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Research Article

Antibacterial effect of Leucocyte and Platelet Rich-Fibrin against *Klebsiella pneumoniae* and *Staphylococcus aureus* – A pilot study

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Abstract: Leukocyte and platelet-rich fibrin (L-PRF) is a platelet concentrate used in diverse areas of tissue healing and regeneration. Thus, its antibacterial properties are promising. In this study, we evaluated the antibacterial effect of L-PRF, in its exudate and membrane forms, against oral cavity opportunistic bacteria *Klebsiella pneumoniae* and *Staphylococcus aureus*. Blood samples were collected from three healthy donors. Blood was centrifuged at 2,700 rpm for 12 minutes. This resulted in an L-PRF membrane, and then, from its compression, an L-PRF exudate. These two forms of L-PRF were placed on Mueller-Hinton Agar previously inoculated with the bacteria. The L-PRF membrane was placed directly into the culture medium, and the L-PRF exudate was impregnated in different quantities onto paper disks (Oxoid). The membrane inhibition values varied between 11 and 13 mm for *K. pneumoniae* and 13 mm for *S. aureus*. For the L-PRF exudate, inhibition only occurred for quantities of 5 and 20 µL of exudate in the case of *K. pneumoniae* and for 20 µL of exudate in the case of *S. aureus*. Thus, L-PRF products effectively inhibit bacterial growth, particularly opportunistic bacteria, such as *K. pneumoniae* and *S. aureus*.

Keywords: leukocyte and platelet-rich fibrin (L-PRF); antibacterial effect; platelet concentrate; oral pathogen; *Klebsiella pneumoniae*; *Staphylococcus aureus*

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Introduction

Living human tissue is full of microorganisms, which, in a state of symbiosis, can protect and benefit the host. Bacterial infections are among the most serious complications that negatively affect wound healing and tissue regeneration. They can infiltrate and colonize the tissues underlying the injury, altering the physiology of the healing process [1]. Blood plays a fundamental role in accelerating tissue regeneration by providing the varied cellular and nutritional composition necessary for this process [2,3]. Leukocyte and platelet-rich fibrin (L-PRF) is one of the four main families of platelet concentrates for surgical use and is frequently used in oral and maxillofacial surgery to improve healing and promote tissue regeneration. A bioproduct, autologous and inexpensive, it is obtained by centrifuging blood. After centrifugation, a clot or membrane of L-PRF is obtained (L-PRFm), with most of the platelets and leukocytes present in the initial collection. Platelets are activated and reinforce the synthesized fibrin

matrix. Leukocytes are trapped within the fibrin network and remain viable. L-PRF is the source of a strong and slow release of growth factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and bone morphogenetic proteins (BMPs), trapped within the fibrin gel, or through the production of new molecules by leukocytes in the membrane [4]. The result of compressing the membrane is an exudate (L-PRFe), which is a liquid concentrate with potential antimicrobial activity. L-PRF can be used in its membrane or exudate form, with the membrane currently being the most widely used. Other forms include buffers and liquid formulations, which can be adapted to specific regenerative needs [1,5,6].

Using L-PRFm and different amounts of L-PRFe, we determined whether there was an antimicrobial effect when in contact with certain bacteria [7,8]. Some studies have evaluated the inhibition by this biomaterial. Still, only a few in the world, in Europe and particularly in Portugal, have investigated its antimicrobial effects, mostly against *Staphylococcus aureus* and none against *Klebsiella pneumoniae* [1,6,9]. *Klebsiella pneumoniae* is an opportunistic bacterial species, especially resistant to antimicrobials in hospitalized patients. It is Gram-negative, facultatively anaerobic, and the most important member of the *Klebsiella* genus. However, knowledge about the pathogenicity of this bacterium is still somewhat limited, and, in the last decade, it has become a clinical and public health threat. A parallel phenomenon of serious community-acquired infections caused by "hypervirulent" *K. pneumoniae* has also emerged, which is associated with strains expressing acquired virulence factors. These distinct clinical concerns have stimulated renewed interest in *K. pneumoniae* research [10]. *Staphylococcus aureus* is an opportunistic and versatile Gram-positive coccus of the *Micrococcaceae* family, which is often found among the microbiota of the skin and the nasal passages of healthy people and can cause various diseases, ranging from acute and destructive infections to chronic and difficult-to-treat infections. *S. aureus* colonizes the nasopharynx of many individuals, but this colonization can be the source of infections ranging from mild superficial skin infections to serious diseases, such as pneumonia or osteomyelitis [11]. Furthermore, these microorganisms are an important leading cause of hospital-acquired infections (HAIs) worldwide, pertaining to the ESKAPE pathogens, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. These microorganisms have relevant intrinsic resistance and expansive capacity to acquire multi-drug resistance, and thus they are prioritized by the World Health Organization (WHO) as a global health threat requiring new antibiotic research and development [12]. This study aimed to evaluate the antibacterial effect of L-PRF by laboratory analysis of the inhibitory effect of L-PRFm and L-PRFe in contact with *K. pneumoniae* and *S. aureus*.

Materials and Methods

Ethics approval

This is an *in vitro* study. All the procedures performed in this study were conducted under the Declaration of Helsinki for biomedical research involving human subjects. All volunteers were approached according to a protocol approved by the IUCS-CESPU Ethics Committee (Ref. CE/IUCS/CESPU-14/21). Written informed consent was obtained from each participant before the procedure.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Participants and design of the laboratory *in vitro* study

To carry out laboratory research, blood was collected from three healthy donors in the morning after fasting. These donors had not received any medication, like antibiotics, antiplatelet therapy, corticosteroids, or other anti-inflammatory drugs during the previous 10 days. The first step was to prepare culture media, defrost bacterial strains, culture in Trypticase Soy agar (Thermo Scientific™ Oxoid™, UK), and incubation (Memmert GmbH, Germany) at 35-37 ºC for 18-24h. This was followed by the preparation of bacterial suspensions in ATB medium (BioMérieux, Marcy-l'Étoile, France) according to the 0.5 McFarland scale and inoculation in Mueller-Hinton Agar (Thermo Scientific™ Oxoid™, UK). The L-PRF concentrate was prepared according to a standard protocol approved by the FDA and CE (CE646982) for clinical practice. L-PRFm and L-PRFe were obtained through compression of L-PRF. For the analyses of antimicrobial activity, L-PRFm and L-PRFe were placed in the culture, as shown in Fig. 1. This study was based on a quantitative and qualitative experimental assay.

Bacterial strains

The *in vitro* susceptibility of two different American Type Culture Collection (ATCC, Manassas, VA, USA) strains, *Klebsiella pneumoniae* (ATCC 43816) and *Staphylococcus aureus* (ATCC 25923), was studied using the Kirby-Bauer agar diffusion method. These strains were selected because of their clinical relevance. They are the main microorganisms involved in persistent infections.

L-PRF preparation

To prepare L-PRF, 9 mL of whole blood was drawn into silica-coated plastic vacuum blood collection tubes without anticoagulant. After collection, blood was quickly centrifuged at 2,700 rpm for 12 min (IntraSpin® System-Intra-Lock International, Boca Raton, FL, USA). After the completion of the cycle, the blood in the tube was separated into three distinct layers: platelet-poor plasma at the top, PRF in the middle, and a red blood corpuscular (RBC) base at the bottom. PRF was then carefully retrieved from the tube, and the RBC base was carefully removed to retain a small part of it in the clot. The clot thus obtained was compressed using an Xpression™ Box (IntraSpin® System-Intra-Lock International-Boca Raton, FL, USA) to form L-PRFm and L-PRFe, which were used for further analysis (Fig. 1).

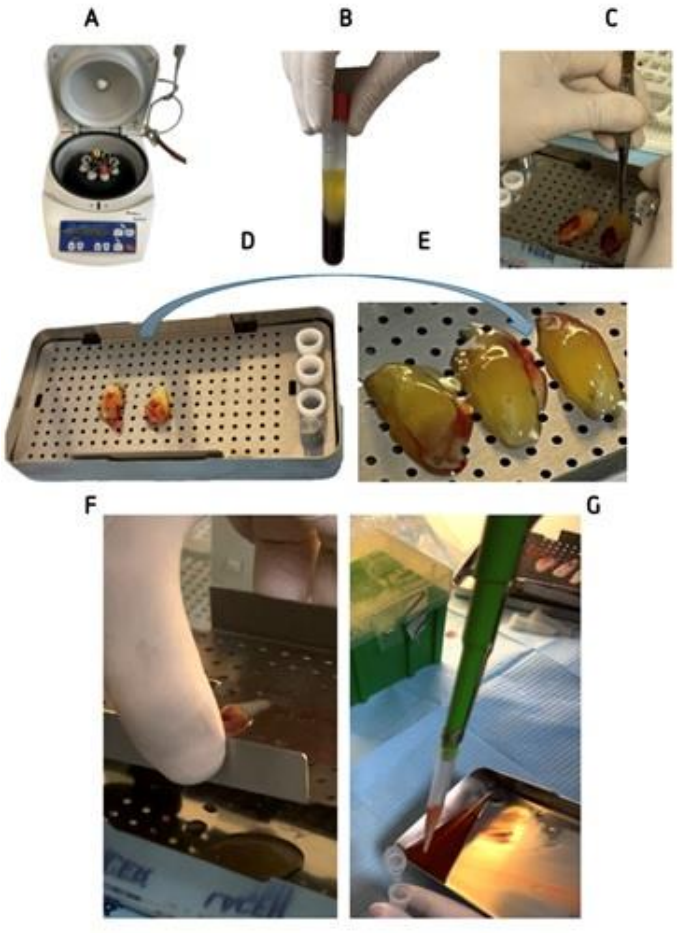

Figure 1. L-PRF preparation. (A) Process centrifuge; (B) Single spin produces three layers: platelet-poor plasma (top), L-PRF (middle), and red blood cells (bottom); (C) L-PRF clots were transferred to a sterile metal surface, and red blood cells were gently scraped away and discarded; (D, E) L-PRF is placed on the grid in the L-PRF box; (F, G) An L-PRF box was used to create the PRF membranes. The serum exudate was collected from the bottom of the box beneath the grid.

Antimicrobial activity

The *in vitro* susceptibility of two different American Type Culture Collection (ATCC) strains, *Klebsiella pneumoniae* (ATCC 43816) and *Staphylococcus aureus* (ATCC 25923), was studied using the Kirby-Bauer agar diffusion method for L-PRFm and the disk diffusion method for L-PRFe.

Results

Antibacterial activity of L-PRFm on *Klebsiella pneumoniae*

Stereomicroscopic images of the inoculated L-PRF membranes are shown in Fig. 2. The results showed an inhibitory action of the L-PRF membrane on *Klebsiella pneumoniae*, with inhibition zones between 13 and 11 mm (Fig. 2a, c, respectively). No inhibition was observed around the membrane in Fig. 2b. In addition to the absence of any kind of inhibition around the membrane, bacterial growth on top of membrane 2 was visible to the naked eye (Fig. 2b). Inhibition varied according to the donor. The inhibition zones were different for each membrane (corresponding to each donor). In membranes with an inhibition

zone, both Fig. 2a and 2c stereomicroscope images, at ×50 magnification, show that the greatest inhibition is in the head zone of the membrane. The results are presented in Table 1.

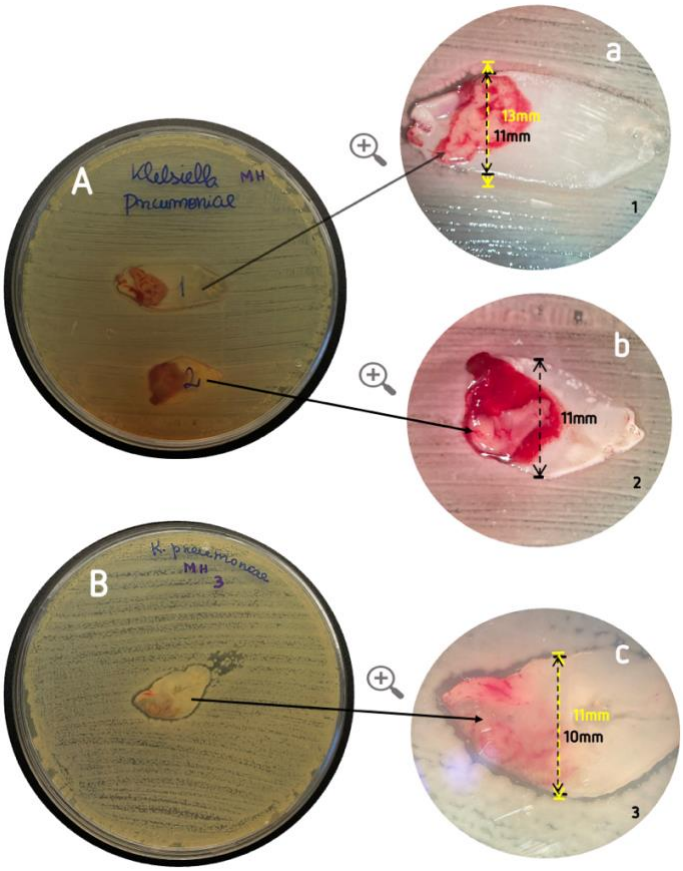

Figure 2. Images of L-PRF membranes at (A, B) ×0 and (a, b, c) ×50 magnifications; numbers 1-3 correspond to membranes from different donors. Measurements were made with Digital Vernier caliper (LIMIT®). (A, B) Plates with L-PRF membranes that had been inoculated on Mueller-Hinton Agar with the *Klebsiella pneumoniae* bacteria; (a, b, c) images of the three L-PRF membranes, taken at 50× magnification.

Table 1. *Klebsiella pneumoniae* inhibition zones, obtained from the membranes of the three different donors.

L-PRF membrane (mm)	Inhibition zone (mm) (measurements obtained vertically between the furthest points formed in the inhibition)
1. 11	13
2. 11	0
3. 10	11

L-PRF: leukocyte and platelet-rich fibrin.

Antibacterial activity of L-PRFm on *Staphylococcus aureus*

Stereomicroscopic images of the inoculated L-PRF membranes are shown in Fig. 3. The results showed an inhibitory action of L-PRFm on *Staphylococcus aureus*, with inhibition zones of 13 mm (Fig. 3a, b). In membranes with an inhibition zone, both Fig. 3a and 3b stereomicroscopic images, at ×50 magnification, show that the greatest inhibition is in the head zone of the membrane. The results are presented in Table 2.

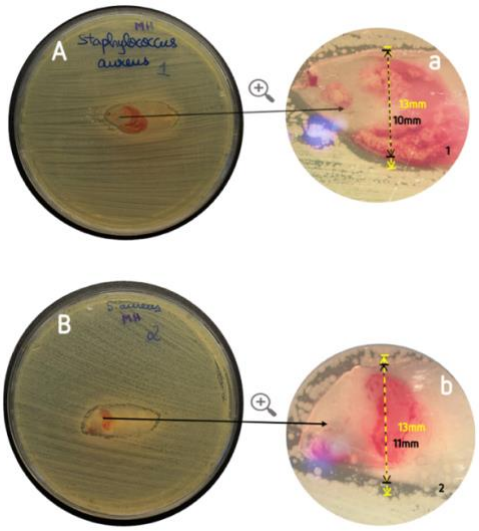

Figure 3. Images of L-PRF membranes at (A, B) ×0 and (a, b) ×50 magnifications; numbers 1-2 correspond to membranes of two of the three different donors. Measurements were made with a Digital Vernier caliper (LIMIT®). (A, B) Plates with L-PRF membranes that had been inoculated on Mueller-Hinton Agar with the *Staphylococcus aureus* bacteria; (a, b) images of the two L-PRF membranes, taken at 50× magnification.

Table 2. *Staphylococcus aureus* inhibition zones, obtained from the membranes of two of the three different donors.

L-PRF membrane (mm)	Inhibition zone (mm) (measurements obtained vertically between the furthest points formed in the inhibition)
1.10	13
2.11	13

L-PRF: leukocyte and platelet-rich fibrin.

Antibacterial activity of L-PRFe on *Klebsiella pneumoniae*

Fig. 4 shows the effect of L-PRFe in contact with *Klebsiella pneumoniae*, according to the cultures taken from different donors (images 1-3). Chlorhexidine (CHX) inhibited all three cultures with inhibition halos of 13 mm. For exudates, only Figs. 4B and 4C showed an inhibitory effect in quantities of 5 µL of exudate, with 7 mm of inhibition, and 20 µL of exudate, with 8 mm. Fig. 4 also includes magnified stereomicroscope images of the inoculated L-PRFe on the disks with an antibacterial effect. The results are presented in Table 3.

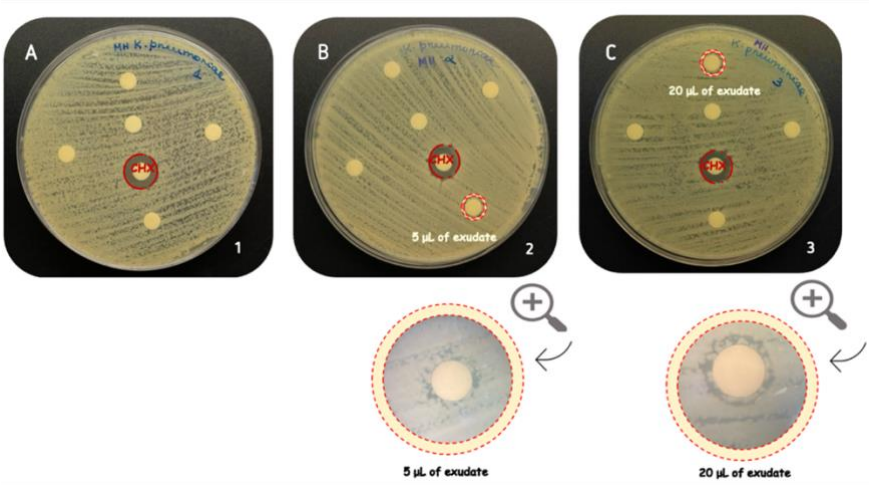

Figure 4. Inhibition by L-PRFe. Images of L-PRF exudate at ×0 and ×50 magnifications; numbers 1-3 correspond to exudates from different donors. (A-C) Images correspond to the triplicate test; measurements were made with a Digital Vernier caliper (LIMIT®).

Table 3. *Klebsiella pneumoniae* inhibition zones, obtained from the exudates of the three different donors.

L-PRF exudate impregnated in the disks	20 µL of exudate (mm)	10 µL of DMSO (C-) (mm)	10 µL of CHX (C+) (mm)	10 µL of exudate (mm)	5 µL of exudate (mm)	2,5 µL of exudate (mm)
1. Inhibition zone	0	0	13	0	0	0
2. Inhibition zone	0	0	13	0	7	0
3. Inhibition zone	8	0	13	0	0	0

C-: negative control; C+: positive control; CHX: chlorhexidine; DMSO: dimethyl sulfoxide; L-PRF: leukocyte and platelet-rich fibrin.

Antibacterial activity of L-PRFe on *Staphylococcus aureus*

Fig. 5 shows the effect of L-PRFe in contact with *Staphylococcus aureus*, according to the cultures taken from different donors (images 1-3). CHX (0.12%) + cetylpyridinium chloride (CPC, 0.05%) inhibited all three cultures with inhibition halos of 19 mm. For L-PRFe, only Fig. 5A and 5C showed an inhibitory effect for 20 µL of exudate, with 7 mm of inhibition in both. Fig. 5 also includes magnified stereomicroscope images of the inoculated L-PRFe on the disks with an antibacterial effect. The results are presented in Table 4.

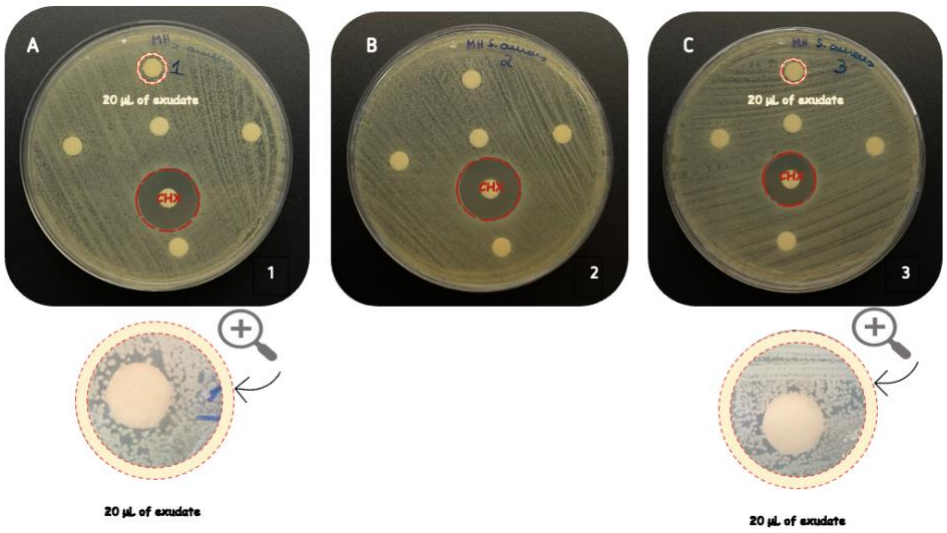

Figure 5. Inhibition by L-PRFe. Images of L-PRF exudate at ×0 and ×50 magnifications; numbers 1-3 correspond to exudates from different donors. (A-C) Images correspond to the triplicate test; measurements were made with a Digital Vernier caliper (LIMIT®).

Table 4. *Staphylococcus aureus* inhibition zones, obtained from the exudates of the three different donors.

L-PRF exudate impregnated in the disks	20 µL of exudate (mm)	10 µL of DMSO (C-) (mm)	10 µL of CHX (C+) (mm)	10 µL of exudate (mm)	5 µL of exudate (mm)	2,5 µL of exudate (mm)
1. Inhibition zone	7	0	19	0	0	0
2. Inhibition zone	0	0	19	0	0	0
3. Inhibition zone	7	0	19	0	0	0

C-: negative control; C+: positive control; CHX: chlorhexidine; DMSO: dimethyl sulfoxide; L-PRF: leukocyte and platelet-rich fibrin.

Discussion

In this *in vitro* study, we analyzed the antibacterial activities of L-PRFm and L-PRFe against pathogenic *K. pneumoniae*, a superbacterium, especially in hospitals, antimicrobial resistant, and *S. aureus*, a bacterium that often causes antibiotic failure. The results of antibacterial activity, evidenced with the aid of stereomicroscopy magnification, supported the hypothesis that both L-PRFm and L-PRFe have an antibacterial effect against *K. pneumoniae* and *S. aureus*. Based on the research carried out leading up to the experimental activity, this is the first study to test the antimicrobial effect of L-PRFm and L-PRFe against *K. pneumoniae*.

This is also the second experimental *in vitro* study to expose *S. aureus* to L-PRF. Feng *et al.* [9] evaluated the inhibitory effect of L-PRF and PRF prepared by horizontal centrifugation (H-PRF) against *S. aureus* for the first time, where the antimicrobial properties of L-PRFe were proven; however, they compared the antibacterial effect of H-PRF and L-PRF. The H-PRF exudates were significantly better than the L-PRF exudates. In agreement, in our study, although the exudate was not obtained by horizontal centrifugation, an inhibitory effect of L-PRFe against *S. aureus* was also proven for exudate amounts corresponding to 20 µL from two different donors. Owing to their ability to release growth factors into cellular tissues, platelet concentrates have been used in different health areas, particularly in dentistry [2]. Therefore, based on the applicability of L-PRF in the craniomaxillofacial region, it is understandable that most of the microorganisms tested are Gram-positive and Gram-negative bacteria from the oral microbiota. These characteristics, such as the fact that *K. pneumoniae* is a Gram-negative bacterium and *S. aureus* is a Gram-positive bacterium, and the corresponding differences in the composition of their cell walls, are important factors in their pathogenicity [13]. Gram-negative bacteria have a thin layer of peptidoglycan and a phospholipid outer membrane with intrinsic and extrinsic proteins, a lipopolysaccharide that participates in the activation of macrophages, B and T lymphocytes, the complement system, the kinin-generating system, the intrinsic coagulation system, and plasminogen [14]. In Gram-positive bacteria, the cell wall has a thick layer of peptidoglycan to which other heteropolymers are firmly attached [15].

The inhibitions in the presence of CHX were variable for *K. pneumoniae* (13 mm) and *S. aureus* (19 mm). In addition, these bacteria are mostly responsible for severe clinical infections [8,10,11,13,15]. For tissue regeneration and healing to take place, various blood elements must be present, such as viable white blood cells, trapped within a high-density fibrin network, which also includes platelets, crucial for cell communication, inflammation, and healing responses; together, they make L-PRF a potentially important element in the clinics [2,3]. Currently, L-PRF is being successfully used as an adjuvant to improve tissue healing; however, its full potential is not fully understood yet. Its versatile and applicable role in different areas is known, but much still needs to be discovered about its antimicrobial power, because *in vitro* studies are based on planktonic bacteria [2,3,16]. For example, in a study carried out by Siawasch *et al.* [1], who evaluated the effect of local and systemic administration of antimicrobials to L-PRF, it was concluded that the administration of antimicrobials such as amoxicillin and metronidazole provided L-PRF membranes with a significantly greater antibacterial capacity against *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*. In this *in vitro* study, the antibacterial effect of L-PRF was determined using two different methods. In our study, the agar diffusion method was used to evaluate the antibacterial activity of L-PRFm from the three different donors.

Regarding the effects on *K. pneumoniae*, two of the donors showed growth inhibition with 11 to 13 mm zones, with the two membranes having initial values, at the time of inoculation, of 10 and 11 mm, respectively. In contrast, one of the donors did not show any inhibitory effect, even showing bacterial growth over the membrane (the initial value of the membrane where no inhibition occurred was 11 mm). An interesting fact to add is that, during the placement of the membrane on the agar surface, due to difficulty in its placement, there was a slight dragging of the membrane along the plate. The results showed that the drag zone, the path that the L-PRFm took until it was stable, inhibited the bacteria. This shows that not only L-PRFm but also the liquid that accompanies it has inhibitory properties. It is not the L-PRFe or the membrane itself but a kind of "serum" that lubricates the L-PRF membrane.

For *S. aureus*, a maximum inhibition of 13 mm was observed. Again, there was variance in the inhibition zone (although the zones of inhibition were both 13 mm, the two membranes had initial values at the time of inoculation of 10 and 11 mm, respectively). Castro *et al.* [17], in their study, demonstrated the antimicrobial activity of L-PRFm against *P. gingivalis* without statistically significant larger inhibition in comparison to other bacteria. In the same way, Melo-Ferraz *et al.* [3] used an L-PRFm to assess antibacterial activity against *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Candida albicans*. L-PRF circular membranes measuring 6 mm were placed on agar plates and showed antibacterial effects, according to results, between 9 and 19 mm, which is a range of inhibition zone values greater than that of our results. Similarly, Feng *et al.* [9] demonstrated the antibacterial effect of L-PRFm based on the resulting inhibition of *S. aureus*.

The disk diffusion method was used to evaluate the antibacterial activity of the L-PRFe. For *K. pneumoniae*, triplicate tests were carried out. There was no inhibition by any disk impregnated with the different amounts of exudate. The disks with 5 and 20 µL of exudate showed inhibitory powers of 7 and 8 mm, respectively. The variation, inhibition, or lack thereof for different or equal amounts of exudate can be explained by the fact that they correspond to different donors. Thus, different amounts of exudate

from different donors will have different inhibitory responses against the same bacteria, which in this case is *K. pneumoniae*. On each plate, the inhibition halo of 10 μL of CHX was 13 mm, higher than the inhibition halo caused by L-PRFe. For *S. aureus*, triplicate tests were performed. In this situation, inhibition occurred for the same quantities of exudate (20 μL). In both cases, the inhibition halo caused by L-PRFe was 7 mm. Again, different amounts of exudate were tested against *S. aureus*, including L-PRFe from the three different donors. On these plates, when 10 μL of CHX were in contact with *S. aureus*, the inhibition halos were larger (19 mm). Therefore, we can hypothesize that there is an inhibitory effect for the exudate [9,16]. The mechanism of action by which the exudate inhibits bacteria when in contact with them is neither described nor known. Whether it works as an antibiotic and destroys the bacterial cell wall or interferes with protein synthesis, among other metabolic processes [16], it has a direct action on their cells, altering their structure or their ability to divide. In the case of *K. pneumoniae*, inhibition halos exist for different amounts of exudate. Even so, considering the triplicates for each bacterium, it is possible to see that, although the inhibition halos caused by L-PRFe are smaller in the case of *K. pneumoniae*, they have great relevance from an inhibitory point of view, since CHX also does not produce inhibition halos greater than 13 mm for this bacterium, and the L-PRFe resulted in halos between 7 mm and 8 mm, approximately two times less. In the case of *S. aureus*, the inhibition halos of L-PRFe were 7 mm, but those of CHX were on average 19 mm, almost three times higher. Rodríguez Sánchez *et al.* [16] evaluated the antimicrobial effect of L-PRFe against planktonic *P. gingivalis* and hypothesized that L-PRF exudate releases hydrogen peroxide and antimicrobial peptides that inhibit *P. gingivalis* growth. Agar plate and planktonic culture experiments showed that the antimicrobial effect of L-PRF exudate against *P. gingivalis* was suppressed by peroxidase or pepsin exposure. Feng *et al.* [9] described the use of a filter paper diffusion method with L-PRFe and demonstrated its antibacterial effect against *S. aureus* and *Escherichia coli*, proving the properties and potential of liquid PRF. With another methodology with the agar diffusion test, Castro *et al.* [17] used L-PRFe to prove the antibacterial effect against pathogens. L-PRFe demonstrated inhibition (mean area of inhibition of 17 ± 2.6 mm^2) only against *P. gingivalis*.

According to the results of the study by Sindhuscha *et al.* [5], who evaluated the antimicrobial effect of injectable platelet-rich fibrin (i-PRF) and L-PRF against oral microbes, where the zone of inhibition of i-PRF was 2.19 mm, the zone of inhibition of L-PRF, as well as that of metronidazole, was 0.14 mm; L-PRF showed an antimicrobial effect equal to that of metronidazole. These values may be related to the lower centrifugation speed, leading to a higher concentration of cells, including leukocytes, in the final platelet concentration before fibrin clot formation.

The methodology of the experimental work and *in vitro* study and its circumstances and limitations are worth mentioning, such as the fact that there are very few studies that have carried out this type of test on the antibacterial potential of L-PRF and, therefore, the lack of supporting references and, consequently, the fact that the best way to work with L-PRFe has not been described; the fact that we only used two bacterial strains, namely planktonic ones, such as *K. pneumoniae* and *S. aureus*; the concentration of exudate we used, since we always worked with quantities and not concentrations, due to failures in previous attempts when we tried to work with minimum inhibitory concentrations (MIC); and the fact that L-PRFe is a product that was obtained from the compression of the fibrin clot and is not actually a serum, whose composition per mL and concentration (as previously mentioned) are known exactly. In addition, L-PRF presents donor-related variability in the work methodology itself, with added variables. When we compare our results with those of Melo-Ferraz *et al.* [3], we realize that there are significant differences in the zones of inhibition caused by the membranes, even considering that different bacteria were involved in this study. The study by Melo-Ferraz *et al.* [3] was carried out before the pandemic, while ours was carried out in a post-pandemic period. This association leads to the hypothesis that SARS-CoV-2 may have altered the human immune response, depressing it at different levels, depending on the individual. This study investigated the antibacterial properties of L-PRF membranes (L-PRFm and L-PRFe) by testing them against the *K. pneumoniae* and *S. aureus* bacterial strains. There was notable variability in the inhibition zones due to donor differences. Specifically, one donor's L-PRF membrane showed no inhibition of *K. pneumoniae*, whereas all membranes inhibited *S. aureus*. This variability is attributed to the differences in bacterial types (Gram-positive vs. Gram-negative) and donor-specific factors. This study highlights the potential of L-PRFm and L-PRFe as autologous alternatives to antibiotics; however, their exact mechanism of action requires further investigation.

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Author Contributions

AFM participated in the study's conception and design, material preparation and data collection, first draft writing, and the manuscript's revision. AMF conceived and designed the work, prepared materials and collected the data, performed analysis and supervision, wrote the first draft of the manuscript, and participated in its revision. CC contributed to the study's conception and design, material preparation and data collection, analysis, supervision, writing the first draft, and manuscript revision. IM and LS

contributed to the study's conception and design, material preparation and data collection, and to the manuscript's revision. MBC, MCM, and PM participated in the study's conception and design, analysis and supervision, and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing interests.

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