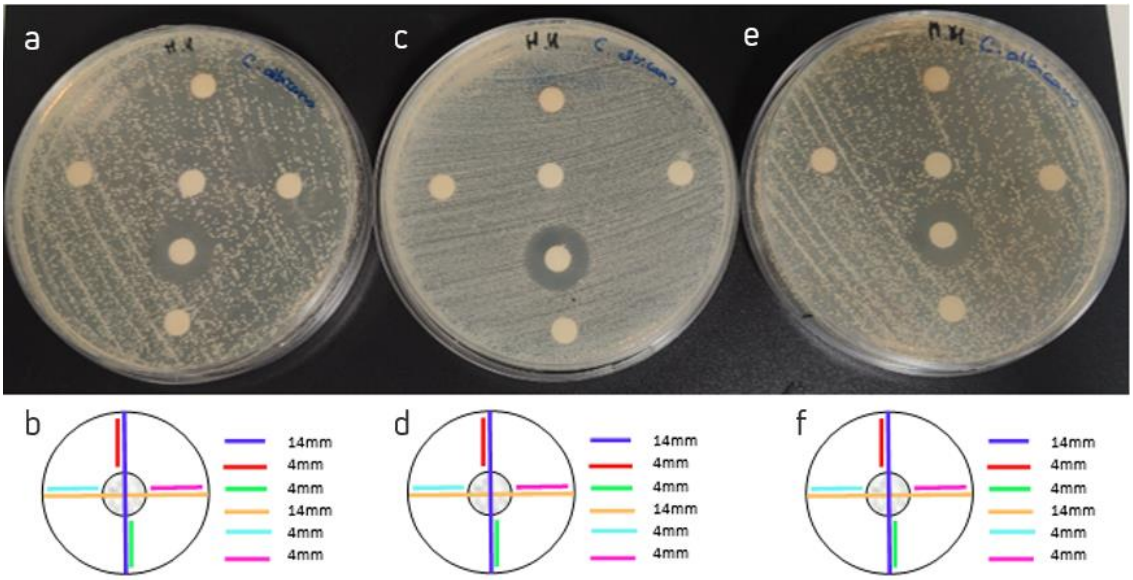

Figure 9 - Antimicrobial properties of different volumes of L-PRF exudate in Mueller-Hinton agar against Candida albicans in a,b and c. L-PRF exudate didn't have an antimicrobial effect. Representation of the inhibition halo obtained by chlorhexidine with different measurements in b,d,f. Image matching: a-b; c-d; e-f.

Table 2 - Zone of inhibition of L-PRF exudate in millimetres.

Sample	***E. coli***					
	L-PRF exudate				C+	C-
	20µl	10 µl	5 µl	2.5 µl		
1	0	0	0	0	16	0
2	0	0	0	0	14	0
3	0	0	0	0	14	0
Mean	0	0	0	0	+/-14.6	0
Standard Deviation	0	0	0	0	0.889	0
Sample	***Candida albicans***					
	L-PRF exudate				C+	C-
	20µl	10 µl	5 µl	2.5 µl		
1	0	0	0	0	14	0
2	0	0	0	0	14	0
3	0	0	0	0	14	0
Mean	0	0	0	0	14	0
Standard Deviation	0	0	0	0	0	0

4.3. Agar diffusion test (L-PRF membrane)

- *Escherichia coli*

The test was conducted on *Escherichia coli* with membranes derived from two volunteers (membrane 1 and membrane 2). The results demonstrated that membrane 1 exhibited an inhibiting halo of 11mm, whereas membrane 2 exhibited no significant inhibition. It is noteworthy that most of the inhibition was observed in the head region of the membrane, which corresponds to the portion of the membrane in direct contact with the red cells (Figure 10).

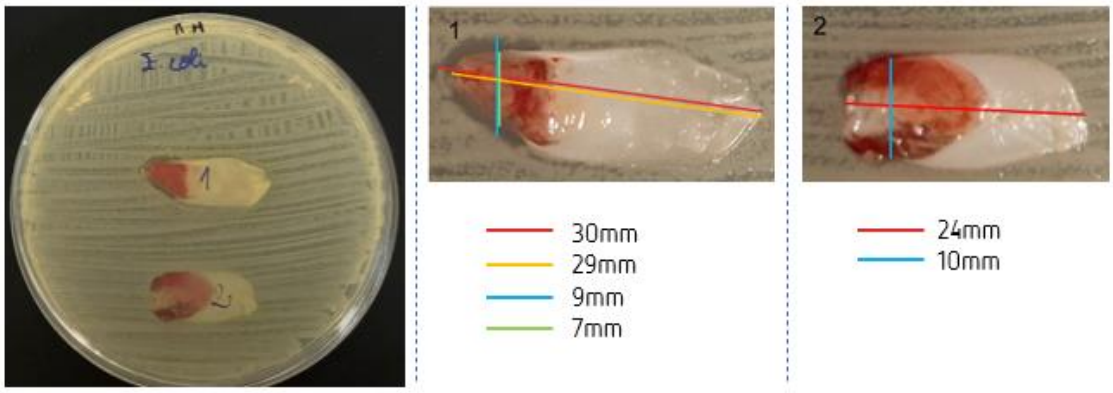

Figure 11 - Antibacterial halos produced by L-PRF membranes on Escherichia coli. Membrane 1 inhibited the bacteria with a halo of 9mm in the head region, in contrast to the second membrane which didn't inhibit Escherichia coli.

- *Candida albicans*

Three membranes from the first subject were tested in three different Petri dishes against *Candida albicans*. Following inoculation, inhibition zones of 11mm, 11mm and 9mm were obtained vertically on the head portion. In Figure 10C&D, a more uniform area of inhibition can be observed, which can be attributed to the dragging of the membrane when it was placed in the culture medium. There is no clear distinction between the head and the rest of the membrane, in contrast to the other two images (Figure 11).

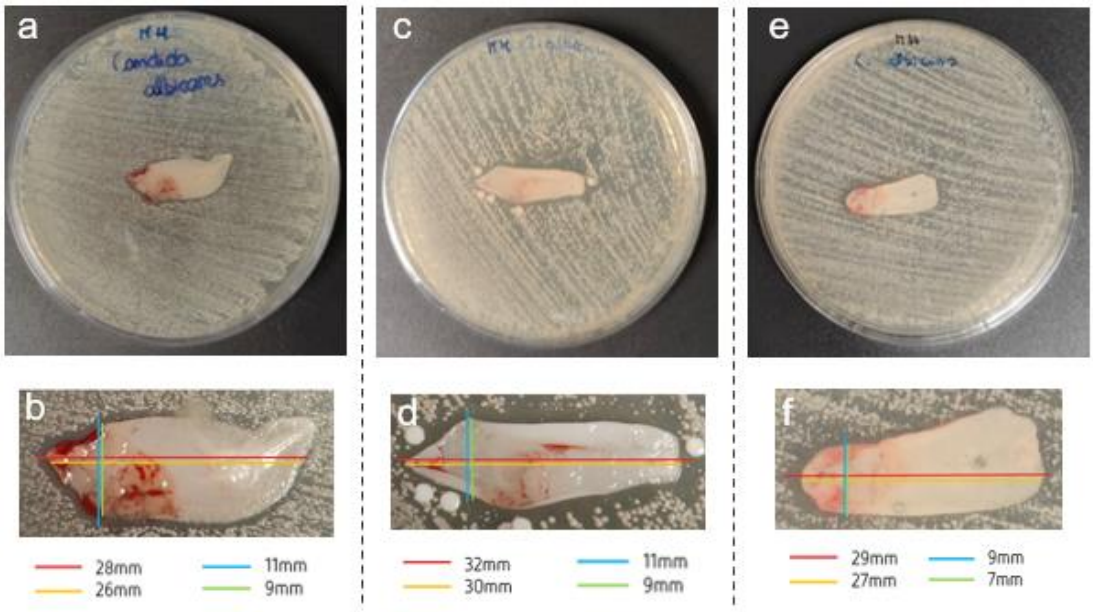

Figure 12 - Antifungal halos of L-PRF membranes on Candida albicans. Different measurements on the 3 membranes. Measurements on the head section a&b - 11mm; c&d - 11mm; e&f - 9mm.

5. DISCUSSION

The oral cavity harbours a plethora of pathogens that can colonise the wound site even after cleansing and lead to infection. Hence, the important role of antimicrobial materials so that the recovery goes smoothly.(10) L-PRF is formed by cellular components of the blood proven to a certain extent to enhance wound healing, help in the regeneration of soft and hard tissue and decrease the state of inflammation. It was then hypothesised that the possibility of an antimicrobial effect was not so far from the truth. A set of molecules, including growth factors such as PDGF, TGF-β, VEGF and IGF, and cytokines like IL-1, IL-4, IL-6 and TNF-α have been studied and appear to protect the surgical site and contribute to the inhibition of certain microorganisms.(4,6,11,12) As a result of a low speed during centrifugation, L-PRF generally has a strong polymerized fibrin matrix that is able to preserve its architecture, not dissolving as easily as platelet rich plasma (PRP), a first-generation platelet concentrate. Therefore L-PRF cells can persist for longer periods of time, releasing the bioactive products for over 7 days.(12)
Furthermore, the mechanical properties of the membrane can be enhanced when pressed.(13)

A study evaluating the antimicrobial efficacy of Platelet-Rich Fibrin (PRF) and Platelet-Rich Fibrin Matrix (PRFM) against various pathogens isolated from root canals found that PRF exhibited a superior inhibitory performance. This difference is likely due to the absence of leukocytes in PRFM, highlighting the essential role of leukocytes in pathogen inhibition and their intrinsic function in the immune response. The study concluded that PRF effectively inhibited bacterial growth but did not suppress fungal biofilm formation.(4,14) Conversely, *Feng et al.* evaluated the inhibitory effect of L-PRF in comparison with H-PRF against *Escherichia coli*. H-PRF exhibited enhanced antimicrobial capacity, which was attributed to the large amounts of leucocytes in the exudate, as PRF solid was less efficient than wet PRF in terms of antimicrobial properties. These results illustrate that the combination of both the membrane and exudate may be more beneficial than the use of only one of the

products. Nevertheless, L-PRF membrane and exudate inhibited *Escherichia coli* and *Staphylococcus aureus*, despite the L-PRF products being obtained through a different protocol from the one used in this study. Both L-PRF and H-PRF exhibited better antimicrobial activity against *Escherichia coli* compared to *Staphylococcus aureus,* demonstrating that the characteristics inherent to the pathogens are important in evaluating the antimicrobial efficacy of PCs.(15) In our investigation, the L-PRF membrane demonstrated inhibitory activity against the study microorganisms, in contrast to the L-PRF exudate, which didn't portray any inhibition. This discrepancy can be attributed to a reduced volume of cellular components in the exudate compared to the membrane.(6,15) Nonetheless, L-PRF exudate had an inhibitory performance against *Porphyromonas gingivalis* according to *Rodríguez Sánchez et al*, who believe that L-PRF exudate releases peroxide and peptides that explain the antibacterial effect previously mentioned.(16)

Our results illustrate a more pronounced inhibition in the head portion of the membrane, as this is where the majority of platelets and leukocytes are concentrated. These results are supported by *Andrade Aldana et al.*, who evaluated the distribution of platelets and leucocytes through three parts of a L-PRF membrane (face, body, and tail), obtained by the same centrifugation system as the one used in this investigation. The investigation piece suggests a decreased of the cellular components towards the tail.(17) In contrast, the work by Feng and associates tested the antimicrobial performance of five portions of the same L-PRF membrane. The portion more effective regarding an inhibitory performance on *Escherichia coli* was L2, which does not correspond to the head of the membrane and is therefore inconsistent with our results.(15) This discrepancy can be explained by the different protocols used to produce L-PRF. The structure and the cells distribution of the PCs will vary, and so will the results, depending on the centrifugation time and the equipment used to produce the PCs.(11)

It is of interest to consider the different aspects of each part of the membrane from a clinical perspective, as each portion can be used for different outcomes, depending on its characteristics. This concept has been carried through in a different PC, named Platelet Rich in Growth Factors (PRGF). PRGF is divided into two distinct fractions: a) PRGF I, which exhibits a lower concentration of platelets and is employed as a membrane for socket

coverage; and b) PRFGII, which exhibits a higher proportion of platelets and growth factors and is implemented to fill the extraction socket following tooth extraction.(10)
A L-PRF membrane was tested on the rough surfaces of contaminated implants, resulting in a significant reduction in the number of bacteria. Approximately 43% of the bacteria appeared to be perforated by the activity of the platelets present in the membrane. Although the article does not specify which bacteria were eliminated, this finding is relevant to this study as *Escherichia coli* can be present in cases of peri-implantitis.(7,18)

There is evidence to suggest that exists an interpersonal variability, particularly regarding the membranes tested on *Escherichia coli*. Only the membrane of one volunteer inhibited bacterial growth on the same Petri dish under the same conditions, whereas the membrane of the other volunteer did not. The age of the subjects may be a significant factor, as stated by *Mamajiwala et al.* The membrane obtained from the blood of a group of people aged 20-34 was found to be more efficient than that of the group aged 50-65. (19) However, this cannot be directly compared with our results, since both volunteers were in the same age group.

While both participants in the study are females, emerging evidence suggests an influence relating to the gender in the biological characteristics of platelet concentrate. In a study conducted by Andrade Aldana and colleagues, analysis of peripheral blood parameters revealed notable differences. While concentrations of platelet, leukocyte, and growth factor were similar between genders, females exhibited significantly higher levels of fibrinogen and BMP-9. Fibrinogen plays a crucial role in the structure, fibrinolysis resistance, and formation time of fibrin clots. Additionally, platelet activation varies among donors. (17) Melo-Ferraz and colleagues employed flow cytometry to investigate platelet activation expression, revealing induced expression of GPIIbIIIa and P-selectin, as well as increased intracellular Ca^{++} mobilization by L-PRF exudate, with activation levels varying among volunteers.(1) These findings underscore the presence of interindividual variability in antimicrobial activity, suggesting that individual characteristics play a role in determining antimicrobial efficacy.

In relation to the broth microdilution test, the results were found to be inconsistent with expectations for *Candida albicans*. In contrast to our predictions, the first two wells of the 96-well plate, corresponding to the two highest concentrations of exudate, exhibited the least degree of inhibition. Conversely, the greatest growth was observed in the first two wells, while the remaining wells demonstrated some turbidity and precipitation, yet did not allow for such accentuated growth. It is known that *Candida albicans* can change its morphology to yeast, pseudo-hyphae and true hyphae depending on its environment. It would therefore be of great interest in future studies to assess whether there is a morphological change in *Candida albicans* after its interaction with the L-PRF exudate, which would have implications for our understanding of the pathological effects of this interaction.(20) This occurrence has been documented by *Castro et al.*, which assessed the antimicrobial properties of L-PRF exudate against *Aggregatibacter actinomycetemcomitans*. The study found an enhanced growth *of A. actinomycetemcomitans* in the presence of the exudate. The researchers suggested that this might be due to the bacteria's potential leucotoxic activity, stimulated by human serum.(6) Reports affirm the possible decrease of platelet aggregation when platelets interact with Candida albicans. These findings suggest the presence of a certain degree of resistance exhibited by the yeast towards platelets.(21)

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that inhibits the visible growth of the tested microorganisms. Although we were unable to assess the MIC for *Candida albicans* and *Escherichia coli* in our study, *Castro et al.* successfully determined the MIC achieved with L-PRF exudate for ATCC strains of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* using the microdilution method.(6)

In dentistry, antibiotic therapy is commonly used to prevent postoperative complications. An innovative method involves using platelet concentrates as carriers for antibiotics to reduce antimicrobial resistance, as well as adverse reactions, by ensuring slow release and findings by *Ozcan et al.* revealed that adding penicillin before PRF centrifugation did not affect the release of essential growth factors for tissue repair. The method significantly

inhibited *Escherichia coli* and Staphylococcus aureus, unlike the systemic antibiotic form.(22)

In a further study, *Straub et al.* tested PRF membranes against several bacteria in patients receiving antibiotic prophylaxis. They found that PRF combined with amoxicillin/clavulanic acid (AMC) was ineffective against *Escherichia coli* but inhibited other microorganisms. However, PRF with intravenous ampicillin/sulbactam (SAM) showed an 11.9mm inhibition zone for *Escherichia coli*, attributed to higher bioavailability. The greatest inhibition was observed for *Streptococcus pneumoniae*, with the control (PRF) enabling *Escherichia coli*. *Bennardo et al.* successfully evaluated the incorporation of vancomycin, gentamicin, and linezolid into tubes for the centrifugation of L-PRF. The results denote that the addiction of vancomycin altered the formation of the PRF membranes, and therefore was excluded from the trial. The linezolid+L-PRF membrane was not very effective against *Escherichia coli* and *Staphylococcus aureus*. In contrast, the gentamicin+L-PRF membrane demonstrated superior antimicrobial activity. Despite the varioations, L-PRF alone exhibited minimal antimicrobial properties.(23)

The addition of certain metals, such as silver, can promote the inhibition of some microorganisms. Nanosilver particles were tested as an enhancer of the antimicrobial properties of PRP and PRF on *Enterococcus faecalis* and *Candida albicans* in *Zafar et al.* article. It has been concluded that the addiction of PRF with nanosilver achieved the greatest inhibition on the growth of *Enterococcus faecalis* followed by PRP with nanosilver, simple PRF and PRP being the most inefficiently, in that order. Concerning *Candida albicans*. neither one of the products had a significant antimicrobial performance.(2) In *Khorshidi et al.* article, L-PRF membrane associated with nanosilver particles had a better inhibitory performance than L-PRF membrane against *Streptococcus viridans* and *Klebsiella pneumoniae.* The stiffness, tensile strength and toughness also seem to be improved by the complementarian nanosilver particles in L-PRF. The association also seem to alter the distribution of leucocytes through the membrane, being more concentrated in the outer layer of the membrane.(13)

Study limitations

The use of ATCC strains in place of clinical case strains does not allow for the evaluation of the true variables associated with each microorganism. Moreover, the study population is relatively small, and there is a scarcity of studies addressing the specific pathogens and themes under consideration.

6. CONCLUSION

The membranes performed an inhibitory effect upon the study microorganisms' results, which concur with other studies on the matter and, therefore the null hypotheses were rejected regarding the L-PRF membranes.

L-PRF exudate had no inhibitory effect on the microorganisms studied, and obtained results that differed from what was expected. The null hypotheses respecting the exudate could not be rejected.

In addition to the inherent antimicrobial properties of L-PRF, it also appears to be a vehicle for delivering antibiotics directly to the surgical wound.

Further research should investigate the antimicrobial activity of L-PRF in a larger study population with clinically isolated strains. The influence of intrapersonal characteristics should also be investigated.

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8. ANNEXES:

8.1. Approval report

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, Crl, no dia 17/06/2021.

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

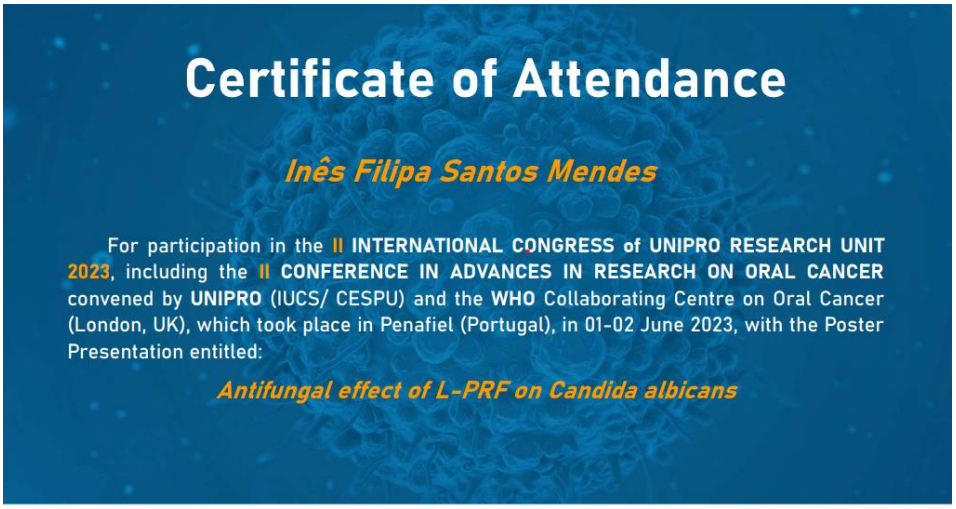
Com os melhores cumprimentos,

CESPU
INSTITUTO UNIVERSITÁRIO
DE CIÊNCIAS DA SAÚDE
Rua Central de Gandra, 1317
4585-116 GANDRA PRD • Portugal
T. +351 224157100 • F. +351 224157 [illegible]
www.cespu.pt

Prof. Doutor José Carlos Márcia Andrade
Presidente da Comissão de Ética do IUCS

8.2. XXXI Jornadas de Medicina Dentária do IUCS/CESPU

8.3. II UNIPRO International Congress 2023



UNIPRO

CESPU INSTITUTO UNIVERSITÁRIO DE CIÊNCIAS DA SAÚDE

Gandra, 01 June 2023

Luís Monteiro

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

8.4. XXXIII Jornadas de Medicina Dentária do IUCS/CESPU

8.5. II UNIPRO International Congress 2023

P 11

Poster 11

Antifungal effect of L-PRF on *Candida albicans*

Inês Mendes [1,ǂ], Laura Silva [1,ǂ] , Ana Francisca Mendes [1,ǂ], Paulo Miller [1,2,ǂ,*], Cristina Coelho [1,2,ǂ], Maria do Céu Monteiro [3,ǂ], Maria Begoña Criado [3,ǂ] and António Melo-Ferraz [1,2,ǂ]

[1] Department of Dental Sciences, University Institute of Health Sciences – CESPU (IUCS-CESPU), 4585-116 Gandra, PRD, Portugal
[2] UNIPRO – Oral Pathology and Rehabilitation Research Unit, University Institute of Health Sciences (IUCS), CESPU, CRL, 4585-116 Gandra, Portugal
[3] TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, 4585-116 Gandra, Portugal
[ǂ] these authors contributed equally to this work

* Correspondence: paulo.miller@iucs.cespu.pt

Abstract

Background: *Candida albicans* is one of the main microorganisms associated with periodontal and endodontic diseases [1]. It is an opportunistic commensal fungus that, in the oral cavity, causes oral candidiasis [2]. Platelet concentrates (PCs) have gained fame in recent years owing to their tissue-regenerating effect and antimicrobial properties [3]. Thus, there are several derivatives, including Leucocyte and Platelet Rich Fibrin (L-PRF), a second-generation platelet concentrate, which differs from the rest due to the presence of a high concentration of leukocytes that degranulate and release cytokines during blood clot formation, promoting protection against inflammation [1,4,5]. **Objective:** To inquire the inhibitory effect of L-PRF on the *Candida albicans* fungus. **Methods:** A search was carried out in PubMed with the expression "Candida albicans (AND) L-PRF". The search covered articles published in English between 2013 and 2022. A total of 4 articles was obtained, from which 2 were used. 1 Google Scholar article was added, as well as 2 articles cited by Melo-Ferraz *et al.*. **Results:** In an *in vitro* study carried out by Melo-Ferraz and associates, the antifungal effect of the L-PRF membrane was tested against strains of the *Candida albicans* fungus by the Kirby-Bauer method on Muller-Hinton agar. An inhibition zone between 12–13 mm was detected [1]. **Conclusions:** An antimicrobial effect by L-PRF was observed on *Candida albicans* and other microorganisms, thus demonstrating the versatility of this compound. More studies must be conducted to confirm its antimicrobial effect, mainly studies with strains isolated from clinical cases.

Keywords: *Candida albicans*; L-PRF

8.6. Bibliographic study results

*Study results of the discussion references. Only studies in vitro were described. *NM- not mentioned*

Author / Year	Title	Aim	Patient nº / age / gender	Intervention type	Microorganisms	Outcomes
Khorshidi et al. 2018 (13)	Does Adding Silver Nanoparticles to Leukocyte- and Platelet-Rich Fibrin Improve Its Properties?	Compare the chances on antibacterial, mechanical, and histologic properties in L-PRF membrane after the adding silver nanoparticles (SNP).	**Patients** : 10 **Age:** 25-35 **Gender:** male	**L-PRF membrane:**9ml blood, 2700 rpm, 12min. **L-PRF membrane + SNP:**nanosilver +saline sonicated at 200, 2min. Addiction to 9ml blood, centrigufated at 2700 rpm, 12 min.	Microflora of saliva – found pathogens: *S. viridans* and *K. pneumoniae*	L-PRF +SNP improves the mechanical properties and antibacterial activity of the L-PRF.
Nagaraja et al. 2019 (3)	Study of antibacterial and antifungal efficacy of platelet-rich fibrin and platelet-rich fibrin matrix.	Assess the antibacterial and antifungal property of platelet-rich fibrin (PRF) and PRF matrix (PRFM).	**Patients** : 6 **Age**: 25-45 **Gender:** *NM	**PRF:** 5ml blood, centrifugated at 400g, 15min **PRFM:**5ml blood. 1st centrifugation 1100g, 6min. 2nd 4500g, 25min	Root canal isolates: Bacteria and Fungi	PRF showed antibacterial property, however, did not perform well against *C. albicans.* PRFM did not show any antimicrobial properties.
Castro et al. 2019 (6)	Antimicrobial capacity of Leucocyte- and Platelet Rich Fibrin against periodontal pathogens.	Evaluate the antimicrobial properties of L-PRF membrane and exudate on periopathogens.	**Patients** :9 **Age:** *NM **Gender:** *NM	**L-PRF membrane and exudate:** 9ml blood, centrifugation 408g, 12 min(Intraspin). Compressed for 1min.	ATCC strains: *P. gingivalis, P. intermedia, F. nucleatum, A. actinomyceremcomitans*	L-PRF inhibited all microorganisms. L-PRF exudate only inhibited *P. gingivalis* on agar plates.Increase growth of *A.a* in the exudate presence.
Puidokas et al. 2019 (10)	Comparative Analysis of Blood Clot, Plasma Rich in Growth	Find if platelet concentrates can be related to	**Patients** : 45 **Age:** 20-24	**PRF:**10ml blood, centrifugated, 1300rpm, 8min.	ATCCS strains: *Staphylococcus aureus*, *Streptococcus pyogenes*,	*S. pyogenes* was the most active in all groups after 3h. The other

	Factors and Platelet-Rich Fibrin Resistance to Bacteria-Induced Fibrinolysis.	resistance to bacteria-induced fibrinolysis.	**Gender:** *NM	**PRGF I&II:**0.2ml sodium citrate+ blood, centrifugated 580g, 8min.	*Streptococcus pneumonia*, *Bacillus cereus*, *Candida albicans*	microorganisms were active in PRF and PRGF II.
Feng et al. 2020 (15)	Antibacterial effects of platelet-rich fibrin produced by horizontal centrifugation.	Compare the antimicrobial properties of PRFs (L-PRF and H-PRF) and correlate with immune cells.	**Patients**:8 **Age:** average of 25 **Gender:** 3 males 5 females	**L-PRF membrane and exudate:**10ml blood, fixed-angle centrifugated 700g,12min **H-PRF membrane and exudate:**10ml blood, horizontal centrifugation 700g, 8min.	ATCC *E. coli*, MG *S. aureus*	Both had notable antimicrobial activities. H-PRF had significantly better performance and more leukocytes. Solid PRF was less efficient than wet PRF.
Mamajiwala et al. 2020 (19)	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.	Evaluate the variations on PRF architecture regarding the centrifugation and different age groups.	**Patients**: 60 **Age:** 20-34, 35-49, 50-65 **Gender:** *NM	**PRF of people in different age groups.** **PRF obtained at different centrifugation speeds:** 3 membranes at 1400, 2800, 3500 rpm, 8min; 3 membranes at 1400, 2800, 3500 rpm, 15min.	Plaque sample from patients.	The patients age and the PRF preparation method influence the PRF properties. The age group 20-34 obtained the best results. PRF membranes were denser when produced at 3500 rpm for 15 min.
Melo-Ferraz et al. 2021 (1)	Platelet activation and antimicrobial activity of L-PRF: a preliminary study.	Analyse the effects of L-PRF on platelet activation, platelet-leukocytes interactions, and antimicrobial activity.	**Patients**: 3 **Age:** *NM **Gender:** *NM	**L-PRF membrane and exudate:** 9ml blood, centrifugated at 2700 rpm, 12min (Intraspin). Compressed with Xpresson Box.	ATCCS strains: *C. albicans, E. faecalis*, *P. aeruginosa*	L-PRF membrane Inhibited the pathogens. L-PRF exudate increased GPIIbIIIa and P-selectin, intracellular Ca^{++} mobilization.
Singh et al. 2021 (14)	Antibacterial and Antifungal Efficacy of Platelet-Rich Fibrin and Platelet-Rich Fibrin Matrix against Root	Evaluate the antimicrobial and atifungal effects of PRF and PRFM on root canal microflora.	**Patients**: 20 **Age**: 20-40	**PRF:**5ml blood **PRFM:**5ml blood Protocol: *NM	Isolates from root canals	PRF had an antibacterial effect but had no antifungal effect. Metaplex had the greatest inhibion of

	Canal Microflora.					the isolates, followed by PRF and then PRFM.
Rodríguez Sánchez et al. 2021 (16)	Antimicrobial Mechanisms of Leucocyte- and Platelet Rich Fibrin Exudate Against Planktonic Porphyromonas gingivalis and Within Multi-Species Biofilm: A Pilot Study.	Evaluate the existence of hydrogen peroxide and antimicrobial peptides release from L-PRF exudate and its effects on *P. gingivalis*.	**Patients**: 1 **Age**:27 **Gender:** Male	**L-PRF exudate:**9ml blood, centrifugated at 408g, 12min. (IntraSpin). Compressed gently.	*P. gingivalis*	L-PRF exudate exhibited an inhibition on *P. gingivalis* in a multi-species biofilme.
Schuldt et al. 2021 (18)	Decontamination of rough implant surfaces colonized by multispecies oral biofilm by application of leukocyte- and platelet-rich fibrin.	Test L-PRF as a disinfectant for rough implant surfaces.	**Patients**: 12 **Age:** mean 33.81 **Gender:** 4 males 8 females	**L-PRF membranes:** blood centrifugated at: 2700rpm,2min; 2400rpm, 4min; 2700rpm,4min; 3000rpm,3min. Compressed for 5min.	Subgingival dental plaque.	L-PRF reduced bacterial numbers on contaminated implants. Platelets were identified and bacteria appeared perforated.
Andrade Aldana et al. 2022 (17)	The impact of gender and peripheral blood parameters on the characteristics of L-PRF membranes.	Describe 3 different zones of a L-PRF membrane and evaluate the impact of gender on its characteristics.	**Patients**: 20 **Age:** mean-29.6±1.8 **Gender:** 10 males 10 females	**L-PRF membrane:** 9ml blood, centrifugated 408g, 12min (IntraSpin). Compressed for 5min.	N/A	Woman released higher levels of BMP-9 and a higher fibrogen level. The face of the membrane it's the richest in cellular components.
Ozcan et al. 2023 (22)	The impact of local and systemic penicillin on antimicrobial properties and growth factor release in platelet-rich fibrin: In vitro study.	Evaluate the antimicrobial properties and growth factors of PRF with the addiction of local and systemic penicillin.	**Patients**: 12 **Age:** 22-26 **Gender:** 6 male 6 female	**PRF:** 7ml blood, centrifugated at 2700, 12min **LAB-PRF:**addiction 0.2ml to the blood.Same protocol above. **SAB-PRF:**2g penicillin orally. 1h after 7ml blood colection. Same protocol above.	*Escherichia coli* and *Staphylococcus aureus*	There wasn't a statistically difference between PRF+ systemic antibiotics and just PRF. Antibiotic addiction before centrifugation was effective without affecting growth factors.
Bennardo et al.	Can platelet-rich fibrin act	Evaluate the role of	**Patients**: 6	**PRF:**9ml blood,	*Escherichia coli*, *Pseudomonas*	Vancomycin was extracted

2023 (23)	as a natural carrier for antibiotics delivery? A proof-of-concept study for oral surgical procedures.	platelet-rich fibrin (PRF) as a natural carrier for antibiotics delivery.	**Age:** >18 **Gender:** *NM	2700rpm, 12min (IntraSpin). **PRF+Vancomycin OR PRF+Linezolid OR PRF+Gentamicin:** Antibiotics were added before centrifugation in different doses. Same protocol above.	*aeruginosa*, *Streptococcus mitis*, *Haemophilus infuenzae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*.	from the study because caused alteration on PRF formation. PRF had antimicrobial effect on all microorganisms. Linezolid-PRF had comparable results with PRF in certain cases. Gentamic-PRF had a massive antimicrobial properties.
Straub et al. 2024 (9)	Impact of aminopenicillin administration routes on antimicrobial effects of platelet-rich fibrin: An in-vitro investigation.	Evaluate the different aminopenicillin administration routes on antimicrobial properties of PRF.	**Patients**: 34 **Age:** 59 (±22) **Gender:** 25 male 9 female	**PRF:**blood centrifugated at 2300rpm, 12min. **PRF+AMC single dose or double dose:**blood collection after 1h. Protocol above. **PRF+SAM:** Blood collection after 15min. Protocol above.	*E. coli, S. aureus, S. pneumoniae, H. Influenzae, P. gingivalis*	Different doses and the type of drug administration were significant.