

Antimicrobial Effect of Leukocyte- and Platelet-Rich Fibrin Against *Aggregatibacter actinomycetemcomitans* Mono-Multiserotypes Model

Efecto Antimicrobiano de la Fibrina Rica en Plaquetas y Leucocitos Contra *Aggregatibacter actinomycetemcomitans* en un Modelo Mono-Multi Serotipos

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ABSTRACT: Leukocyte- and platelet-rich fibrin is a widely used platelet concentrate for periodontal surgery procedures. Many benefits are described regarding its use, such as antimicrobial properties. The objective of this study was to evaluate the antimicrobial effects of the different zones of this platelet concentrated against the most prevalent serotypes of *Aggregatibacter actinomycetemcomitans* in an *in vitro* mono-multiserotype model. Three patients who were treated at a School of Dentistry in the city where the researchers reside, were included. Modified direct contact method tests and results were analyzed using multivariate logistic regression analysis. In the modified direct contact method test, a decrease in bacterial count was found at time 1, but at time 2, the count increased for all serotypes and zones of L-PRF. It can be noted that the areas with more cellular content in leukocytes and platelet-rich fibrin are the areas with the most antimicrobial power. This platelet concentrate would have better results with serotype c. At time point 2, it is likely to act as a growth promoter of *A. actinomycetemcomitans*.

KEY WORDS: *Aggregatibacter actinomycetemcomitans*, dental implants, infections, platelet-rich fibrin

INTRODUCTION

Platelet concentrates are autologous products obtained from centrifugation of a patient's own blood (Dohan Ehrenfest *et al.*, 2009). In this procedure, the active components of the blood are collected and prepared in a way that is clinically usable, for example, in the form of a solution or gel that can be placed on a surgical site for tissue regeneration purposes (Bielecki *et al.*, 2012; Bielecki & Dohan Ehrenfest, 2012a). Platelet concentrates are classified into P-PRP (pure platelet-rich plasma), L-PRP (leukocytes and platelet-rich plasma), P-PRF (pure platelet-rich fibrin), and L-PRF (leukocytes and platelet-rich fibrin) (Dohan Ehrenfest *et al.*, 2010).

L-PRF has several advantages: it is an inexpensive, simple, and an atraumatic technique (Anwandter *et al.*, 2016) that does not use anticoagulants. Its polymerization is slow and natural, and the fibrin network has a three-dimensional configuration, which allows the retention of cytokines for longer periods of time (7-11 days) and in the form of a membrane with great elasticity and flexibility (Kumar & Shubhashini, 2013; Khiste & Naik Tari, 2013).

L-PRF is attributed to properties that favor the surgical site, such as modulating the inflammatory response, increasing the effectiveness of regeneration,

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angiogenesis, and neovascularization, and decreasing pain and edema postoperatively (Choukroun *et al.*, 2006).

Several studies have analyzed L-PRF by zone, according to its cell concentration (Kobayashi *et al.*, 2012; Bootkrajang *et al.*, 2020; Dias *et al.*, 2020). Different nomenclatures have been used to identify these zones. This study used the nomenclature proposed by Dohan Ehrenfest *et al.* (2018), where the head, body, and tail are used for classification of the different zones. The head is the closest to the red zone, followed by the body, and then the tail.

Within the L-PRF zones, it has been noted that the density and organization of the fibrin network increase as they move away from the red zone. As they approach the red zone, there are more cells. Thus, the tail has higher fibrin organization and density, and the head has more cells (Kobayashi *et al.*; Bootkrajang *et al.*; Dias *et al.*).

Despite the noise caused by platelet concentrates in the overall dental community, there is little evidence of their antimicrobial effect (Moojen *et al.*, 2008).

A common surgery in the dental community, implants, may have early or late infections (Aboltins *et al.*, 2014). From sites where dental implants have failed, we most often find: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Peptostreptococcus micros*, *Campylobacter rectus*, *Fusobacterium spp.* and *Capnocytophaga spp.* among others (Quirynen *et al.*, 2002).

A. actinomycetemcomitans is a Gram-negative, non-motile, facultative anaerobe coccobacilli and capnophilic bacteria (Slots & Ting, 1999; Newman *et al.*, 1976). This bacterium is part of the oral cavity microbiota in healthy individuals (Flores, 2011) and it has been found in 90 % of patients with localized aggressive periodontitis and 30-50 % of patients with severe chronic periodontitis (Raja *et al.*, 2014). Infection by this bacterium leads to an inflammatory immune response, which causes an imbalance in the normal tissues of the periodontium, such as the gum, periodontal ligament, cement and alveolar bone, ultimately causing loss of the tooth piece (Herbert *et al.*, 2016). This bacterium not only affects tooth pieces but can also colonize titanium surfaces, causing peri-implant inflammation and bone loss (Nelson & Thomas,

2010). There are seven serotypes of *A. actinomycetemcomitans* (Yang *et al.*, 2005) but serotypes a, b, and c are the most frequently isolated from the oral cavity (De Bruyn *et al.*, 2013).

It is important then to determine the antibacterial effect of L-PRF and its different zones, which could be used as a protocol for periodontal surgeries and dental implants, to reduce post-surgical infections. Thus, the objective of this study was to evaluate the antibacterial effect of the different areas of L-PRF against the most prevalent serotypes of *A. actinomycetemcomitans* in an *in vitro* mono-multiserotype model.

MATERIAL AND METHOD

Design. This study used a randomized experimental research type. The investigation was carried out with ASA I patients ages 25-45 who were treated at the School of Dentistry in the city where the researchers reside. Patients who had smoked or used antibiotics in the three months prior to the evaluation were excluded from the study. Patients who were not willing or capable of providing written authorization were also excluded. The research work was reviewed by the bioethics committee of the Faculty of Dentistry in the city where the patients resided (N° 30/19) and conducted in compliance with the conventions of the Declaration of Helsinki adopted by the World Medical Association.

Selection and sample size. Volunteers were recruited through convenience sampling. Potential participants were patients of students specializing in periodontics who had scheduled surgeries with the use of L-PRF. Usually, after surgery, some membranes are left over and are discarded. The intention was to use those excess membranes (if any) for the study. Each patient was asked for use of his or her leftover L-PRF membranes. Each participant provided informed consent. It should be noted that no extra punctures were performed, nor was additional blood taken for the study; only L-PRF membranes that were considered for surgery and were not necessary were utilized.

Protocol for obtaining L-PRF. Tubes (Vacuette®, USA) of 9 mL were filled with a patient's blood and without anticoagulants, then centrifuged (EBA-20, Hettich®, Canada). The centrifuge was calibrated to 2,700 revolutions in 12 minutes (Dohan Ehrenfest *et*

al., 2006). The precise amount of blood depended upon what the provider deemed necessary for the patient's surgery.

When centrifugation was finished, the clot was separated from the red elements with an anatomical clamp and a modeling compound spatula, leaving all excess inside the tube. Clots were left in the PRF box to obtain membranes (Figs. 1 and 2) (Dohan Ehrenfest *et al.*, 2010).

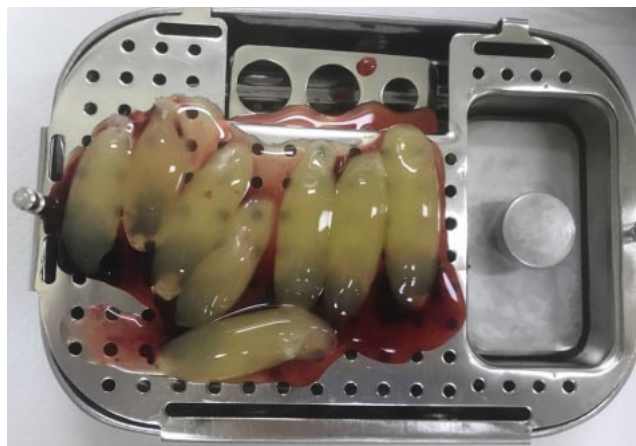


Fig. 1. Clots of L-PRF.



Fig. 2. L-PRF membranes.

Modified direct contact test. The strains of *A. actinomycetemcomitans* American Type Control Culture (ATCC) serotypes a (ATCC® 43717™), b (ATCC® 43718™), and c (ATCC® 43719™) were incubated separately in TSBV agar in anaerobiosis at 37 °C for 48 hours.

After this culture was made, an isolated colony was selected. Then, it was transferred to a tube with 5 mL of liquid brain heart infusion (BHI) medium

supplemented with 10 % equine serum (Oxoid®, Basingstoke, UK) with the help of a sowing handle. This broth was reincubated at a temperature of 37 °C for 48 hours. The turbidity of the inoculum was then adjusted with liquid heart infusion medium to 0.5 on the McFarland scale with the help of an Oxoid turbidometer (Fisher Scientific Company®, Ottawa, Canada) to obtain approximate suspensions of 1×10^8 UFC/mL. Individual suspensions of each serotype and a multispecies suspension were used for the study. After suspensions of the three serotypes were obtained, a multispecies suspension was performed by adding 5 mL of each inoculum to a tube, i.e., serotypes a, b, and c. The tubes were shaken in a Vortex Super Mixer stirrer (Lab-line Instruments, Inc.®, Melrose Park, USA) to achieve homogeneity. Then, the modified direct contact test was performed according to the protocol described by Zhang *et al.* (2009).

With a scalpel handle and leaf number of 15, each membrane was divided into three segments: head, body, and tail. To obtain these segments, each membrane was measured and divided into three equal zones. A 96-well microtiter plate (Sarstedt, Inc.®, Newton, NC) was maintained vertically, and a fixed-size area on the sidewall of the wells was covered with approximately a similar amount of each L-PRF segment, tacking them to the site with an anatomical clamp.

After the mix and L-PRF segments were properly placed, 1- and 24-hour intervals were tested.

To test the antibacterial activity of L-PRF and each specific segment, 10 μ l of the multispecies suspension was placed on each segment for each of the three groups (head, body, and tail). It was also done with 10 μ l of serotype a suspension, serotypes b, and c. In addition, positive and negative monitoring was carried out.

Six groups were held: positive control (multispecies inoculum plus antibiotic), negative control (serotypes a, b, c, and m plus water, without L-PRF), a (inoculum serotype a plus L-PRF), b (inoculum serotype b plus L-PRF), c (inoculum serotype c plus L-PRF), and m (multispecies inoculum plus L-PRF) (Fig. 3).

After incubation in 100 % humidity at 37 °C at time intervals of 1 and 24 hours, 215 μ l of tryptic soy broth (TSB) (Oxoid, Basingstoke, UK) was added to each well. The initial inoculum in all groups was 4×10^6 UFC/mL. After mixing with a micropipette for 1 minute, the bacterial suspension of each well was transferred and diluted 4 times in series in TSBV.

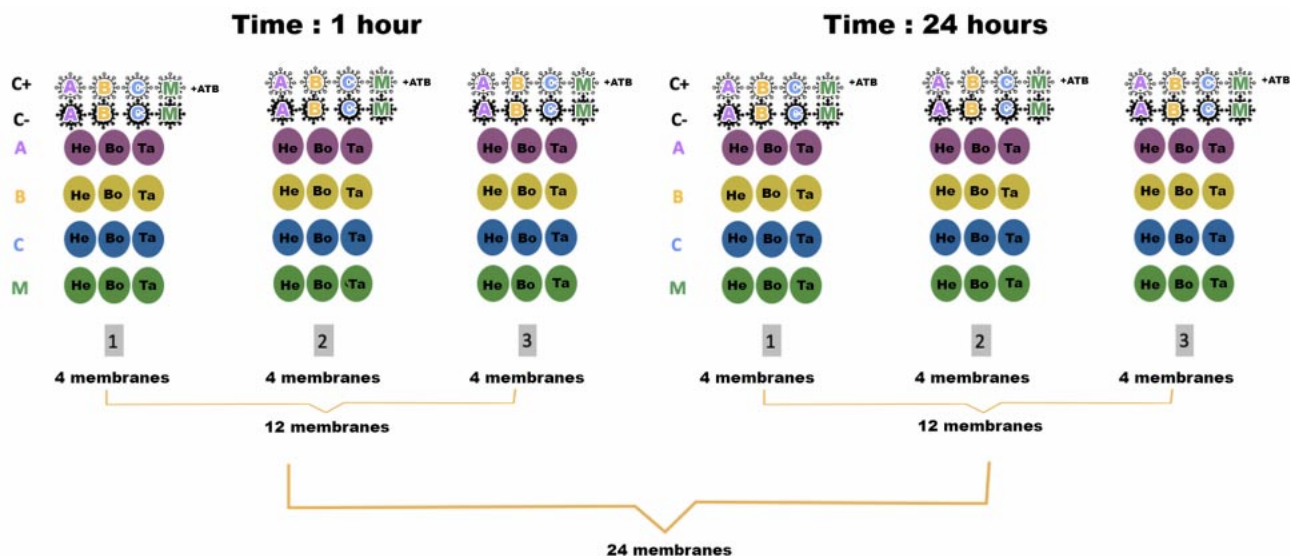


Fig. 3. Modified direct contact test groups.

To evaluate the survival kinetics of bacteria, petri plates with BHA heart brain agar (Oxoid®, Basingstoke, UK) were divided into five parts and plated in each segment, with approximately 3 to 4 drops corresponding to 20 μ L of the undiluted suspension and 4 serial dilutions. It was important that the agar plates were completely dry so that the droplets were absorbed almost instantly. The plates were then incubated at 37 °C for 24 hours, and a colony count was then performed with colony counter equipment (BOECO®, Hamburg, Germany).

Statistical analysis. The study was based on descriptive statistics by presenting central trends and dispersion measures. The data were flushed into an Excel spreadsheet based on logarithms. For analysis of data through tables and graphs, IBM SPSS 25 software was used.

RESULTS

The initial inoculum in all groups was $6,6 \times 10^6$ UFC/mL. At the first time point (1 hour), Fig. 4 (a and c) shows that serotypes a and c decreased their bacterial concentration in all areas. Serotype b, Fig. 4 (b), also shows a decrease in its head and body zones but an increase in its tail zone. For serotype m (Fig. 4 (d)), decreased concentrations in the body and tail and an increase in the head were observed.

For timepoint 2 (24 hours), the concentration increased in all zones (head, body, and tail) for serotypes a, b, c, and m relative to time 1 (Fig. 4). The difference between the unit-forming colonies at the initial inoculum (time 0) and time 1 was statistically significant (Table I).

Table I. Colony forming unit (in Log values) for times 0, 1 (1 hour), y 2 (24 hours).

Time	Mean	Mean Rank	p (ANOVA)
0	6.60	0.54 B	
1	5.15	0.31 A	=0.04
2	5.92	0.31 AB	

DISCUSSION

This study sought to assess the potential for antimicrobial properties that could occur in the different zones of L-PRF. Through the modified direct contact test, the antimicrobial potential of L-PRF and its different zones was evaluated against different strains of *A. actinomycetemcomitans* serotypes a, b, c, and a multispecies serotype (with serotypes a, b, and c). This test was performed at two time intervals: 1 hour and 24 hours.

Several studies explored the antimicrobial effect of L-PRF, but few studies have analyzed the effect in detail and in relation to periodontal bacteria (Yang *et al.*, 2015; Badade *et al.*, 2016). In addition, it is difficult

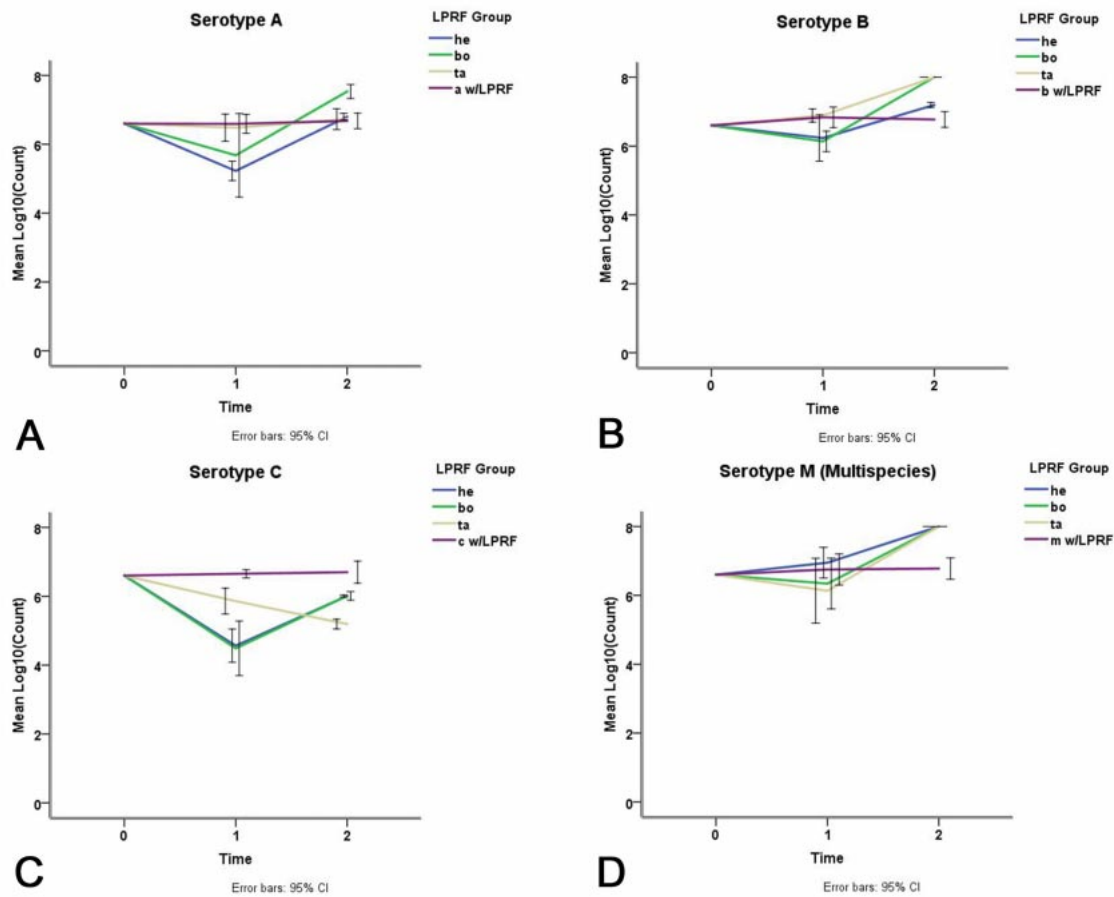


Fig. 4. Bacterial growth for different times: 0, 1 (1 hour), 2 (24 h) and zones: he (head), bo (body), ta (tail), with their respective negative control groups: a w/LPRF (serotype a without L-PRF), b w/LPRF (serotype b without L-PRF), c w/LPRF (serotype c without L-PRF), m w/LPRF (serotype m without L-PRF). (A) Serotype a, (B) serotype b, (C) serotype c, (D) serotype m.

to correlate the available studies due to the varied nomenclatures of platelet derivatives and the different protocols available for formulation.

At time 1, our results showed that the area with the most antimicrobial power was the head, followed by the body, and finally the tail. This could be explained by the cellular and fibrin configuration of each zone where the head is the zone with more cellular content decreasing to the tail (Dias *et al.*). This cellular content refers to platelets and leukocytes in their defense against the host (Róz'alski *et al.*, 2013; Bielecki & Dohan Ehrenfest, 2012b). Thus, in the area of the tail, more bacterial growth was found for serotypes a, b, and c. Reports of the antibacterial properties of the different areas of L-PRF against different serotypes of *A. actinomycetemcomitans* could not be found, suggesting that these are not yet fully understood. In

the present study, the area of the head was the one that had the least growth in serotype a, with similar results in the body area in serotypes b and c. On the other hand, a totally opposite result was given for serotype m, where the head was the zone with the most growth, followed by the body, and then the tail.

Yang *et al.* (2005) demonstrated that PRP allowed inhibition of *P. gingivalis* and *A. actinomycetemcomitans* but not *Fusobacterium nucleatum*. One study found no antimicrobial L-PRF activity against *P. gingivalis* and *A. actinomycetemcomitans* when compared to PRP, which would not agree with the results obtained at time 1. The authors suggested that the strongest bacterial inhibition of PRP when compared with L-PRF could be due to calcium chloride (Badade *et al.*). In some PRP techniques for activating platelets and inducing fibrin formation, thrombin or calcium chloride is added to the preparation

(Arnoczky *et al.*, 2013). Calcium Chloride could inhibit microbial growth (Lawrence *et al.*, 2003). Another study, where bacterial plaque samples were taken from patients with gingivitis, showed opposite results; PRF demonstrated significant antimicrobial activity when compared to PRP, which would be similar to what was seen in our results at time 1 (Karde *et al.*, 2017). The study authors did not speculate on the cause of this difference between PRP and PRF.

In the current study, at time 1, L-PRF had bacteriostatic power against some strains of *A. actinomycetemcomitans*. These results contrast with the findings of Castro *et al.* (2019) where researchers did not find an inhibition of this bacteria, and when in contact with L-PRF exudate, *A. actinomycetemcomitans* increased. It is important to note that Castro and colleagues performed their analysis in relation to a polymerase chain reaction and in L-PRF exudate, which differs from our analysis. In the Kour *et al.* (2018), study, the antimicrobial power of PRF and PRP against *A. actinomycetemcomitans* was evaluated. Both tested positive for antimicrobial properties, which was more pronounced when using PRP. The researchers attribute this difference to the fact that platelets and cytokines in PRF are found within the fibrin mesh and will be released when it disintegrates, for which a longer measurement time may be necessary.

The most aggressive serotype in this study was multispecies, which was a mixture of equal amounts of serotypes a, b, and c. In many patients with periodontitis, coinfection of two or three serotypes of *A. actinomycetemcomitans* are found (Rylev & Kilian, 2008). In the Rojas *et al.* (2018) study, it was found that in coinfection by serotypes a and b, serotype a works by decreasing the immunostimulation potential of b.

The serotype that, by itself, was the most aggressive in this study was b, followed by a and then c. This is in accordance with the Rojas *et al.* (2020), study, where researchers evaluated the immunogenic potential of different serotypes, and b was seen to have the greatest immune stimulator potential. Similarly, another study (Monasterio *et al.*, 2018), which analyzed the potential to produce alveolar reabsorption, found that the most pathogenic serotype was b. In addition, the LPS of this serotype has been shown to be a more potent immunogen than other serotypes (Díaz-Zúñiga *et al.*, 2015). Serotype b has been associated with periodontitis (Haubek *et al.*, 2008), and some studies associate serotypes b and c with an increased risk of

periodontitis in the future (Pietiäinen *et al.*, 2018). Despite evidence from multiple studies, the correlation between serotypes and periodontal conditions remains confusing (Brígido *et al.*, 2014).

For time 2 (24 hours) in the present study, an increase in the growth of all serotypes (a, b, c, and m) and in all areas of the L-PRF (head, body, tail) was found (by comparison with the negative control). That is, L-PRF acted as a promoter of the growth of *A. actinomycetemcomitans*, which is consistent with Castro *et al.* findings, where they attribute this effect to human serum stimulating leucotoxic activity in this bacterium. This would also be aligned with the study of Tang-Siegel *et al.* (2016), which found that some strains of *A. actinomycetemcomitans* respond to human serum by showing rapid growth.

The study has several limitations. First, it was an *in vitro* experiment, and as such, it cannot exactly reproduce the complexity of the *in vivo* conditions (Lorian, 1989). Additionally, the patient sample was quite small, and the relationship of L-PRF was analyzed with only a single periodontal bacterium.

It would be interesting to conduct an ELISA study to search for antimicrobial peptides in L-PRF in order to relate an antimicrobial effect. To the best of our knowledge, this is the first study to analyze the microbiological potential of different areas of L-PRF.

CONCLUSIONS

For the first time, it can be noted that the areas with more cellular content, that is, regions of the head and body, are the ones with the most antimicrobial power. L-PRF would have better results with serotype c. At time 2, L-PRF is likely to act as a growth promoter of *A. actinomycetemcomitans*.

LANATA-SELINGUE, N.; BETANCUR, D.; BELLO-TOLEDO, H.; GONZÁLEZ-ROCHA, G.; LUENGO, L. & SÁNCHEZ-SANHUEZA, G. Efecto antimicrobiano de la fibrina rica en plaquetas y leucocitos contra *Aggregatibacter actinomycetemcomitans* en un modelo mono- multi serotipo. *Int. J. Odontostomat.*, 15(4) :1035-1042, 2021.

RESUMEN: La Fibrina rica en Leucocitos y Plaquetas es un concentrado plaquetario ampliamente utilizado en procedimientos quirúrgicos periodontales. Muchos beneficios se describen con respecto a su uso, tales como propieda-

des antimicrobianas. El objetivo de este estudio fue evaluar los efectos antimicrobianos de las diferentes zonas de este concentrado plaquetario frente a los serotipos más prevalentes de *Aggregatibacter actinomycetemcomitans* en un modelo mono-multi serotipo in vitro. Se incluyeron tres pacientes que fueron tratados en la Facultad de Odontología de la ciudad donde residen los investigadores. Se utilizó para su análisis una prueba de contacto directo modificado. En la prueba de contacto directo modificado, se encontró una disminución en el recuento bacteriano en el tiempo 1, pero en el tiempo 2, el recuento aumentó para todos los serotipos y zonas de L-PRF. Se puede observar que las áreas con mayor contenido celular en la Fibrina rica en Leucocitos y Plaquetas son las áreas con mayor poder antimicrobiano. Este concentrado de plaquetas tendría mejores resultados con el serotipo c. En el tiempo 2, es probable que actúe como un promotor del crecimiento de *A. actinomycetemcomitans*.

PALABRAS CLAVE: *Aggregatibacter actinomycetemcomitans*, implantes dentales, infecciones, fibrina rica en plaquetas.

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